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THÈSE

présentée par :

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Discipline/ Spécialité :

Sciences du vivant/Aspects moléculaires et cellulaires de la biologie

Régulation de l'apoptose par les microARN du virus associé au sarcome de Kaposi

THÈSE dirigée par :

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Mr GRUNDHOFF Adam	Professeur, Université de Hambourg
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en particulier pour un de ces articles, tous les résultats des expériences auxquelles j'ai participé se sont révélés être négatifs et n'ont donc pas été publiés dans ce dernier ; cependant pour le travail effectué et les conclusions de ces expériences qui ont permis de faire avancer l'étude en éliminant certaines hypothèse, j'ai pu bénéficier de mon nom parmi les co-auteurs. Merci encore pour m'avoir donné ces opportunités de collaborer avec votre équipe et pour nous avoir fait pleine confiance en suivant aveuglément nos avis et suggestions d'expériences sur la partie du travail dont nous nous occupions. Merci à ***Ghada*** *pour tout le travail que nous avons pu effectuer ensemble dans la joie et la bonne humeur dans le cadre de cette collaboration, qui était toujours nerveuse et impatiente pendant que les manip tournaient en attendant les résultats -à chaque fois à rester par ex. devant le luminomètre pendant la lecture des plaques, à regarder arriver chaque mesure une par une-, et surexcitée à chaque résultat positif! C'est grâce au travail mené avec elle que j'ai pu obtenir pour la première fois mon nom sur une publication, au cours de mon Master 2 ; toujours pleine d'optimisme, très souriante, enthousiaste, tenace et emplie de gentillesse, à régulièrement faire de petites attentions. J'ai vraiment apprécié travailler toi, merci pour tout! Cette collaboration a également impliqué* ***Lucas****, un très bon ami, avec qui après avoir étudié ensemble sur les bancs de la fac nous nous sommes souvent délecté à travailler ensemble (et à d'autres moments au contraire!) - ce qui a impliqué beaucoup de grands moments de rires "gras" en plein travail. Merci à toi pour ce travail productif (même si pas toujours!) que nous avons réussi (malgré tout!) à mener ensemble ; c'est un honneur pour moi d'avoir mon nom sur un article premier auteur d'un de mes proches amis! L'escalier de l'institut se sera "rappelé" de nous pendant un certain moment, lors de cette fameuse soirée de travail que l'on n'oubliera pas! J'espère que le bourdonnement de Vienne dans tes oreilles s'est enfin arrêté!*

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Finalement, nous avons monté avec d'autres doctorant quelques projets pédagogiques, dont en particulier le plus mémorable, un stand de vulgarisation scientifique à la fête de la science sur la biodiversité des microorganismes. Ce fut un travail d'équipe de longue haleine et épuisant, mais aussi effectué avec des amis et dont nous avons été très fier. Merci à ***Saïd Hasanoui****, un ami également, pour son attention particulière qu'il nous a accordé en tant qu'un des organisateurs de cet événement. Merci à* ***David Halter****, avec qui nous avons monté, encadré ce projet et réparti le travail à effectuer pour "l'équipe", et qui est un excellent ami rencontré à la fac. Merci également pour toutes les discussions, réflexions et points de vue que l'on a pu échanger ensemble sur ce qu'était réellement une thèse, quant à nos impressions et ressentis respectifs, soit à s'en complaire, ou par autres moments comme tout doctorant à s'en plaindre, et aussi à échanger nos avis et conseils pour nos perspectives professionnelles respectives à suivre. Merci à* ***Sarah*** *qui est également une bien bonne amie de fac qui a effectué sa thèse comme moi à l'IMBC, qui a participé elle aussi à ce projet de vulgarisation et y a fourni un travail considérable. Un grand merci bien sûr aussi à toi pour le soutien que tu as pu me fournir pendant la rédaction de ma thèse, et m'avoir aidé à me remotiver quand j'ai pu fatiguer par moments.*

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Merci beaucoup pour tout à tous!

ABREVIATIONS LIST

Ago: Argonaute
AIDS: acquired immunodeficiency syndrome
BAC: bacterial artificial chromosome
BCBL: body cavity-based lymphoma
Bcl: B-cell lymphoma
BclAF1: Bcl2-associated transcription factor 1
BKV: human BK polyoma virus
BLV: bovine leukemia virus
bp: base pair
BPCV: bandicoot papillomatosis carcinomatosis virus
CASP: Caspase
CDS: coding DNA sequence
ceRNA: competing endogenous RNA
circRNA: circular ncRNA
CLIP: crosslinking and immunoprecipitation
Cyc: Cyclin
DCL1: Dicer-like 1
DE: delayed-early
DGCR8: DiGeorge syndrome critical region gene 8
DNA: deoxyribonucleic acid
ds: double-stranded
EBV: Epstein-Barr Virus
eIF: translation initiation factor
Exp5: Exportin-5
FLIP: FLICE-like inhibitory protein
GPCR: G-protein-coupled receptor
HBP1: HMG-box transcription factor 1
HCMV: human cytomegalovirus
HCV: hepatitis C virus
HHV: human herpesvirus
HITS-CLIP: high-throughput sequencing coupled with ultraviolet crosslinking and immunoprecipitation
HIV: human immunodeficiency virus
HPV: human papilloma virus
HSV: herpes simplex
HvAV: Heliothis virescens ascovirus
HVS: herpesvirus saimiri
HzNV-1: Heliothis zea nudivirus 1
ICP: infected cell protein
IE: immediate-early
IFN: interferon
IL: interleukin
ILTV: avian infectious laryngotracheitis virus
IP: immunoprecipitation
IRF: interferon regulatory factor
JCV: human JC polyoma virus
KapB: Kaposin B
kb: kilobase
KS: Kaposi's sarcoma
KSHV: Kaposi's sarcoma-associated herpesvirus
L: late
LANA: latency-associated nuclear antigen
LAT: latency-associated transcript
LECA: last eukaryotic common ancestor
LMP1: latent membrane protein 1
LTIII: latency III
MCD: multicentric Castleman's disease
MCMV: mouse cytomegalovirus
MDV: Marek's disease herpesvirus
MHV-68: murine gammaherpesvirus 68/Murid herpesvirus 68
MICB: major histocompatibility complex class I-related chain B
MID: middle
miRNA*: passenger strand or star strand
miRNA: microRNA
moRNA: miRNA-offset RNA
mRNA: messenger RNA
muPyV: murine polyomavirus
ncRNA: non-coding RNA
NK: natural killer
nt: nucleotide
ORF: open reading frame
PABP: polyA-binding protein
PAR-CLiP: photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation
PAZ: Piwi Argonaute Zwille
PEL: primary effusion lymphoma
piRNA: PIWI-interacting small RNA
Pol: RNA polymerase
pRB: retinoblastoma protein
pre-miRNA: precursor miRNA
pri-miRNA: primary miRNA transcript
PUMA: p53 up-regulated modulator of apoptosis
qRT-PCR: quantitative reverse transcription-polymerase chain reaction
RBP: RNA binding protein
RdRP: RNA-dependent RNA polymerase
RISC: RNA-induced silencing complex
rLCV: rhesus lymphocryptovirus
RNA: ribonucleic acid
RNAi: RNA interference
RNAse: ribonuclease
RRV: rhesus rhadinovirus
RTA: replication transcriptional activator
siRNA: short-interfering RNA
SMAD: similar to mothers against decapentaplegic
snoRNA: small nucleolar RNA
SV40: simian virus 40
TE: transposable element
TGF-β: transforming growth factor beta
THBS1: thrombospondin 1
TLR: toll-like receptor
TNF: tumour necrosis factor
TPA: 12-O-tetradecanoylphorbol-13-acetate
TR: terminal repeat
TRBP: HIV-1 TAR RNA-binding protein
tRNA: transfer RNA
TWEAKR: TNF-like weak inducer of apoptosis receptor
UTR: untranslated region
v: viral
VA RNA: adenovirus viral associated RNA
VEGF: vascular endothelial growth factor
VSR: viral suppressors of silencing
VZV: varicella-zoster virus
WT: wild type

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INTRODUCTION

1. Foreword

The discovery of the RNA silencing phenomenon at the end of the 20th century revealed a previously overlooked layer of regulation, which dramatically increased the complexity of the molecular biology's dogma (Figure 1). One of the key features of RNA silencing conserved in most eukaryotes such as plants, worm, fungi, insects and vertebrates is the involvement of small RNA species of 19 to 30 nucleotides (nt) in length that act as specificity determinants for the regulation of gene expression at the RNA and/or DNA level.

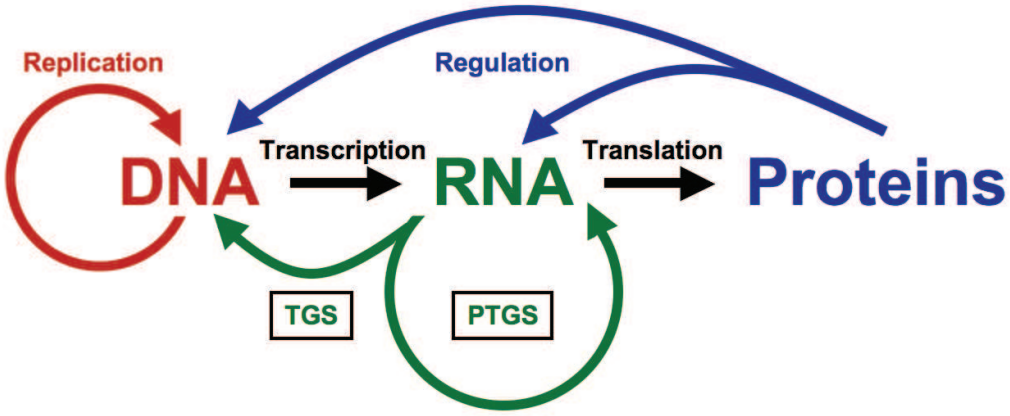

Figure 1: Schematic representation of molecular biology's dogma. RNA silencing is symbolised by the green arrows in the diagram. TGS: Transcriptional Gene Silencing; PTGS: Post Transcriptional Gene Silencing.

In 1990, following the introduction in the petunia of a transgene encoding the chalcone synthase (CHS), a protein involved in the production of the compounds responsible for the coloration of petals, Napoli *et al.* first described the phenomenon of co-suppression. Indeed, in addition to the expected more intense purple colour than in wild type (WT) petals, some transgenic plants and their progeny showed partially white petals. Thus, they further showed that in the altered white flowers, the expression of both the endogenous and the introduced CHS genes were "extinct" or "silenced" at the post-transcriptional level by an unclear mechanism (Napoli et al., 1990). Later on, this co-suppression phenomenon, which has been referred to as post-transcriptional gene silencing (PTGS), was linked to a naturally occurring innate antiviral plant mechanism where viruses were also shown to be initiators and targets of gene silencing (Ratcliff et al., 1997). Further studies then described this antiviral mechanism in other organisms such as insects and worms (Wang et al., 2006a; Wilkins et al., 2005). Viruses infecting these organisms have in turn developed various kinds of viral suppressors of silencing (VSR), as a counter defence mechanism. Their presence in viral genomes is a

signature of virus targeting by RNA silencing in hosts able to deploy it as an innate antiviral defence (reviewed in (Ding and Voinnet, 2007; Obbard et al., 2009)).

The key involvement of double-stranded (ds)RNA in this mechanism was first established in plants (Baulcombe, 1996; Dougherty and Parks, 1995; Metzlaff et al., 1997). Fire and Mello then showed that the introduction of long dsRNA in *Caenorhabditis elegans* leads to the degradation of homologous transcript by a process coined RNA interference (RNAi) (Fire et al., 1998). In 1999, Hamilton and Baulcombe demonstrated that this phenomenon was mediated by small RNA species of about 25 nt in length (Hamilton and Baulcombe, 1999). These small RNAs derived from dsRNAs were shown to act as a guide to silence complementary RNAs. They were further characterised and named short-interfering (si)RNAs by Elbashir and Tuschl (Elbashir et al., 2001a). It was then further shown by the Tuschl laboratory, that delivery of synthetic siRNA duplexes in mammalian cells allows to bypass the unspecific effects of long dsRNA in these cells and enabled to specifically silence RNAs harbouring homologous sequences through RNAi (Elbashir et al., 2001b). Nevertheless, it still remains to be formally proven whether mammals possess an antiviral RNAi defence system (reviewed in (Umbach and Cullen, 2009; Voinnet, 2005)). Another role that has also been described for some classes of siRNAs in plants and in fission yeast is their involvement in transcriptional gene silencing (TGS) (Figure 1) of heterochromatin by DNA and histone methylation. RNA silencing can thus also protect cells against repeat and transposable elements (TEs), both by degrading their transcripts and by preventing their expression through heterochromatin formation. A similar function for RNAs of 24 to 32 nt, termed PIWI-interacting small RNAs (piRNAs), has been described in insect and mammalian germ cells (reviewed in (He et al., 2011; Lippman and Martienssen, 2004; Obbard et al., 2009). It is worthy of noting that while the RNA silencing process include both TGS and PTGS, the RNAi phenomenon only refers to the regulation mechanisms linked to PTGS.

Simultaneously to the research on PTGS, the Ruvkun and Ambros laboratories characterised a gene involved in the developmental timing of the nematode *C. elegans* and termed *lin-4*. The product of this gene is a small non-coding (nc)RNA that acts by pairing to complementary sites in the 3' untranslated regions (UTRs) of another RNA, *lin-14*, to regulate its translation (Lee et al., 1993; Wightman et al., 1993). A few years later, Ruvkun and collaborators described another similar small RNA, encoded by the *let-7* gene, also involved in developmental fate of cell lineage in *C. elegans* (Reinhart et al., 2000). These regulatory

ncRNAs were thus coined small temporal (st)RNAs and were first thought to be a unique feature of worms, until it was discovered that *let-7* was widely conserved during evolution in a variety of other organisms, including humans (Pasquinelli et al., 2000). As a consequence, a frenzied search for other RNAs similar to let-7 started, which led to the identification of 100 small RNAs in *Drosophila*, *C. elegans* and human, and therefore to the definition of micro (mi)RNAs by Tuschl, Bartel and Ambros' laboratories (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). To date, the number of miRNAs deposited in the miRNA registry miRBase (http://www.mirbase.org) (Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011) exceeds 25,000 mature miRNA sequences distributed in more than 190 species, including plants, worms, insects, mammals, but also, viruses. Indeed, viruses are parasites well known for continuously adapting to their host, which is reflected by their capacity to hijack the cellular machinery. It is thus not surprising that some viruses evolved to turn RNA silencing great regulatory potential to their own advantage by expressing miRNAs. These allow viruses to establish favourable cellular environments for their replication and maintenance in the cell in a non-immunogenic manner. The first virus-encoded miRNAs were identified by the Tuschl laboratory in cells latently infected with the Epstein-Barr virus (EBV) (Pfeffer et al., 2004). This discovery paved the way for the identification of numerous other viral miRNAs and for a whole new axis of research in the RNA silencing field.

2. Regulation by small non-coding RNAs and miRNAs evolution in eukaryotes

2.1. RNA silencing: a conserved process of genetic regulation among eukaryotes

Gene regulation by small ncRNAs is a mechanism widely conserved across the different eukaryotic phyla, and is essential for processes as diverse as development, apoptosis, stem cell renewal, differentiation and maintenance of cellular integrity (reviewed in (Baulcombe, 2004; Carrington and Ambros, 2003; Lippman and Martienssen, 2004)). These key ncRNA regulators can be distributed into three major classes, based on their precursors nature, their biogenesis, and their functions: siRNAs, miRNAs, and piRNAs. siRNAs are mainly involved in the defence against viruses and TEs, miRNAs in the regulation of eukaryotic gene expression, and piRNAs in the maintenance of TEs silencing in germline cells (reviewed in (Röther and Meister, 2011; Siomi and Siomi, 2009)). These different classes of small RNAs associate with multiple protein components to form effector ribonucleoprotein complexes, which regulate the expression of perfectly or partially complementary target transcripts. A key component found in all these complexes is a member of the Argonaute (Ago) family of proteins. This family is subdivided into three clades, the Agos which interact with siRNAs and miRNAs, the PIWI proteins that associate with piRNAs, and the Wago proteins, a clade constituted entirely of worm Agos, generally occupied by "secondary siRNAs", and for which the roles are still not clearly understood (reviewed in (Chapman and Carrington, 2007; Farazi et al., 2008; Jinek and Doudna, 2009; Joshua-Tor and Hannon, 2011)).

Phylogenetic analyses, based on the observation that RNA silencing has been described in each of the eukaryotes supergroups (Excavata, Chomalveaolata, Archeaplastida and Unikonta), led to the conclusion that the last eukaryotic common ancestor (LECA) possessed a relatively complex RNA silencing machinery. The minimal machinery consisted of an Ago-like protein and/or a PIWI-like protein, a dsRNA-specific ribonuclease (Dicer), and a RNA-dependent RNA polymerase (RdRP). It seems likely that the ancestral RNA silencing system functioned primarily in defence against genomic parasites such as TEs, via nuclear PIWI-based pathways, and against viruses, via cytoplasmic, Ago-centered pathways. This idea is supported by the presence of an RdRP, being primarily involved in siRNA amplification, but not in miRNA pathways. RNA silencing machinery thus would have evolved and diverged during eukaryotic evolution as an extension of this defence role, giving rise to processes showing a limited taxonomic distribution, such as RNA-directed methylation, e.g. in *Arabidopsis thaliana* (Zilberman et al., 2003), DNA elimination, e.g. in *Tetrahymena thermophilia* (Lee and Collins, 2006; Liu et al., 2004b), and meiotic silencing by unpaired

DNA, e.g. in *Neurospora crassa* (Shiu et al., 2001), and to more flexible innovations such as the several kinds of endogenous siRNAs (endo-siRNAs), and miRNAs for the regulation of gene expression. The absence of RNA silencing, has only been observed in some unicellular species that possess relatively small genomes, such as *Saccharomyces cerevisiae*, that may be affected by a limited number of genomic parasites and/or likely have alternative means to control them, which could explain why these species lost this process when diverging. This is not a definite explanation though, since some budding yeasts like *Saccharomyces castellii* and *Candida albicans* do possess a RNA silencing machinery (Drinnenberg et al., 2009). It could thus be that in special cases, the loss of RNA silencing might provide a specific evolutionary advantage on the short term. On the other hand, RNA silencing seems to have become essential in many multicellular eukaryotic lineages, which we can infer by the lethality of Dicer mutations due to the involvement of miRNAs in the regulation of development. Indeed, miRNAs as a class of genes were present early in evolution of animals, with the example of miR-100 being shared between eumetazoans (Grimson et al., 2008). A dramatic expansion of the miRNA repertoire then occurred during evolution of metazoans and is associated to the bilaterian innovation, with 34 miRNAs conserved between protostomes and deuterostomes. Additional expansions are observed at the base of vertebrate lineage, and in the lineage leading to the placenta mammals (Figure 2) (reviewed in (Berezikov, 2011; Cerutti and Casas-Mollano, 2006; Shabalina and Koonin, 2008)).

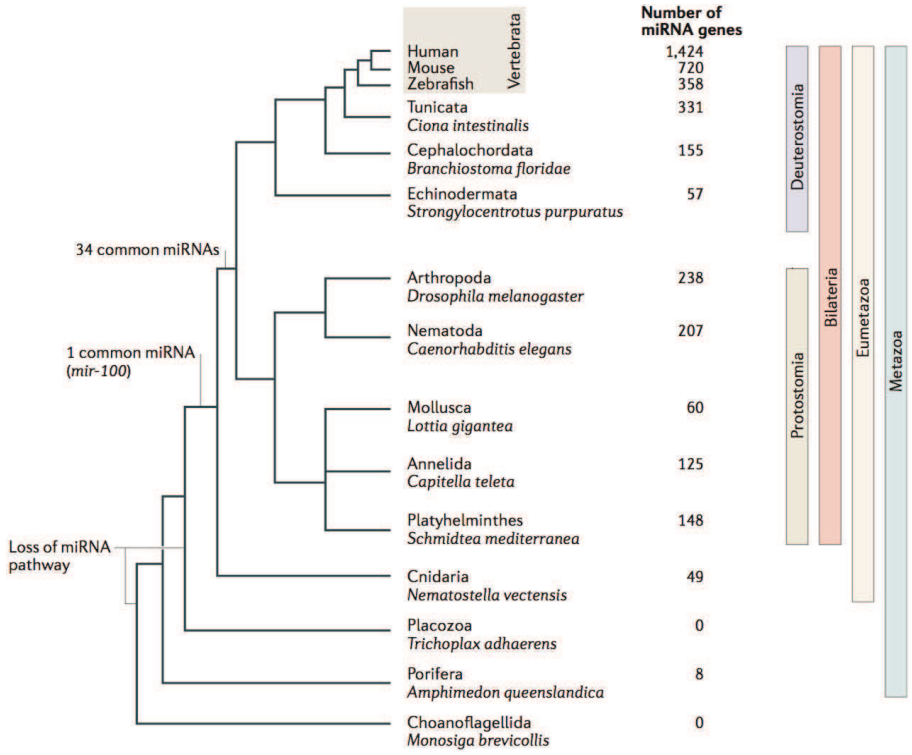

Figure 2: Distribution of miRNA genes in the animal kingdom. Adapted from (Berezikov, 2011).

However, it remains unclear whether some kind of proto-miRNA already existed in LECA, or if miRNAs appeared independently in plants and animals kingdoms after their divergence during evolution.

2.2. Evolutive origin of miRNAs

MiRNAs, which act as post-transcriptional regulators, are encoded in the genomes of unicellular and multicellular eukaryotes. MiRNAs sharing similar sequences are attributed the same identifying number and grouped into families, the members of which are sometimes regulated differentially and expressed in tissue-specific patterns. These miRNA genes have extensive sequence homology in their mature miRNA sequence, which is identical or nearly identical from one species to another, or within a species (Ambros et al., 2003). The miRNA families represent paralogous sequences, which derived from an orthologue miRNA by gene duplication. Two types of duplication events can occur: local tandem duplication, where the duplicate gene remains located in the same transcript, opposed to non-local tandem duplication, which results in a new miRNA remote location, often on a different chromosome (Hertel et al., 2006). Gene duplication followed by subfunctionalization or neofunctionalization processes are considered as the major source of expansion of the miRNA repertoires (Figure 3A) (Ruby et al., 2007).

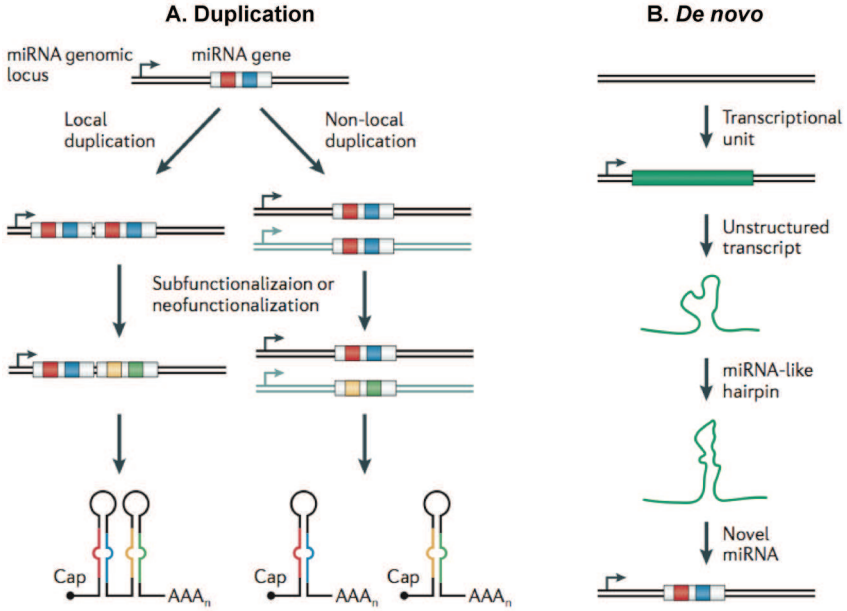

Figure 3: Expansion of the miRNA repertoire. (A) Processes of subfunctionalization or neofunctionalization following miRNA gene duplication. Sequences changes in the mature miRNA and miRNA* leading to the emergences of novel miRNA sequences are represented by the colours. (B) *De novo* emergence of miRNAs. Adapted from (Berezikov, 2011).

The latter processes explain how novel miRNAs can be generated by deriving from already established miRNAs genes, but does not explain what are the evolutionary mechanisms that could have led to the emergence of *de novo* miRNA (Figure 3B) and to the acquisition of their targets. This question is related to the concern whether emergence of a proto-miRNA system occurred in LECA, or later during evolution, independently in plant and animal kingdoms. Thereby, various aspects of plant and animal miRNA biology have to be explored in order to give some elements of response to this interrogation.

2.2.1. Contrasting miRNA pathways in plant and animals

MiRNAs pathways in animals and in plants share common features, but also present specific aspects. Here is an overview of these pathways in which I emphasised on the differences described in the two kingdoms, in attempt to answer to the question whether plant and animal miRNAs derive or not from the same common ancestor. To this end, I introduced a certain number of notions about miRNA biogenesis, mode of action and subsequent targeting strategies in animals among which some are described in more details later. For a detailed description of plants miRNA pathway, please refer to (Baulcombe, 2004; Voinnet, 2009).

2.2.1.1. A brief overview of canonical miRNA biogenesis in plant versus animal

MiRNAs derive from long primary miRNA transcripts (pri-miRNA) harbouring imperfect stem-loop structures, which undergo a multistep maturation process involving type III ribonuclease (RNAse) enzymes such as Dicer. In animals, the first step that occurs in the nucleus involves the RNAse III Drosha, which gives rise to a small hairpin precursor miRNA (pre-miRNA) (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). This pre-miRNA, after nuclear export by a member of the exportin family of proteins, Exportin-5 (Exp5) (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003), is then recognised in the cytoplasm by Dicer for a second step of processing, in order to obtain a small RNA duplex, miRNA/miRNA* (Bernstein et al., 2001; Hutvágner et al., 2001; MacRae et al., 2006; Zhang et al., 2004). The mature miRNA contained in this duplex is then loaded into the effector RNA-induced silencing complex (RISC), which invariably contains a member of the Ago family. The other strand, referred as "passenger strand", or "star strand" (miRNA*) is usually degraded (Carmell et al., 2002). By contrast, in plants, both steps are achieved by the Dicer-like 1 (DCL1) enzyme and are restricted to the nucleus in specialised

subnuclear compartments called D-bodies (Fahlgren et al., 2007; Fang and Spector, 2007; Park et al., 2005; Song et al., 2007). Prior to, or after the nuclear export of the small RNA duplex by an orthologue of Exportin-5, HASTY (Park et al., 2005), an additional step of maturation occurs. This step, absent in animals, consists in the 2'-O-methylation of the miRNA/miRNA* duplex 3' terminal nucleotides by the HEN1 protein (Yu et al., 2005); it is not clear whether this step is achieved in the nucleus or in the cytoplasm. This modification prevents 3' uridylation of mature miRNAs that accelerates their turnover (Figure 4) (Li et al., 2005).

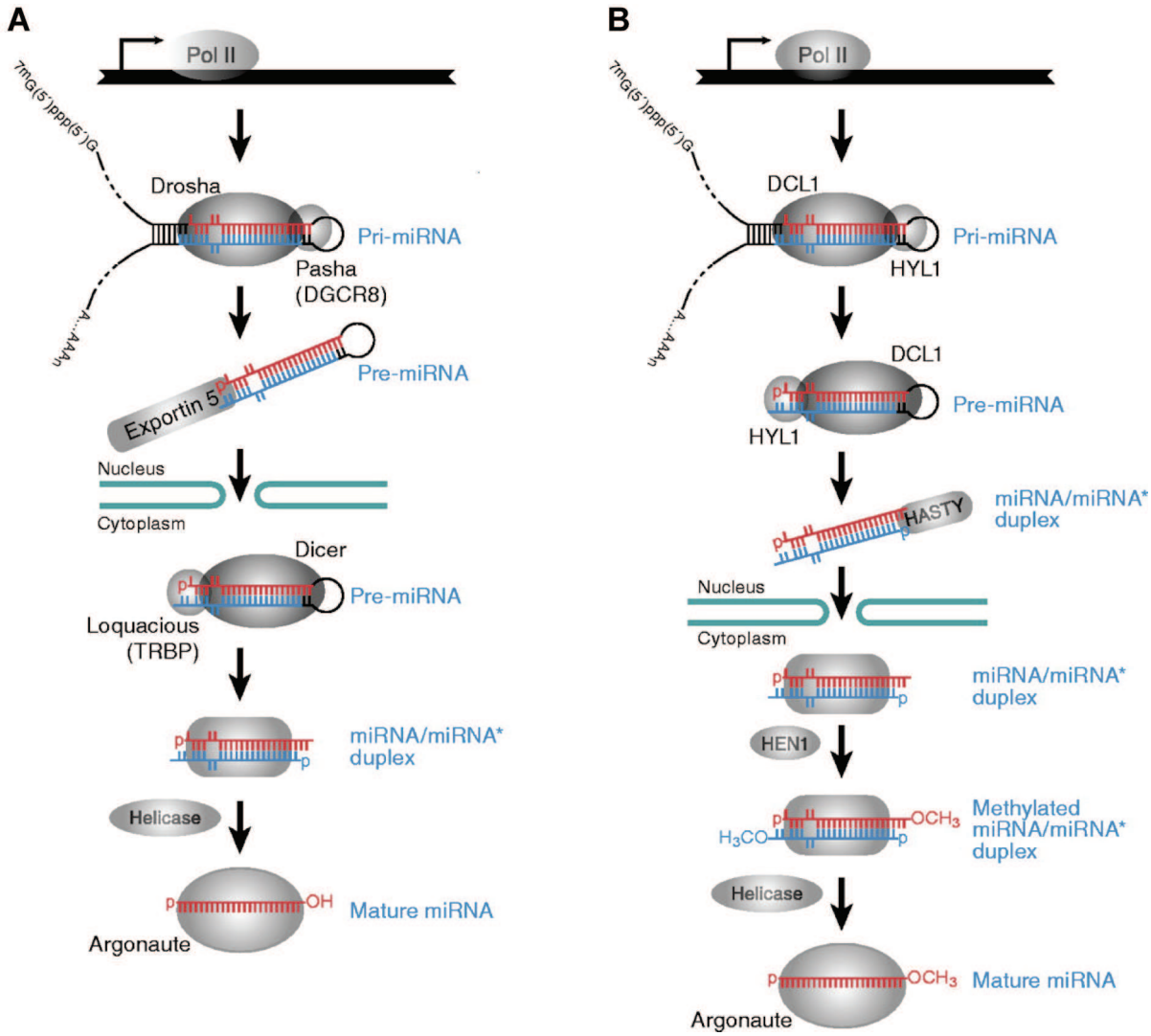

Figure 4: The miRNA biogenesis pathway. (A) Animal miRNA biogenesis. The pri-miRNA is generally transcribed by Pol II, and presents stem-loop structures. In the nucleus, the combined action of Drosha and DGCR8 drives the production of a pre-miRNA. Exp5 allows its export through the nuclear pore to the cytoplasm where it is cleaved by Dicer associated with its co-factor TRBP to generate a miRNA duplex. One strand is then incorporated into the Ago-containing RISC complex. (B) Plant miRNA biogenesis. The pri-miRNA presents stem-loop structures and is transcribed by Pol II. In the nucleus, the combined action of DCL1 and HYL1 drives the production of a miRNA/miRNA* duplex, which is then methylated by HEN1 to genereate a mature miRNA/miRNA* duplex. The exportin HASTY allows its export to the cytoplasm where it is incorporated into RISC. Mature miRNAs are indicated in red; miRNA* strands are in blue. Adapted from (Du and Zamore, 2005).

The length of pri-miRNAs and thus of the resulting processed pre-miRNAs in plants -DCL1 first cleavage generally occurring at the base of the pri-miRNA hairpin (Kurihara and Watanabe, 2004)- is variable, ranging from 70 to several hundred bases (Szarzynska et al., 2009; Xie et al., 2005). Most plant pri-miRNAs produce a single miRNA/miRNA* duplex, but some loci give rise to multiple duplexes (Addo-Quaye et al., 2009; Bologna et al., 2009; Zhang et al., 2010). Animal pre-miRNAs length is more constant, usually around 70 nt (Lee et al., 2002); however some examples of longer pre-miRNAs up to 200 nt have been described in *Drosophila* (Ruby et al., 2007). Although animal pre-miRNAs processing results in one miRNA/miRNA* duplex, it has been recently described in *Ciona intestinalis* that some small RNA species could be generated from sequences immediately adjacent to a pre-miRNA. These have been termed miRNA-offset (mo)RNAs. They are 20 nt long products resulting from an RNAse III processing activity, Drosha being probably involved, but their biological role is still unclear and has to be further investigated (Shi et al., 2009). Such moRNAs have also been described in *Drosophila melanogaster* (Ruby et al., 2007), in mouse embryonic stem cells (Babiarz et al., 2008), and human (Langenberger et al., 2009).

2.2.1.2. Non-canonical miRNAs

Recent studies showed that other sources of RNA transcripts could be shaped by evolution into hairpins recognised by the miRNA processing machinery. These non-canonical miRNAs bypass the first step of processing, having been already folded with pre-miRNA-like features. Transfer (t)RNAs can generate products with miRNA-like features (reviewed in (Pederson, 2010)). These tRNA-derived small RNAs have been described in human to be preferentially loaded onto Ago3 and Ago4 RISC complexes and restricted to the cytoplasm (Haussecker et al., 2010). It is interesting to note that such tRNA-derived non-conventional small RNAs are also found in organisms where the RNA silencing machinery has been almost totally lost upon evolution like *Trypanosoma cruzi* (Garcia-Silva et al., 2010). Small nucleolar (sno)RNAs can also generate miRNA-like sequences (reviewed in (Scott and Ono, 2011)). It has been shown that a subset of snoRNAs, in addition to acting in the nucleus at the level of modification and processing of ribosomal (r)RNAs and small nuclear (sn)RNAs, could be exported to the cytoplasm and then processed by Dicer to give rise to 20-22 nt long small RNAs. These RISC-loaded small RNAs are efficiently able to regulate either a reporter RNA or an endogenous target (Ender et al., 2008; Scott et al., 2009). Finally, some cases of pre-miRNA derived from introns borders have been described in *Drosophila* and *C. elegans* as

well as in mammals. These so-called mirtrons are excised in a Drosha-independent manner, by direct splicing. After splicing, the resulting branched intron folds into a secondary structure that mimics the features of a pre-miRNA, the 3' end of the "pre-miRNA" being defined by the 3' splice site. It is then recognised and processed by Dicer after its export into the cytoplasm (Berezikov et al., 2007; Ruby et al., 2006).

One of the main issues to characterise these non-canonical miRNAs as functional with a significant biological role, is to distinguish them from degradation by-products that could have been randomly incorporated into RISC complex (Berezikov et al., 2010). Therefore, it is important for these types of non-canonical miRNAs to assess that they are able to regulate targets at their physiological levels of expression.

Among these different types of non-canonical miRNAs, only mirtrons have also been described in plants with only one example reported (Zhu et al., 2008). Because plant miRNAs maturation relies upon a single Dicer-like protein to generate the miRNA/miRNA* duplex from a pri-miRNA transcript, the pre-miRNA-like structures that give rise to these non-canonical miRNAs may be very poorly, if at all, recognised by the plant DCL1 complex. Therefore, the emergence of these non-canonical miRNA "precursors" is not excluded in plants, but they are not likely to represent suitable candidates for selection and are likely to disappear rapidly.

In rare cases, pre-miRNA processing can be Dicer-independent such as for miR-451. Its maturation has been shown to rely on Ago2, via endogenous cleavage of the pre-miRNA to generate an intermediate 3' end, which is further trimmed giving rise to its mature form (Cheloufi et al., 2010; Cifuentes et al., 2010).

2.2.1.3. Genomic arrangement of miRNA genes

Plant miRNA-encoding loci comprise independent, non-protein coding transcription units; miRNAs encoded within introns are rare (Axtell et al., 2007; Rajagopalan et al., 2006). About one-fifth of the annotated miRNAs from *A. thaliana*, rice and poplar are tandem-clustered within up to 10 kilobases (kb). Most of these clusters (61% to 90% in these species), encode identical mature miRNAs, suggesting that they result from tandem duplication in order to increase the dosage of a specific miRNA under the control of one single promoter (Merchan et al., 2009). The clusters that encode for more than one mature miRNA family are rare and are not conserved.

The genomic distribution of miRNAs in animals is significantly different from those observed in plants. By contrast with plants, miRNAs derived from exonic parts of protein-coding genes such as UTRs or coding DNA sequences (CDSs) have been described, but still remains rare (Berezikov et al., 2011; Han et al., 2009). In addition, although many derive from stand-alone non-protein-coding-loci, approximately 30% are encoded within introns (Baskerville and Bartel, 2005; Rodriguez et al., 2004). This arrangement permits miRNAs to benefit from the *cis*-regulatory elements that direct the expression of the host messenger (m)RNA, thus explaining why they would be a privileged location for *de novo* miRNA hairpins emergence. Consistent with this hypothesis, younger, species-specific miRNAs are more often located in introns than ancient, conserved miRNAs. Campo-Paysaa *et al.* argue that "*Exaptations are defined as traits that, although evolved for one particular function, subsequently come to serve another - here, the evolution of a novel miRNA from a more ancient intron is an exaptation given that the intron predates the origin of the miRNA, and the selective reason(s) for the origin of introns is not related to the inclusion of miRNAs*", and recently termed "intronic exaptation" the evolution of miRNAs in intronic sequences (Campo-Paysaa et al., 2011). Nevertheless, intronic miRNAs can also be transcribed autonomously under the control of their own promoter (Aboobaker et al., 2005; Isik et al., 2010; Martinez et al., 2008). The differences between these two types of intronic miRNAs have still to be investigated from an evolutive point of view, to better understand if different processes are responsible of their emergence, or if the autonomous intronic miRNAs derive from the first ones.

Clustering is also a common feature of animal miRNAs: more than 40% of miRNAs in nematode, flies and mammals are clustered in their respective genomes. Another remarkable difference with plants miRNAs, is that most of the animal miRNA clusters encode for totally unrelated mature miRNAs, with only 5 to 20% of the clusters exclusively composed of duplicated miRNAs (Altuvia et al., 2005; Axtell et al., 2011; Bartel, 2004). Therefore, amplification of specific miRNA levels by tandem duplication is not sufficient to explain the composition of animal miRNA operons. This suggests that the emergence of animal miRNAs is privileged by location close to a pre-existing miRNA. Different studies have shown that Drosha processing of pri-miRNAs transcripts might occur co-transcriptionally (Ballarino et al., 2009; Kim and Kim, 2007; Morlando et al., 2008), and it was demonstrated that miRNA specific promoters enhanced processing efficiency (Ballarino et al., 2009). Physical proximity of a novel miRNA hairpin to an established one could thus enhance its processing by facilitating its access to Drosha, compared to "solo" emergent miRNA. This might be particularly important for the processing of new miRNAs harbouring suboptimal hairpins.

Supporting this hypothesis, newly evolved miRNAs have been described in proximity to strongly conserved animal miRNAs (Berezikov et al., 2011). This model would also explain the discrepancies observed between plant and animal miRNA clusters, due to the absence of a Drosha homologue in plants.

2.2.1.4. Target recognition by plant and animals miRNAs

Plant miRNAs pair to their target transcripts with a high degree of complementarity, perfect of near-perfect, which greatly eases the identification of their targets (Llave et al., 2002; Rhoades et al., 2002). Their binding sites can be found on the entire length of the mRNA sequence: in 5'UTR, open reading frame (ORF) and 3'UTR, with similar repression efficiency. In addition, miRNA binding sites can be found within non-protein-coding transcripts, which suggests no preferential RNA context for plant miRNA-directed regulation (German et al., 2008; Rhoades et al., 2002).

By contrast, animal miRNAs only need partial complementarity with the targeted mRNA, their 5' region being usually determining in this respect. This region is called the "seed" and corresponds to the 5' proximal part of the small RNA, more precisely nt 2 to 7 or 8 (Brennecke et al., 2005; Lai, 2002; Lewis et al., 2003). Additional features, such as an adenosine following the seed match, the location within the mRNA, proximity to other miRNA binding sites, and the accessibility of binding sites depending on the degree of local secondary structure also influence the target sites activity (reviewed in (Bartel, 2009). Another notable difference with plant miRNAs is the localisation of target sites among the mRNAs, and their associated mode of regulation and efficiency. Although there is clear evidence of evolutionary selection of miRNA binding sites within CDSs, or even in 5'UTRs (Lee et al., 2009a; Schnall-Levin et al., 2010; Zhou et al., 2009), most of the well studied target sites occur in 3'UTRs, suggesting a preferential location of these sites in 3'UTRs. However this postulate could be hampered by initially biased methods developed for the identification of animal miRNAs target sites, which were based on the first characterised ones. Indeed, the initial target sites identified for the first described miRNA, lin-4, were found in the 3'UTR of the *C. elegans lin-14* mRNA (Lee et al., 1993; Wightman et al., 1993). Subsequent studies in *Drosophila* development identified arrays of conserved 3'UTR motifs of approximately 7 nt, which mediate post-transcriptional repression of Notch genes (Lai and Posakony, 1997; Lai et al., 1998). Following the characterisation of *Drosophila* first miRNAs (Lagos-Quintana et al., 2001), these motifs turned out to represent the binding sites to their 5'

extremities (Lai, 2002), which later on were commonly referred to as the seed. In parallel, several studies that identified targets of the initially characterised miRNAs, strengthened this idea of a targeting in 3'UTR (Abrahante et al., 2003; Brennecke et al., 2003; Lin et al., 2003; Moss et al., 1997; Reinhart et al., 2000; Slack et al., 2000). Thus, prediction algorithms were developed, which scan the 3'UTRs of mRNAs for miRNA binding sites (Enright et al., 2003; John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003; Stark et al., 2003), and subsequent investigations in order to identify miRNA targets mainly focused on searching these sites in 3'UTRs. Interestingly, from the rare validated examples of animal miRNA targeting the mRNA 5'UTR, some induced an increase rather than a repression of the target expression (Ørom et al., 2008; Tsai et al., 2009). On the other hand, it has also been shown that miRNA targeting the 5'UTRs of transcripts could also induce their repression (Lytle et al., 2007; Moretti et al., 2010). Noteworthy, animal miRNAs targeting the 3'UTR for translational repression (their main mode of action, detailed in section 3) provides a stronger efficiency of repression. It was indeed reported that the efficacy of CDS or 5'UTR sites appears to be reduced owing to competition with ribosomes (Gu et al., 2009; Moretti et al., 2010). Thus, this observation argues for a selective advantage of the 3'UTR localisation for miRNAs binding sites.

As opposed to the animal kingdom, plant miRNAs mostly trigger the cleavage of their target RNAs in a Ago-catalysed reaction after almost perfectly pairing (Addo-Quaye et al., 2009; German et al., 2008; Llave et al., 2002). It should be noted though that this idea has been re-evaluated, as there is now ample evidence that translational inhibition seems to be widespread in plants, involving the decapping enzyme VARICOSE (Brodersen et al., 2008; Chen, 2004; Gandikota et al., 2007). Nonetheless, cleavage of the target mRNA in plants could explain why no preferential location for the binding sites is required along the transcript. In animals, even if some targets undergoing slicing have been described, they still remain very rare examples (Karginov et al., 2010; Shi et al., 2009; Yekta et al., 2004).

About 30% and up to 60% of animal transcripts have been predicted to contain miRNA binding sites (Friedman et al., 2009; Grün et al., 2005; Jan et al., 2011; Krek et al., 2005; Lewis et al., 2005; Ruby et al., 2007). Genome-wide transcriptome (Guo et al., 2010; Lim et al., 2005) and proteome (Baek et al., 2008; Selbach et al., 2008) approaches have provided experimental support to these predictions. An important consideration about miRNA targeting in animals is that it often happens in a cooperative manner. Thus, the degree of translational repression may increase exponentially with the number of binding sites within a target mRNA. Indeed, many 3'UTRs have conserved target sites for different miRNAs, enabling

combinational control of these mRNAs. This is of particular interest for the fine-tuning of cell- or developmental stage-specific target genes through different combinations of miRNAs expressed (Krek et al., 2005; Lewis et al., 2005). In the case of plants, miRNA targeting strategy seems radically different: less than 1% of *Arabidopsis* transcripts are predicted or known to be miRNA targets, and no pervasive combinatorial control has been described; nearly all plant miRNA targets are targeted by only one miRNA via one binding site (Fahlgren et al., 2007; Addo-Quaye et al., 2008). Nevertheless, there is also room for combinatorial regulation in plants, but this involves the generation of an additional class of small RNAs, generated in a miRNA-dependent manner, the trans-acting small interfering (tasi)RNAs (Brodersen and Voinnet, 2006; Voinnet, 2009). We will not describe these RNAs in details here, as it is outside the scope of this manuscript.

Thus, plant and animal kingdoms seems to have adopted radically different strategies for the use of miRNAs in gene expression regulation. Whereas animal miRNAs are implied in a wide and complex net of subtle regulation of the transcriptome, plants appear to have focused on stronger regulatory effects on key target genes. These two strategies are strongly related to the pairing requirements and their resulting mode of actions, and might reflect, either a deeply divergent evolutionary route from a common ancestor, or different evolutionary emergences of the two systems from the inherited proto- RNA silencing machinery.

2.2.1.5. A miRNA-like regulation in the last common ancestor of plants and animals?

Although the structure and mode of action of animal and plant miRNAs differ substantially, they share the same homologous key proteins involved both in miRNA and siRNA pathways, suggesting that they derive from the same proto- RNA silencing system. Plant and animal miRNAs repertoires each possess some extremely conserved miRNAs families and targets, such as *MIR166/165* family present in the last common ancestor of land plants (Floyd and Bowman, 2004) and *let-7* family in bilaterian animals (Pasquinelli et al., 2000, 2003). Both of these conserved families are crucial in differentiation and development, but no cross-kingdom conservation of miRNAs is known. Furthermore, miRNAs genomic distribution in plants and animals strikingly differs, with for example a clustering tendency in animals, whereas plants miRNAs are mostly scattered over the genome, thus suggesting different evolutionary mechanisms that led to their emergence and/or spreading in their respective genome. Finally, the many non-canonical mechanisms that have also been shown to convert precursor

transcripts into miRNAs, suggest that different evolutive processes can lead to the emergence of new miRNAs in organisms that possess RNA silencing machinery. Taking in account these considerations altogether, it seems more relevant that miRNA regulation evolved independently in each kingdom after their divergence from an ancestral RNA silencing pathway that have been adapted to generate miRNAs from endogenous inverted repeat transcripts as a convergent strategy of genetic regulation. However there's still no hard evidence that can exclude the possibility of a miRNA-like regulation in LECA that strongly diverged during evolution to respectively fit the best with these two fundamentally different eukaryotic forms of life.

2.2.2. Evolutionary mechanisms for *de novo* miRNAs emergence and target acquisition

The prerequisite for the emergence of a novel miRNA is a transcribed genomic locus that can be evolved to produce a correct secondary structure, which is recognisable by the RNA processing machinery. As RNA easily folds into non-perfect secondary structures, the evolution of a new miRNAs gene appear to be more likely than the emergence of novel protein-coding gene (Chen and Rajewsky, 2007). Thus, the miRNAs constituting the known repertoires in eukaryotes would be of polyphyletic origin, having evolved from distinct genomic sources, as discussed below.

Here I will present the different models of emergence of *de novo* miRNAs in eukaryotes, which are intimately related to their mode of recognition and regulation of the target-transcripts.

2.2.2.1. Inverted duplication model

For several non-conserved plant miRNAs, it has been observed that not only the mature miRNA, but also adjacent regions of pre-miRNAs show complementarity to their mRNA target. This suggest that these new miRNAs evolved by inverted duplication of a protein-coding sequence, followed by mutations and sequence changes that would then have led to imperfect hairpin structures. Finally, over time, the extensive homology to the parental transcript would be lost with the genetic drift, leaving only the mature miRNA sequence with complementarity, which could explain why this extensive complementarity of pre-miRNAs is only observable for non-conserved, young miRNAs (Figure 5) (Allen et al., 2004; Rajagopalan et al., 2006; Wang et al., 2006b). Consistent with this model, studies using high-

throughput sequencing techniques, identified many new non-conserved miRNAs families in *Arabidopsis* with extended complementarity of their hairpins to their targets mRNAs (Fahlgren et al., 2007, 2010). The selection of the newly evolved miRNAs, if beneficial for the plant regulatory network, would occur during a transitional stage, where the small RNA precursors would first be imprecisely processed by one or more of the siRNA-generating DCL enzymes. Indeed, the initial precursor would harbour a perfect stem-loop structure, which is not suitable for the precise requirements of canonical miRNA processing by DCL1 (Axtell et al., 2011). This hypothesis is supported by numerous examples of recently emerged plant miRNAs processed by DCL2, DCL3 or DCL4 instead of, or in addition to the miRNA-specific DCL1 (Chellappan et al., 2010; Rajagopalan et al., 2006; Vazquez et al., 2008; Wu et al., 2010). It is thus tempting to speculate that processing by siRNA-generating DCL enzymes of the newly born miRNAs could reflect ancestral mechanisms of the plant RNA silencing machinery metabolising endogenous hairpin structures, and which led to the innovation of miRNA-based regulation.

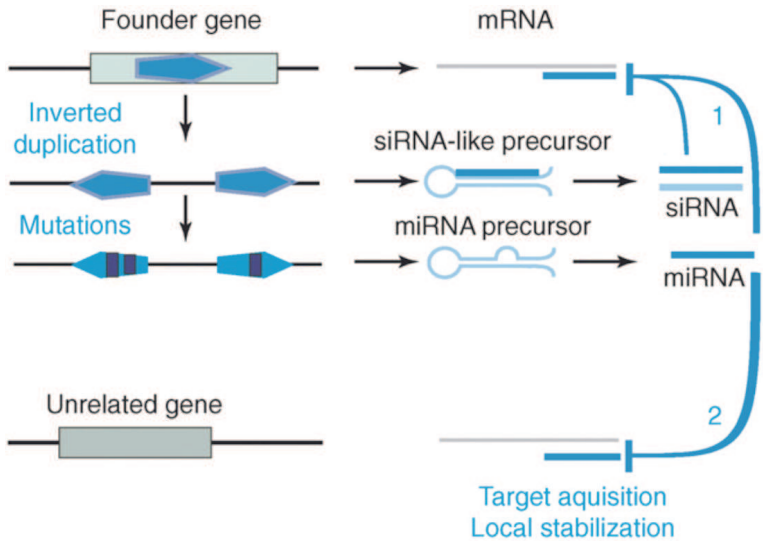

Figure 5: Inverted duplication model. Adapted from (Shabalina and Koonin, 2008).

The inverted duplication model is an attractive model for plants miRNAs as it accounts for the near-perfect complementarity required between the miRNA and its target, which may minimise off-target effects of novel miRNAs. However, this model seems less relevant for animal miRNAs because the length of the complementary sequence is much smaller in the binding sites. Indeed, very few described animal miRNA exhibit "plant-like" extensive complementarity. The most interesting example is probably miR-196a: the chromosomal proximity of *miR-196a-1* to its target gene, *HOXB8*, is similar to those observed for several

young plant miRNA genes to their targets, thus suggesting that *miR-196* genes evolved from a local duplication of *HOX* genes (Mansfield et al., 2004; Yekta et al., 2004).

2.2.2.2. MiRNAs deriving from transposable elements

A substantial proportion of the human genome (approximately 45%) is composed of TEs or of their inactive remnants. Considering the invasion of eukaryotic genomes by TEs through their ability to move and/or replicate to another genomic location, they are commonly viewed as genomic parasites, also termed "selfish genetic elements". Indeed, they can produce deleterious effects in host genomes by generating insertion mutations into genes, or disrupting *cis*-regulatory elements. Additionally, they can alter gene expression when inserted in proximity due to the transcriptional regulatory elements they carry. They have been implied in human diseases (e.g. cancer) (reviewed in (Belancio et al., 2008; Cordaux and Batzer, 2009; Han and Boeke, 2005; Jordan et al., 2003; Sinzelle et al., 2009). There are two major classes of TEs: DNA transposons and retrotransposons. DNA transposons can excise themselves from the genome, move as DNA and insert themselves into new genomic sites. Retrotransposons replicate through RNA intermediates that are retrotranscribed and inserted in new genomic locations. Although they represent a major source of genomic instability - with the negative consequences one can imagine-, they have been more recently reconsidered for their significant contribution to genome evolution, and are now recognised as an important source of genomic innovation (reviewed in (Belancio et al., 2008; Cordaux and Batzer, 2009; Han and Boeke, 2005; Jordan et al., 2003; Sinzelle et al., 2009).

A certain number of human miRNAs precursors, among which some are highly conserved, have been shown to contain sequences derived from TEs and repeat elements (Borchert et al., 2006; Piriyapongsa et al., 2007; Smalheiser and Torvik, 2005). Several miRNA precursors in this group contain *LINE-2*-like TE sequences, and hairpins from these miRNAs transcripts arise from two adjacent, inverted LINE-2 elements (Hertel et al., 2006; Smalheiser and Torvik, 2005). TEs often carry inverted or direct terminal repeats (TRs), and insertion of TEs with direct TRs near or into each other can result in inverted gene arrangement. Transcription across such elements might thus be a source of hairpins that could potentially result in the formation of miRNA-like precursors. Part of target specificity of the TE-derived miRNAs could then arise from the insertion of the "parental" TEs into transcribed genes (Figure 6). Indeed, bioinformatics analyses show that numerous miRNAs have complementarity to conserved LINE-2 TEs or *Alu* repeat elements found in the 3'UTRs of human mRNAs

(Piriyapongsa et al., 2007; Smalheiser and Torvik, 2005, 2006). Another more direct route is the processing of miniature inverted-repeat-transposable elements (MITEs) transcribed into miRNA, as they possess a high potential to form extremely stable miRNA-like hairpin structures (Piriyapongsa and Jordan, 2007) and the derived mature miRNA could target complementary repeat-related sequences, thus exhibiting "plant-like" extensive complementarity to targets. Such types of MITEs-deriving miRNAs have also been described in plants (Piriyapongsa and Jordan, 2008), thus suggesting a shared route of evolution between plants and animals for the emergence of novel miRNAs. Finally, the study of the miRNAs in the human chromosome 19 miRNA cluster (C19MC) revealed that they are interspersed among *Alu*. These miRNAs derive from these repetitive-elements, formed from *Alu* 3' sequences (Borchert et al., 2006). Consistently, a recent study using bioinformatics approach identified 278 human miRNA genes that overlapped with repeats deriving from both DNA transposons and retrotransposons (Yuan et al., 2011).

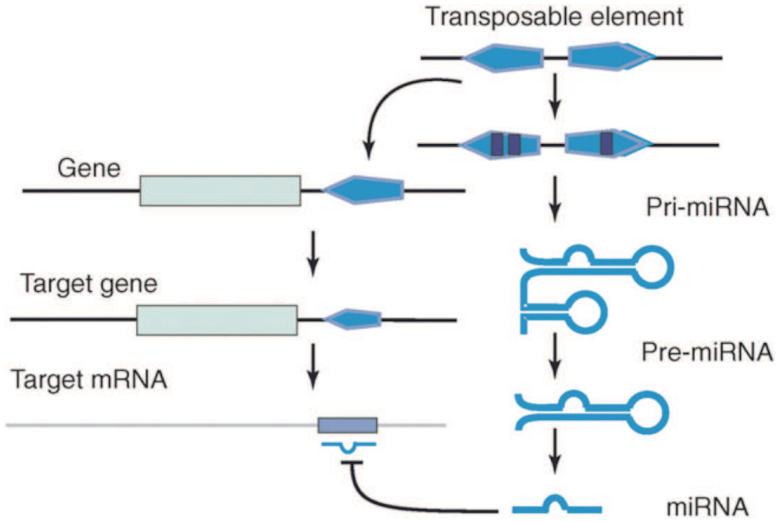

Figure 6: Origin of miRNAs from genomic repeats or transposable elements. Adapted from (Shabalina and Koonin, 2008).

2.2.2.3. snoRNA and tRNA-derived miRNAs

As discussed above in section 2.2.1.2, non-canonical miRNAs can derive from snoRNAs and tRNAs. It is thus intuitive to imagine that duplication and subsequent evolution of genes encoding for classical snoRNAs and tRNAs could lead to new miRNAs genes, with an intermediary stage where they still exert a dual function as in the cases described in the literature.

Concerning snoRNAs, as reviewed in (Scott and Ono, 2011), it is interesting to mention that in addition to the existence of dual function sno-miRNAs, some share similar features with

miRNAs in their precursor sequences and structures, or even for some in their genomic context. Based on computational studies, an interesting hypothesis suggests that some of the existing snoRNAs and miRNAs could have both derived from a common ancestral snoRNA molecule, itself derived from TEs. Indeed, snoRNAs are believed to be more ancient than miRNAs, with some members found in Archaea where miRNAs are absent. However this does not exclude that some miRNAs could have emerged directly from TEs, or due to the similarities of some miRNAs and snoRNAs precursor structures, that some dual function sno-miRNAs (or even snoRNAs), could have evolved from miRNAs. In this review, Scott and Ono discuss another interesting hypothesis in which the snoRNA and RNA silencing processing machinery could have a common evolutive origin, due to the similarities mentioned above, the similar set of processing enzymes, as well as binding partners and subcellular localisation shared by both systems. Whether these similarities result from convergent evolution, or from a common evolutive origin remains an unanswered question.

2.2.2.4. *De novo* generation of miRNA hairpins and random selection model

Because relatively few animal miRNAs seems to have derived from their targets (e.g. TE-derived miRNAs), a predominant idea is that miRNA birth in this kingdom mostly occurs *de novo* from unstructured transcribed sequences or pre-existing hairpins (e.g. tRNAs or snoRNAs) (Figure 3).

Svodoba and Di Cara observed that for many miRNAs, no targets had been identified, and therefore that the number of biologically active miRNAs could be much smaller than the number of potential miRNAs produced from miRNA-like hairpin precursors. They thus proposed a two-step model for the generation of new miRNAs, which they called the random selection model. In the first step, emergence of new potential miRNAs occurs through RNA hairpins that progressively gain the requirement to be processed as a functional RISC-loaded miRNA via random mutations. The second step consist in the miRNA selection following random target acquisition: if the pairing results in positive selection, it is then maintained, and point mutations in the precursor and targeting sequence will then respectively increase the processing efficiency of the miRNA and strengthen the interaction with its target (Figure 7) (Svoboda and Di Cara, 2006).

This model can be supported by the conclusions of various recent studies. Indeed, these past years, genome-wide studies have provided convincing evidence that the majority of the animal euchromatin is transcribed - so called pervasive transcription (Birney et al., 2007;

Manak et al., 2006). These findings offer the perspective of a consequent pool of non-coding transcripts that can evolve into primary transcripts for the production of short RNAs (Kapranov et al., 2007). In favour of this hypothesis, Bentwich *et al.,* in a bioinformatics screen, identified around 11 millions potential hairpin structures encoded by the human genome (Bentwich et al., 2005). Therefore, the problem of creating *de novo* miRNAs might be less a matter of creation of new stem-loop structures, but rather for these hairpins to be transcribed and to fold with the appropriate features recognised by the miRNA biogenesis machinery (Chen and Rajewsky, 2007). Thus, before being processed efficiently as "true", conserved miRNAs, emerging hairpins encoding miRNAs-like loci would first represent transitional forms gradually evolving toward a "perfect" miRNA structure (Liu et al., 2008). Indeed, annotations studies in *D. melanogaster* identified such candidate hairpins that present evidence for miRNA biogenesis (multiple reads, existence of star species, presence in Ago immunoprecipitates), but which also do exhibit patterns of random RNA breakdown products, suggesting they are only partially processed by the miRNA biogenesis machinery (Berezikov et al., 2011). Additionally, it has been shown in *Drosophila* evolution a dynamic of birth and death of new miRNAs, where only a subset of them (2,5%), were retained by natural selection to be moderately or highly expressed (Lu et al., 2008a), and that recently emerged miRNA genes such as *miR-310s* cluster and *miR-303* exhibit fast adaptively-evolving sequences harbouring the signature of positive selection (Lu et al., 2008b).

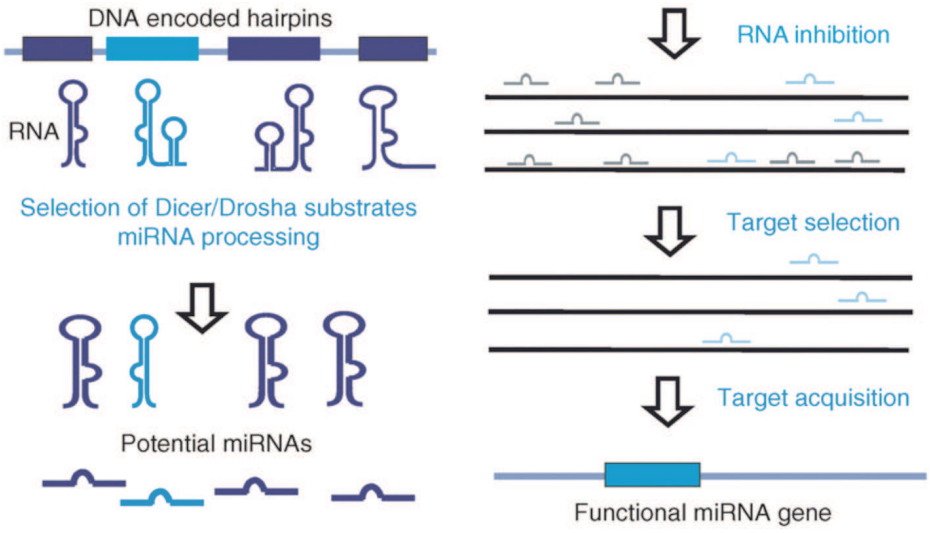

Figure 7: Random selection model of miRNA origin. Potential miRNA genes are selected from hairpins encoded in the genome. Random targeting of transcripts by potential miRNAs could be deleterious, with only a few targets being selectively neutral or advantageous. Acquisition and expression of a potential novel miRNA gene can occur only when, by chance, it is not strongly deleterious. Adapted from (Shabalina and Koonin, 2008).

2.2.2.5. Transcriptional control model of new miRNAs

The propensity of animal miRNAs to target a large number of transcripts renders counterintuitive the notion that the introduction of a new miRNA would have no detrimental consequence on the organism's fitness. Indeed, it was observed by transcriptome analysis that introduction of siRNAs -initially designed to be highly specific of their targeted genes- could result in the repression of numerous off-targets with partial identity to the siRNA (Jackson et al., 2003). More strikingly, *in vivo* studies in *D. melanogaster* showed that replacing the *miR-310s* cluster by the *Drosophila pseudoobscura* sequence, had a strong deleterious impact on viability with a significant number of genes misexpressed (Tang et al., 2010). On the other hand, the evolution of animal 3'UTRs has been described to be under selective pressure to maintain or to avoid miRNA binding sites (Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005). Indeed, genes involved in essential cellular processes harbour short 3'UTR that are specifically depleted of miRNA binding site to avoid their regulation (Stark et al., 2005). In addition, the existence of anti-target genes -i.e. an anticipated class of tissue-specific highly expressed genes, which need to avoid regulation by co-expressed miRNA through depletion of the binding sites (Bartel and Chen, 2004)- has been demonstrated (Farh et al., 2005).

Thereby, a major issue, which has been raised by Chen and Rajewsky, of *de novo* miRNAs gene emergence remains unanswered in the above presented models: "*Because the minimal binding site of an animal miRNA is short, a new miRNA should be able to target many mRNAs simply by chance, and many of these interactions are likely to be selectively deleterious, as is the case for all types of mutations. (...) These observations raise the question of how a new miRNA could ever be acquired without seriously impairing the fitness of the organism*" (Chen and Rajewsky, 2007). They proposed a transcriptional control model of new miRNAs, which postulates that novel miRNAs are initially expressed weakly, in specific tissues or at specific developmental stages. Taking into account the combinatorial mode of action of miRNAs, i.e. that multiple sites of the same miRNA or a combination of multiple binding sites from different miRNAs are needed to generate strong repression of a mRNA, these low expressed novel miRNAs should have minimal repressive effects on their targets. This would allow natural selection to purge deleterious-target sites from the transcriptome, while maintaining the physiologically beneficial ones and/or fixing newly emerging ones. Once this process would be completed, the level and spatial range of the new miRNA expression could be increased (Chen and Rajewsky, 2007). Therefore, this model explains how emergence of novel miRNAs with their set of *de novo* acquired targets can occur with minimal detrimental consequences and, as proposed earlier by Bartel and Chen,

allow new networks of beneficial regulations to progressively take place, as a flexible driving force of metazoan evolution (Bartel and Chen, 2004).

This model is in good agreement with the random selection model, where the purging phase would comprise the evolution of the miRNA targeting sequence to strengthen and/or loosen up beneficial interactions, followed by mutation in the pre-miRNA structure to improve its processing efficiency and increase the expression level of the mature miRNA. Consistently, studies have shown that the more recently acquired miRNAs in *Drosophila* are more likely to be expressed at weak levels compared to strongly conserved ones (Berezikov et al., 2011; Lu et al., 2008a; Ruby et al., 2007). In addition, another study comparing the expression of miRNAs in human and chimpanzee brains had allowed the identification of 447 new miRNAs among which many were non-conserved beyond primates, and that harboured low expression profiles (Berezikov et al., 2006). Finally, an implication of this model is that miRNAs that show low levels of conservation and that are weakly expressed are probably still in the purging phase of their evolution and might not yet have acquired targets that they regulate at significant biological levels (Berezikov, 2011).

Regarding plant miRNAs, this model seems less relevant knowing that they commonly have only one target with much higher pairing requirement specificity. Thus, any potential detrimental influence of an emerging miRNA on its target might be simply solved by increasing the activity or expression of this target (Axtell et al., 2011). However, despite the fact that evolution of plant miRNA loci is mostly thought to occur by inverted duplication, some studies comparing two closely related species of *Arabidopsis* suggested that young miRNA loci arose from inverted repeats of random intergenic sequences (Fahlgren et al., 2010; Felippes et al., 2008; Ma et al., 2010). Such plant miRNAs emerge without pre-existing targets -supported by the fact that for many of them no target has been identified- and thus are likely to disappear rapidly due to neutral mutational drift (Fahlgren et al., 2010; Ma et al., 2010). Nevertheless, it has been proposed that targets can occasionally be captured, such as it was demonstrated for MIR447 and MIR856, which validated targets are distinct from their loci of origin (Fahlgren et al., 2007, 2010).

3. Animal miRNAs: biogenesis, mode of action and targeting functions

3.1. Biogenesis of miRNAs in mammals

Although many aspects of animal miRNAs biogenesis remain unclear and still have to be investigated, these last years, several studies have allowed a better understanding of its mechanisms. As some differences in miRNA biogenesis exist within the animal kingdom, here I focused on the current knowledge on mammalian miRNA biogenesis, but it has to be kept in mind that some of the below detailed mechanisms and processes have been inferred from studies in invertebrates such as for example *D. melanogaster* or *C. elegans*.

3.1.1. Generation of the pre-miRNA and export into the cytoplasm

pri-miRNAs are generally transcribed by RNA polymerase (Pol) II (Cai et al., 2004; Lee et al., 2004) and in rare cases by Pol III (see section 6.2). In a manner similar to mRNAs, the pri-miRNA is capped and poly-adenylated after splicing (Cai et al., 2004), and can contain one to multiple pre-miRNAs (Lee et al., 2002; Rodriguez et al., 2004). Pri-miRNAs folds into a typical hairpin structure with a long imperfect stem of approximately 33 base pairs (bp) and flanking single-stranded RNA segments at its base, which is recognised and processed by the RNAse III Drosha (Han et al., 2006; Lee et al., 2003; Zeng et al., 2005). Drosha is the catalytic protein of the microprocessor complex and its major co-factor is the DiGeorge syndrome critical region gene 8 (DGCR8) protein. DGCR8 recognises and binds the stem-loop precursor, allowing the exact positioning and cleavage by Drosha about 11 bp from the base of the pri-miRNA to give rise to the pre-miRNA (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). The pre-miRNA is folded into a hairpin of about 70 nt in length, with 2 nt overhangs at the 3' end generated upon Drosha cleavage (Lee et al., 2002). Exp5 then recognises these specific features of the pre-miRNA and transports it into the cytoplasm through the nuclear pore in a Ran-GTP dependant manner (Figure 4) (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Exp5 was first identified as a nuclear export factor for dsRBPs (Brownawell and Macara, 2002) as well as for the adenovirus VA1 RNA (Gwizdek et al., 2003). Its role in the pre-miRNA export was later on independently described by three groups (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003).

3.1.2. Dicing of the pre-miRNA into miRNA/miRNA* duplex

Once into the cytoplasm, the pre-miRNA is recognised by Dicer. It was first described that this enzyme binds via its Piwi Argonaute Zwille (PAZ) domain the 3' overhang of the pre-miRNA prior to its cleavage near the terminal loop by the two RNAse III catalytic domains into an RNA duplex of approximately 22 nt with 2 nt 3' overhangs (Figure 4) (Bernstein et al., 2001; Hutvágner et al., 2001; MacRae et al., 2006; Zhang et al., 2004). More recently it was shown that in metazoans Dicer also anchors the 5' end of the pre-miRNA through a basic motif (5' pocket), which recognises its 5'-terminal phosphate group with the cleavage site being principally determined by the distance from this end (Park et al., 2011). Dicer has been described to interact during this step with the HIV-1 TAR RNA-binding protein (TRBP) and with the PACT protein. These two cofactors are not required for the processing activity itself, but it seems that they have a role in facilitating RISC assembly and their depletion strongly affects mature miRNAs accumulation in the cells. They are considered to constitute the RISC-loading complex in association with Dicer and Ago proteins (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). Therefore, some argue that RISC assembly is an event coupled with dicing -independent of ATP-, but others argue that these processes uncoupled and ATP-dependent are (Yoda et al., 2010).

In rare cases, pre-miRNA processing can be Dicer-independent such as for miR-451. Its maturation has been shown to rely on Ago2, via endogenous cleavage of the pre-miRNA to generate an intermediate 3' end, which is further trimmed giving rise to its mature form (Cheloufi et al., 2010; Cifuentes et al., 2010).

3.1.3. Mature strand selection and loading into RISC

After processing by Dicer, the mature or "guide" strand, which possesses a biological function, is incorporated into the Ago-containing RISC - thus referred to as "miRISC". In some cases the miRNA*, which is usually degraded, can also be incorporated (Figure 4) (Carmell et al., 2002). Initially, regarding the mechanism of strand selection, it was shown that the extremity of the duplex with the weakest thermodynamical pairing defines the 5' of the guide strand (Khvorova et al., 2003; Schwarz et al., 2003). Consistently, recent studies have shown that Dicer, through its helicase domain, could sense the thermodynamic stability of the duplex's ends (Noland et al., 2011; Sakurai et al., 2011; Welker et al., 2011). Noland *et al.*, thus proposed a model in which the RNA duplex, following dicing, is repositioned within Dicer complexes in an orientation to allow correct guide strand incorporation into RISC

(Noland et al., 2011). Finally, it seems that in animals a 5'U tends to improve the loading of the guide strand. This strand selection mechanism is distinct from its contribution to weakening base pairing at the 5′-end (Seitz et al., 2011).

Whereas in *Drosophila* mismatched miRNA duplexes and perfectly matched siRNAs duplexes are actively sorted respectively into AGO1- and AGO2-RISC complexes (Förstemann et al., 2007; Tomari et al., 2007), in human, all four Ago proteins (Ago1-4) incorporate miRNAs indiscriminately of their sequence, showing very similar preferences for the structures of small RNA duplexes (Liu et al., 2004a; Meister et al., 2004; Yoda et al., 2010). Central mismatches promote RISC loading, and thus their features are reminiscent of *Drosophila* AGO1 (Yoda et al., 2010). Among the four human Ago, only Ago2 has been shown to be capable of miRNA- and siRNA-mediated target cleavage through an RNAse H-like fold (Liu et al., 2004a; Meister et al., 2004). Ago proteins are multidomain proteins that contain an N-terminal, a PAZ, a middle (MID), and a PIWI domain. They adopt a bilobate architecture, with one lobe formed by the N-terminal and PAZ domains, and the other by the MID and PIWI domains (Jinek and Doudna, 2009). The RNAse H-like fold is found in the PIWI domain (Song et al., 2004). MID and PIWI domains junction forms a binding pocket for a deep anchoring of the 5' monophosphate group of the miRNA first nucleotide (Ma et al., 2004; Parker et al., 2005), the 3' end being reversibly bound to the PAZ domain in a preformed hydrophobic pocket (Lingel et al., 2003, 2004; Ma et al., 2004; Song et al., 2003; Yan et al., 2003). It is interesting to note that slicing has been shown to occur between the bases of the target paired with nt 10-11 of the small RNA (Elbashir et al., 2001a, 2001c) and that modelling of a full length siRNA-target duplex gave consistent results for this specificity, placing the target RNA in the slicer catalytic site of Ago, at a fixed distance measured from the 5' end of the guide strand. Perfect complementarity around these base pairs would thus probably ensure correct orientation of the scissile phosphate group of the target in the active cleavage site (Jinek and Doudna, 2009).

The process during which Dicer substrates are loaded into RISC is not yet fully understood, but the incorporation of a single strand of the duplex, either siRNA or miRNA, seems to be a stepwise process. Recent studies and inferred models have proposed that it involves two steps: a first step with the physical association of the duplex with Ago, and a second step of activation during which the passenger strand is eliminated by Ago, or in the presence of accessory proteins (Gu et al., 2011a; Kawamata and Tomari, 2010; Ye et al., 2011). Indeed, after association with the diced substrate, an active process of elimination by cleavage of the miRNA* has been described for Ago1 and 2, which depends on the intrinsic slicer property of

Ago proteins (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005; Wang et al., 2009a). In addition, it has also been suggested that before their binding by Dicer, some pre-miRNAs could interact with Ago2, which would result in the cleavage of the pre-miRNA hairpin 12 nt from its 3' end. Thus, this would facilitates the nicked miRNA* removal after pre-miRNA dicing (Diederichs and Haber, 2007). On the other hand, many studies have shown that RISC-loading requirements differ for miRNAs and siRNAs. Indeed, most miRNA duplexes contain mismatches, thus preventing Ago-mediated slicing, and Ago proteins that lack slicer activity are unable to cleave the passenger strand. Even for Ago2-loading of siRNAs, it was demonstrated that slicing enhances strand separation, but this is not an absolute requirement, as a "bypass mechanism" exists when cleavage by the small RNA is not feasible (Matranga et al., 2005). Therefore, an RNA helicase activity is thought to mediate the unwinding and removal of the passenger strand of the miRNA duplex, and this step could be performed by Dicer or by a specific helicase, such as for example P68 or RNA Helicase A (Robb and Rana, 2007; Salzman et al., 2007).

3.2. Mode of action miRNAs in animals

As introduced in section 2.2.1.4, animal miRNAs exhibit a clearly distinct main mode of action compared to plant miRNAs, with most of them recognising their targets via imperfect base pairing. In this part I detailed some important considerations about the features of target recognition by animal miRNAs and briefly discussed our current general knowledge for their subsequent main modes of action, to finish with a description of models attributing different classes to miRNA targets and the respective roles of their functional interactions with miRNAs.

3.2.1. Target recognition

Due to their imperfect mode of pairing with their target sites, an outstanding problem in the animal miRNA field is deciphering the features of these sites allowing their recognition by miRNAs, and how do miRNAs specifically recognise some sequences of partial complementarity, while they do not for some others that harbour similar sequence patterns. In order to answer these issues, which complicate the prediction of binding sites, many efforts have been realised by studying the typical patterns of miRNA binding sites, but also the

additional factors that influence their interaction with miRNAs, or miRISC mechanistic of assembly with a target RNA, which are discussed below.

3.2.1.1. Characteristics of animal miRNAs target sites

The vast majority of animal miRNAs recognise their targets via partial duplexes presenting mismatches and multiple bulged nucleotides. The most studied motif, thus inferred as the most common one and referred to as canonical miRNA binding sites, is perfect base-pairing between a 6-mer seed (nt 2-7 of the miRNA) and the target site, together with either an adenosine opposite to miRNA nucleotide 1, or an additional base pair (7-mer seed) involving the 8th nucleotide of the miRNA (Figure 8A) (Bartel, 2009). Consistently, it has been shown that seed pairing could be both necessary and sufficient for miRNA regulation -the repression being hindered by G:U woobles in the seed match- and that the 3' region-pairing of the miRNA with the target site is rarely determinant (Brennecke et al., 2005; Doench and Sharp, 2004). Moreover, crystal structures support this hypothesis of the seed region as a crucial determinant of the specificity of target recognition by miRNAs, as it was shown that in miRISC positions 2 to 6 of the guide strand are exposed and free to base pair, and that nt 2 to 10 adopt a stacked helical conformation, the seed region being pre-organised to initiate base-pairing with the target strand. These studies also showed that the first nucleotide is not available for pairing, being buried in between the MID and the PIWI domains (Ma et al., 2005; Parker et al., 2005; Wang et al., 2008b). In addition, biochemical studies have determined that the 5' end affinity of the miRNA to its target is stronger compared to the other regions, indicating that the seed seems to primarily mediate the initial miRISC-target association, followed by eventual annealing of the rest of the guide strand to the target (Ameres et al., 2007; Haley and Zamore, 2004). However, in some cases, it has been shown that target recognition allowed mismatches in the seed pairing, then generally compensated by extensive 3' end complementarity (Figure 8B) (Brennecke et al., 2005), as described for example in *C. elegans* for *let-7* target site in *lin-41* 3'UTR (Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004). The features of such sites could have an important role for miRNAs that are expressed under specific conditions to regulate their targets (e.g. at precise development stages), so that they can avoid regulation by paralogous miRNAs sharing the same seed sequence. Indeed, imperfect seed pairing with the target would prevent regulation by the other miRNA family members, while the 3'-compensatory unique complementarity to the targeting miRNA would enable its specific regulation. Consistently, *let-7* possesses 3

paralogues sharing the same seed sequence but differing in their 3' region, and that are expressed during earlier developmental stage, upon which *lin-41* repression would probably be detrimental for the worm development (Bartel, 2009). More recently, "centered sites" have been described where the target sites lack both perfect seed and 3'-compensatory pairing, thereby harbouring a 11-12 bp contiguous complementarity with the middle region of the miRNA (Shin et al., 2010). Finally, examples of functional binding sites that do not correspond to any of the above-described ones have also been documented in the literature (Figure 8B) (Pasquinelli, 2012).

Although the characterisation of these different types of miRNA binding sites give good insights for miRNAs target recognition requirements, they are not sufficient to explain all of miRNA targeting specificity. Therefore, also considering that they pinpoint a certain flexibility in targeting rules, other factors beyond binding capacity mediate functional target interactions *in vivo* (Bartel, 2009; Pasquinelli, 2012).

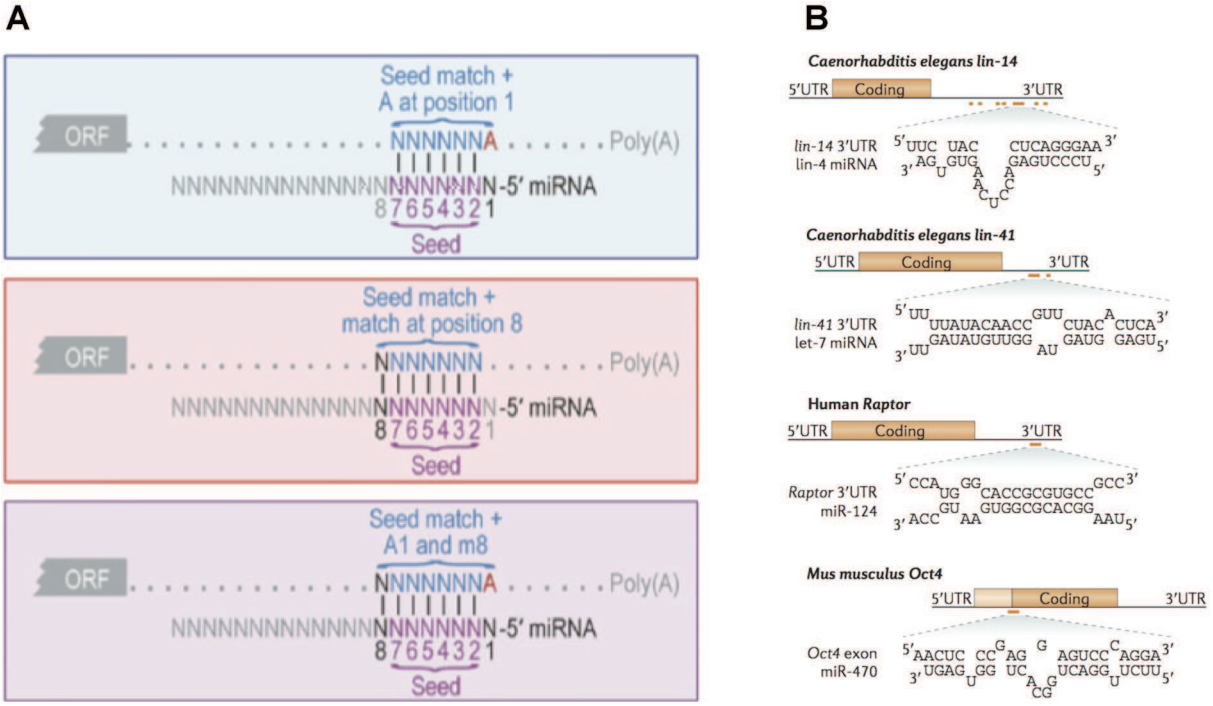

Figure 8: Characteristics of animal miRNAs target sites. (A) Canonical miRNA binding sites. The three types of canonical miRNA binding sites are represented. Please refer to the text for more details. Vertical dashes indicate contiguous Watson-Crick pairing. Adapted from (Bartel, 2009). (B) Examples of functional miRNA target sites. Examples of each type of miRNA target site described in the text are represented: *C. elegans let-7* canonical target site in *lin-14* 3'UTR; *C. elegans* let-7 imperfect seed pairing 3'-compensatory target site in *lin-41* 3'UTR; human miR-124 centered target site in *Raptor* 3'UTR; mouse miR-470 atypical target site in *Oct4* exon. Adapted from (Pasquinelli, 2012).

3.2.1.2. Influence of the binding site sequence context

As introduced in section 2.2.2.5, expression of miRNAs can lead to selective avoidance in co-expressed transcripts that need to avoid miRNA regulation. Selective avoidance leads to the depletion of approximately 50% of the sites with 7-8 nt matches to co-expressed miRNA seed regions, but not 100%, as it would be expected if these sites, regardless of their sequence context, were sufficient to allow target recognition. Furthermore, reporter assays have revealed that identical sites in certain 3'UTR contexts could lead to target repression, while not in others. Therefore, the RNA context is a supplementary factor, which influences the faculty of a miRNA to repress an mRNA that present a characteristic miRNA target site (Bartel, 2009; Farh et al., 2005). Indeed, Grimson *et al.*, in a study to investigate the determinants beyond the target site sequence requirements, came up with a list of these factors such as AU-rich nucleotide composition in the site's vicinity for a more accessible, weaker secondary structure; positioning in the 3'UTR of the target site at least 15 nt from the stop codon to avoid competition of the RISC complex with the ribosome; positioning away from the center for long UTRs, as they would have more opportunities to form occlusive secondary structures with segments from either side; proximity of seed matching mRNA region to residues pairing to nt 13-16 of the miRNA; and proximity with sites of co-expressed miRNAs for cooperative, combinatorial repression of the target (Grimson et al., 2007).

Some other characteristics of the UTR context, or *trans*-regulatory factors that bind to sequence-specific motifs in the proximity of binding sites, could also influence miRNAs' ability to target typical characteristic sites and to repress the associated RNA (Pasquinelli, 2012). Indeed, one example of RNA binding protein (RBP), Deadend 1 (DND1), has been demonstrated in the zebrafish to bind at the vincinty of miR-430 binding sites in some of its target genes, and to decrease the miRNA repressive-activity by reducing its accessibility to these sites (Kedde et al., 2007). This protein is specifically expressed in primordial vertebrate germ cells, thus explaining how upon zebrafish early development certain targets of miR-430 could escape from its regulation in these cells, but not in somatic cells (Mishima et al., 2006). Therefore, one can imagine that other cell-specific, or stimuli-dependent expression-induced RBP with similar roles could exist. Alternatively, other factors, which remain to be identified, could open the target RNA secondary structure to enhance miRISC accessibility, tether miRISC complex in the proximity of specific binding sites, or strengthen miRISC interaction with its binding site after its recognition.

3.2.1.3. Assembly of the miRISC-mRNA ternary complex

The first complete mechanistic models elaborated for the assembly of miRISC with a target RNA gave insights of how perfect, or nearly-perfect complementary target was recognised and subsequently cleaved by the silencing machinery. Two models have been proposed: the fixed-end model, and the two-state model (Filipowicz, 2005; Tomari and Zamore, 2005). These models take into account the topological constraint on the guide-target interaction imposed by the binding of both ends of the guide strand to Ago, which would then limit in a first step the extent of base-pairing to less than one helical turn (11 bp), with its bases exposed and free to pair with a target RNA, and could thus explain the limits of the seed region to nt 2 to 8 of the guide RNA (Figure 9A) (Filipowicz, 2005; Jinek and Doudna, 2009).

In the fixed-end model, both ends of the guide RNA remain bound to Ago during slicing, and Ago adopt a conformation that sets the PIWI and PAZ domains further apart to stretch the guide strand, in particular its 3' region. The nucleotides of the seed region, still presented on the PIWI surface in a quasi-helical form would initiate the pairing with the target RNA, thus leading to the propagation of the helix toward the 3' region, which would in turn bring back Ago in a more compact conformation. Subsequent cleavage and release of the target RNA would then be accompanied by the return of Ago to the extended conformation (Filipowicz, 2005). The second model, the two-state model, postulate that miRISC complex would first be engaged in a "double-anchor state" were the guide strand, still bound by both extremities in Ago, would be ready to engage interaction with the target RNA using the 5'-proximal seed nucleotides. To allow base pairing of the downstream region of the guide to the mRNA, its 3' extremity would need to break down its interaction with the PAZ domain, leading to a "single-anchor state". The 3' region of the guide, thus available for base pairing with the target RNA, would permit the duplex to form a two A-helical Watson-Crick paired duplex, leading to the cleavage of the target. Re-anchoring of the 3' end, possibly coupled with the ejection of the cleaved product, would achieve the cycle and bring back miRISC to the first state (Figure 9A) (Filipowicz, 2005; Tomari and Zamore, 2005).

It is interesting to note that observations from crystal structures of a duplex of a seed-containing DNA guide and non-cleaved target RNA complexed with Ago showed that the seed segment adopts A-form-like helical turn conformation, with both ends of the guide anchored in the complex, and that Ago adopts a more open conformation to accommodate the target RNA, via a rotation of the N-terminal and PAZ domains away from the lobe containing MID and PIWI domains (Wang et al., 2008c). Therefore, these observations could be in agreement with both models, as a cleavage-competent complex envisaged by the fixed-end

model, or as an intermediate form of the two-state model. Crystal structures of cleavage-inactive Ago in complex with perfect matching guide-target RNA duplex will be necessary to determine which of these models fits the best with the reality (Jinek and Doudna, 2009). In addition, both models are also in agreement with an *in vitro* and *in vivo* study on human RISC, which concluded that seed-pairing to the binding site is essential to allow a 3' extended association of the miRISC-contained guide with its target. In this study, Ameres *et al.* proposed that seed mismatches or inaccessibility would immediately lead to RISC dissociation with the target site (Ameres et al., 2007).

In animals, as the vast majority of miRNAs pair with their targets via partial complementarity, these models have to be adapted to their mode of recognition. Ameres *et al.* conclusions are not consistent with the 3' compensatory miRNA binding sites, which present mismatches in the seed pairing (or the later on discovered centered sites). Thus, considering that the study's results were based on a siRNA-RISC cleaving complex (therefore Ago2-containing), they raised the possibility that discrepancies could exist between the different human Ago, and that they could differ from each other in how the guide-RNA, and which of its region, is exposed, therefore enabling it to direct different Ago complexes to distinct binding sites. Alternatively they suggested that additional *cis*-regulatory factors could stabilise *in vivo* energetically unfavourable miRISC-mRNA interactions (Ameres et al., 2007). Bartel, considering the 3' region of the guide strand, suggested that this pairing would occur, as proposed in the two-state model, after the release the 3' end of the guide from Ago, but without the miRNA wrapping around the mRNA, as animal miRNA targets tend to skip contiguous pairing to the central residues (Figure 9B) (Bartel, 2009).

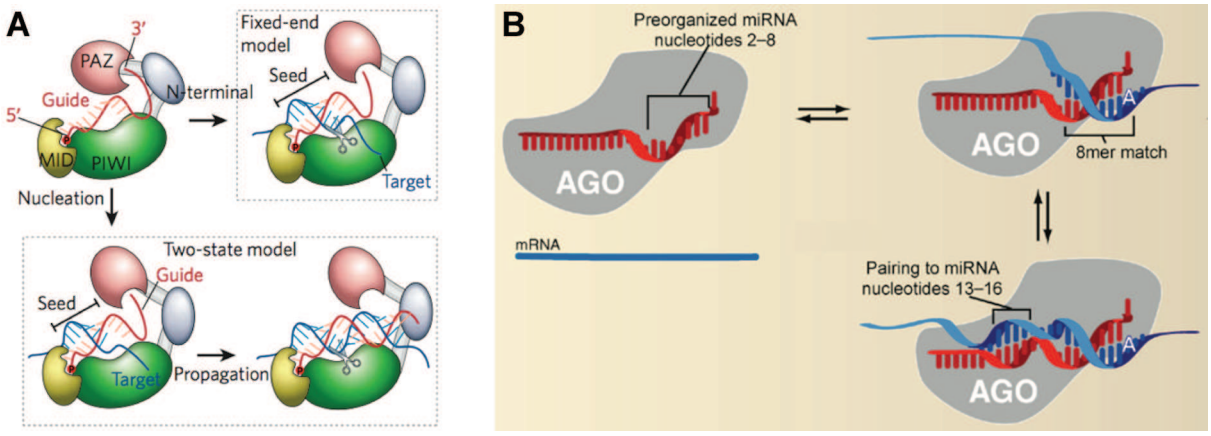

Figure 9: Assembly of the miRISC-mRNA ternary complex. (A) The fixed-end and the two-state models. Adapted from (Jinek and Doudna, 2009). (B) Model of assembly in animals. Adapted from (Bartel, 2009). Please refer to the text for the figures descriptions.

3.2.2. Mechanism of miRNA-mediated target repression

Even though there are a few examples of miRNAs regulation by target cleavage, translation repression is the main mechanism of regulation observed for mammalian miRNA-targeted mRNAs (introduced in section 2.2.1.4).

Partially depending on the degree of complementarity between the transcript and the targeting miRNA after miRISC binding, the target mRNA may undergo two different fates. In case of a perfectly matching binding site, the transcript will be most of the time -only if RISC contains an Ago2 (Hutvágner et al., 2001; Meister et al., 2004)- cleaved and subsequently degraded by exonucleases. Upon incomplete pairing, the mRNA will undergo translational repression. Translational repression can occur in different manners from inhibition of translation initiation, to mRNA decay by 5'-to-3' exonucleases, a process induced by deadenylation followed by decapping of the transcript (reviewed in (Eulalio et al., 2008)). Indeed, since it was demonstrated that translationally inhibited mRNAs were also undergoing destabilisation, the hypothesis arguing that miRNA-induced regulation of their targets was mostly due to translational repression rather than transcript destabilisation has been revised (Baek et al., 2008; Selbach et al., 2008). It is not yet fully understood by which exact mechanism translational control by miRISC action occurs, but some recent findings have suggested that many different steps could be targeted.

In a study in 2005, from the observation that M^7G-cap-independent translation is not subject to repression and by analysing polysome fractions from which miRNAs and their associated targets are absent, Pillai *et al.* first showed that the miRNA-mediated translation inhibition occurs at the initiation step, and that the cap structure is required (Pillai et al., 2005). This process probably involves the displacement of the translation initiation factor (eIF) 4E, the cap-interacting factor of the eIF4F complex. Post-initiation mechanisms have also been described, such as repression of the assembly of the 80S complex, or arrest of the translation elongation before completion of the nascent polypeptide chain. Indeed, a study has shown that miRNAs and their targets could in fact also be detected in the polysome fraction, leading to the hypothesis that translation arrest could occur by ribosome drop off (Petersen et al., 2006). However, this study remains controversial, as it was not reproducible in a subsequent study where the let-7 targets *lin-41* and *daf-12* were found to be depleted in the polysome fraction (Ding and Großhans, 2009).

Another possibility to prevent mRNA translation is to reduce the transcript's availability for the translation machinery and/or to induce its decay. To this aim, RNA sequestration and decay usually takes place in cytoplasmic processing, or GW182 bodies (referred to as P-

bodies). The miRISC-mRNA complex can then be translocated to these cytoplasmic foci, which contain the Ago-interacting GW182 protein, the CAF1-CCR4-NOT deadenylase complex, the decapping enzymes DCP1 and DCP2, some decapping activators, as well as RNA helicases (for review see (Eulalio et al., 2009a; Fabian and Sonenberg, 2012)). Interestingly, the sequestration of repressed mRNAs in GW/P-bodies is reversible in certain stress conditions such as serum starvation (Bhattacharyya et al., 2006). The deadenylation step can be mediated by the interaction of miRISC with GW182 (Behm-Ansmant et al., 2006), which recruits the CCR4-NOT1 deadenylase complex (Eulalio et al., 2009b) and requires the polyA-binding protein (PABP) recruitment (Figure 10) (Fabian et al., 2009).

Another subcellular compartment, the mulitivesicular body -a specialised late endosomal compartment-, has been proposed to contribute to miRNA function, or miRISC turnover, as they have been shown to be enriched in GW182, Ago2 and some miRNAs, but not DCP1 (Lee et al., 2009b; Gibbings et al., 2009). However, its function still has to be more deeply investigated.

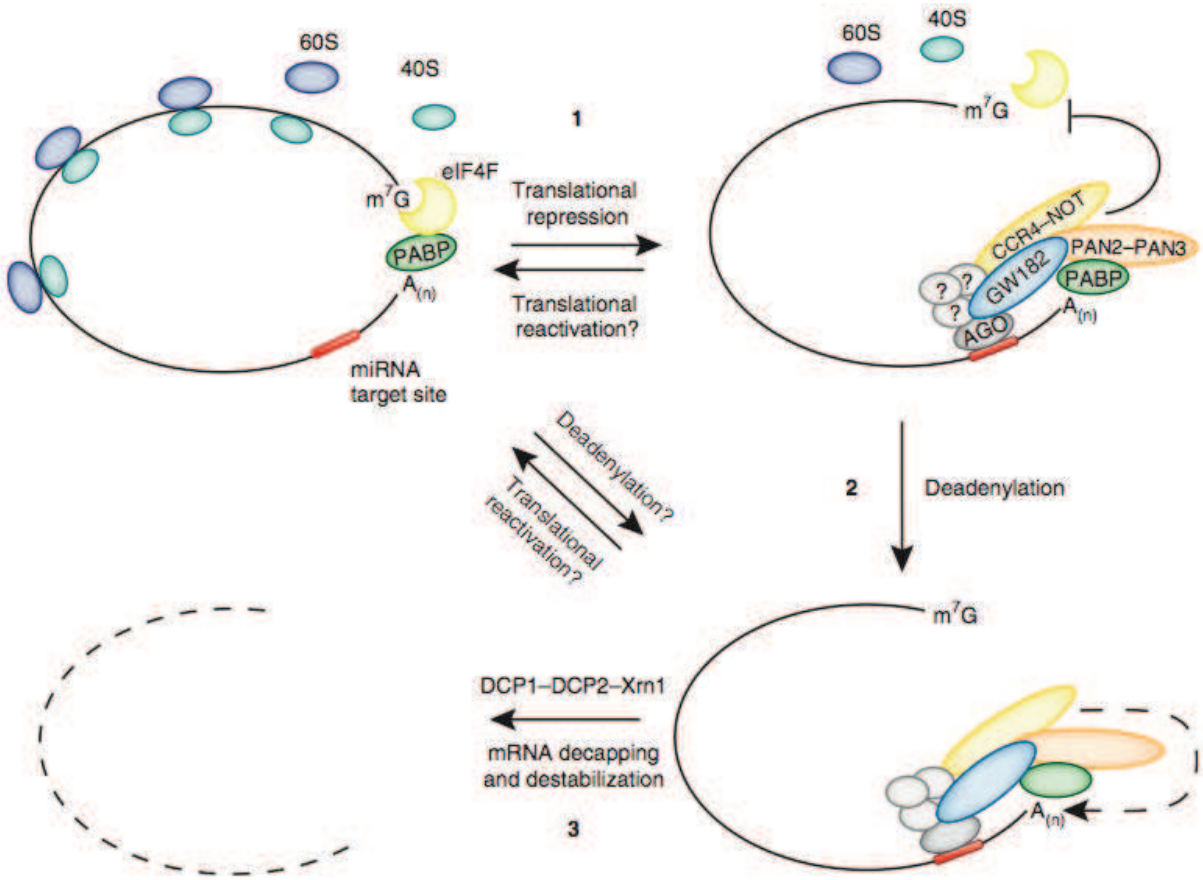

Figure 10: Temporal model of miRNA-mediated gene silencing. According to this model, the miRISC complex in interaction with GW182 protein, inhibits translation through the displacement of eIF4E, the cap-interacting factor of the eIF4F complex, and through GW182 interaction with PABP, which might results in the decircularisation of the mRNA (1). GW182 recruits the CCR4-NOT and PAN2-PAN3 complexes that mediate the deadenylation of the mRNA (2). Finally, DCP1-DCP2 decapping complex remove the 5′-terminal cap (m7G) from the mRNA, which is thus degraded by the 5′-3′ Xrn1 exonuclease. Adapted from (Fabian and Sonenberg, 2012).

3.3. Roles of functional interactions of miRNAs with their targets

Considering the potential ability of one miRNA to target hundreds of different genes, it has been soon assumed that these target genes should fall in different categories, depending on the function of the gene product and on the subsequent potential consequences of its targeting on the cell's and organism's physiology. Here I present below some models categorising miRNA targets into different classes, which highlights the importance of determining whether the functional interaction of a miRNA with a specific target gene does indeed have a physiological relevance on the organism.

3.3.1. The micromanager model

In 2004, Bartel and Chen proposed the micromanager model, in which miRNA targets fall in 3 categories termed "switch targets", "tuning targets", and "neutral targets" (Figure 11) (Bartel and Chen, 2004).

The switch targets are targets that should not be expressed in a specific cell type or developmental stage to avoid detrimental consequences to the organism (e.g. *C. elegans lin-24*, *lin-28* and *lin-41*). These targets take advantage of the miRNAs to dampen protein production to inconsequential levels, and represent the equivalent of a discrete off-switch. They comprise the target transcripts that remain from a previous environmental or developmental stage (such as maternally deposited mRNAs for example), or which arise from leaky transcription (Bartel and Chen, 2004). Consistently, a recent study by Mukherji *et al.* has demonstrated that miRNAs establish a threshold level of target mRNA below which protein production is highly repressed (Mukherji et al., 2011); the roles of miRNAs in reducing the transcriptional noise and in enforcing homeostasis are reviewed in (Ebert and Sharp, 2012). The tuning targets encode for gene products that need to be expressed above a precise, minimal low level below which they would no longer exert their required effect, but if overly expressed in particular cell types, would have undesirable effects. These genes thus take advantage of the miRNA milieu to achieve optimal expression in each cell type. The third class of described targets comprises mRNAs that fortuitously base pair with miRNAs, but their consequent protein expression alteration would be tolerated or offset by feedback mechanisms. They are termed neutral targets as there would be no selective pressure to maintain or decrease the pairing interaction with their targeting miRNAs (reviewed in (Bartel and Chen, 2004)).

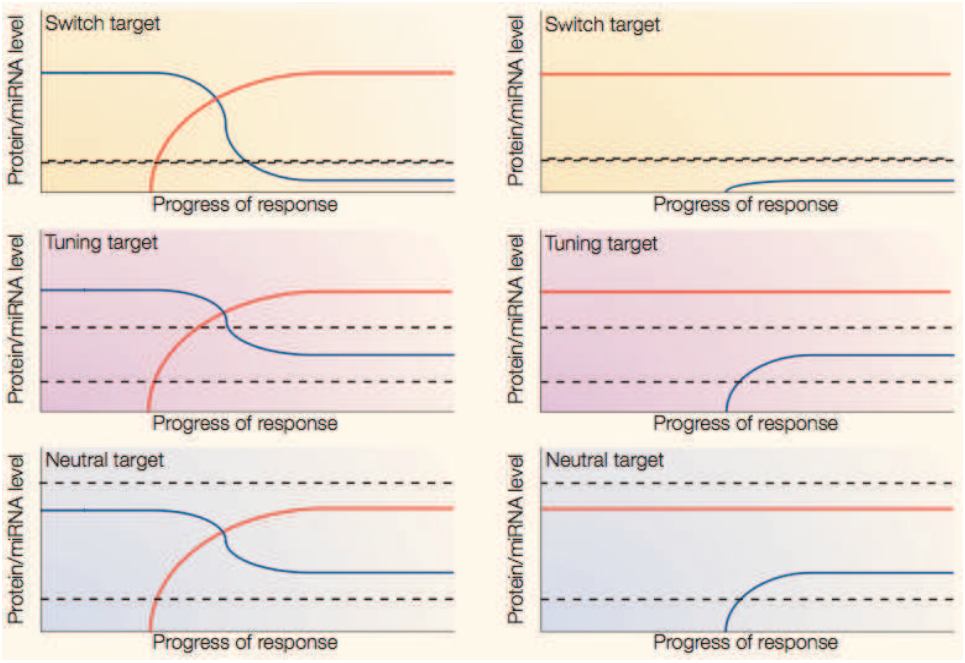

Figure 11: The micromanager model. The dashed lines indicate critical thresholds of protein expression; above the upper line the protein level would be undesirably high and below the lower line the protein no longer exerts its effect. In the top panels the two thresholds are at the same level. Left panels represent the induction of miRNAs expression (in red) in a pre-established protein context (in blue); right panels represent the induction of protein expression in a pre-established miRNA context. Adapted from (Bartel and Chen, 2004).

The latter class of neutral target are also included in the model of transcriptional control of new miRNAs proposed in 2007 by Chen and Rajewsky (described in section 2.2.2.5), in which these neutral targets of newly emerged miRNAs would be maintained after the purging phase of the deleterious targets (Chen and Rajewsky, 2007). However, it remains unclear why such neutral targets would be maintained without any selective pressure. Indeed, one would expect that over time, neutral evolution would result in mutations within the binding sites of these neutral targets, so that they would loose their interaction with the targeting miRNA.

3.3.2. A role for neutral targets: the pseudotargets

In 2009, Seitz proposed a redefinition of miRNAs targets (Seitz, 2009). He based his model on the following observations: (i) first, although computational predictions suggest that each miRNA regulates ten or hundreds of targets, consistent with genome-wide approaches which have shown that miRNAs effectively tune expression of most of their targets (numbered as hundreds), genetic studies have shown a physiological role for only a handful of miRNA targets. (ii) Secondly, miRNA-mediated repression (rarely exceeding 2 fold) is lower than most of the well-tolerated, intrinsic variations in gene expression. (iii) Finally, miRNA targets

are well conserved among closely related species, but for many, differ greatly between distant animals (for more details, please refer to (Seitz, 2009)).

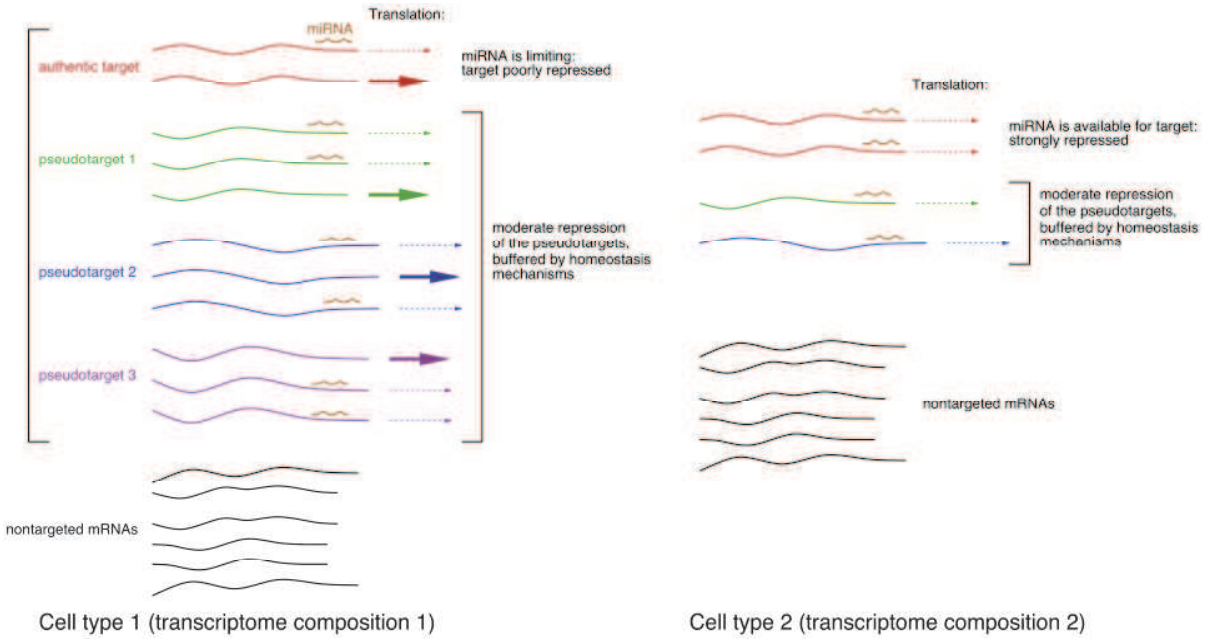

Figure 12: Model for miRNA regulation by pseudotargets. The only physiological effects of the miRNA are due to the repression of authentic targets (red). In cell type 1, the miRNA is titrated by pseudotargets (green, blue, and magenta); authentic targets are poorly repressed. In cell type 2, in which few pseudotargets are present, the miRNA is available; authentic targets are strongly repressed. Adapted from (Seitz, 2009).

Therefore, he postulated that many of the computationally identified miRNA targets may actually represent competitive inhibitors of miRNA function, coined pseudotargets, which would thus sequestrate miRNAs to prevent them from binding to their authentic targets. This hypothesis explains why the "neutral targets" described by Bartel and Chen (discussed above) and harbouring the typical patterns recognised by bioinformatics algorithms would indeed be under selective pressure to conserve their miRNA binding sites. But their targeting would only result in modest downregulation with inconsequential effects, buffered by homeostatic mechanisms. He noted that interestingly, the activity of some of the best-characterised miRNA targets -through *in vivo* studies- is really sensitive to alteration of their expression. His model then postulates that authentic miRNA targets would be repressed with a physiological-resulting function in a cell-specific manner where few of these pseudotargets would be expressed, whereas in environments where many pseudotargets would be co-expressed, the miRNA(s) would be titrated out and would not be able to exert a sufficient alteration of their expression. The authentic targets, in these conditions would then also participate to the miRNA expression dilution, therefore also playing a pseudotarget role; this alternative pseudotarget role for authentic targets depending of their expression context, is of crucial consideration when many authentic targets are co-expressed as substrates competing

for the same miRNA(s) (Figure 12). Finally, the non-conservation of miRNAs binding sites in pseudotargets between distant species is simply explained by the fact that the important role of a pseudotarget is to sequester a miRNA, independently of its gene product, as long as it is not affected by the miRNA repressive effect. Therefore, only the overall quantity of pseudotargets would be needed to be conserved, but not the specific pseudotargets themselves, conferring a flexibility for the transcripts that harbour the titrating-binding sites (Seitz, 2009).

According to this model, the neutral targets defined by Bartel and Chen would fall in the pseudotargets class, and the switch and tuning targets would represent two subclasses of the authentic targets.

3.3.3. The competing endogenous RNAs models

In 2011, Salmena *et al.* proposed a modified, generalised model of the pseudotarget hypothesis (Salmena et al., 2011). They combined the model from Seitz, and results obtained in recent studies, which showed that the human genome is pervasively transcribed and that the non-coding part of the genome is of crucial importance in dictating the greater complexity of higher eukaryotes. Indeed, some long ncRNAs and pseudogenes, most of them without any identified biological function, are targeted by miRNAs and compete with authentic targets (Cesana et al., 2011; Salmena et al., 2011). In this hypothesis, they defined RNAs that can bind miRNAs as "competing endogenous RNA" (ceRNA), which act as a competitor of other target RNAs, resulting in a dilution of the miRNAs activity. In this reverse function, where the miRNA targets regulate miRNAs activity, ceRNAs can cross talk through their ability to compete for miRNA binding. As a consequence, their activity would form a wide regulatory network across the transcriptome. In support this hypothesis, two very recent studies identified long circular ncRNAs (circRNAs), which bear multiple miRNA binding sites but are completely resistant to miRNA-mediated target destabilisation; they thereby function as natural miRNA sponges (i.e. competitive inhibitors) by sequestrating them and suppressing their activity (Ebert et al., 2007; Hansen et al., 2013; Memczak et al., 2013). This model greatly expands the functional genetic information in the human genome, in which ceRNAs play important roles in pathological conditions, such as cancer (for more details, please refer to (Salmena et al., 2011).

4. Methods for identifying and validating miRNA targets

The first characterised targets were the ones bound by let-7 and lin-4 miRNAs in *C. elegans*. These miRNAs have been discovered by analysis of mutant worms, which presented developmental defects (Lee et al., 1993; Reinhart et al., 2000). Their target were afterward identified by genetic screens, either through the examination of overexpression-mutants harbouring similar mutant phenotypes, of mutants with opposite phenotypes, or of double mutants that rescue the miRNA loss-of-function phenotype (Reinhart et al., 2000; Slack et al., 2000; Wightman et al., 1993).

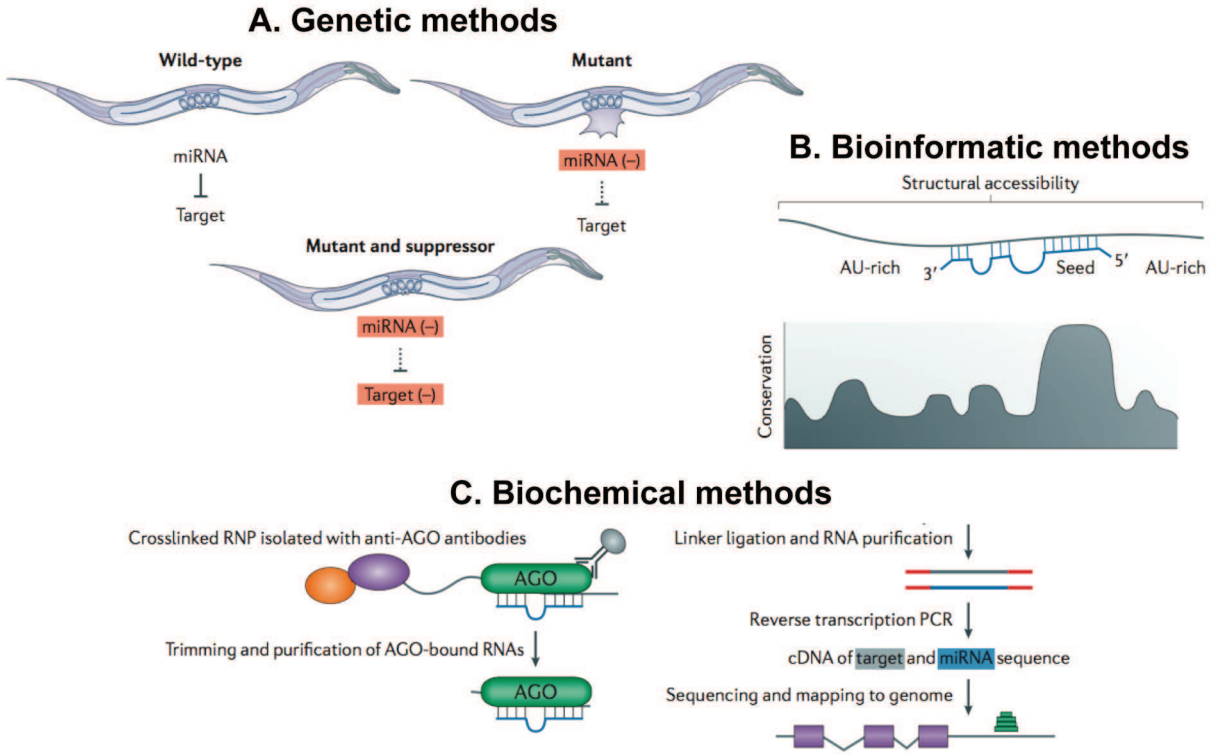

Figure 13: Strategies for identification of miRNA targets. Genetics (A), bioinformatic predictions (B) and biochemical methods (C) are three frequently employed approaches to identify miRNA targets. For more details, please refer to the text. Adapted from (Pasquinelli, 2012).

Genetic approaches for miRNAs targets identification confer an important advantage, as the characterised targets are physiologically relevant genes in terms of regulation by miRNAs. However, these approaches do not allow distinguishing between direct and indirect targets. Further sequence analysis in the candidates target gene and binding site validation have to be performed, and as a consequence, this method can present difficulties for the identification of suppressors of the mutant phenotype if the latter implies the contribution of many miRNA targets (Pasquinelli, 2012). In addition, while genetic screens for phenotypical analysis are feasible in some animals such as *C. elegans*, or *Drosophila*, in mammals they represent a

greater technical challenge (alternatively they can be performed via knock-down experiments in cells through the use of siRNA libraries) (Figure 13A).

With the growing repertoire of identified miRNAs, it became rapidly evident that other, genome-wide approaches had to be developed in order to efficiently identify miRNA targets. The better understanding of animal miRNA biology allowed the development on one hand, of bioinformatics tools for predicting binding sites and target genes, and on the other hand of experimental procedures such as genome-wide transcriptome and proteome analyses, or biochemical methods to analyse the genes altered by miRNA expression (Figure 13B and C). Such approaches result in the predictions of numerous putative miRNAs targets. These methods are thereby generally followed by candidate approaches, by cherry picking in the list of putative targets, candidate genes that have a relevant biological function in the context of the study, and which are further analysed by experimental validation. For a detailed review of methods for the experimental validation of specific miRNA targets, please refer to (Kuhn et al., 2008).

4.1. Predictions methods of miRNA targets

4.1.1. Computational methods

Numerous algorithms have been developed in order to predict miRNA targets (Table 1). These programs take into account diverse criteria, most of them relying on empirical-derived conclusions aiming at reducing false-positive predictions or increasing the signal-to-noise ratio. These criteria consist in elements such as base pairing pattern, thermodynamical stability of the miRNA-target RNA duplex, comparative sequence analysis to check conservation, and search for multiple target sites (Figure 13B) (reviewed in (Min and Yoon, 2010; Watanabe et al., 2007)).

Base-pairing criteria rely on the search of the specific patterns that have been described for characteristic miRNA binding sites, and rare are the bioinformatic tools, which do not include requirement for a perfect seed-match (Bartel, 2009; Min and Yoon, 2010; Pasquinelli, 2012; Watanabe et al., 2007). However, some algorithms such as miRanda (John et al., 2004), or DIANA-microT (Kiriakidou et al., 2004) do allow to define weak binding of the miRNA 5' end, or a tolerance for G:U wobble in the seed-match. The next aspect taken into account is the thermodynamic property of the miRNA-mRNA duplex, obtained by prediction of the free energy of binding. Nonetheless, it is difficult to determine the appropriate threshold of free

energy, as the data sets of known miRNA-mRNA duplexes are limited, and as low free energy (stable binding) is not always a reliable prediction of miRNA target genes. Therefore, it is necessary to consider other features (Watanabe et al., 2007). Indeed, it has been shown in a study that thermodynamic restrictions can be removed without lowering the specificity of the algorithms, by incorporating evolutionary conservation derived from multiple sequence analysis (Lewis et al., 2005). Evolutionary conservation of the miRNA binding sites within 3'UTR of closely related species is widely used by algorithms to refine the list of predicted targets (Bartel, 2009; Pasquinelli, 2012; Watanabe et al., 2007). Algorithms thus in a first step identify orthologous 3'UTR sequences and then perform conservation analysis across related species (Watanabe et al., 2007). Finally, as discussed in section 2.2.1.4, one target gene is often targeted by multiple miRNAs. Based on this observation, Starck *et al.* proposed to check for multiple target sites and therefore to take in account the number of target sites for predictions (Stark et al., 2005), a feature used by many of the existing algorithms (Bartel, 2009), and which is one of the two basic requirements of the PicTar alogorithm (Krek et al., 2005).

In addition, some algorithms allow the search for non-conserved binding sites, since a lot of them have been demonstrated to exist and some of them could thereby represent important species-specific repression (reviewed in (Bartel, 2009)), as for example the targets of the newly emerging species-specific miRNAs (discussed in section 2.2.2.5). Additional criteria are considered by some of the bioinformatics predictions tools, such as the suggestion by Robin *et al.* to consider the folded structure of the mRNA for miRNA accessibility. This approach also allows to ignore evolutionary conservation requirements (Robins et al., 2005). The PITA algorithm (probability of interaction by target accessibility) has been designed following this consideration (Kertesz et al., 2007). An alternative method consists in analysing the sequence context surrounding the site: the TargetScan algorithm searches for non-conserved binding sites surrounded by AU-rich regions (Grimson et al., 2007).

Some databases also propose a combination of the target predictions with other features, such as for example, expression profiling of both miRNAs and their putative mRNA targets according to cell and/or tissue specificity, functional annotations of the predicted targets, and/or the cellular pathway in which they are involved (reviewed in (Min and Yoon, 2010; Watanabe et al., 2007)).

While most of the algorithms rely on the criteria described above, another type of algorithm as been developed by Elefant *et al.*, which does not take into account evolutionary conservation and conventional binding patterns of characteristic miRNA target sites (e.g.

perfect seed-match). This algorithm, RepTar, relies on the identification of repetitive elements in 3'UTRs, which are then tested to identify miRNAs that can base-pair with them through thermodynamical stable interaction. Once the list of these repetitive binding sites is established, the algorithm scans the 3'UTRs (or even CDS if desired), to search for single occurrence of these sites. Therefore, RepTar allows the prediction of conventional, but also non-conventional targets sites, such as seed-match with G:U wobbles, 3' compensatory sites with mismatches in the seed-pairing, or also the recently characterised centered sites (Elefant et al., 2010).

Tool	Clades	Criteria for Prediction and Ranking	Reference
		Site Conservation Considered	
TargetScan	Mammalian/vertebrate	Stringent seed pairing, site number, site type, site context (which includes factors that influence site accessibility); option of ranking by likelihood of preferential conservation rather than site context	(Friedman et al., 2009)
TargetScan	Fly, worm	Stringent seed pairing, site number, site type	(Ruby et al., 2006, 2007)
EMBL	Fly	Stringent seed pairing, site number, overall predicted pairing stability	(Stark et al., 2003)
PicTar	Mammalian/vertebrate, fly, worm	Stringent seed pairing for at least one of the sites for the miRNA, site number, overall predicted pairing stability	(Krek et al., 2005)
Miranda	Mammalian/vertebrate, fly, worm, others	Moderately stringent seed pairing, site number, pairing to most of the miRNA	(John et al., 2004)
miRBase Targets	Mammalian/vertebrate, fly, worm, others	Moderately stringent seed pairing, site number, overall pairing	(Griffiths-Jones et al., 2008)
PITA Top	Mammalian/vertebrate, fly, worm	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility	(Kertesz et al., 2007)
		Site Conservation Not Considered	
TargetScan	Mammalian/vertebrate	Stringent seed pairing, site number, site type, site context (which includes factors that influence site accessibility)	(Grimson et al., 2007)
PITA All	Mammalian/vertebrate, fly, worm	Moderately stringent seed pairing, site number, overall predicted pairing stability, predicted site accessibility	(Kertesz et al., 2007)
RNA22	Mammalian/vertebrate, fly, worm	Moderately stringent seed pairing, matches to sequence patterns generated from miRNA set, overall predicted pairing and predicted pairing stability	(Miranda et al., 2006)

Table 1: Comparison of tools for predicting animal miRNA targets. Adapted from (Bartel, 2009).

A strong bias subsists in most of the algorithms, as the majority of them are restricted to the analysis of 3'UTRs and do not include prediction of binding sites in the CDS or even in 5'UTRs; a feature which is more or less linked to the historical identification of the first target sites (discussed in section 2.2.1.4), and to the evolutionary conservation pattern of UTRs compared to CDSs (Bartel, 2009; Rigoutsos, 2009). Nevertheless there are some exceptions,

and recent studies, through the use of the algorithm *rna22,* have shown that miRNAs can extensively target CDS (Lal et al., 2008; Tay et al., 2008a, 2008b) (Miranda et al., 2006). Computational predictions therefore represent trivial methods to predict a large number of miRNA targets, but they are still not perfect, and most algorithms produce widely divergent predictions with various degrees of false positives and false negatives that are difficult to determine (Min and Yoon, 2010; Thomson et al., 2011). Thus, to definitely assess the predicted target as real targets, they need experimental biological validation, which are by contrast challenging and labour-intensive. One intermediary step to scale down the number of false-positive predictions is the use of large-scale experimental approaches, such as genome-wide analyses, or biochemical methods. However, bioinformatics analyses remain in most of the cases an inevitable step for miRNA target prediction, as they are part of the rare methods allowing determination of the precise binding sites. Therefore, it has to be kept in mind that by contrast with computational approaches, most of the experimental methods described below are not sufficient to establish a list of putative miRNA targets, and generally need to be combined with bioinformatics.

4.1.2. Genome-wide RNA and protein expression analyses

Genome-wide analyses are often employed to study the global impact of miRNA expression (Baek et al., 2008; Guo et al., 2010; Jovanovic et al., 2010; Lim et al., 2005; Selbach et al., 2008). In 2005, Lim *et al.* showed that following transfection of miRNAs, changes in mRNAs expression profile were observed, of which some could be linked to direct targeting by these miRNAs (Lim et al., 2005). At this time, animal miRNAs that bind with partial complementarity to their targets were only thought to act by repressing the translation step, and this was one of the first studies to show that they also act by affecting mRNA stability. It was later confirmed in a study on let-7 et and lin-4 targets in *C. elegans* (Bagga et al., 2005). Following these observations, several groups attempted to correlate expression of miRNAs (by their inhibition or overexpression) with global mRNA expression of their targets (Rajewsky, 2006). However, as it has been described in a study that combined microarray profiling with proteomics, some miRNA targets are repressed without detectable changes at the mRNA level. Thus, transcriptome analyses do not allow the systematic detection of all the targets of a given miRNA. Nonetheless, targets that showed more than one third of repression at the protein level also underwent mRNA degradation, and for the highly repressed ones, mRNA destabilisation appeared to be the major component of repression (Baek et al., 2008).

Proteome analyses using quantitative mass spectrometry approaches -stable isotope labelling with amino acids in cell culture (SILAC) (Baek et al., 2008; Selbach et al., 2008), or two-dimensional differentiation in-gel electrophoresis (Muniyappa et al., 2009; Zhu et al., 2007)- therefore confer the advantage of analysing the ultimate effect of miRNAs on their target transcripts, by measuring the expression of hundreds of proteins at once, but are not sensitive enough to detect low abundance proteins that may also be under the control of miRNAs. This point will require improvement of the technologies in the future. Another alternative, consists in assaying indirectly protein production through ribosome profiling, where association of mRNAs with ribosome indicate their translation state (Pasquinelli, 2012; Thomson et al., 2011). Through deep sequencing of ribosome-protected mRNA fragment, this technique allows in addition of quantitative measurement of the transcripts being translated, the determination of mRNA sequences associated with the ribosome (Ingolia et al., 2009). Thus, in the case of translation initiation inhibition by a miRNA, an accumulation of ribosomes at the 5' end of the mRNA is observed. A recent study has demonstrated the efficiency of this technique for application in the miRNA field, and has concluded that more than 84% of the miRNA-regulated targets at the translational level exhibited decreased level of mRNA, by comparing the ribosome profiling data obtained with mRNA expression analysis through deep sequencing (Guo et al., 2010).

Although these high-throughput approaches enable the analysis of the global impact of miRNA expression and the identification of the cellular pathways targeted, the deregulated genes represent both direct and indirect targets. Thus, in order to obtain a list of putative targets, some bioinformatics filter have to be applied (Thomson et al., 2011). As animal miRNAs regulate modestly the expression of their targets, a first filter can be applied by removing all the genes that are repressed at levels higher than what is commonly known for animal miRNA-regulated targets in the literature, and then, a search for potential binding sites (e.g. search for perfect seed-matches) in the list of the remaining down-regulated genes has to be performed. As a consequence, some of the real targets will be lost, such as particular cases (e.g. potentially upregulated targets, or highly repressed genes such as cleaved targets), or targets harbouring binding sites not detected by the algorithm employed, and false positive targets will be found in the list due to the bioinformatics binding sites predictions intrinsic limits, as discussed above.

Nonetheless, these experimental genome-wide approaches allow the detection of genes, for which the expression is *de facto* altered as a result of miRNA expression, by contrast with the biochemical methods that will be discussed below (Pasquinelli, 2012).

4.1.3. Biochemical methods

Biochemical methods in order to predict putative miRNA targets genes are the more recently developed approaches, and rely on the miRNAs' interactions with the binding sites of the targeted transcripts.

Earlier approaches consisted in immunoprecipitation (IP) of the miRISC complex, isolation of the associated mRNAs, followed by the identification of the targeted transcript by microarray analysis or cDNA cloning and sequencing (Figure 13C) (Beitzinger et al., 2007; Easow et al., 2007; Hendrickson et al., 2008; Karginov et al., 2007; Landthaler et al., 2008; Zhang et al., 2007). More recently derived-methods aim at isolating the sequences in the endogenous RNAs that are in direct interaction with the miRISC complex, such as ultraviolet crosslinking and immunoprecipitation coupled to deep sequencing (CLIP-seq) or high-throughput sequencing coupled with ultraviolet crosslinking and immunoprecipitation (HITS-CLIP). Intact animals, tissues or cells are irradiated with ultraviolet light, which crosslinks protein-RNA interactions. Immunopurification of miRISC complexes is then followed by an RNAse treatment to isolate the RNAs protected by miRISC components, which consist in the miRNA-associated RNA fragments. Theses isolated RNAs are then cloned and subjected to the newly developed next-generation sequencing platforms, providing nucleotide-level resolution of the sequences, and subjected to bioinformatics analyses (Chi et al., 2009; Leung et al., 2011; Zisoulis et al., 2010). A modified version of these CLIP approaches, termed photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLiP), include the incorporation of a photoactivable ribonucleoside analogue such as 4-thiouridine in the RNAs (by incubating the cultured cells with it), which can lead to a single T to C mutation at the site of the crosslink, thereby marking and indicating the surrounding sites of targeting (Figure 13C) (Hafner et al., 2010).

Another biochemical approach to enrich for miRNA targets is to transfect cells with biotinylated miRNA mimics, and to isolate the miRNA-mRNA complexes from cell lysates using streptavidine beads. This technique has been successfully applied in *Drosophila* and mammalian cells, associated to microarray for the analysis of the enriched mRNAs, and represents the advantage, compared to RISC IP, of pulling down targets for a single miRNA of interest (Ørom and Lund, 2007; Ørom et al., 2008).

These biochemical approaches confer the advantage of an increased sensitivity and the ability to identify, on a large-scale, endogenous target mRNA transcripts or even the target sequence within the mRNA. They allow strong alleviation of the biases that result from the above-described methods to obtain putative miRNA targets lists, as for example the identification of

non-canonical binding sites harbouring imperfect seed-pairing (Pasquinelli, 2012; Thomson et al., 2011). Moreover, it has been confirmed through these studies that CDS regions were extensively bearing miRNA target sites and that some binding sites (less than 5%) were mapping to 5'UTRs (Chi et al., 2009; Hafner et al., 2010). However, by contrast with genome-wide approaches insuring the analysis of altered gene expression resulting from miRNAs expression, the detection of an RNA bound by miRISC does not guarantee that it is effectively regulated. Therefore, additional experiments have to perform to validate the putative identified targets genes as functional miRNA targets (Pasquinelli, 2012).

4.2. Validation of miRNA targets as biologically relevant targets

As discussed previously in different sections of this manuscript, demonstrating that a given miRNA binds a target RNA is not sufficient to assess the latter RNA as an effectively regulated target. In addition, even if the target is validated, it still remains to be characterised as a functional, biologically relevant targeted gene.

Moreover, some of the experimental predictions for miRNA targets can turn out to be artefacts. Indeed, miRNAs overexpression in order to detect their targeted genes can cause low affinity binding sites to appear functionally important. On top of that, the use of miRNA mimics transfection to overexpress some miRNAs, considering that mimics bypass the natural mechanisms of miRNA biogenesis, can result in the over-incorporation of the passenger strand into RISC and mediate off-target effects. Also, numerous overexpression experiments are often performed in cell environments that are artificial to the natural context of expression of the miRNA (e.g. in easily transfectable cell-lines such as HeLa, or HEK293), where cell-specific natural targets can be missed, whereas other normally non-co-expressed targets can be detected. As an alternative to overexpression, some of the resulting problems can be avoided by the use of miRNA-specific antisense oligonucleotides inhibitors, with the goal to achieve a differential expression of the miRNA within physiological limits (reviewed in (Thomson et al., 2011)).

Therefore, a first step for validation of a predicted miRNA target is to assess its effective miRNA-mediated regulation by some of the experimental methods described below, the second step consisting in the confirmation of a relevant functionality of the characterised-regulation in a given biological process.

4.2.1. Experimental validation of a predicted miRNA target gene

Gene-specific experimental validation of animal miRNA-mRNA regulation can be achieved by different well-established techniques such as qRT-PCR (quantitative reverse transcription-polymerase chain reaction), northern blot, luciferase reporter assays, western blot, ELISA (enzyme-linked immunosorbent assay), and *in situ* immunocytochemistry.

Generally, the first step, which is extensively employed, consists in reporter assay validation where a luciferase reporter is fused to the candidate 3'UTR and the miRNA of interest is overexpressed or inhibited. To validate the direct effect of miRNA expression, a loss of regulation of the reporter constructs has to be observed following mutation of the miRNA predicted binding sites. Luciferase reporter assays confer the advantage of the possibility to screen numerous candidate genes at the same time with the many kits specifically developed by commercial companies for miRNA targets analysis. However, before one can perform the actual reporter assay, the cloning step for the construction of the luciferase reporters can be quite labour intensive (Kuhn et al., 2008; Thomson et al., 2011). Indeed, as inferred from Grimson *et al.* study (Grimson et al., 2007), it is an important consideration to clone the whole UTR of the candidate transcript, and not only the few tens or hundreds nt surrounding the site, as it could result in alleviating, or even getting rid of the potential structural accessibility constraints of the site, and thereby "forcing" its pairing with the miRNA, giving rise to false positive validation. On the other hand, if cooperative regulation of other miRNAs is needed, the regulation would be lost in the absence their binding sites, and then could result in false negatives.

As luciferase reporter assays still represent an artificial technique, confirmation of the endogenous target regulation needs to be obtained by other methods, either at the mRNA level by qRT-PCR (in the case of important mRNA destabilisation or mRNA cleavage, northern blot is possible), and/or at the protein level by western blot, ELISA, or *in situ* immunocytochemistry (Kuhn et al., 2008; Thomson et al., 2011). Alternatively, mRNA destabilisation can be observed by assaying its polyadenylation state (Pasquinelli, 2012; Sallés et al., 1999). As these methods do not allow distinguishing between direct and indirect miRNA targets, specific inhibition of targeting miRNAs has to be assayed. As discussed above, for consistent results and ultimate validation of the target transcript regulation, these specific inhibition assays on the endogenous target should be performed in the natural, or as close as possible to the physiological context of co-expression of miRNAs and their target transcripts (Kuhn et al., 2008; Thomson et al., 2011).

In the specific cases of cleavages of the mRNA target, RNA ligase mediated-5′ rapid identification of cDNA ends (5′ RLM-RACE) technique can be employed (reviewed in (Thomson et al., 2011), an approach which allowed to show that the *HOXB8* transcript is cleaved by miR-196 (Yekta et al., 2004).

4.2.2. Importance of biological function assessment of characterised miRNA-mediated regulation

Considering Seitz's model, the derived ceRNAs model, and Chen and Rajewsky's model of transcriptional control of new miRNAs (discussed in section 3.3), most of the functionally miRNA-mediated regulated targets will thus not have any physiological role through the alteration of their gene product expression (e.g. pseudotargets, or newly emerged miRNA targets for miRNAs being still in the purging phase). Moreover, as a consequence of the pseudotargets hypothesis, an authentic target in a specific cell type could show no physiological effect through its targeting in another cellular context. It is thus extremely important to ultimately assess a demonstrated miRNA-mediated regulated target as a true biologically relevant miRNA target. This has to be rigorously assayed for a physiological role in the natural context of its co-expression with the targeting miRNA(s).

Therefore, once a given miRNA has been experimentally confirmed to be involved in the regulation of a target gene, the last step consists in demonstrating that this regulation results in into changes in biological functions. Depending on the targeted gene of interest, biological assays could include studying the impact of the miRNA regulation on signaling pathways, cell proliferation, cell differentiation, cell death, cell migration, etc. In order to obtain consistent results, it is important to note that phenotypic changes that may be assayed in the studied biological pathway are indirect measures of the miRNA effect on its targeted gene products. As a consequence, these phenotypic assays have to be accompanied by direct assays on the target gene products, to confirm that the biological changes that may be observed, effectively result from the regulation of the target gene through the miRNA activity (Kuhn et al., 2008).

Cellular miRNAs have generally evolved to target networks of genes involved in specific cellular pathways. The identification of their individual repressed targets and the confirmation of a functional-associated role through the observation of an associated phenotype to exclude the pseudotargets, can thus help to assign them the cellular pathway in which they are each involved. An interesting example is the *miR-17~92* cluster, which encode many miRNAs that have evolved to target different related pathways in relation with cellular proliferation, cell

survival or differentiation inhibition (see (Olive et al., 2010) for a review). This miRNA cluster has therefore been coined *oncomir 1*, given its role in cancer.

During the course of my thesis, I studied the link between cancer development and the expression of virus-encoded miRNAs upon infection. Among the many different factors responsible for cancer, viruses have been long-standing culprits, and up to 12% of cancer are associated with viruses. In particular, my work has focused on the study of one specific oncogenic virus named Kaposi's sarcoma-associated herpesvirus (KSHV), which encode a cluster of miRNAs. It is thus an interesting question, whether KSHV miRNA cluster could play a role in the virus' oncogenic properties, and even be qualified, akin to miR-17~92, as an oncomir. In the following section, we will describe the virus and its associated pathogenesis.

5. Kaposi's sarcoma-associated herpesvirus

Kaposi's sarcoma (KS) was first described by a Hungarian dermatologist, Moritz K. Kaposi, in 1872, as an idiopathic purplish pigmented skin cancer affecting elder male patients in Vienna, now referred as classic KS (Figure 14) (Olive et al., 2010). KS was first thought of as a rare, slow progressing neoplasm, which affected mainly elderly men of Mediterranean and Eastern European region. However it emerged in the early 1980s, with the acquired immunodeficiency syndrome (AIDS) pandemic caused by the human immunodeficiency virus (HIV), as the most common AIDS-associated cancer (Ganem, 2007; Cai et al., 2010). This endemic KS is much more aggressive and can rapidly be fatal, being associated with significant morbidity and mortality. The observation that KS occurs predominantly among patients who acquired HIV by sexual routes (e.g. homosexual and bisexual men) rather than those who acquired HIV by a parenteral route (e.g. haemophiliacs and children), suggested the existence of a pathogen transmitted through sexual contact, prompting the search for such an agent by several groups in the early 1990s (reviewed in (Ganem, 2007; Cai et al., 2010)).

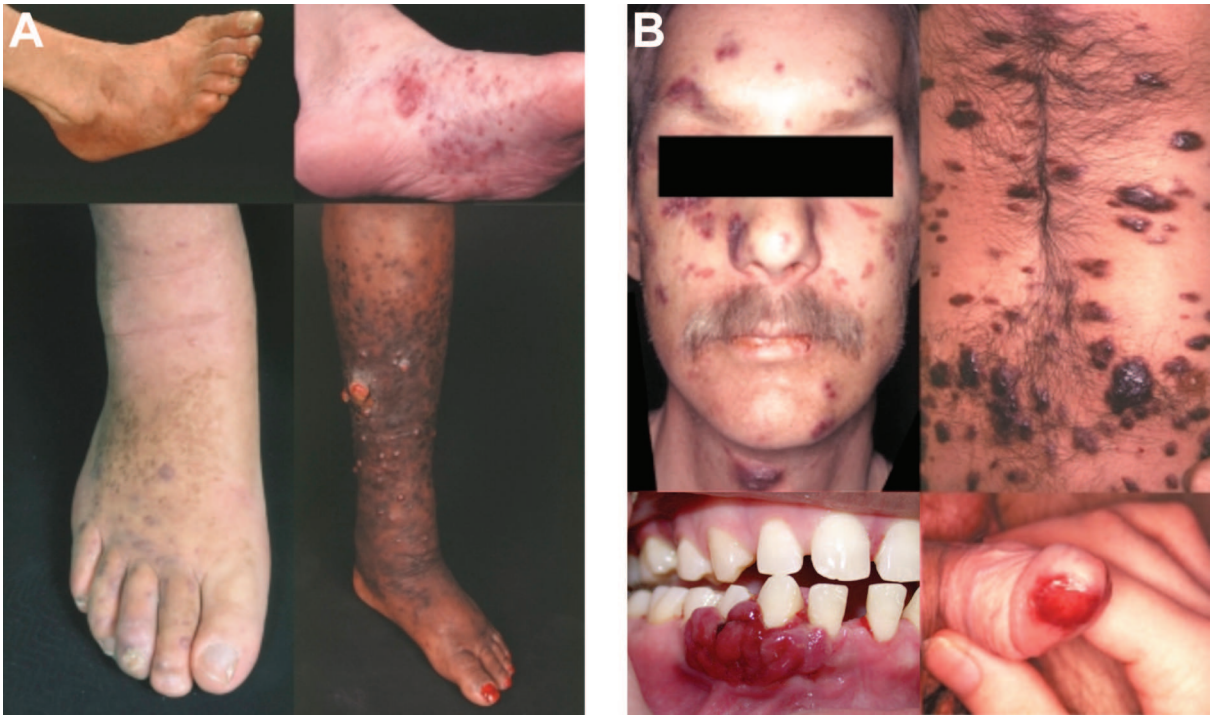

Figure 14: Kaposi's sarcoma lesions. (A) Epidemiological forms of KS. Upper left: classic KS; brownish, livid, confluent plaques and papules on the forefoot and toes. Lower left: iatrogenic KS; multiple livid papules and nodules on the dorsum of the foot. Upper right: AIDS-KS; red-purple macules and papules on the sole and foot. Lower right: endemic African KS; multiple confluent nodules and plaques on the thigh with prominent oedema of the lower thigh and ankle. (B) Manifestations of AIDS-KS. Upper left: multiple livid, irregular papules and plaques. Upper right: disseminated purple-black nodules and tumours on the chest. Lower left: lobulated and nodular purple colour mass involving the gum. Lower right: violaceous well demarcated plaque with prominent borders on the tip of the glans penis. Adapted from (Hengge et al., 2002; Restrepo and Ocazionez, 2011).

In 1994, Chang and Moore's group, using a PCR-based technique (representational difference analysis) to search for DNA sequences invariably present in KS lesions but absent in normal tissue, successfully identified a new herpesvirus as the causative agent of KS. Being the eighth human herpesvirus discovered, it was thus called human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (Chang et al., 1994).

5.1. The herpesvirus family

The *Herpesviridae* family consists in a large group of animal viruses that are ubiquitous to vertebrate species. These viruses predominantly infect mammals and birds, although a certain number of species also infect lower vertebrates such as reptilian, amphibian, fish and some invertebrate hosts. In addition to HHV-8, which I just mentioned, seven herpesviruses have been identified so far: herpes simplex 1 and 2 (HSV-1 and HSV-2, or HHV-1 and HHV-2), varicella-zoster virus (VZV or HHV-3), EBV (or HHV-4), human cytomegalovirus (HCMV or HHV-5), and human herpesvirus 6 and 7 (HHV-6 and HHV-7) (Figure 15A) (Davison, 2007).

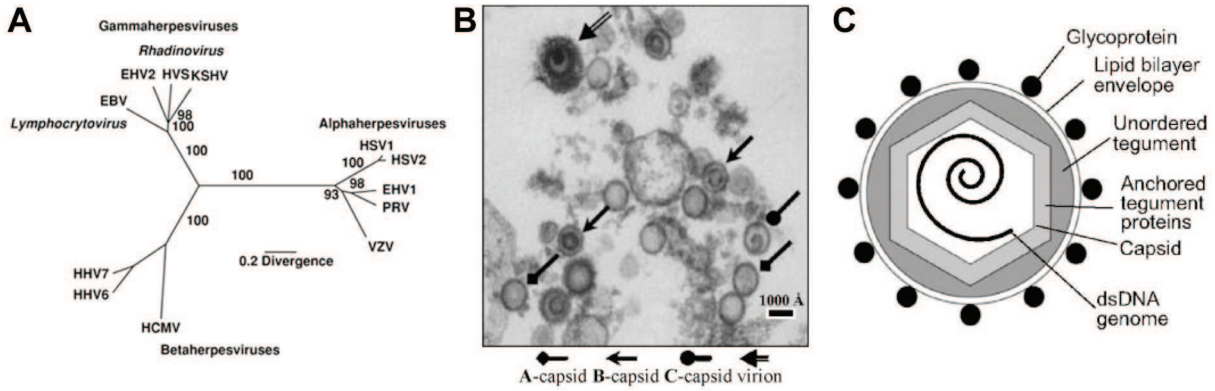

Figure 15: The *Herpesviridae* family. (A) Phylogenetic tree for selected herpesviruses. Adapted from (Moore et al., 1996). (B) Electron microscopy of virions and different kinds of capsids observed in human tissues infected with HCMV. (C) Schematic diagram illustrating the multilayer organisation of the herpesviruses viral particule; please refer to the text for description. Adapted from (Liu and Zhou, 2007)

Herpesviruses are characterised as large enveloped viruses with linear dsDNA genomes ranging from approximately 130 to 250,000 bp. The viral particles are spherical with a diameter ranging from 200 to 300 nm and are composed of a core, a capsid, a tegument and an envelope. The core represents the packed dsDNA molecule encased in a highly ordered icosahedral-shape nucleocapsid of about 125 to 130 nm in diameter. The nucleocapsid is surrounded by a partially ordered layer of several proteins called the tegument, but its structure and function are not fully understood. The tegument is in turn enclosed in the

envelope, which arises from the cellular membrane modified and decorated by different kinds of viral glycoproteins, and which composition is highly species-specific and responsible for viral attachment and entry to host cells (Figure 15B and C) (reviewed in (Liu and Zhou, 2007; Mocarski, 2007)).

Like other virus families, through a long co-evolution with their host, these viruses have adapted very specific mechanisms to evade host defences and usurp the cellular machinery. They also all share a unifying biological property, which is their ability to persist indefinitely over time in their hosts by shutting down their own lytic replication and establishing a latent infection. By contrast, a persistent infection is an attenuated form of acute infection, for a long-term maintenance of the virus into the reservoir host and during which it will continuously produce virions and spread, but with minimal triggering of immune responses and cytocidal effects. Latent infections can be seen as particular extreme cases of persistent infection, as they may occasionally lead to recurrent symptoms provoked by the virus reactivation and particles production, which thus represent an efficient mean of dissemination within the host population. Each herpesvirus occupies its own unique biological niche within the host in terms of their sites of primary infection and latency, and consequently causes diverse kinds of symptoms. Because of the long co-evolution of these viruses and their hosts, severe symptoms are usually restricted to very young or immunocompromised individuals, respectively for their poorly developed or compromised cellular immune response (reviewed in (Cohrs and Gilden, 2001)).

The *Herpesviridae* family is classified in three subfamilies *alphaherpesvirinae* (*Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus* genera), *betaherpesvirinae* (*Cytomegalovirus*, *Muromegalovirus* and *Roseolovirus* genera) and *gammaherpesvirinae* (*Lymphocryptovirus* and *Rhadinovirus* genera) on the basis of their genome sequence and organisation, and biological characteristics (Figure 15A) (Davison, 2007). G*ammaherpesvirinae* subfamily has been identified in many different animal species and was first distinguished by a cellular tropism for lymphocytes. EBV is a *lymphocryptovirus,* whereas KSHV is a *rhadinovirus*, as are its close relatives herpesvirus saimiri (HVS) and rhesus rhadinovirus (RRV), respectively infecting primates from the Old and New World. A striking property shared by many members of this subfamily, is to induce lymphoproliferative disorders and cancers. Tumours caused by EBV and KSHV include lymphoproliferative diseases and lymphomas, but also include tumours from other tissue types, e.g. of epithelial or endothelial origin. Current evidence has shown that both latent and lytic genes of these herpesviruses participate in viral

oncogenesis through transforming and/or paracrine mechanisms (reviewed in (Damania, 2004; Longnecker and Neipel, 2007)).

5.2. KSHV epidemiology and virus transmission

Four epidemiological forms of KS have been described: classic KS affecting elderly men of Mediterranean or eastern European Jewish ancestry; endemic KS non-related to HIV pandemic, existing in parts of Central and Eastern Africa and often affecting children; iatrogenic KS, developing after an organ transplant in individuals under immunosuppressive therapy; and epidemic or AIDS-associated KS, a major AIDS-defining malignancy. In the Western world, AIDS-associated KS is predominantly found in homosexual men, whereas in Africa, epidemic KS is commonly affecting both sexes since the spread of HIV (Figure 14) (reviewed in (Mesri et al., 2010)). The two aggressive and rapidly fatal lymphoproliferative diseases linked with KSHV-infection are frequently observed in HIV-infected patients (Cai et al., 2010).

Although KSHV transmission by blood transfusion or organ transplantation has been documented, it is likely that for the different described KSHV-associated diseases, the most probable route of transmission of the virus for mother-to-child transmission is through saliva, as for HIV-infected homosexual men by oral-oral, oral-genital, or oral-anal sexual contact. Viral DNA can be detected in seminal fluid, but infrequently and with a low copy number compared with saliva, making sexual transmission uncertain, whereas extensive prepubertal infection by KSHV strongly indicates non-sexual transmission (reviewed in (Ambinder and Cesarman, 2007; Ganem, 2007; Mesri et al., 2010)). Indeed, these conclusions from epidemiological studies are consistent with the fact that the virus can replicate *in vitro* in primary oral-derived epithelial cells (Duus et al., 2004).

5.3. KSHV associated diseases

KSHV is associated with three forms of neoplasia ranging from polyclonal hyperplasia to monoclonal monoplasia, and which are KS, primary effusion lymphoma (PEL), (also referred to as body cavity-based lymphoma (BCBL)), and a specific form of the multicentric Castleman's disease (MCD), called plasmablastic MCD (Cai et al., 2010). In contrast to KS, the rarity of PEL and MCD has made it difficult to definitely establish KSHV as the etiological agent of these disorders by comparative epidemiologic studies, although the

relationship is widely accepted in the field. Indeed PEL tumours are always observed in the context of KSHV infection, the viral genome is always detected in every cells of the tumour, and PEL cell survival *in vitro* requires the expression of KSHV latency genes. Concerning MCD, KSHV genomes are regularly found in affected B-cells, strong correlations exist between the viral load and MCD exacerbations, and an epidemiologic relationship between KS and MCD had already been well-described years before the discovery of KSHV (reviewed in (Ganem, 2007)).

5.3.1. Kaposi's sarcoma

KS is a vascular tumour that is by far the most important KSHV associated disease. Although KS bears sarcoma in its name, it cannot be defined as a classical cancer. Unlike classical tumours, which are composed and derive predominantly of single cell types, KS lesions contain different cell types. These tumours display extensive neoangiogenesis, infiltrating inflammatory cells, erythrocytes, endothelial cells, and the so-called "spindle cells" thought to be derived from KSHV-infected endothelial cells. Attempts to grow *in vitro* infected tumour cells directly from KS lesions have been unsuccessful, and result in the loss of the virus after few passages. This might be due to the fact that spindle cells appear to require the products of inflammatory cells for survival and/or growth. However, even if KS do not harbour classical tumour characteristics, it can sometimes be locally or systematically invasive. The most aggressive form of KS is the AIDS-associated KS, which occurs throughout the body, such as skin of face, torso, the extremities, and mucous membranes of the oral cavity. It differs from the classic form of KS, which is strictly localised to the lower extremities (Figure 14). Gastrointestinal lesions may be associated with haemorrhage, diarrhoea, or obstruction; while pulmonary lesions may lead to fatal respiratory compromise (reviewed in (Ambinder and Cesarman, 2007; Cai et al., 2010; Ganem, 2007)).

5.3.2. Primary effusion lymphoma

In contrast to KS, PEL represents a more typical neoplastic process. PEL is a classical malignancy, deriving from post-germinal center B-cells of monoclonal origin, and characterised by effusion of the lymphoma in visceral cavities (pleura, pericardium, peritoneum). Infiltration of solid organs is sometimes also observed. Every cell in the tumours harbours the KSHV genome, and is also frequently, but not always, co-infected with EBV.

Unlike KS-deriving infected cells, the malignant cells in PEL are fully transformed, cultivable *in vitro*, and efficiently induce tumours in nude mice. It is a rare, very aggressive and rapidly fatal disease, with a mean survival diagnosis of 5 to 7 months, occurring in patients in end-stage AIDS (Cai et al., 2010; Ganem, 2007).

5.3.3. Multicentric Castleman's disease

Plasmablastic MCD is a rare, polyclonal and non-neoplastic lymphoproliferative disorder characterised by expanded germinal centers with B-cell and KS-reminiscent endothelial vessels proliferation within involved lymph nodes, and which contains large plasmablastic cells. It is an aggressive systemic illness with multiple centers of origin, involving many lymph node groups and spleen, and a malignant presentation. The disease occurs at increased frequency in AIDS-patients and can be occasionally seen in HIV-negative patients, for which only 40 to 50% harbour KSHV DNA in the lesions. Dysregulated levels of interleukin-6 (IL-6) production are considered to be the underlying cause of MCD pathophysiology. IL-6 is an inhibitor of B-cell programmed cell death (apoptosis), which reduces B-cell turnover, and also an autocrine growth factor for some B-cell neoplasia. The secretion of its viral analogue vIL-6 by infected cells appears to be the cause of MCD in KSHV-positive patients, and is likely responsible for the B-cell hyperplasia surrounding uninfected B-cells. Lytic viral replication may be a feature of MCD, as lytic antigens are more frequently expressed in KSHV-infected cells in MCD than in KS or PEL. Plasmablastic MCD is clinically severe, with most patients dying within 2 years, and is characterised by a variety of disorders, such as recurrent fevers, lymphadenopathy, hepatosplenomegaly, autoimmune haemolytic anaemia, and sometimes progresses to lymphoma or Kaposi's sarcoma (Ambinder and Cesarman, 2007; Cai et al., 2010; Ganem, 2007). Indeed, these conclusions from epidemiological studies are consistent with the fact the virus can replicate *in vitro* in primary oral-derived epithelial cells (Duus et al., 2004).

5.4. Experimental systems for the study of KSHV infection

5.4.1. Animal Models

KSHV field has for long suffered from the absence of any physiologically relevant animal model that could be successfully infected by the virus, as no natural host other than human had been reported.

Some studies have been performed in mouse with the goal of generating murine models. Two endothelial cell lines stably carrying the KSHV genome have been generated, the first by infection of telomerase-immortalised HUVECs (TIVE-LTC) and the second by transfection of a KSHV bacterial artificial chromosome (BAC36 - described below) into mouse bone marrow endothelial-lineage cells (mECK36), and have succeeded to generate KSHV-infected tumours when injected into nude mice. It is worth noting that both systems suggest that KSHV tumour formation requires both latent and lytic viral gene expression, in contrasts with classic viral-induced tumours such as EBV-driven lymphoma or human papillomavirus (HPV)-associated cervical cancer, respectively in which lytic or abortive infection plays little, if any, role (An et al., 2006; Mesri et al., 2010; Mutlu et al., 2007). The mECK36 system is of particular interest, as the cell line induces lesions resembling KS in mice, with characteristic viral and host transcriptome. It demonstrates the *de novo* tumourigenicity of KSHV infection in normal mouse cells, showing that the virus readily provides a survival advantage to cells *in vivo* (Mutlu et al., 2007). Other attempts, aiming to establish a more physiologically relevant *in vivo* model of KSHV infection of human cells, have used different types of humanised immunodeficient mice injected intravenously or directly into the implant with purified virus. These models resulted especially into infection of B-cells, mimicking KSHV natural tropism, with an early phase of lytic replication accompanied and followed by sustained latency, but failed to generate KS-like tumours; no spread to mouse tissue was observed, and no disease state developed in infected animals (Dittmer et al., 1999; Parsons, 2006). Finally, Jones *et al.* have recently developed a new interesting mouse model, which pathological and viral features resemble those in KS tumours. Indeed, they succeeded in transforming primary rat embryonic metanephric mesenchymal precursor cells by infecting them with KSHV. In effect, the cells, which are multipotent cells, were reported as being reprogrammed following infection as expressing, in addition to mesenchymal markers, hallmark vascular endothelial and lymphatic endothelial markers. Then, when introduced into nude mice, transformed cells efficiently induced tumours. The resulting tumours often manifested as reddish lesions and dissemination to visceral organs, and consisted of proliferating spindle cells with vast inflammatory infiltrates and neoangiogenesis. KSHV infection of tumourous cells was characterised at the latent stage, with some cells undergoing spontaneous lytic infection (Jones et al., 2012).

Recently in 2009, Chang *et al.* reported a successful zoonotic transmission of KSHV into the common marmosets *Callithrix jacchus*, a New World primate. Viral infection was established both through intravenous injection and oral inoculation. Although a lower efficiency was observed for oral infection, one orally infected animal developed a characteristic KS-like

tumour, with infiltration of leukocytes by spindle cells expressing both latent and lytic viral proteins. This study thus provides the first animal model, which significantly recapitulates the important aspects of KSHV infection in humans, and will permit a better comprehension of the disease's physiology (Chang et al., 2009).

5.4.2. Cell culture systems

The first successful attempts of cultivation of KSHV-infected cells were performed with PEL cell lines explanted from AIDS-patients; many such cell lines have now been established from EBV-positive and EBV-negative PEL, and they tend to grow readily in cell culture. Although the majority of PEL cells harbour latent KSHV genomes, from which only a small fraction of viral genes are expressed without any viral production, in most experimental culture, 1% to 5% of cells spontaneously display lytic replication, in which the whole genome is expressed and results in the release of infectious virions. The titers of virus particles thus obtained in the culture supernatant is generally low, but can strongly be increased by chemical treatments with drugs such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA or PMA) phorbol ester, or the histone deacetylase inhibitors sodium butyrate or valproic acid. Following such treatments, 15% to 30% of the cells are switched into lytic replication, and virus stock obtained after purification from induced PEL cells can be used to establish *de novo* infection in other cell lines or animals. PEL-derived virus stocks have thus allowed the *in vitro* screening of multiple different cell lines for susceptibility to KSHV infection, and have shown that most *de novo* infection *in vitro* results in immediate entry into latency and no lytic growth (reviewed in (Ganem, 2007)).

Although fibroblasts and epithelial cells have not been reliably observed to be infected *in vivo*, these cell types are readily susceptible to KSHV infection *in vitro* but display no phenotypic changes on latent infection. By contrast, primary endothelial cell lines, which are also susceptible to *in vitro* infection, undergo important morphological changes upon KSHV latent infection, such as cell elongation, and rearrangement of their actin cytoskeleton, thus resembling the spindle cells of KS. Early after infection, a substantial amount of lytic infection is observed, and efficient spread of the virus occurs through cells on the dish, in contrast to most other *in vitro* culture systems, where horizontal transmission has been reported to be relatively poor. Within few days, the viral spread subsides, resulting in a fully latent culture. Importantly, endothelial cells lines are not immortalised by the infection. B-

cells lines, despite their willing susceptibility to infection *in vivo*, are the most refractory to infection *in vitro* (reviewed in (Ganem, 2007)).

5.4.3. Genetic analysis

As opposed to the other herpesvirus subfamilies, *gammaherpesvirinae* are viruses very difficult to genetically engineer by homologous recombination. The critical step seems to be an inefficient rescue of the recombinant viral genome, which may reflect an inefficient virion infectivity and spread. The basis for this inefficiency is unknown, so no rational solution exists to solve the problem. However, a few teams have succeeded with great efforts in the construction of mutant KSHV, by persisting in laborious screening and amplification of many clones. Two successful general strategies have been employed so far (Ganem, 2007).

In 2001, Vieira *et al.* performed homologous recombination directly by transfection in PEL cells to generate a recombinant KSHV expressing a selectable marker and a green fluorescent protein (GFP) cassette (Vieira et al., 2001). In 2002, Gao's laboratory has achieved the cloning of KSHV genome from infected BCBL-1 cells by inserting a BAC into the viral genome by homologous recombination. The recombinant BAC plasmid, named BAC36, was then transfected into HEK293 epithelial cell line, and infectious virions produced by induction with TPA. The KSHV-BAC was then showed to efficiently serve for KSHV genetic analysis as the starting point for additional rounds of homologous recombination in *Escherichia coli*, with the subsequent successful generation of a KSHV deletion mutant (Zhou et al., 2002). However, recent studies have shown that BAC36 contains a 9 kb duplication of the KSHV genome, which is located within the unstable TR region at the extremities of the viral genome. This duplication includes two viral ORFs, between which is actually located the BAC vector backbone of BAC36 that was effectively initially designed to integrate between the latter ORFs, but within their original location in the central region of the KSHV genome. This duplication, in addition of this TR-localisation of the BAC vector backbone could explain why BAC36 has frequently been observed to undergo homologous recombination events, which result in large deletions of the KSHV genome. To solve this issue, a new KSHV-BAC, BAC16, which shows minimal sequence variations compared to other KSHV strains, was then recently successfully generated from another PEL cell line, and thus can efficiently serve for knockout and recombinants studies on KSHV (Brulois et al., 2012).

5.5. Viral replication

5.5.1. Genome structure and organisation

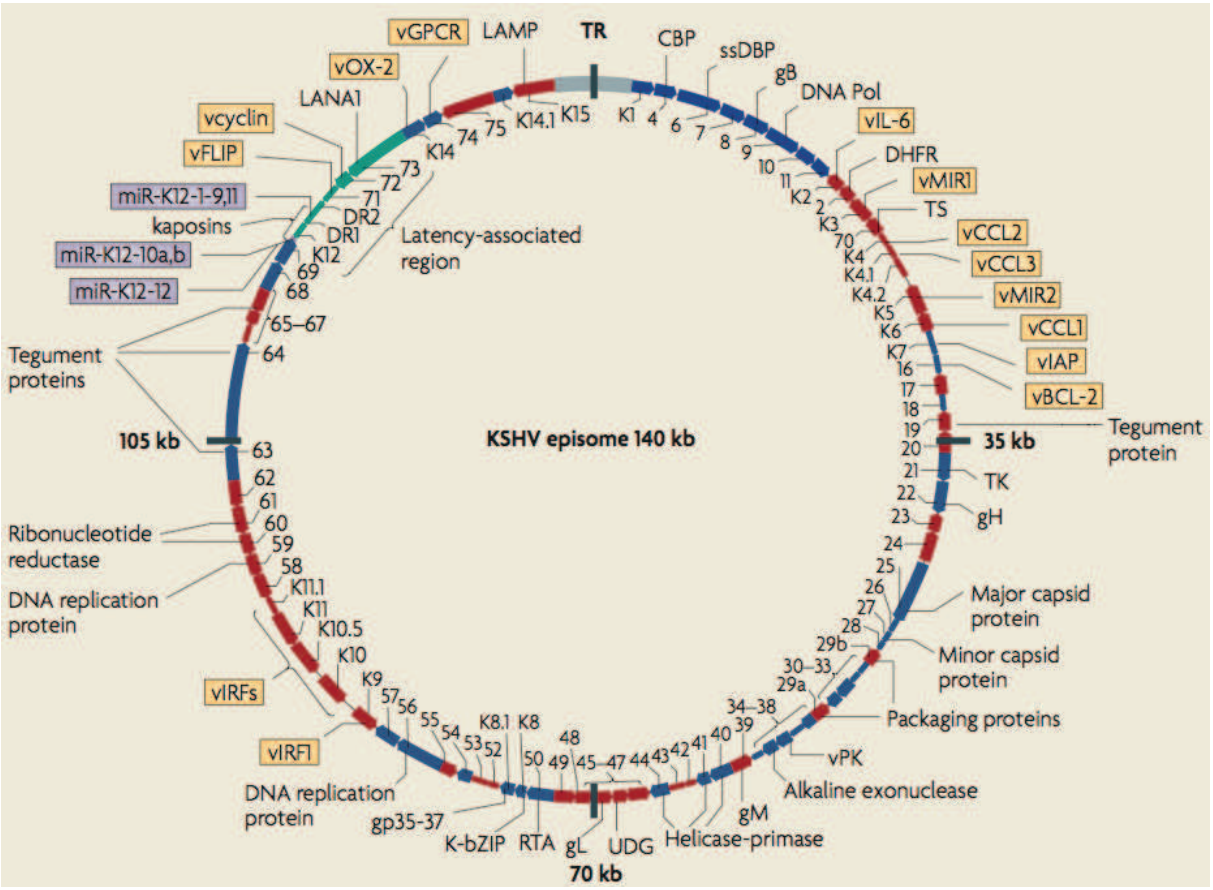

Figure 16: The KSHV episome. KSHV encodes 87 ORFs and at least 12 pre-miRNAs (purple boxes). Identified ORFs and encoded proteins are indicated. Putative latent transcripts are indicated in green, and cellular orthologues in yellow. Adapted from (Mesri et al., 2010).

Comparison of the KSHV sequence with other gammaherpesviruses reveals four major blocks of highly conserved genes encoding the replicative functions and structural proteins of the virions, with many of these conserved across alpha- and betaherpesviruses subfamilies. When KSHV genome was annotated, the closest relative sequence known was HSV. Thus, KSHV genes are consecutively named after the HSV counterparts, knowing that in the latter virus ORFs are numbered in their consecutive order from left to right (ORF1 to ORF75). KSHV genes that are not homologous to HSV are given the prefix "K" (ORF K1 to K15, numbered from left to right); they are interspersed between the conserved genomic blocks. Many of these KSHV specific genes encode signaling molecules and include cellular orthologues. Indeed, a striking feature of KSHV is the number of ORFs that have cellular homologues (at least 16, e.g. vIL-6, viral homologues of central dicysteine (CC) chemokines and of interferon regulatory factors (IRFs)) (Figure 16) (reviewed in (Ganem, 2007; Moore and Chang, 2001)).

5.5.2. Overview of the viral cycle

The first step in KSHV infection is the binding of the virion to cellular receptors through its envelope glycoproteins. The specific cellular receptors of KSHV are not well known, but it was shown that binding of virion is initiated by interaction with heparan sulfates, subsequently followed by interaction with secondary receptors, for which integrin $\alpha 3\beta 1$ and cysteine/glutamate transporter xCT are thought to play an important role; interestingly, it was shown that xCT exists as a heterodimer with the molecule CD98 and the complex has been described to associate with integrin $\alpha 3\beta 1$ (reviewed in ((Ganem, 2007)). Following binding, the capsid and tegument are delivered to the cytosol through a fusion of the envelope and cellular membrane. The capsid is then transferred to the nuclear envelope, and the viral genome subsequently delivered into the nucleus across nuclear pores (Figure 17). The genome is then circularised -TR regions are believed to play an important role for circularisation-, and then becomes organised into chromatin via host histones as a suitable template for host's Pol II (reviewed in ((Ganem, 2007)).

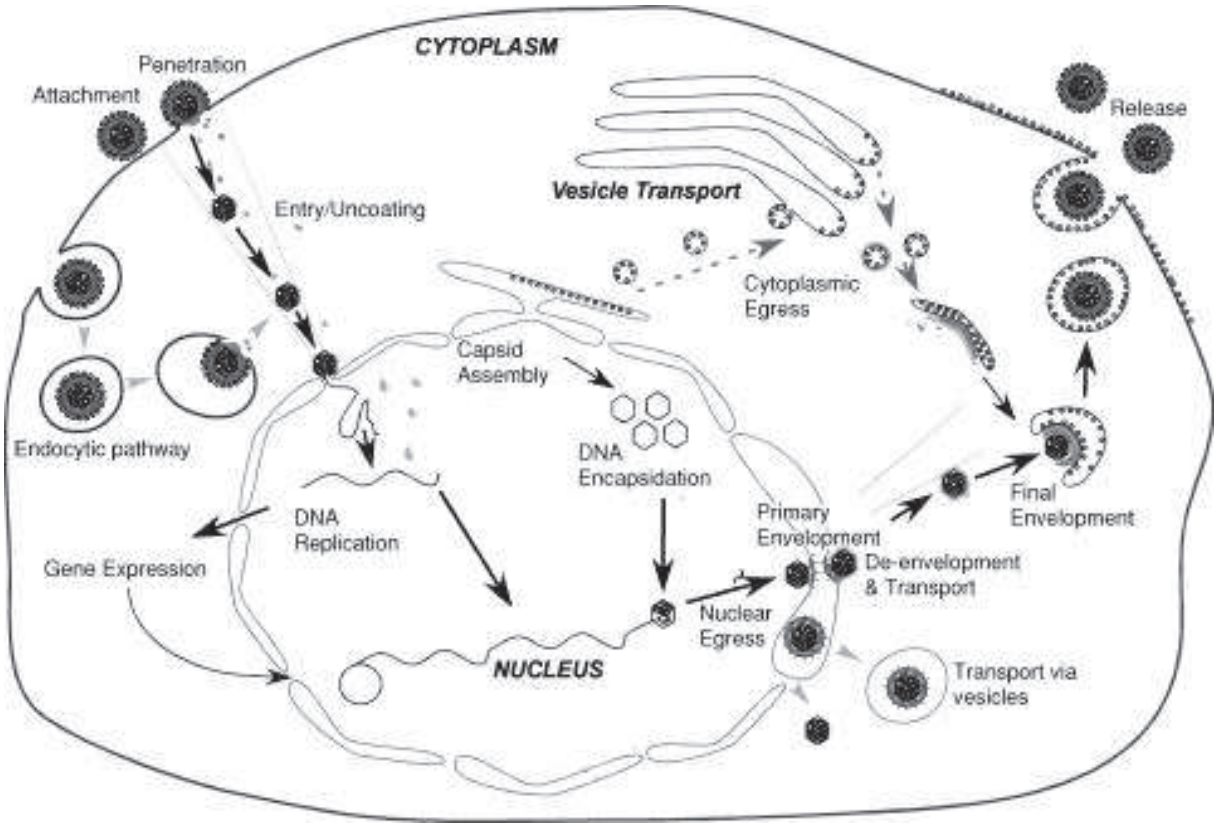

Figure 17: The viral replication cycle. After attachment, the entry involves direct fusion to the cell membrane or endocytosis. Once the nucleocapsid is in the cytoplasm, it migrates to the nucleus and the DNA is released into it, where viral gene expression and replication occur. Early lytic genes include those encoding viral proteins required for DNA replication or viral gene expression, whereas late lytic genes are those encoding viral structural proteins, such as envelope and capsid proteins that are required for assembly of viral particles. Finally, after capsid assembly and envelope acquisition, mature virions are released out of the cell. Adapted from (Mocarski, 2007)

As all other herpesviruses, KSHV can thus alternate between two modes of replication: latency, during which the circularised viral episome is passively replicated in tandem with host cell DNA by the host cell replication machinery, and without any virion production; and lytic replication, with replication of viral DNA by the virus-encoded polymerase, followed by encapsidation into infectious virions (Figure 17) (Ganem, 2007) - the latter results in cell killing (lysis) primarily through apoptosis (Yu et al., 1999); the possibility of virion production without cell death has not been extensively investigated.

5.5.2.1. Latency

From the results obtained in most experimental situations of *de novo* infections in established cell lines (see above), latency seems to be the default cycle from viral entry, where most infected cells display profiles of viral transcripts and proteins characteristic to latency 24 to 48h post-infection. In latency, viral expression is heavily restricted and only few of the viral genes are expressed. As a result no virus production occurs in the latently infected cells (Ganem, 2007). An important role for the gene product of ORF73, also called LANA (for latency-associated nuclear antigen) -a large multifunctional protein for which antibodies are detected in the sera of KS patients- has been described, as binding to DNA specific motifs of the TR regions and tethering viral episomes to mitotic chromosomes, thus triggering semiconservative replication probably by recruitment of host DNA replicative machinery via its interaction with origin recognition complex (ORC) factors. The entire nuclear viral episome is then maintained in the host cells and replicated at low copy number, and genomes distributed to progeny cells upon cell mitosis (reviewed in (Lieberman et al., 2007)).

Most of what we know of KSHV latent gene profiles of expression comes from studies in cultured PEL cell lines; it is thus possible that latent gene expression in PEL is not representative of other forms of latency, but for the most part expressed genes detected in these cells correspond to the ones observed in other contexts such as cell lines infected *de novo* with KSHV and primary KS tumours. Nonetheless, there are also reasons to believe that the latency program is not invariant. Indeed, a specific latent gene product of PEL cell lines has been described, vIRF3 (also called LANA-2), which is not expressed in spindle cells. Moreover, in EBV infection, at least three transcriptional latency programs have been described (reviewed in (Ganem, 2007)). In PEL cells, most of the latency transcripts derive from one specific latency region of the viral genome located between ORF K12 and ORF74, and at least two latent promoters have been found to be active in this interval (Figure 16)

(Ganem, 2007). For more details about the latency gene products and their functions, please refer to (Ganem, 2007; Moore and Chang, 2001).

Latent cells can be reactivated in lytic replication by a variety of exogenous signals, that are not yet fully characterised in authentic *in vivo* infections (reviewed in (Lukac and Yuan, 2007)).

5.5.2.2. Lytic cycle and viral replication

Lytic infection involves the expression of virtually the entire genome, through a highly choreographed transcription cascade. A specific viral transactivator encoded by ORF50 and known as RTA (replication transcriptional activator), which belongs to the first set of genes expressed -known as the immediate-early (IE) genes-, acts as a master control switch (called "switch protein") that governs the cascade of transcriptional activation of downstream genes. Transcription of genes involved in virus replication and encapsidation is subsequently activated either directly by RTA or indirectly by secondary transcriptional regulators in a controlled series of delayed-early (DE) and late (L) waves of viral genes expression. However, a regulation of KSHV genes can occur by tissue-specific cell-signaling pathways, and some genes expressed in some tissues during latency may be upregulated during lytic replication in other cell types. The newly synthesised viral genomes are incorporated into nascent viral capsids, which then acquire their tegument by yet unknown processes, and finally tegumented capsid bud through host membranes decorated by KSHV glycoproteins, in a not yet well documented series of steps to acquire the viral envelope (Figure 17) (reviewed in (Ganem, 2007; Lukac and Yuan, 2007)).

Similarly to the situation observed in PEL cell lines where a subset of the cells continuously undergo lytic cycle, analysis of gene expression in studies on tumour biopsies reveals that both latent and lytic genes expression occurs *in vivo* (Table 2). Such observations lead to the conclusion that both latent and lytic gene expression products seem to have a role in the oncogenesis associated with KSHV infection (reviewed in (Damania, 2004)).

Malignancy	Proteins expressed in most tumour biopsies	Proteins expressed in few tumour biopsies
Kaposi's sarcoma	LANA, vCyc, vFLIP, vIRF-1, Kaposin	vIL-6, K1, vGPCR
Primary effusion lymphomas	LANA, vCyc, vFLIP, vIRF-3/LANA-2, Kaposin, vIL-6 (~40%)	K1, vGPCR
Multicentric Castleman's disease	LANA, vCyc, vFLIP, vIL-6 (~40%)	K1, vGPCR

Table 2: Viral proteins expressed in KSHV-associated tumour samples. Adapted from (Damania, 2004).

5.6. Model for viral oncogenesis

5.6.1. General considerations on oncogenic viruses

Viruses inducing cell transformation and tumour formation fall in different taxonomic groups. All oncogenic RNA viruses belong to the retrovirus family and have generally developed transforming capacity by the acquisition and modification of cellular genetic material (for more details on oncogenic RNA viruses please refer to (Nevins, 2007)). DNA tumour viruses can be grouped in seven different categories encompassing both non-enveloped and enveloped viruses (Table 3). The oncogenic potential of these DNA viruses is tightly linked to their viral replication genetic strategy, and most of them require a long latent period to initiate tumour growth. Tumour formation is not an inevitable consequence of virus infection, but rather reflects the multistep nature of oncogenesis, in which each step is an independent and irreversible change that progressively tends to deregulate cell growth. Thus, viral infection constitutes only one of many steps such as aberrant cell cycle progression, apoptosis evasion, sustained angiogenesis, self-sufficiency in growth signal and insensitivity to anti-growth signals, and finally tissue invasion and metastasis (Nevins, 2007). Tumour development alone is not sufficient to explain why viruses would encode oncogenes. In fact, although tumourigenesis increases the total number of infected cells, thus increasing the virus number, the virus is generally non-transmissible being latent, or in a defective state. Furthermore, the shortening of the host's lifespan as a result of tumourigenesis is particularly disadvantageous for the virus as it reduces the opportunities of transmission to new hosts. Tumour formation indeed occurs in only a subset of the infected hosts (e.g. dead-end, non-native, or immunocompromised hosts) and is not likely to be an essential stage of the viral life cycle; it thereby represents a biological accident. This conclusion thus leads to question the real benefit (if any) for oncogenic viruses to conserve their encoded oncogenes (Ganem, 2007).

Virus family or Subfamily	Examples	Tumour Types
Adenoviridae	All types	Various solid tumours
Hepadnaviridae	Hepatitis B	Hepatocellular carcinoma
Herpesviridae	Epstein-Barr virus	Nasopharyngeal carcinomas, Burkitt's lymphoma
Papovaviridae		
Polyomaviruses	SV40, polyoma	Various solid tumours
Papillomaviruses	Human papillomavirus	Papillomas, carcinomas
Poxviridae	Shope fibroma	Myxomas, fibromas

Table 3: DNA oncogenic viruses. Adapted from (Nevins, 2007).

Most tumour viruses cause cell transformation by directly or indirectly inhibiting two major tumour suppressor proteins, pRB (retinoblastoma protein) and p53, which induce feedback

loops on each other. These two proteins control an important cell growth checkpoint, which guides the progression of the cell cycle to the G1 phase. In addition, p53 plays a key role in triggering the intrinsic apoptosis pathway through activation of pro-apoptotic signaling molecules such as Bax. By interfering with these two proteins, oncogenic DNA viruses alter the cellular environment and drive the naturally quiescent host cells to enter the S phase. This is a key step to understand the link between the replication strategies of these viruses and their oncogenic potential. Indeed, the cell will enter in a proliferating state, therefore inducing expression of genes necessary for DNA replication, which allow nucleic acids to be synthesised for viral replication. In addition, viral proteins that inhibit apoptosis permit optimal maturation and release of infectious virions, by delaying the cell death induced by viral replication. Under normal conditions of infection by oncogenic viruses, this cell cycle deregulation is of no consequence as the infected cells eventually die, or their hyperproliferation and cancerous potential is controlled and attenuated by the host immune system. However, impairment of the replicative infection i.e. in dead-end, non-native hosts or infection of immunocompromised hosts may contribute, via expression of viral oncoproteins, to cellular transformation by deregulating cell proliferation and blocking programmed cell death (reviewed in (Ganem, 2007; Nevins, 2007)).

A last point to take into account is the fact that cells use tumour suppressor proteins in order to prevent and eliminate viral infections. Innate immune system signaling (e.g. Jak-STAT, IRF) induced upon detection of a viral pathogen induces an antiviral state in the cell that activates key tumour suppressor checkpoints. For example, interferon (IFN) signaling induces pRB activation through p21, which then leads to G1/S cell cycle arrest. On the other hand, IFN also sensitises cells to p53-mediated apoptosis, which might kill virus-infected cells. Furthermore, the extrinsic pathway of programmed cell death can also be activated via Fas-Fas ligand (FasL, tumour necrosis factor (TNF) family) signaling pathway induced by natural killer (NK) cells to kill virus-infected cells. Modulation of these pathways by viruses might thus also inadvertently participates to cell transformation (Ganem, 2007).

For a detailed overview of DNA tumour viruses and specific roles of their oncoproteins upon viral infection and cell transformation, please refer to (Nevins, 2007; Saha et al., 2010). For more details on oncogenic properties of gammaherpesviruses, please refer to (Damania, 2004).

5.6.2. Mechanisms of KSHV-induced oncogenesis

Despite the variety of oncogenes specifically encoded by KSHV, some are functionally similar to those found in well-established model tumour viruses.

The viral homologue of D-type cyclins, vCyclin (vCyc - ORF72) and LANA inactivate pRB, similarly to adenovirus E1A and E7 oncoproteins. Other known *in vitro* transforming KSHV proteins that are found to be expressed in some tumour biopsies (Table 2), such as K1, viral G protein-coupled receptor (vGPCR - ORF74), and vIRF-1 may also induce mitogenic signals that complement or substitutes for vCyc activity in the infected tissue (reviewed in (Damania, 2004; Ganem, 2007)).

vCyc has also been shown to interact with cyclin-dependent kinase (CDK)-6 to trigger apoptosis, but this event can be then inhibited by the viral B-cell lymphoma (vBcl)-2 protein (ORF16). Nonetheless, the latter is minimally expressed in KS lesions and is markedly induced by TPA treatment of PEL cell lines as a lytic gene. The p53-inhibitory properties of LANA make this protein the leading candidate for pRB-induced apoptosis inhibition, similarly to adenovirus E1B and E6 proteins. The other KSHV oncogenes with anti-apoptotic activities acting downstream of p53, including *vbcl-2*, *virf-1* and *vil-6*, could also play this role, but LANA is the only one of these proteins that is always detected and regulated together with vCyc in infected cells and tumours. Furthermore, two additional KSHV proteins have been shown to inhibit the extrinsic apoptotic pathway mediated by caspase (CASP)8. Viral FLICE-like inhibitory protein (vFLIP - K13), which is ubiquitously expressed in KSHV-infected cells, possesses a single death-effector domain required for CASP8 interaction but cannot interact with the downstream apoptotic signaling proteins. It therefore acts as a dominant competitor for CASP8 substrates, thus inhibiting its activity and preventing NK cells from killing KSHV-infected cells (reviewed in (Damania, 2004; Ganem, 2007)). Moreover, LANA-2, which is only but ubiquitously expressed in PEL cell lines, has also been shown to inhibit CASP8 mediated apoptosis (Esteban et al., 2003), and thus reinforce the inhibitory action of vFLIP.

Finally, many of the KSHV proteins with important putative tumourigenic activities are only expressed in a small subset of KSHV lytically infected cells. The expression of K1, vIRFs, vIL-6, the viral-encoded CC chemokine ligands (vCCLs), vGPCR and K15 leads to cell proliferation and survival, secretion of the angiogenic vascular endothelial growth factor (VEGF), and other pro-angiogenic and inflammatory cytokines such as angiopoietin 2 (ANGPT2), IL-6 and IL-8. vIL-6 is thought to have an important paracrine stimulating role of latently infected cells in the KS lesions, by inducing secondary VEGF secretion and spindle

cell proliferation and survival. On the other hand, PEL cells are dependent on vIL-6 (and consequently on KSHV episome maintenance in the cells) in an autocrine manner. Indeed, infected B cells attempt to induce their own apoptosis through intracellular inner defence mechanisms in response to the viral infection, and thus become dependent for their survival on the vIL-6 that they secrete and that interferes with the pro-apoptotic intrinsic signals by inducing B-cell survival. Therefore, vIL-6 acts as counterselection factor of the cells that could progressively loose the KSHV episome upon cellular division after some generations, as this lost would then lead to a recovery of the vIL-6-inhibited pro-apoptotic intrinsic signals initiated. Interestingly, stably expressed vGPCR oncoprotein also induces secretion of VEGF in murine cells, and mice expressing transgenic vGPCR develop angiogenic lesions that resemble KS. In addition, co-injection of vGPCR expressing-cells in nude mice has been demonstrated to be necessary to induce tumour formation by cells expressing only KSHV latent genes. As this lytic gene is expressed at low levels in KS lesions, paracrine mechanisms of VEGF-mediated angiogenesis would then reflect its role in KS oncogenesis (reviewed in (Damania, 2004; Ganem, 2007; Mesri et al., 2010)).

A molecular interplay between both latent and lytic viral genes seems thus to contribute to KSHV-induced oncogenesis. Nevertheless, lytic infection is unlikely to have any direct role in the pathogenesis, being associated to viral replication and cellular lysis. One of the well-accepted theories in the field is that the oncogenic potential of latent genes needs to be enhanced in a paracrine manner by both host and viral growth factors and cytokines supplied by the small percentage of lytically infected cells to result in KSHV-induced tumour development. The importance of lytically infected cells in KSHV-induced pathogenesis is supported by the fact that interrupting lytic replication by immune reconstitution or by anti-lytic herpes antiviral drug (e.g. gancyclovir) can inhibit or prevent KS development (Damania, 2004; Mesri et al., 2010).

Among the other factors that could be contributing to oncogenesis, the recently discovered miRNAs have been the subject of intense research in the past years.

6. miRNAs and viral infections

Viruses are characterised as obligate intracellular parasites and are currently considered as non-living nucleoprotein entities that can only replicate in living cells. Although their can appear as rudimentary infectious agents, they constitute an extremely elaborated form of parasitism. Indeed, they possess the genetic material necessary for the synthesis of their own components but are unable to express its information - being deprived of energetic metabolism and complete replication, transcription and translation system. They thus need to hijack the cellular machinery of their host to insure replication and spread, which usually turns out to be deleterious for the infected cell. In a way, they constitute small fragments of genetic code, which only goal is to multiply by parasiting cells to disseminate in the environment. Through their long co-evolution with and adaptation to their hosts, they have developed several ways to alter the cellular environment in favour of their viral cycle and to prevent the innate immune response from eliminating them from the infected organism. It is therefore not surprising that viruses take advantage of all the diverse cellular regulatory pathways such as interfering with the cellular miRNA regulatory network, or at the other end of the spectrum, by encoding their own miRNAs.

6.1. Virus-host interactions involving cellular miRNAs

In some cases, cellular miRNAs expression is deeply altered upon viral infection, which can result from both innate immune antiviral response and viral alteration of the cellular environment. Although no study has yet been able to demonstrate that RNAi could act as an innate antiviral mechanism in vertebrates, some cellular miRNAs have been characterised as playing antiviral roles. The Voinnet laboratory was the first to demonstrate that a cellular miRNA can impede viral replication, with the characterisation of the antiviral role of miR-32 upon primate foamy retrovirus (PFV-1) infection in human cells, through direct targeting of its viral mRNA (Lecellier et al., 2005). Pedersen *et al.*, showed that IFN-ß could perturb the expression of numerous cellular miRNAs, out of which eight have sequence-complementarity within the hepatitis C virus (HCV) RNA genome and whose artificial overexpression partially reproduces the antiviral effects of IFN-ß (Pedersen et al., 2007). Similarly, miRNA:RNA virus interactions have been reported with the targeting of vesicular stomatitis virus (VSV) RNAs by miR-24 and miR-93 in mice, where Dicer knockdown results in hypersensitivity to the virus (Otsuka et al., 2007). In addition, miR-24 seems to play an important role in virus-host interactions as its expression is up-regulated upon ascovirus and EBV infections

(Cameron et al., 2008a; Hussain and Asgari, 2010)., In the same study, Cameron *et al*. observed the up-regulation of miR-27a, which is on the same cluster as miR-24, upon EBV infection (Cameron et al., 2008a). The regulation of miR-27 seems to be common in viral infections. Indeed, it was later observed that this miRNA was strongly down-regulated by HVS and the mouse cytomegalovirus (MCMV) (Buck et al., 2010; Cazalla and Steitz, 2010; Marcinowski et al., 2012). Interestingly, in both cases the regulation occurs directly at the level of the mature miRNA stability and requires pairing to a transcript of viral origin. Because it was also shown that overexpression of miR-27 is deleterious for MCMV (Buck et al., 2010), it is therefore tempting to speculate that in this particular case, the cellular miRNA regulation might be a counter-measure developed by the virus.

Other examples of miRNA down-regulation exist, but they do not always involve a direct antiviral role of the miRNA. Sometimes, a given miRNA inhibition favours the viral life cycle and pathogenicity. This is the case for miR-100 and miR-101 expression modulation by HCMV. These two miRNAs have been proposed to regulate rapamycin (mTOR) signaling components (Wang et al., 2008a), which are involved in metabolism, growth and survival, and which are already manipulated by many herpesviruses, including HCMV (reviewed in (Buchkovich et al., 2008)). HPV oncoproteins E6 and E7 indirectly inhibit miR-203 expression (McKenna et al., 2010; Melar-New and Laimins, 2010), and E6 miR-34a expression (Wang et al., 2009b). This effect occurs via the modulation of the mitogen-activated protein (MAP) kinase/protein kinase C (PKC) pathway and/or of p53, leading in a an increase in cell growth. Interestingly, p63, which is targeted by miR-203 and whose repression results in cell cycle arrest, seems to play a key role in the activation of HPV DNA genome amplification and L gene expression, therefore implicating miR-203 repression as a critical element in HPV life cycle (Mighty and Laimins, 2011). Another example is the E6-mediated repression of miR-218 expression in high-risk HPV-16, but not in low-risk HPV-6. This results in the increase of miR-218-target laminin 5 β3 (LAMB3) expression, and may enhance cell migration and tumourigenicity (Martinez et al., 2007). Similarly, HIV-1 can act negatively on the oncogenic miR-17~92 cluster, which contains miR-17-5p and miR-20a. The latter target p300/CBP-associated factor (PCAF), a proposed cofactor of the HIV-1 Tat transactivator (Triboulet et al., 2007).

The global down-regulation of miRNA expression upon viral infection has been less frequently observed in mammalian cells, but it can happen. Some viruses can impact negatively on essential factors of the miRNA biogenesis machinery. Indeed, HIV-1 down-regulates Dicer in monocytes via the viral gene *vpr* (Coley et al., 2010). The adenovirus viral-

associated (VA) RNA expressed at 10^8 copies per cell can saturate the function of Exp5, thus blocking pre-miRNA nuclear export, and hence pre-miRNA processing (Lu and Cullen, 2004). It can also act as a competitive inhibitor of Dicer function (Andersson et al., 2005). Finally, vaccinia virus (VACV) infection results in a decrease in Dicer expression (Grinberg et al., 2012), and in the active degradation of the cellular miRNAs induced by the viral poly(A) polymerase (Backes et al., 2012). For viruses establishing long-term persistent or latent infections, such kind of alteration of cellular miRNA expression profiles may perturb the normal cellular functions, including cell fate maintenance, and may thus lead to oncogenesis (Pfeffer and Voinnet, 2006).

On the other hand, some cellular miRNAs can promote viral life cycle and are positively required by the virus. The most striking and well-studied example of a cellular miRNA promoting viral replication is the liver-specific miRNA miR-122. Indeed, this miRNA is essential for HCV genome replication by positively regulating HCV RNA accumulation through its binding of the viral genome 5'UTR (Jopling et al., 2005, 2008). It has been proposed that miR-122 targeting of HCV RNA might stimulate its translation by enhancing the association of ribosomes with the viral RNA (Henke et al., 2008). Another hypothesis that emerged more recently is that the binding of miR-122 (and most likely Ago2) at the very 5' end of the genome acts as a protection from cellular factor (Machlin et al., 2011). Therefore, HCV liver-specific tropism is partly dependent on the expression of miR-122 and specific blocking of this miRNA offers a very promising therapeutic perspective (Haussecker and Kay, 2010; Lanford et al., 2010). Another interesting case is the targeting of HIV-1 mRNAs 3' ends by a cluster of cellular miRNAs including miR-28, miR-125b, miR-150, miR-223 and miR-382. The resulting translational inhibition of the viral RNAs plays a role in the maintenance of HIV-1 latency in cultivated resting CD4+ T cells, thus serving as a latent viral reservoir (Huang et al., 2007).

Specific cellular miRNAs have also been found to be up-regulated by some viruses in order to promote their replication cycle, and/or participate in the associated pathology. Enteroviruses (EVs) provide an interesting example: by strongly inducing the expression of miR-141, which represses the cellular translation factor eIF4E, these viruses re-route the translation machinery for their exclusive usage. Indeed, they rely on an internal ribosome entry site (IRES) for translation and thus do not need eIF4E, required for cap-dependent mRNA translation. MiR-141 overexpression therefore promote the shutoff of host protein synthesis to favour the IRES-dependent expression of EVs mRNAs (Ho et al., 2011). Several herpesviruses, including KSHV, up-regulate miR-132 by more than 10-fold. As a consequence, miR-132

targeting of the transcriptional co-activator p300 exerts a negative effect on IFN-stimulated genes and thus facilitates viral replication, as inhibition of miR-132 results in a significant two-fold drop in viral replication (Lagos et al., 2010). Finally, several miRNAs are induced upon EBV infection. During latency III (LTIII), EBV-encoded latent membrane protein 1 (LMP1) activates the *miR-146a* promoter, leading in a reduction in the expression of a group of IFN-responsive genes. MiR-146a is known to be induced by agents that activate the IFN response pathway, suggesting a function of negative-feedback loop to limit the innate immune response (Cameron et al., 2008b). MiR-29b is also induced by LMP1, and results in the down-regulation of T-cell leukemia 1 (TCL1) oncogene, thus correlating with the cytostatic and growth-relenting properties of LMP1 in Burkitt's lymphoma (BL) cells (Anastasiadou et al., 2010). The most appealing case of cellular miRNA up-regulation by EBV LMP1 is provided by the dramatic overexpression of miR-155 (more than 100-fold) within days of infection, which suggests a key role for miR-155 in EBV-mediated signaling in part through transcriptional regulatory factors modulation (Gatto et al., 2008; Yin et al., 2008). Moreover, overexpression of miR-155 is associated with oncogenic transformation in various B-cell lymphomas and other types of cancer (reviewed in (Croce, 2009)). A critical role of LMP1-mediated miR-155 induction in the establishment of EBV infection has been demonstrated, as the specific inhibition of this cellular miRNA was found to result in cell cycle arrest and apoptotic death of lymphoblastoid cell lines (LCLs) in culture (Linnstaedt et al., 2010).

Whereas EBV induces miR-155 expression, a remarkable observation for two other oncogenic herpesviruses, KSHV and the avian Marek's disease herpesvirus (MDV), has been made. These two viruses both encode viral orthologs of the cellular miR-155 that down-regulate a similar set of cellular mRNAs (Gottwein et al., 2007; Morgan et al., 2008; Skalsky et al., 2007; Zhao et al., 2009). This underlines the importance of this miRNA-mediated gene targeting for the viral replication cycle of these viruses. Interestingly, numerous KSHV-infected B cell lines display decreased levels of miR-155, suggesting that expression of miR-K12-11 may be substituting for miR-155. Another example of a viral ortholog of the cellular miRNA miR-29a was recently shown for the bovine leukemia virus (BLV) (Kincaid et al., 2012). Again, miR-29a overexpression in human chronic lymphocytic leukaemia (CLL) bears striking phenotypic resemblance to BLV-associated tumours in cattle (Kincaid and Sullivan, 2012).

6.2. Virus-encoded miRNAs

Virus Family or subfamilly	Vernacular Name	Pre-miRnas	Genome size (kb)	Host	References
Alphaherpesvirinae	Herpes simplex virus	17	152	Human	1
	Herpes simplex virus 2	18	155	Human	2
	Herpes B Virus	13	157	Simian	3
	Pseudorabies virus	11	143	Swine	4
	Varicella zoster virus	0?	125	Human	5
	Infectious bovine rhinotracheitis HV	10	140	Cattle	6
	Infectious laryngotracheitis virus	7	165	Avian	7
	Marek disease HV 1	14	180	Avian	8
	Marek disease HV 2	18	164	Avian	9
	Herpesvirus of turkeys	17	161	Avian	10
Betaherpesvirinae	Human cytomegalovirus	12	230	Human	11
	Rhesus monkey cytomegalovirus	13	221	Simian	12
	Mouse cytomegalovirus	18	235	Murine	13
	Rat cytomegalovirus	24	230	Murine	14
	Human herpesvirus 6	4	162/168	Human	15
	Human herpesvirus 7	?	145	Human	
Gammaherpesvirinae	Epstein-Barr virus	25	172	Human	16
	Squirrel monkey HV, HV saimiri	3	155	Simian	17
	Mouse HV strain 68 (MHV-68)	15	135	Murine	18
	Rhesus lymphocryptovirus	36	131	Simian	19
	Kaposi's sarcoma-associated virus	12	170/210	Human	20
	Rhesus rhadinovirus	15	171	Simian	21
	Sheep-associated malignant catarrhal fever of cattle HV	8	135	Ovine	22
Polyomaviridae	Simian virus 40	1	~5	Simian	23
	BK virus	1	~5	Human	24
	JC virus	1	~5	Human	25
	Merkel cell polyomavirus	1	~5	Human	26
	Simian agent 12 lymphotrophic papovavirus (LPV)	1	~5	Baboon	27
	Mouse polyomavirus	1	~5	Murine	28
(*Polyomaviridae-papillomaviridae*)	Bandicoot papillomatosis carcinomatosis virus type 1	1	~7	Bandicoot	29
Formally *Baculoviridae*	*Heliothis zea* nudivirus	2	228	Insect	30
Iridoviridae	Singapore grouper iridiovirus	15	140	Fish	31
Ascoviridae	*Heliothis virescens* ascovirus	1	186	Insect	32
Baculoviridae	Bombyx mori nucleopolyhedrosis virus	4	128	Insect	33
Adenoviridae	Human adenovirus type 5	2	36	Human	34
Retroviridae	Human immunodeficiency virus, HIV	1*	~9.7	Human	35
Orthoretrovirinae	Bovine leukemia virus	5	~8.4	Cattle	36
Flaviviridae	West Nile virus	1	11	Insect	37

Table 4: Virus-encoded miRNAs. Viruses known to encode miRNAs are listed in order of viral family or subfamily genus, along with their common names. The number of pre-miRNAs encoded by each virus is listed along with approximate genome lengths and the host organism. * conflicting reports (1-3 miRNAs). Adapted from (Tuddenham and Pfeffer, 2013).

References: 1. (Cui et al., 2006; Jurak et al., 2010; Umbach et al., 2008, 2009); 2. (Jurak et al., 2010; Tang et al., 2008, 2009; Umbach et al., 2010a); 3. (Amen and Griffiths, 2011; Besecker et al., 2009) ; 4. (Anselmo et al., 2011; Wu et al., 2012); 5. (Umbach et al., 2009); 6. (Glazov et al., 2010); 7. (Rachamadugu et al., 2009; Waidner et al., 2009); 8. (Burnside et al., 2006; Yao et al., 2008); 9. (Waidner et al., 2009; Yao et al., 2007); 10. (Waidner et al., 2009; Yao et al., 2009); 11. (Dunn et al., 2005; Grey et al., 2005; Pfeffer et al., 2005; Stark et al., 2012); 12. (Hancock et al., 2012); 13. (Buck et al., 2007; Dölken et al., 2007); 14. (Meyer et al., 2011); 15. (Tuddenham et al., 2012); 16. (Cai et al., 2006a; Grundhoff et al., 2006; Pfeffer et al., 2005; Zhu et al., 2009); 17. (Cazalla et al., 2011); 18. (Pfeffer et al., 2005; Reese et al., 2010; Zhu et al., 2010); 19. (Cai et al., 2006a; Riley et al., 2010; Walz et al., 2010); 20. (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005); 21. (Schäfer et al., 2007; Umbach et al., 2010b); 22. (Levy et al., 2012); 23. (Sullivan et al., 2005); 24. (Seo et al., 2008); 25. (Seo et al., 2008); 26. (Lee et al., 2011; Seo et al., 2009); 27. (Cantalupo et al., 2005); 28. (Sullivan et al., 2009); 29. (Chen et al., 2011); 30. (Wu et al., 2011); 31. (Yan et al., 2011); 32. (Hussain et al., 2008); 33. (Singh et al., 2010); 34. (Aparicio et al., 2006, 2010; Xu et al., 2007); 35. (Ouellet et al., 2008); 36. (Kincaid et al., 2012); 37. (Hussain et al., 2012).

Since the initial discovery of the first virally-encoded miRNAs in EBV (Pfeffer et al., 2004), a large number of viruses has been described to encode viral miRNAs. The advent of high-

throughput sequencing of small RNA derived libraries has been very useful in establishing viral miRNomes. To date, more than 500 mature miRNAs of viral origin are listed in the literature (Table 4) (for a complete description of the virally encoded miRNAs, please refer to (Grundhoff and Sullivan, 2011; Skalsky and Cullen, 2010; Tuddenham and Pfeffer, 2013).

Intuitively, one can speculate that, given the fast rate of evolution of viruses and thus the chances of random appearance of inverted hairpins in their genomes, many viruses would have evolved for the expression of miRNAs as a regulatory strategy. Indeed, they can confer great advantages to viruses, as they require relatively little coding capacity, are non-immunogenic, can evolve rapidly to target new transcripts, and can regulate individually to varying degrees a great number of host or viral targets with high specificity. Therefore they can represent an adapted solution to the genomic size limitation constraints of many viruses and to establish a cellular environment favourable to viral replication without triggering the innate and adaptive immune response (Grundhoff and Sullivan, 2011; Skalsky and Cullen, 2010).

Nonetheless, only few virus families have been found to expresses miRNAs. Most of the viral miRNAs that have been identified to date are encoded by DNA viruses, and only very few examples of miRNAs deriving from RNA viruses have yet been described. The number of viral miRNA genes varies greatly among the different viruses, ranging from one in polyomaviruses to up to 36 in the rhesus lymphocryptovirus (rLCV) (Table 4). For several viruses that replicate in the cytoplasm (e.g. *Poxviridae, Flaviridae*, *Picornaviridae* and HCV), the restriction of Drosha availability to the nucleus can be a tempting explanation for their lack of miRNAs but is not entirely satisfactory as they could have developed ways to bypass the need for nuclear microprocessor cleavage, i.e. by encoding proteins with Drosha-like function, or by encoding non-canonical miRNAs. Furthermore, excision of a miRNA from a RNA virus would mean the cleavage and destabilisation of its genome/antigenome and be deleterious for its integrity, which therefore results in a strong counterselection against the evolution of miRNAs in RNA viruses (Grundhoff and Sullivan, 2011; Skalsky and Cullen, 2010). On the other hand, taking into account the subtle regulation operated by miRNAs, it is also conceivable that virus-encoded miRNAs may have a minor impact on gene expression during lytic infections given the robust changes that occur in host and viral gene expression. Moreover, when accounting for protein half-life, which is in average greater than 10 hours in stressed chordates cells, compared to the kinetics of *de novo* expression of miRNAs (around 4 to 6 hours), viral miRNAs may only exert their regulation on a limited number of host and/or viral targets during the life cycle of most viruses, which is generally accomplished in less than

12 hours with the exception of chronic viral infections. Therefore, upon *de novo* lytic infection, the only relevant targets of virus-encoded miRNAs would be newly transcribed genes or genes encoding proteins with unusually short half-lives (Kincaid and Sullivan, 2012; tenOever, 2013).

It is thus not surprising that most viral miRNAs have been identified in viruses with a DNA component to their replication cycle, a nuclear replication, and the ability to undergo long-term persistent infections.

6.2.1. MiRNAs encoded by DNA viruses

As mentioned above, DNA viruses are the viruses that encode most of the viral miRNAs that have been identified, and among them, the vast majority belong to viruses of the herpesvirus family; the latter express the highest number of known viral miRNAs both in terms of absolute number and average number per virus (Table 4). Given the ability of herpesviruses to establish life-long latent infection, it makes perfect sense that they would express miRNAs, as they need to evade and block the host innate or adaptive immune responses in the long term, which entitles a minimal expression of potentially antigenic viral proteins. Indeed, most herpesviruses express their miRNAs during latency, and some of them play a role in the maintenance of this latent stage and/or in the switch from viral production to the latency. The most striking examples are HSV-1 and -2, for which the only abundant viral gene product expressed during latency is a long ncRNA, the latency-associated transcript (LAT), which was shown to encode for miRNAs (Umbach et al., 2008) and is involved in the control of early gene expression (please refer to section 6.3.3).

Viral miRNAs have been described in 6 of the 8 human herpesvirus species, considering that HHV-7 has not yet been tested (Table 4). Surprisingly, in contrast with the other human neurotropic herpesviruses (i.e. HSV-1 and -2), and even with other animal varicelloviruses such as bovine herpesvirus 1 (BoHV-1) and suid herpesvirus 1 (SuHV-1) (Anselmo et al., 2011; Glazov et al., 2010), deep sequencing efforts failed to identify any viral sequence corresponding to potential miRNAs in VZV, the etiological agent of chicken pox and shingles (Umbach et al., 2009). Several non-human herpesvirus families have also been shown to encode for viral miRNAs, such as murine, simian, bovine and a large number of avian herpesviruses. From these animal herpesviruses, it is interesting to note that the murine gammaherpesvirus 68 (or Murid herpesvirus 68 - MHV-68), and HVS have evolved to express miRNAs by Drosha-independent processing, thus reflecting the advantages and

importance of viral miRNA expression in the herpesviruses life cycle (Bogerd et al., 2010; Cazalla et al., 2011).

In addition to the herpesviruses, the other DNA viruses that encode miRNAs also have a nuclear lifecycle and the ability to undergo latent and/or persistent infections. These viruses include several members of the polyomavirus family (Cantalupo et al., 2005; Lee et al., 2011; Seo et al., 2008, 2009; Sullivan et al., 2005, 2009), as well as adenoviruses, which express non-canonical VA RNA-derived miRNA-like molecules (mivaRNA) (Aparicio et al., 2006, 2010; Xu et al., 2007). In addition, a virus from the iridovirus family that infects grouper fish (Yan et al., 2011), and viruses infecting insects belonging to the ascovirus and baculovirus families and to the unclassified baculiform *Nudivirus* genus also express miRNAs (Hussain et al., 2008; Singh et al., 2010; Wu et al., 2011). Finally, although HPVs fall in the category of the viruses most likely to encode miRNAs, it was demonstrated, such as for VZV, that at least HPV-31 appears to be devoid of such RNAs (Cai et al., 2006b). The absence of miRNAs in HPVs, though, is controversial and requires further investigations, as Gu *et al.* predicted several miRNAs that could be expressed in other HPV genotypes and claimed that HPV-18 encodes at least one miRNA, which validation still lacks evidence (Gu et al., 2011b). In addition, bandicoot papillomatosis carcinomatosis virus (BPCV) types 1 and 2 -two closely related viruses comprising a fascinating group of marsupial viruses that are thought to be derived from an ancient recombination event between a polyomavirus and a papillomavirus- express one miRNA from a long non-coding region located in between their polyomavirus-derived and papillomavirus-like regions (Chen et al., 2011).

This exponentially growing repertoire of viral miRNAs, which predominantly concerns the DNA virus families cited above, therefore suggests a strong selection pressure that drives viruses able to undergo latent and/or persistent infections to evolve in expressing their own miRNAs.

Although counterintuitive, it seems that a handful of RNA viruses might be well using a similar strategy of miRNA expression, despite the potential negative effects on their genomic fitness and the lack of access to the nucleus for some of them.

6.2.2. MiRNAs encoded by RNA viruses

Initially, for the reasons detailed above, viruses with RNA genome were thought to be unable to express miRNAs. This idea was supported by many attempts using low or high-throughput sequencing of RNAs extracted from cells infected with RNA viruses such as HCV, yellow

fever virus (YFV), West Nile virus (WNV), poliovirus, or influenza A virus, and which failed to identify virally encoded miRNAs (Parameswaran et al., 2010; Pfeffer et al., 2005; Umbach et al., 2010c). However, its has been reported by several groups that some RNA viruses could be engineered to express functional miRNA precursors without impairing their viral replication and thus demonstrating that even cytoplasmic viruses could produce miRNAs from non-canonical Drosha-independent precursors but also, more surprisingly, from canonical miRNA precursors (Rouha et al., 2010; Shapiro et al., 2010; Varble et al., 2010).

It has been reported that the retrovirus HIV-1, which possess a DNA stage in its infectious cycle, may encode miRNAs (reviewed in (Houzet and Jeang, 2011)), but their validity is still controversial due to their low abundance, their lack of conservation between strains, their unknown biological function and also due to some contradictory results obtained in other studies (Lin and Cullen, 2007; Pfeffer et al., 2005). It is only very recently that the first concrete example of a natural miRNA expressed from an RNA virus was described with WNV. Indeed, when infecting mosquito cells, this virus expresses a miRNA from a stem-loop structure in its 3′UTR. Consistently with previous studies, the small RNA has not been detected in infected mammalian cells (Hussain et al., 2012). It is worth noting that this RNA virus belongs to the *Flaviridae* family, and that its life cycle is fully restricted to the cytoplasm. Soon after, Kincaid *et al.* have demonstrated the existence of a conserved cluster of 5 miRNAs expressed by the oncogenic BLV retrovirus, that are transcribed by Pol III. This RNA retrovirus has thus evolved in producing its miRNAs from small subgenomic Pol III transcripts that can directly and efficiently be processed by Dicer, thus avoiding the cleavage of its genome/mRNA by Drosha. The authors therefore predicted that Pol III-transcribed miRNAs could be a common feature of retroviruses, which will thus require further investigations (Kincaid et al., 2012). In addition, as cited in section 6.1, the authors could show in their study that one of these miRNAs is an orthologue of the B-cell oncomiR miR-29a, and seems to have a role in BLV-induced tumourigenesis.

6.2.3. Conservation of viral miRNAs

Intuitively, one might speculate that viral miRNAs that play an important role during viral replication and pathogenesis ought to be under a strong evolutionary pressure. This is indeed the case when comparing genomic sequences of different KSHV isolates, for which the miRNAs are generally highly conserved (Marshall et al., 2007). However, some isolates carrying mutations that affects the processing of some KSHV miRNAs have also been

reported (Gottwein et al., 2006; Umbach and Cullen, 2010). Similar high levels of conservation also seem to exist between the different strains of other human herpesviruses, such as EBV and HCMV (Cullen, 2010). However, things get different when comparing more distantly related viruses. Indeed, only the closely related EBV and rLCV or HSV-1 and -2 have been described to show some level of conservation for some of their respectively encoded-miRNAs, as have RRV and Japanese macaque herpesvirus (JMHV) (Cai et al., 2006a; Jurak et al., 2010; Umbach et al., 2010b; Walz et al., 2010). The existence of such conserved miRNA homologues between these related herpesviruses thus argue for an important role in viral cycle. By contrast, the other closely related viruses from the same families do not share any miRNA sequence homology, although the genomic location of particular regions that appears as hot spots for miRNA genes are generally conserved, as for example for KSHV and RRV, MDV-1 and -2, or different polyomaviruses (Burnside et al., 2006; Schäfer et al., 2007; Seo et al., 2008; Yao et al., 2007).

It is therefore likely that viral miRNAs have generally co-evolved with their hosts through sequence divergence of pre-existing miRNA stem-loop structures, in addition to some occasional duplication events, rather than from *de novo* generation of miRNA hairpin (Walz et al., 2010). The lack of conservation of viral miRNA sequences could be explained by different reasons such as the higher rate of mutations and faster evolution in viruses when compared to eukaryotes, which also means a rapid adaptation to their host and their cellular environment, and could thus reflect the different evolution histories and host specificity of viruses. However, sequence divergence between two viral miRNAs of related viruses does not necessarily imply the non-conservation of their targeted genes and thus their functionality. Indeed, these miRNAs could target two distinct regions in the same transcript, or different gene products of the same cellular pathway, then resulting in a similar phenotype. Furthermore, evolution of some viral miRNAs could be accelerated by selective avoidance from their cellular targets, as maintaining these target sites is not expected to be beneficial for the host. Therefore, alterations in the target site could be the first event to occur, followed by the viral miRNA acquiring compensatory mutations to maintain its binding to its mRNA-target (Cullen, 209; Walz et al., 2010).

In any case, the fact that viral miRNAs sequences are generally not evolutionary conserved results in a particularly challenging task for the prediction and identification of their targets. Indeed, as discussed in section 4, most of the target predictions algorithm rely heavily on sequence conservation; it has been only recently that efficient experimental procedures independent of computational predictions tools have been developed, thus allowing an

exponential increase in the number of identified viral miRNA targets, and a better understanding of virus-encoded miRNAs functions in these viruses replication life cycles and pathogenesis.

6.3. Virus-encoded miRNAs functions

The elucidation of viral miRNAs functions has long been a very challenging and laborious task taking in account a certain number of difficulties in identifying their targets and biological roles. Indeed, compared to cellular miRNAs, the total number of viral miRNA targets that have been the subject of complete studies is still low, and is even very rare when it comes to *in vivo* studies (Table 5 and Table 6). Indeed, as discussed above, the development of efficient viral target computational prediction tools offers one of the main challenges to solve, and this difficulty has considerably slowed down the advances in this field. In addition, it has to be taken in account that a lot of the viral miRNAs were only recently discovered and have simply not yet been the subject of deeper functional studies. Moreover, another issue is the lack of easily accessible animal models for some viruses, due to the non-conservation of encoded miRNAs by human viruses in non-primate animal virus models (e.g. HCMV vs MCMV). Finally, a last problem consists in the low number of miRNA mutant viruses available, especially when considering gammaherpesviruses and the difficulties to engineer such mutants (discussed in section 5).

The recently developed high-throughput techniques now at least allow a better apprehension of the different targeted pathways with the general overview brought by the viral miRNA targetomes obtained. Several groups have yet realised independent studies using these approaches in EBV- and KSHV-infected B-cell lines and thus have provided extensive lists ranging from 44 to more than 2000 putative cellular targets of these viruses' miRNAs (Dölken et al., 2010a; Gottwein et al., 2011; Riley et al., 2012; Skalsky et al., 2012). Taken together, the results of theses studies show that these viral miRNAs regulate pathways such as innate immunity, cell proliferation and cell survival, consistently with the cellular pathways in which fall the viral miRNA targets identified in other studies (Table 6). Although these approaches are very powerful and reliable tools to identify miRNA binding sites on a large scale (e.g. 80% of the tested predicted targets by Gottwein *et al.* were validated), one has to keep in mind, as discussed in section 4, that they do not constitute an ultimate proof of physiologically relevant miRNAs targets, and that additional functional assays have to be performed to this aim.

In some few cases however, target assignment is facilitated by the existence of viral analogues of cellular miRNAs sharing a common set of cellular targets (see below), or even by the antisense location in the viral genome of some miRNA genes to ORFs. The latter case allowed the first identifications of viral miRNA targets.

6.3.1. Models of viral miRNA function

Two classes of virus-encoded miRNAs can be distinguished: analogues of host miRNAs and viral-specific miRNAs (Figure 18A and B). Viral orthologues of host miRNAs, such as KSVH miR-K12-11 and MDV miR-M4, which share homology with the cellular miR-155, or BLV miR-B4, an orthologue of miR-29a (see section 6.1), have evolved to mimic host miRNAs by sharing the same seed sequence or even more extensive sequence similarity with their cellular counterpart. Therefore, considering the importance of the seed region into tethering miRNAs to their binding sites, they can take advantage of at least a major part of the pre-existing cellular network of targets regulated through the same docking sites by their analogous host miRNAs. These regulatory cellular networks are likely to have evolved into well-defined functions for the modulation of specific cellular pathways, and have already been purged from their undesired off-targets, through the evolutionary processes described in the model proposed by Chen and Rajewsky detailed in section 2.2.2.5 (Figure 18A) (Grundhoff and Sullivan, 2011; Kincaid and Sullivan, 2012). So far there is only one example of an entire cellular pre-miRNA sequence that seems to have been captured from its host by a virus (i.e. the herpesvirus of turkeys (HTV) miR-H14-3p) (Waidner et al., 2011), despite the well-known ability of herpesviruses to pirate cellular genes and to subvert them to their own purposes. This fact could be explained by the short length of a seed sequence, which makes it easier for a virus to evolve into mimicking cellular host miRNAs by acquiring a stretch of 6-7 nt rather than by capturing a 70-80 nt sequence. Nonetheless, a minority of virus-encoded miRNAs seems to have evolved into analogues of cellular miRNAs, and given that more than 50% of seed-sharing between virus- and host-encoded miRNAs could have arisen by chance, it seems likely that only a fraction of them are really encoded with the aim to mimic host miRNAs and their regulative function. Viral analogs of cellular miRNAs thus require evidences to be characterised as "authentic" functional analogs, as it was done for example for miR-155 and KSHV/MDV viral analogs (Grundhoff and Sullivan, 2011).

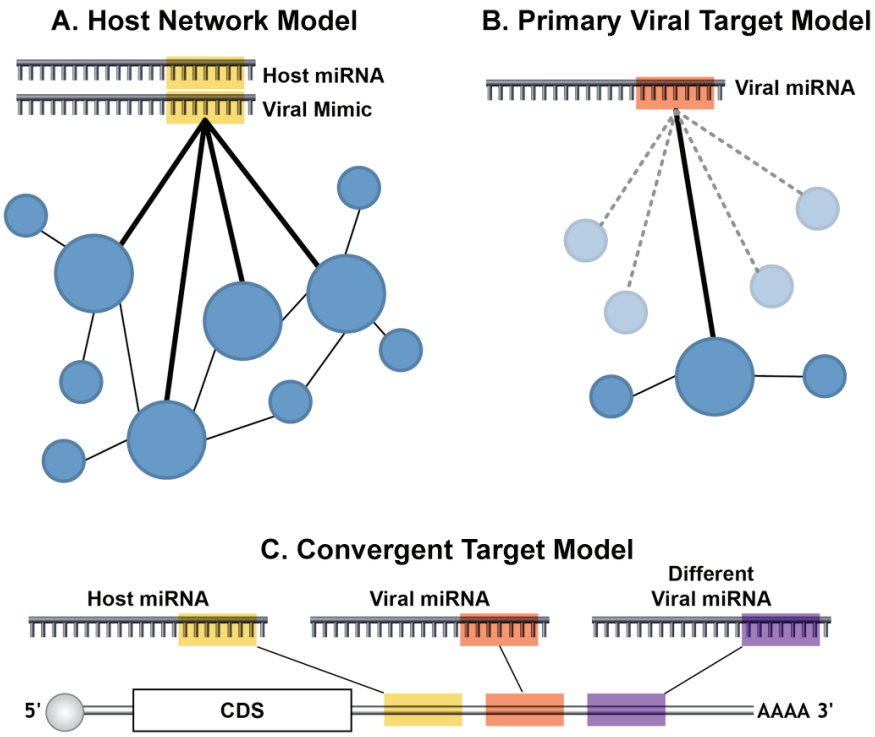

Figure 18: Models of viral miRNA function. Models (A), (B), and (C) are detailed in the text. Adapted from (Kincaid and Sullivan, 2012).

Owing to the greater number of non-analogous viral miRNAs, which are thus not predicted to bind to pre-existing conserved but to novel viral miRNA-specific docking sites, Grundhoff and Sullivan suggested a predominant model for viral miRNA targeting regulatory strategy, which they called the primary viral target model (Figure 18B) (Grundhoff and Sullivan, 2011; Kincaid and Sullivan, 2012). In this model, most virus-encoded miRNAs regulate virus-specific networks of genes, as opposed to the host networks, which are regulated by host miRNAs or by viral analogs of cellular miRNAs. They proposed that, with the exception of a few cases in which the viral-specific miRNAs have been shown to functionaly regulate an important number of targets involved in a specific pathway, the main function of virus-encoded miRNAs would be to regulate a very small number of important host and/or viral transcripts. Given the mode of targeting by miRNAs, similarly to experimentally introduced siRNAs, an exogenously non-orthologous introduced miRNA is likely to bind hundreds of targets (discussed in 2.2.2.5). Within theses targeted mRNAs, only a small fraction would be functionaly relevant targets for the viral life cycle, and the majority of the others would be either neutral, or even disadvantageous off-targets. As long as the expression of the viral-specific miRNA would result in an overvall beneficial effect for the virus, the disadvantageous targets might thus be tolerated (Grundhoff and Sullivan, 2011; Kincaid and Sullivan, 2012). This model could at first seem intriguing: why would the virus not take full

advantage of the miRNA propensity to regulate hundreds of targets? To answer this question one has to consider the undesirable nature of viral miRNAs for the host; if these miRNAs are beneficial for the viral infection, they are detrimental for the infected host. As a consequence, as suggested by Walz *et al.* and mentioned in the above section, its seems likely that cellular binding sites of viral miRNAs that are not subjected to evolutionary conservation constraints are altered by mutations to loose their binding affinity with viral miRNAs and that in turn viral miRNAs have to adapt their sequence to these changes to keep their interaction with their targets and thus their function (Walz et al., 2010). Whereas it is reasonable to imagine that a viral miRNA could easily manage to adapt to only one target site evolving that way, it would be much more complex for the latter to adapt to several binding sites diverging at the same time without loosing some of these interactions, and over and above, impossible when considering a multiplicity of sites ranging from tens to hundreds. Therefore, in this regard, the primary viral target model makes perfect sense. One might thus speculate that non-analogous viral miRNAs, which functionally target a large number of transcripts involved in specific pathways, bind sequences in UTRs that are the purpose of important evolutionary pressure, such as recognition sites for host regulatory proteins, sequences involved in important secondary structures, partially overlapping docking sites of cellular miRNAs, or even CDS. Indeed, as supporting examples, one EBV and one KSHV miRNA bind at two different sites in MICB 3'UTR that respectively overlap with miR-376a and miR-433 target sites (Nachmani et al., 2010), or several cases of viral miRNAs targeting cellular CDSs have been demonstrated for KSHV and EBV miRNAs (Dölken et al., 2010a; Skalsky et al., 2012).

As the majority of viral miRNAs are thus each expected to function by binding one or few target sites, this highlights the importance for theses small RNAs to function in a cooperative manner (as detailed in section 2.2.1.4 for animal miRNAs) with other virus-encoded or cellular miRNAs to efficiently repress one given transcript. Convergent targeting of the same transcript by different viral miRNAs, and/or transcripts that are already targeted by host miRNAs seems effectively to be a strategy for which viruses have opted (Figure 18C). Indeed, a striking example comes from the high-throughput analysis of Riley *et al.* that reported the binding of cellular miRNAs to 90% of the 1664 3'UTRs that were found to be bound by the 12 most abundant EBV miRNAs via distinct target sites. Half of these were targets of the oncogenic miR-17~92 miRNA cluster, including gene involved in apoptosis and cell cycle. However, these results have to be interpreted with caution as they arise from an HITS-CLIP approach, which thus do not prove that these interactions are biologically functional (Riley et al., 2012). Interestingly, miR-17~92 has also been shown to target EBV

LMP1 3'UTR (Skalsky et al., 2012), which is also regulated by some of EBV miRNAs (see below in section 6.3.3). Furthermore, evolutionary divergent viruses appear to have deployed the same miRNA regulatory function, which is nicely illustrated by KSHV and EBV miRNAs that share a common set of targets, as shown in Ago2 PAR-CLiP analysis of B-cells infected either by KSHV alone or co-infected with EBV (Gottwein et al., 2011), and by the example of MICB targeting by KSHV, EBV and HCMV miRNAs (detailed in section 6.3.4).

6.3.2. Non-canonical functions

When considering virus-encoded miRNAs, especially viral-specific miRNAs that do not share any cellular analogue, all possible functions must require some attention and need to be investigated. For example, viral miRNAs which are found clustered in genomic regions close to viral origins of replication or antisense to ncRNAs could have novel functions linked to these particular loci (reviewed in (Kincaid and Sullivan, 2012)). Additionally to the *trans* regulation of their targets, the processing of some miRNAs can result in a *cis* regulation, as reported in the autoregulative function of the Drosha-DGCR8 complex that cleaves hairpin structures embedded within the DGCR8 transcript, and which leads to the transcript destabilisation but without any derived miRNA produced (Han et al., 2009). Indeed, several viruses encode pre-miRNAs embedded in *cis* within the sense strand of ORFs and/or the UTRs of transcripts. For example, two KSHV miRNAs are processed from the Kaposin B (KapB - ORF K12) CDS and 3'UTR of its mRNA, resulting in a decrease in the levels of KapB protein expression during latency. KapB transcripts lacking the Drosha cleavage sites lead to higher levels of KapB protein and thus to increased cell death, consistently to the cytotoxicity associated with the protein expression. Interestingly, lytic infection induces a decrease in Drosha levels, which thus partially alleviates this *cis* regulation (Lin and Sullivan, 2011). As another example of *cis* regulation linked to miRNA processing, during EBV LTIII the processing of miR-BHRF1-1 from the LTIII BHRF1 RNA results in the presence of an intron in the transcript that thus blocks its translation. In addition, the LTIII BHRF1 RNA possesses in *cis* a splicing inhibition sequence upstream of the BHRF1-1 pre-miRNA to insure that splicing will not occurs prior to the miRNA excision, thereby favouring the miRNA processing at the expense of the BHRF1 protein expression. Notably, upon lytic replication the BHRF1 mRNA is transcribed from another promoter, which transcription site lies within the pre-miR-BHRF1-1 sequence thus allowing correct splicing of the transcript and its translation into BHRF1 protein, as it does not contain neither the splicing inhibition

sequence nor the Drosha cleavage site upstream of the intron (Xing and Kieff, 2011). Therefore, as for KapB the result is similar for BHRF1, with in both cases less protein produced during the latent phase but due to different processes, each linked to the *cis* excision of the pre-miRNAs by the microprossessor complex. Many cellular processes have been discovered through the study of viruses. Non-canonical regulative functions could thus also exist for some cellular miRNAs, and will probably require further investigations.

6.3.3. Viral targets

The currently known viral targets of virus-encoded miRNAs are described in the Table 5. One of the first viral miRNA targets identified was the simian virus 40 (SV40) large T antigen (TAg) transcript, cleaved by the perfectly complementary antisense-encoded miRNA miR-S1 (Sullivan et al., 2005). Similarly, this function has been conserved for other polyoma viral miRNAs, which are expressed at late times of infection and thus exert an autoregulatory role on early lytic gene expression (Cantalupo et al., 2005; Lee et al., 2011; Seo et al., 2008, 2009; Sullivan et al., 2009). It is worthy to note that contrary to polyomaviruses, BPCV types 1 and 2 do not encode their miRNA antisense to their polyomavirus-derived early genes, but from their non-coding regions located downstream of the papillomavirus-like region and transcribed in the same orientation. Nonetheless, their miRNA possesses a convergent function to the polyomavirus-encoded miRNAs role in the attenuation of lytic gene expression (Chen et al., 2011) This regulation of the infectious cycle may play a role in evading the adaptive immune response *in vivo*. Indeed, cells infected with a mutant SV40 lacking miR-S1 exhibited an enhanced sensitivity to lysis by cytotoxic T-cells and trigger less cytokine production in culture (Sullivan et al., 2005). The targeting of the EBV oncoprotein LMP1 by EBV BART miRNAs could have a comparable function in evading the antiviral response by modulating the growth inhibition and sensitisation to apoptosis mediated by LMP1-induced NF-κB signaling (Lo et al., 2007; Ramakrishnan et al., 2011). However, concerning the role of polyomaviruses miRNAs in evading the immune adaptive system, the results obtained with SV40 could not be confirmed *in vivo* with a murine polyomavirus (muPyV) miRNA mutant. Indeed, no major differences were observed in cytotoxic T-cells response, in secretion of the IFN-γ cytokine, and in viral clearance in the experimentally inoculated mice (Sullivan et al., 2009). These results therefore suggest that the main role of the conserved *Polyomaviridae* miRNA-mediated autoregulation of early genes still remains to be investigated.

Another putative role for polyomaviruses miRNAs could be the regulation of the balance between lytic infection and persistence (Kincaid and Sullivan, 2012). Indeed, viral gene targeting by virus-encoded miRNAs could impact the latency/persistence balance through the down-regulation of genes which are directly involved in the latent/lytic switch and thus in the maintenance of a latent or latency-like state in infected cells.

Similarly to SV40 miR-S1, an EBV-encoded miR-BART2 is transcribed antisense to the gene coding for BALF5, a subunit of the viral DNA polymerase. BALF5 transcript cleavage induced upon forced expression of the small RNA during lytic replication is responsible of a 20% reduction in the viral titers produced by EBV-infected cells, and thus attributes to miR-BART2 a role in maintaining the latency by inhibiting the transition from latent to lytic viral replication (Barth et al., 2008). This mode of regulation in the control of viral latency is also used by alphaherpesviruses. Some of the miRNAs encoded by HSV-1 and -2 LAT transcripts are expressed antisense to the IE infected cell protein (ICP)0 transactivator and to the key virulence factor ICP34.5 (required for efficient viral replication in neurons *in vivo*) (Tang et al., 2008, 2009; Umbach et al., 2008). The same has been observed for the avian infectious laryngotracheitis virus (ILTV) miR-I5, which mediates repression of the ICP4 transactivator (Waidner et al., 2011). In addition, the involvement of herpesvirus miRNAs in the control of viral IE genes to regulate the entry into productive stage is also described in a more "classical" miRNA-targeting mode of regulation via seed pairing such as for the neurotropic HSV-1- and MDV-mediated downregulation of ICP4 (Strassheim et al., 2012; Umbach et al., 2008), or also for betaherpesviruses- (HCMV) and gammaherpesviruses- (KSVH) induced repressions of respectively IE1/IE72, and RTA transactivators (Bellare and Ganem, 2009; Grey et al., 2007; Lin et al., 2011; Murphy et al., 2008). EBV Zta and RTA transactivators are also repressed by the expression of EBV-miR-BART6-5p, as well as the EBNA2 viral oncogene, which is required for the transition from immunologically less responsive type I and type II latency to the more immunoreactive type III latency. However, in this case, direct targeting by the miRNA has not yet been demonstrated (Iizasa et al., 2010). Apart from herpesviruses, a role in the control of latency has also been described for miRNAs encoded by viruses infecting insects. *Heliothis virescens* ascovirus (HvAV) miR-1, similarly to EBV miRNA-mediated repression of BALF5 directs post-transcriptional degradation of its own DNA polymerase I, although through imperfect pairing, and thus regulates the virus replication (Hussain et al., 2008). This regulation could results in attenuating the generally lethal infection of this virus in order to establish a persistent infection of the host, the latter which would thus serve as a reservoir for the virus spread (Kincaid and Sullivan, 2012).

Finally, *Heliothis zea* nudivirus 1 (HzNV-1), as HSV-1 and -2, express a ncRNA, the persistency-associated gene 1 (*pag1*) transcript, which is the only viral transcript detectable during viral latency and from which are processed two distinct miRNAs. These two miRNAs target and degrade the transcript of one of the viral major early genes, *hhi1*, which is responsible for the establishment of productive viral infection. This regulation has been formally demonstrated to be directly responsible of HzNV-1 latency induction (Wu et al., 2011).

Target	Function	Virus (miRNA)	References
ICP0	Viral immediate-early transactivator	HSV-1 (H2-3p), HSV-2 (III)	(Tang et al., 2009; Umbach et al., 2008)
ICP4	Viral immediate-early transactivator	HSV-1 (H6), MDV-1 (M7-5p), ILTV (I5)	(Strassheim et al., 2012; Umbach et al., 2008; Waidner et al., 2011)
RTA	Viral transactivator	KSHV (K12-5, -7-5p, -9*), EBV (BART6-5p)	(Bellare and Ganem, 2009; Iizasa et al., 2010; Lin et al., 2011; Lu et al., 2010)
BALF5	Viral DNA polymerase	EBV (BART2)	(Barth et al., 2008)
UL28, UL32	Viral genes involved in cleavage/packaging of viral DNA	MDV-1 (M4)	(Muylkens et al., 2010)
TAg	Viral large Tumour antigen	BKV (B1-5p, -3p), JCV (J1-5p, -3p), SV40 (5p, 3p), BPCV1 (B1), BPCV2 (B1)	(Chen et al., 2011; Seo et al., 2008; Sullivan et al., 2005)
IE1/IE72	Immediate-early gene	HCMV (miR-UL112-1)	(Murphy et al., 2008)
DNA pol. I	Viral DNA polymerase	HvAV (miR-1)	(Hussain et al., 2008)
hhi1	Major early gene	HzNV-1 (246, -2959)	(Wu et al., 2011)
LMP1	Latent membrane protein, oncogene	EBV (miR-BART9, miR-BHRF1-1)	(Lo et al., 2007; Ramakrishnan et al., 2011)
EBNA2	Viral transactivator	EBV (miR-BART6-5p)	(Iizasa et al., 2010)

Table 5: Examples of viral targets of virus-encoded miRNAs. Adapted from (Tuddenham and Pfeffer, 2013).

Considering the above detailed lines of evidence, its seems therefore obvious that viruses encoding miRNAs have taken advantage of their own small RNAs in the aim to attenuate, or even shut down, the expression of key lytic viral genes to promote and maintain latency/persistence. A possible important part of this viral strategy to maintain the latency state could also be the control of leaky transcripts expressed at low-level. Indeed, in addition to the increase of antigenicity that could be provoked by such leaky transcription, some low abundance lytic-promoting transcripts such as transactivators may be sufficient to initiate the cascade of expression of lytic genes and thus could induce an "undesirable" entry into lytic phase. As miRNAs have an important role in enforcing homeostasis by repressing inappropriate low-level leaky transcripts (discussed in section 3.3.1), enforcing latency against low-level viral gene expression noise could also very well fit with the viral miRNA functions in the maintenance of latency phase (Kincaid and Sullivan, 2012). The role of viral miRNAs in latency establishment and maintenance pave the way for very interesting

therapeutic perspectives, in particular when considering herpesviruses infections. Indeed, it is plausible that blocking the viral miRNAs could be used as a strategy to force these viruses to enter into the lytic stage, and combined to the use of drugs blocking their replication, it could thus eventually allow to purge the latent reservoir.

Furthermore, the control of latency by virus-encoded miRNAs is not only achieved by the targeting of viral genes. Indeed, several KSHV-encoded miRNAs have been shown to target cellular genes, which results in enhanced latency (Lei et al., 2010; Liang et al., 2011; Lu et al., 2010) (reviewed in (Grundhoff and Sullivan, 2011)) such as demonstrated with a deletion mutant of most of the viral miRNAs for which viral lytic replication was significantly enhanced as a result of reduced NF-κB activity (Lei et al., 2010), and similar to some viral proteins expressed during latency, viral miRNAs also modulate the immune response through the targeting of cellular transcripts.

6.3.4. Cellular targets

Table 6 lists examples of virus-encoded miRNAs cellular targets that are described in this section, and belong to the three main cellular pathways regulated by viral miRNAs. Indeed, as mentioned in section 6.3.1, viruses encoding miRNAs are likely to have evolutionary converged to functionally regulate the same cellular pathways in the aim to establish a cellular environment favourable for their viral life cycle, in particular during the latent/persistence phase they settle.

To this end, they first need to evade the host antiviral defences to avoid viral clearance of the infected organism by the immune system. One striking example of gene regulation involved in the cell-mediated innate immunity is one of the first targets that was identified, through the RepTar algorithm (described in 4.1.1), the major histocompatibility complex class I-related chain B (MICB) gene (Stern-Ginossar et al., 2007). Initially discovered as a HCMV miR-UL112 target, it was then demonstrated to be also targeted by EBV miR-BART2-5p and KSHV miR-K12-7 via different binding sites (Nachmani et al., 2009). MICB is a stress-induced immune ligand for the NK cells NKG2D receptor, and its repression by these viruses reduces NK-mediated recognition and killing of infected cells. Similarly, human JC and BK polyoma viruses (JCV and BKV) miRNAs also have converged to modulate NK-mediated killing of infected cells by downregulating another stress-induced ligand, ULBP3, which is also recognised by NKG2D (Bauman et al., 2011). Interestingly, NKG2D is also expressed by various T-cells subsets, which thus suggests that the miRNA-mediated repression of MICB

and ULBP3 could also be involved in escaping detection by the adaptive immune system. In addition, other targets involved in the recognition of infected cells by the innate immune system, such as critical factors of toll-like receptor (TLR) signaling (IRAK1 and MYD88), or in the attraction of specialised immune cells implied in the control of viral infection, such as chemokines (CXCL-16, RANTES and CXCL-11), have been described to be repressed by viral miRNAs expressed by different herpesviruses (Table 5).

In addition to regulating immune response, viral miRNAs have also been implicated in the regulation of cell death and apoptosis. This aspect will be further developed in the Results section.

Seto *et al.*, in a study using engineered EBV miRNA-mutants, have analysed the phenotypic changes observed upon primary B cells infection, in comparison with WT EBV. They could show that the miRNAs encoded by EBV's *BHRF1* locus prevented spontaneous apoptosis induced by EBV infection, but also that these miRNAs are implicated in a strong induction of B-cell proliferation during the early phase of infection (Seto et al., 2010). Notably, this study was the first to definitely show that EBV miRNAs contribute to its transforming capacity, and was soon after followed by an independent study by Feederle *et al.*, who confirmed the previous results. Indeed the latter showed that an EBV mutant lacking the 3 BHRF1 miRNAs had its B-cell transforming capacity reduced by more than 20-folds and that infected cells with the mutant virus displayed slower growth, exhibiting a 2-fold reduction in the percentage of cells entering into the S phase. In addition, they observed a stronger expression of EBV's latent genes and proteins in the mutant virus-infected cells. These observations lead them to suggest that this miRNA cluster can at the same time increase the viral overall load in the infected host (i.e. via an increased proliferation of the infected cells), and reduce the viral antigenic load, two important features for the life-long persistency of latently infected cell into the host (Feederle et al., 2011).

The last category of genes which undergoes repression by virus-encoded miRNAs thus comprise genes involved in cell cycle regulation/oncogenesis, which deregulation is beneficial for the viral lifecycle and that can also have a role in the viral pathogenesis. One of earliest examples is the *thrombospondin 1* (THBS1) gene targeting by four of the twelve miRNAs encoded by KSHV, and whose expression had previously been reported to be downregulated in KS lesions. THBS1 is a matricellular protein involved in cell-to-cell adhesion and cell-matrix adhesion (a property that is typically lost in metastasic tumourous cells), and possesses anti-proliferative and anti-angiogenic activity. Its targeting by KSHV miRNAs could then participate to proliferation of KS tumour cells, but also to the

angiogenesis observed in KS lesions. Additionally, it was also described as a strong stimulator of monocytes recruitment to vascular injury and regulator of T-cell migration through extracellular matrix. Its repression is thus of particular interest for the virus, as it may also participate in immune evasion of KSHV-infected cells (Samols et al., 2007). KSHV miRNAs have also been shown to regulate other genes with a function in cell proliferation, such as p21 targeting by miR-K12-1, thus preventing p53-mediated cell cycle arrest in PEL cells (Gottwein and Cullen, 2010), and SMAD5 (similar to mothers against decapentaplegic 5) repression by miR-K12-11, which de-sensitise PEL cells to the cytostatic effect of transforming growth factor beta (TGF-β) signaling, thus reinforcing the repercussions of the epigenetic silencing of TGF-β type II receptor mediated by viral LANA (Liu et al., 2012). Grey *et al.*, using a RISC-IP approach identified many genes involved in cell cycle and tumour progression as being targeted by HCMV miR-US25-1, suggesting a viral-specific function for the miRNA in this pathway. Interestingly, the binding of the miRNA to these gene transcripts occurs in their 5'UTRs, and mediates efficient repression. In addition, they could show that deletion of miR-US25-1 led to an overexpression of G1/S-specific CycE2 (CCNE2). HCMV induces the expression of CCNE1 and CCNE2 upon infection probably to drive resting G0 cells into the G1/S phase, and into which the virus blocks them to create a cellular environment conducive for DNA replication. As overexpression of CycE has been linked to an increase in sensitivity to apoptosis, the virus might thus negatively regulate CCNE2 to generate a fine balance in the protein induction. Following *de novo* infection, when comparing the WT and mutant virus during time course experiments, the authors observed that miR-US25-1 exerted its repression on CCNE2 only 48h post-infection. This could be linked to the levels of the miRNA, which increase during the progression of the viral infection, but these observations, in addition of an unimpaired viral replication of the mutant virus, thus challenges the hypothesis of an involvement of this regulation in the lytic replicative cycle of virus. Alternatively, it was then proposed that CCNE2 repression, in association of the targeting of genes involved in cell differentiation, could play a role during latency in the aim to manipulate the production of cells generated by the latently infected haematopoietic stem cells to favour certain cell types such as monocytes and macrophages (Grey et al., 2010). Finally as a last example, the recently identified retroviral orthologue of miR-29a, BLV miR-B4, was shown, as its cellular counterpart, to target the tumour suppressor HMG-box transcription factor 1 (HBP1), which is characterised as a cell cycle inhibitor, and whose inhibition could be related to the oncogenic nature of this virus (Kincaid et al., 2012). Modulation by viral miRNAs of the expression of genes involved in cell cycle

arrest and of genes which when repressed lead to cellular proliferation, in addition to benefit to the viral lifecycle, also represents an important advantage for the maintenance and spread of the virus into the host during latency. Indeed, as the latent viral genomes are only passively replicated upon cellular division of the infected cells, the regulation of these genes thus also leads to an increase in the total number of cells bearing the viral genome in the host, which thereby represents a greater virus reservoir. This can thus result in the production of higher virion titers upon lytic reactivation, for a more efficient dissemination into the environment and transmission to new hosts.

Cellular Pathway			
Target	**Function**	**Virus (miRNA)**	**References**
		Cell-mediated immunity	
MICB	Host stress-induced NKG2D-ligands	HCMV (UL-112-1), EBV (BART2-5p), KSHV (K12-7)	(Nachmani et al., 2009; Stern-Ginossar et al., 2007)
ULBP3		BKV (B1-3p), JCV (J1-3p)	(Bauman et al., 2011)
CXCL-16	Chemokines	MCMV (M23-2)	(Dölken et al., 2010b)
RANTES		HCMV (UL-148D)	(Kim et al., 2012)
CXCL-11		EBV (BHRF1-3)	(Xia et al., 2008)
IRAK1, MYD88	TLR Signaling	KSHV (K12-1, K12-9) KSHV (K12-5, K12-11)	(Abend et al., 2012)
		Cell cycle regulation, oncogenesis	
G1/S Cyclin E2	Cyclin	HCMV (US25-1)	(Grey et al., 2010)
p21	Cell cycle inhibitor	KSHV (K12-1)	(Gottwein and Cullen, 2010)
SMAD5	Mediator in TGF-β signaling	KSHV (K12-11)	(Liu et al., 2012)
THBS1	Tumour suppressor and anti-angiogenic factor	KSHV (K12-1, -3-3p, -6-3p, -11)	(Samols et al., 2007)
HBP1	Tumour suppressor	BLV (B4)	(Kincaid et al., 2012)
		Cell survival, apoptosis	
BACH1	Regulator of transcription	KSHV (K12-11)	(Gottwein et al., 2007; Skalsky et al., 2007)
BCL6		EBV (BART3, 9, 17-5p)	(Martín-Pérez et al., 2012)
TWEAKR	TNF weak inducer of apoptosis receptor	KSHV (K12-10a)	(Abend et al., 2010)
PUMA	Pro-apoptotic factors	EBV (BART5)	(Choy et al., 2008)
SMAD2		MDV (M3)	(Xu et al., 2011)
BclAF1		HCMV (UL112-1) KSHV (K12-5, -9, -10a, -10b) EBV (BART17-5p)	(Lee et al., 2012; Riley et al., 2012; Ziegelbauer et al., 2009)
Bim		EBV (BART Cluster I & II)	(Marquitz et al., 2011)

Table 6: Cellular pathways targeted by virus-encoded miRNAs. Adapted from (Tuddenham and Pfeffer, 2013).

Such studies, that have allowed assigning to the currently known viral miRNAs some of their targets and thus that have shed light on viral miRNA functions, have been highly informative in deciphering virus-associated pathologies, particularly cancer. Indeed, miRNA targeting of genes involved in apoptosis induction and cell survival, cell cycle and proliferation, and

innate and adaptive immune response (e.g. that leads to clearance of abnormal cells such as infected cells, but also cancerous cells), is of particular interest when considering the oncogenic viruses encoding miRNAs, such as MDV, BLV, EBV, and KSHV, and is a clear indicator that viral miRNAs actively participate in the onset of tumourigenesis. Nonetheless, the *in vivo* relevance of such targeting remains to be determined to ultimately confirm the potential involvement of virus-encoded miRNAs in oncogenesis.

6.3.5. *In vivo* roles

Most of the studies described above were carried out in cell culture, which sometimes tends to give very different results from what is observed in physiological relevant infections. A good illustrating example is detailed in 6.3.3, with the miRNA mutant muPyV infection of mice that failed to confirm the results of enhanced sensitivity to T-cells recognition obtained in cell culture with the miRNA mutant SV40. It is therefore of prime importance to be able to confirm inferred functions from *in vitro* studies in *in vivo* models, where in addition the virus encounters a broad array of cell types and immune responses that cannot be addressed *in vitro*. As mentioned earlier in the introduction of this section, this is a major issue for human herpesviruses (e.g. EBV and KSHV) and even for some other animal viruses encoding miRNAs, as a large number of them have a very restricted host range and thus cannot be conveniently studied in animal models that are easily accessible in laboratories.

Dölken *et al.* have been the first to report an *in vivo* functional phenotype of a miRNA mutant MCMV. By introducing deletions and point mutations in the viral genome, they generated a virus deficient in the expression of two miRNAs, miR-m21-1 and miR-M23-2. They could show that the viral titers measured in salivary glands -an organ essential for virus persistence and horizontal transmission- of C57BL/6 mice infected by the mutant virus were undergoing roughly a 100-fold reduction compared to WT virus. By depleting the mice of both NK and T-cells, they could then revert the observed phenotype, therefore arguing for a role of these miRNAs in evading both the innate and adaptive immune system, and also for the establishment of a persistent infection considering the role of the organ during infection, in which was observed this phenotype. The targeting by miR-M23-2 of CXCL16 chemokine, which is involved in the recruitment of cells expressing the CCRC6 receptor, can be a potential candidate responsible of this phenotype (Dölken et al., 2010b).

It is interesting to cite a study that preceded the discovery of virus-encoded miRNAs, in which was analysed a spontaneous deletion mutant of MHV-68 that had lost a region of

approximately 9.5 kb that encompasses all the 15 miRNA genes encoded by this virus, as well as four ORFs. This mutant did not show any defect in viral replication when cultured *in vitro*, but induced a more severe acute infection *in vivo*, in particular in the lungs, leading to an increased inflammatory response and thus an accelerated viral clearance in the host. In addition, once establishment of latent infection was achieved, the level of latent virus infection were substantially lower for the mutant than for WT (Macrae et al., 2001). Although it would be tempting to associate these observations to viral miRNAs potential function in the control of viral latency and evasion of the immune system, one has to keep in mind that these results cannot be directly imputed to the absence of MHV-68 miRNAs expression, due to the concomitant deletion of the four ORFs in the mutant strain analysed. At least, it demonstrates that MHV-68 miRNAs are not essential for either lytic or latent infection, but that they still could play a role in their modulation (Cullen, 2011).

HSV-2, the etiological agent of genital herpes, exhibits the ability to efficiently infect guinea pig thus allowing its *in vivo* study. An engineered miR-H6 mutant HSV-2 was recently show to provoke significantly reduced neurological complications of acute infection in infected guinea pigs. However, the sole mutation of miR-H6 did not impede either the establishment of HSV-2 latency or the frequency of recurrences (Tang et al., 2011).

The role of MDV-1 orthologue of cellular miR-155, miR-M4, has been described *in vivo* by Nair laboratory. MDV-1 miR-M4 function was in this study assessed in the natural context of infection in chicken, which allowed examination of the potential oncogenic role of the viral miRNA. By using a series of mutant viruses, the authors have observed that the deletion or mutations of the six miRNAs in MDV cluster 1 did not affect viral replication but abolished the oncogenicity of the virus. The phenotype seemed only to result from the loss of miR-M4 expression, as its deletion or a 2-nucleotide mutation in its seed sequence was sufficient to inhibit viral tumourigenesis, the latter phenotype that was then rescued by the miRNA revertant virus, and more strikingly, by a virus expressing the cellular gga-miR-155. This study is of a particular interest in the field, as it is the first demonstration that assigns to a virus-encoded miRNA a direct in vivo role in oncogenesis in a natural infection model. Additionally, the authors have shown that the attenuated viruses defective in the miRNA cluster 1 expression could serve as an effective vaccine against virulent MDV infection, therefore highlighting the therapeutic potential of the inhibition of viral miRNAs expression (Zhao et al., 2011).

Finally, concerning human herpesviruses with a very restricted host range such as KSHV, it remains nonetheless possible to study certain aspects of the consequences of viral miRNAs

expression in humanised mouse models, such as Boss *et al.* did to study K12-11 and to validate miR-155 in vivo as its functional cellular analogue in NOD/LtSz-scid IL2Rγ^{null} mice. They could show a similar significant expansion of the CD19+ B-cell population in the spleen, and trough the use of antagomirs the downregulation by both miRNAs of C/EBPβ, a transcriptional regulator of IL-6, which is involved in B-cell lymphomagenesis and which repression could partially explain the observed phenotype (Boss et al., 2011). Similarly, the roles of KSHV miRNAs during in vivo infection could also be studied using humanised and/or nude mice models of infection (or even the common marmosets), such as described in section 5.4.1, and combined with the delivery of antagomirs and/or using KSHV miRNA mutant strains, such as the miRNA deletion mutant engineered by Gao laboratory (Lei et al., 2010).

RESULTS

In the next sections I will only focus on the results I obtained during my thesis that were published in PLOS Pathogens in an article attached below. In annexes are attached four other articles from studies performed with some of our collaborators, and in which I was included. One of these studies deals with the identification by RISC-IP of EBV and KSHV miRNAs targets (Dölken et al., 2010a) and is cited and commented several times in the different parts of this manuscript. The three others articles are outside of the scope of the viral miRNAs field, as they deal with the roles of cellular miRNAs in the context of rheumatoid arthritis, and therefore are not further described in this manuscript (Alsaleh et al., 2009; Philippe et al., 2012; Semaan et al., 2011).

1. KSHV miRNAs

1.1. Identification

Following the discovery of the EBV miRNAs, several groups then focused on the identification of other new virus-encoded miRNAs. KSHV miRNAs were thus independently fully enumerated by multiple groups. By the use of small RNA cloning strategies, Pfeffer *et al.*, Cai *et al.*, and Samols *et al.* cloned cDNAs from PEL cell lines and identified 11 pre-miRNAs derived from a region adjacent to the K12 ORF, which were termed as miR-K12-1 to miR-K12-11 (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). Several of these pre-miRNAs were shown to be processed into 2 matures miRNAs from both arms of the pre-miRNA hairpin (referred as 5p or 3p to indicate its origin from the 5' or 3' segment of the pre-miRNA). Grundhoff *et al.* took advantage of a different approach that they designed to identify miRNAs in genomes of small size (less than 2 Mb) without the use of cDNA cloning, and which is based on bioinformatics predictions of pre-miRNAs structures, followed by an array probing for the candidate hairpins, and then northern blot validation of the candidate miRNAs. In addition to the identification of most of the already known pre-miRNAs, they discovered a novel KSHV pre-miRNA hairpin, which is processed into the mature miR-K12-12 form (Grundhoff et al., 2006).

KSHV pre-miRNAs are all clustered into its latency-associated region, more specifically in the latently transcribed *Kaposin/ORF K12* locus (Figure 19). Ten of the 12 hairpins are

located within the intron of the major latent Kaposin transcript, whereas the remaining two others, which encodes miR-K12-10 and -12, are located in the CDS and 3'UTR of *Kaposin* gene and thereby modulate the expression of the resulting protein (detailed in section 6.3.2 of the introduction).

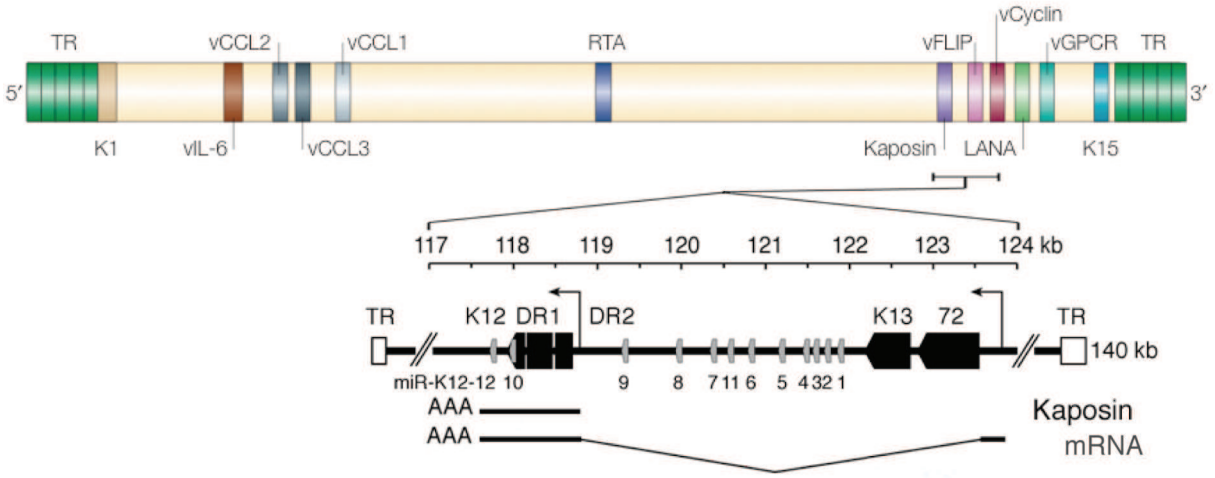

Figure 19: Genomic localisation of KSHV encoded miRNAs. Above is represented KSHV linearised genome. The first (K1) and last (K15) ORFs adjacent to the TRs are indicated. Other viral genes involved in transformation, modulation of signaling and maintenance of latency are also indicated, together with the viral cytokines. Adapted from (Damania, 2004). Below is represented the genomic fragment encoding KSHV miRNAs within the latency associated region. KSHV miRNAs encoding regions are shown as gray arrows, the two alternate promoters for kaposin transcripts as black arrows, the kaposin transcripts as black lines, and the intronic region in the larger transcript as a break in the line. DR1 and DR2 indicate (G+C)-rich direct repeats 1 and 2. The large black arrows indicate known ORFs. Adapted from (Pfeffer et al., 2005).

1.2. Features of the mature miRNA sequences

It is worthy to note that pre-miRNA-K12-10 gives rise to 2 mature forms derived from the same strand of the stem-loop (termed miR-K12-10a and miR-K12-10b) following RNA editing by ADARs at a single position in its seed sequence (Gandy et al., 2007; Pfeffer et al., 2005). Furthermore, using large scale sequencing of KSHV latently infected cells, mature miRNAs arising from each strand of all the 12 pre-miRNAs can be detected, and in addition, the expression of moRNAs and of antisense miRNAs have also been recently reported; their functions are currently unknown. The sequence reads of the latter were relatively low, and they are principally detected during the lytic cycle (Lin et al., 2010; Umbach and Cullen, 2010). Despite a generally good conservation at the 5' end of the mature miRNAs upon their expression in infected cell lines, some variations have been also reported, and a variant of miR-12-10a has been shown to function as an analogue of a cellular miRNA (see below); variability of additional bases at the 3' end is more frequent, but it is yet unknown if it can affect the function of these miRNAs (Gottwein et al., 2011; Lin et al., 2010; Umbach and

Cullen, 2010). In total, at least 25 forms of mature miRNAs derive from the cluster of 12 KSHV pre-miRNAs.

In addition to miR-K12-11, which is an orthologue of cellular miR-155 (detailed in section 6 of the introduction), miR-K12-6-5p also shares seed homology with human miR-15a and -16 but this sequence homology misses the first nt of the viral miRNA seed (i.e. nt 3 to 10 of miR-K12-6-5p are identical to nt 2 to 9 of miR-15a/16) (Skalsky et al., 2007). MiR-15a and -16 are both associated with tumour suppressor activity, and a potential analogous function shared between the viral, and the two cellular miRNAs still need to be demonstrated. Moreover, such analogous function resulting from a "shifted" seed identical to a cellular miRNA seed sequence could be possible, as it has been reported by PAR-CLiP for a KSHV-encoded miRNA that partially mimics cellular miR-142-3p function. Indeed, miR-K12-10a possesses variant forms with an additional nt in 5' (thus shifting its original seed sequence) which has a 7-mer seed region identical to a 5' one-nt shorter form of miR-142-3p. Finally, miR-K12-10a also shares seed homology with miR-rR1-15, expressed from one of the 15 pre-miRNAs encoded by KSHV's animal model, RRV (Umbach et al., 2010b).

1.3. Expression patterns

Although most of the studies on the expression of KSHV miRNAs have been conducted in a limited number of PEL cell lines, some teams have employed different models of study ranging from other cultured cell lines (e.g. immortalised endothelial cell lines, numerous diverse PEL cell lines) to clinical samples (e.g. peripheral-blood mononuclear cells, saliva, or biopsy samples from patients with classic KS, AIDS-KS, or MCD), as well as animal models. Viral miRNAs have been shown to be expressed both during the latent and lytic stages, and can be detected in infected tissues or in the lesions associated with virus pathology (Hansen et al., 2010; Marshall et al., 2007; O'Hara et al., 2009; Umbach and Cullen, 2010).

Some variability was reported in pre-miRNA expression levels across different PEL cell lines and KS biopsies, but the greater differences were observed when comparing different models of infection, with patient-derived samples and cell lines showing a greater expression of the viral pre-miRNAs expression compared to *in vitro* infection models (O'Hara et al., 2009). Sequence analysis of KSHV pri-miRNA regions, pre-miRNAs and miRNAs from 5 PEL cell lines and 17 diverse types of clinical samples revealed 2 distinct clusters of the intronic pri-miRNA sequences (a major and a variant cluster), but a high degree of conservation of the pre-miRNAs and miRNAs sequences. Interestingly, this conservation was observed across

disease states and geographical locations, thus suggesting that KSHV miRNA genes are under tight selection *in vivo* and suggest that they have an important role for the virus cycle, and associated pathogenesis. Nonetheless, some variability that could affect processing was observed in some of the pre-miRNAs sequences (Marshall et al., 2007). This correlates with the expression variability observed in the study from O'Hara *et al.*, and with some observation reported in previous studies. Indeed, it was previously shown by Gottwein *et al.* that the BCBL-1 PEL cell line presented a very low expression of miR-K12-5 compared to another PEL cell line (BC-1). This is caused by a single nt polymorphism in the pre-miR-K12-5 expressed in BCBL-1 cells, which therefore importantly alters its processing (Gottwein et al., 2006). These results were also confirmed by the Sullivan laboratory in a deep sequencing analysis, in which they observed that pre-miR-K12-5 was the fourth most abundant KSHV pre-miRNA that was cloned, but that its processed mature form was one of the lowest expressed KSHV miRNAs (Lin et al., 2010). Pre-miR-K12-9 also shows a high degree of variability, in particular in the PEL cell lines and was even hyper-mutated in one of them (BCP-1), suggesting that this miRNA is not essential for maintenance of the virus in cell culture (Marshall et al., 2007). A lack of miR-K12-9 expression has later on been observed in another PEL cell line (BC-3) through in depth analysis of KSHV miRNAs expression, and sequence analysis confirmed extensive mutations of the pre-miRNA (Umbach and Cullen, 2010).

To date, there is no evidence of a specific promoter for the cluster of KSHV miRNAs, which would allow the expression of KSHV miRNAs independently from an intron or from the Kaposin transcript. The transcription of the pri-miRNA seems to be driven by the same promoters that also drive the expression of the transcript encoding Kaposin, vFLIP, vCyc. The expression of the pri-miRNA is currently reported to be always associated to a transcript that also contains at least the Kaposin CDS (Cai and Cullen, 2006; Pearce et al., 2005). Nonetheless, studies that have compared viral miRNA expression during latent and lytic phases have shown a significant increase in pre-miR-K12-10 and -12 expression during lytic infection (Lin et al., 2010; Samols et al., 2005); this is due to an RTA-inducible unspliced Kaposin transcript expressed as a DE lytic gene (Ganem and Ziegelbauer, 2008). The biological relevance of such a higher expression of these two miRNAs is yet unknown. Indeed, although KSHV miRNAs are also expressed during lytic infection, it is currently unclear if they could play a role in the replicative cycle, when compared to the robust shutoff of host gene expression induced by viral lytic proteins such as the KSHV shutoff and exonuclease protein (SOX - ORF73) (Glaunsinger and Ganem, 2004) (discussed in section

6.2 of the introduction), and it has even been suggested that a long ncRNA expressed antisense to the latency-associated region during the lytic phase could act as a miRNA sponge to inhibit miRNA accumulation or function during lytic infection (Chandriani et al., 2010).

KSHV miRNAs are abundantly expressed, as they can represent from 30% to about 70% of all miRNAs in a cell (Gottwein et al., 2011; Lin et al., 2010). As suggested by Pfeffer and Voinnet, such high expression levels of viral miRNAs could saturate the processing machinery, leading to a global downregulation in the expression of cellular miRNAs, and thus to a perturbation of cellular homeostasis, which could be linked to oncogenesis (discussed in section 6.1 of the introduction). However, there seems to be enough room to accommodate a certain number of extra miRNA precursors, and that with some exceptions (e.g. adenovirus VA RNA), cellular miRNA levels do not seem to be globally affected by the expression of virus-encoded miRNAs.

Finally, even if most of the KSHV miRNAs are expressed from the same transcripts, their relative abundances are dramatically different (Gottwein et al., 2011; Umbach and Cullen, 2010). Deep sequencing analysis of the BC-3 PEL cell line reported miR-K12-4-3p and miR-K12-3 as the two most abundant KSHV miRNAs, with respectively 78% and 12% of the KSHV miRNA sequence reads obtained, whereas the nine other miRNAs (miR-12-9 being not expressed in BC-3) ranged from 2.4% to 0.19% of the total number of sequence reads (Umbach and Cullen, 2010). However, this study did not look specifically at the miRNAs ioncorporated into RISC, but the overall miRNA population present in the cells, and therefore these results cannot be definitely stated as representative of KSHV miRNAs activity. Indeed Gottwein *et al.*, in their PAR-CLiP study, observed a different distribution of the viral miRNAs loaded into Ago2. In BC-3 cells, miR-K12-4-3p was still the most represented, with this time 33% of all reads pertaining to KSHV miRNAs, and was followed by miR-K12-6-3p with 20% of the viral miRNA total (3.5% in Lin *et al.* study). On the other hand, miR-K12-3 was surprisingly the lowest in the number of sequence reads (0.17%) when compared to the other non-cited above non-star species, which accounted for up to 6.2% of all KSHV miRNA sequence reads (Gottwein et al., 2011). However, it is noteworthy that the number of reads for a given sequence (e.g. miRNA) obtained by deep sequencing is not always representative of the real absolute quantities of the sequences analysed. Indeed, it was recently demonstrated that some cloning bias could exist in the small RNA libraries generated, thus altering quantitation (Sorefan et al., 2012). Nevertheless, these results still remain good indicators of the discrepancies in the levels of expression of KSHV miRNAs, as when considering the star sequences of the miRNAs, their reads numbers are very low.

These different expression levels might be caused by distinct efficiencies of pre-miRNA processing, due for example to their more or less optimal hairpin structure, accessibility for the microprocessor complex within the cluster, secondary structures, or by additional cellular or viral factors involved in the processing or even in the stability of the mature miRNAs. It would be thus tempting to speculate that the different levels of expression of KSHV miRNAs could reflect their evolutionary apparition within the cluster as discussed in section 2.2.2, and therefore that they would be indicators of their functional relevance. However, other factors have to be taken in to account for such considerations, such as the fact that viral miRNAs are not under the same selection pressure compared to their cellular counterparts (see section 6.2.3), in addition to the fact that the different types of functional viral miRNAs (i.e. analogues of cellular miRNAs, or viral-specific miRNAs) are subjected to different evolutionary constraints. Therefore, to be able to establish a link between such discrepancies in the levels of expression of viral miRNAs and their biological functionality, a better comprehension of their targeting functions is required.

2. Function of KSHV miRNAs and their potential involvement in viral oncogenesis

The functions of KSHV miRNAs will not be extensively described in this section, as many such examples of them have been detailed in section 6 of the introduction. For a complete specific overview on the function of KSHV miRNAs please refer to (Ziegelbauer, 2011). Nonetheless, I briefly introduce in this section the problematic of my thesis subject, which is linked to the functions of KSHV miRNAs, and more specifically to their cellular targets that can be associated to viral oncogenicity.

As introduced in section 6, one of the major concerns in the study of viral miRNAs is their potential link with oncogenesis. Indeed, several evolutionary divergent viruses that encode miRNAs possess oncogenic properties, such as for example the polyomavirus SV40, the retrovirus BLV, the papilloma/polyoma-like virus BPCV, or herpesviruses including the alphaherpesvirus MDV, and the gammaherpesviruses EBV and KSHV.

When I started to work on my thesis subject in 2007, very little was known on viral miRNA targets, and only a limited number of targets had been identified. With the few data that were already available, viral miRNAs were already thought to play a role in establishing for the virus a favourable cellular environment for its replication. Taking into account the ability of herpesviruses to undergo latency, the oncogenic nature of KSHV, and the fact that its miRNAs are part of the latency-associated region, we hypothesised that some KSHV miRNAs would target genes involved in oncogenesis, such as: immune evasion, cell cycle progression and cellular proliferation, apoptosis, and cell survival. This idea was supported by a study published before the discovery of viral miRNAs in which the authors could show that overexpression in rat cells of a fragment of 4.4 kb that contained the region encoding for the KSHV miRNAs, was inducing tumour formation (Muralidhar et al., 1998). Since these early experiments, the relationship between viral miRNAs and oncogenesis has been nicely demonstrated *in vivo* for MDV miRNAs (detailed in section 6.3.5 of the introduction). In addition, the study on KSHV miRNA expression by O'Hara *et al.* (described in the above section) permitted them to infer from their results that the levels of KSHV miRNAs increased linearly with the degree of transformation of endothelial cells (O'Hara et al., 2009).

3. Cellular targets of viral miRNAs involved in the control of apoptosis and cell survival

The targets described in this section are listed in Table 6. Controlling cell death is a key feature in order to potently transform a cell. A first case of gene with pro-apoptotic properties, which seems to be an important target of herpesviruses miRNAs, is the Bcl2-associated transcription factor 1 (BclAF1). It was first reported as being regulated by KSHV miRNAs (Ziegelbauer et al., 2009), and later on, it was recently shown to be also targeted by HCMV and EBV miRNAs (Lee et al., 2012; Riley et al., 2012). The importance of this protein as a viral target is underlined by the fact that HCMV immediately directs its proteasomal degradation upon viral entry via two viral proteins, which are packaged within virions for rapid delivery into the cells on infection (Lee et al., 2012). Although this gene is categorised as a pro-apoptotic factor, its precise function, especially upon viral infection remains to be elucidated (reviewed in (Sarras et al., 2010)). Indeed, repressing this protein with viral miRNAs did not result in any significant effects on apoptosis protection, however, a sensitisation to lytic reactivation was shown (Ziegelbauer et al., 2009). In addition, Lee *et al.* have demonstrated a role for BclAF1 in inhibiting HCMV gene expression and replication (Lee et al., 2012). Nonetheless, other targets of viral miRNAs have readily shown to be involved in the inhibition of programmed cell death in infected cells. For example, the EBV-encoded BART miRNA cluster also represses a pro-apoptotic gene from the Bcl-2 family, Bim (Marquitz et al., 2011). Of note, miR-BART5 targets the p53 up-regulated modulator of apoptosis (PUMA), whose repression was shown to render EBV-infected cells less sensitive to proapoptotic agents (Choy et al., 2008). Abend *et al.* have also implicated a KSHV miRNA in the repression of the TNF-like weak inducer of apoptosis (TWEAK) receptor (TWEAKR). This regulation was demonstrated in endothelial cells expressing KSHV miR-12-10a, and resulted in a protection of IFN-γ stimulation to apoptosis. Also, the authors observed a decrease in the expression level of the proinflammatory IL-8, as well as the monocyte chemoattractant protein 1 (MCP-1) in response to TWEAK, thereby implying both direct and indirect regulative functions of this viral miRNA in apoptosis induction and of the immune response respectively. In addition, TWEAKR protein levels were shown to be lower in a lymph node biopsy sample from a KS-infected patient than in normal lymph node tissues (Abend et al., 2010). MDV miR-M3 represents another example of a functional role for viral miRNAs in the inhibition of pro-apoptotic signals, through its repression of SMAD2 expression, which is a component of the TGF-β signaling pathway. SMAD2 induction, upon TGF-β-stimulation mediates the expression of death-associated protein kinase (DAP-kinase), and this triggers mitochondrial-based pro-apoptotic events. MDV miR-M3 has thus been

shown through SMAD2 repression to inhibit cisplatin-induced apoptosis (Xu et al., 2011). Furthermore, genes involved in cell survival have also been described as targets of viral miRNAs, such as the transcriptional repressors BCL6 or BACH1 (Table 6). Targeting of such genes involved in cell survival/apoptosis by viral miRNAs makes perfect sense as they can be implicated in antiviral mechanisms via the intrinsic or extrinsic induction of apoptosis, respectively mediated by the cell itself or by extracellular signals (e.g. cytokines, death receptor activation by cells of the immune system).

4. KSHV miRNAs target Caspase 3 and regulate apoptosis

A candidate-based approach that followed prediction of KSHV miRNAs targets by microarray coupled to bioinformatics, allowed us to identify CASP3 as a KSHV miRNA target. Subsequently, we thus showed that this targeting was of biological relevance and that KSHV miRNAs targeting CASP3 were involved in an inhibition of drug-induced apoptosis. During the course of my thesis, the number of viral targets identified increased exponentially, and as we just saw in the previous section, some other viral miRNA targets involved in apoptosis or cell survival have been identified.

The results I obtained during my thesis were published in PLOS Pathogens in an article that is attached here. The latter is in great part the fruit of my work conducted under the supervision of Sébastien Pfeffer but this study also involved the collaboration of several teams and colleagues. The results presented from experiments performed by myself, are listed below.

- Figure 1B to E*
- Figure 2*
- Figure 4
- Figure 5
- Figure 6
- Figure 7**, ***
- Figure 8C***
- Figure S1
- Figure S6
- Figure S7
- Figure S8

* The results of Figure 1D and 2B were obtained with the help of Frédéric Gros.

** The cell extracts I assayed in Figure 7D were obtained by Johanna Viiliäinen.

*** The qRT-PCRs of Figure 7F and 8C were performed by Georg Malterer on cell extracts I prepared.

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Kaposi's Sarcoma Herpesvirus microRNAs Target Caspase 3 and Regulate Apoptosis

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Abstract

Kaposi's sarcoma herpesvirus (KSHV) encodes a cluster of twelve micro (mi)RNAs, which are abundantly expressed during both latent and lytic infection. Previous studies reported that KSHV is able to inhibit apoptosis during latent infection; we thus tested the involvement of viral miRNAs in this process. We found that both HEK293 epithelial cells and DG75 cells stably expressing KSHV miRNAs were protected from apoptosis. Potential cellular targets that were significantly down-regulated upon KSHV miRNAs expression were identified by microarray profiling. Among them, we validated by luciferase reporter assays, quantitative PCR and western blotting caspase 3 (Casp3), a critical factor for the control of apoptosis. Using site-directed mutagenesis, we found that three KSHV miRNAs, miR-K12-1, 3 and 4-3p, were responsible for the targeting of Casp3. Specific inhibition of these miRNAs in KSHV-infected cells resulted in increased expression levels of endogenous Casp3 and enhanced apoptosis. Altogether, our results suggest that KSHV miRNAs directly participate in the previously reported inhibition of apoptosis by the virus, and are thus likely to play a role in KSHV-induced oncogenesis.

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Introduction

The development of cancer is linked to six major hallmarks that explain how cells transgress from a normal to a neoplastic state, including (i) sustained proliferative signaling, (ii) evasion of growth suppression, (iii) activated invasion and metastasis, (iv) enabled replicative immortality, (v) induced angiogenesis and (vi) resistance to cell death [1]. There is ample evidence that programmed cell death or apoptosis functions as a barrier to cancer development (reviewed in [2]). Many different factors, including environmental ones, contribute to the origin and progression of cancer. For example, infection by microbial pathogens sometimes leads to tumor development. Several viruses have been recognized as causal agents of specific types of cancer, and up to 20% of all human cancers are associated with single or multiple viral infections. One such oncogenic virus is Kaposi's sarcoma-associated herpesvirus (KSHV), the primary etiological agent of Kaposi's sarcoma, which is a highly angiogenic tumor most probably arising from the endothelium and developing primarily in immunocompromised individuals. KSHV-infection is also associated with aggressive lymphomas such as primary effusion lymphoma and multicentric Castleman's disease [3]. Like many viruses, KSHV has been shown to inhibit apoptosis, and possesses a truly impressive arsenal to do so (reviewed in [4,5]).

Viruses have acquired an extraordinary capacity to evolve and adapt to their host, which translates into an incessant battle between the infected organism and the virus. One of the latest discoveries reflecting this continuous arms race is that certain mammalian viruses encode for miRNAs. In mammals, miRNAs constitute one of the most important classes of regulatory RNAs

Author Summary

MiRNAs are small, non-coding RNAs that regulate gene expression post-transcriptionally via binding to complementary sites in target mRNAs. This evolutionary conserved regulatory system is present in most eukaryotes, and it has recently been shown that certain viruses have evolved to express their own miRNAs. Due to their non-immunogenic nature, viral miRNAs represent an efficient tool for the virus to control its environment. Here we show that KSHV miRNAs are involved in the control of apoptosis both when expressed in stable cell lines and in the context of viral infection. Using a microarray based approach we identified putative cellular targets, among which the effector caspase 3 is targeted by three of the viral miRNAs. Finally, we showed that blocking these miRNAs in infected cells resulted both in increased Casp3 levels and a higher apoptosis rate. These findings indicate that miRNAs of viral origin are key players in cell death inhibition by KSHV.

[6,7]. Their biogenesis involves the processing of a large primary transcript into a stem-loop pre-miRNA, ultimately leading to the mature single stranded ~22 nt miRNA (reviewed in [7–11]). This functional miRNA is incorporated into an RNA-induced silencing complex (RISC) that invariably contains a member of the Argonaute protein family. Once loaded, the active RISC can be directed towards its messenger RNA target to regulate, predominantly negatively, its translation (see references [12,13] for review). The fact that target RNAs are frequently destabilized justifies the use of large-scale approaches to look at global changes in transcriptomic profiles as a way to identify miRNA targets [14]. To date, the vast majority of reported miRNA/mRNA interactions involve binding of the miRNA to the 3′ untranslated region (UTR) of the transcript through an imperfect base-pairing mechanism in which nucleotides 2 to 8 of the miRNA (the seed) appear to play an important role [15]. However, other types of interactions, such as binding in the coding sequence or in the 5′ UTR, or with bulges in the seed region, have also been reported [16–18].

The use of small non-coding RNAs such as miRNAs to regulate gene expression makes perfect sense for viruses, allowing them to modulate the cellular environment in a non-immunogenic manner [19]. The first virus-encoded miRNAs were identified in Epstein-Barr virus [20], and subsequent studies concluded that many herpesviruses, including Kaposi's sarcoma herpesvirus (KSHV) encode miRNAs (reviewed in [21]). KSHV has been shown to encode 12 miRNAs [22–25], which are clustered in the vicinity of the major KSHV latency transcript, K12. KSHV-miR-K12-1 to miR-K12-9, and miR-K12-11 are located in the intron of the larger kaposin transcript, while miR-K12-10 maps to the coding region, and miR-K12-12 resides within the 3′ UTR of the K12 coding sequence. Some cellular targets of KSHV miRNAs have been identified, mostly for miR-K12-11, which shares an identical seed sequence with the cellular miRNA miR-155 [26,27].

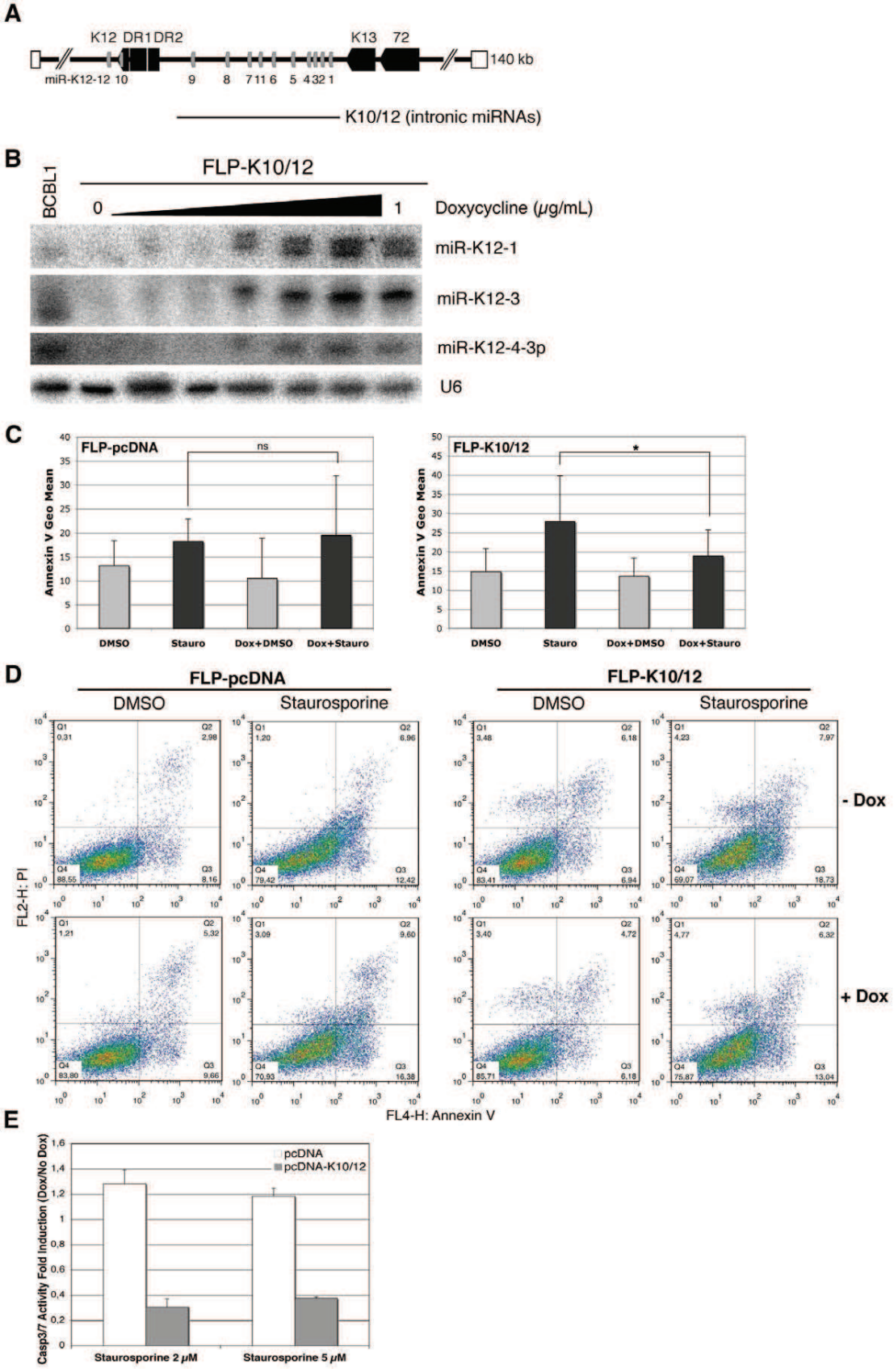
Here, we show that KSHV miRNAs also contribute to the inhibition of apoptosis in infected cells. We show that cell lines expressing KSHV miRNAs are less sensitive to both caspase-dependent and -independent apoptosis induction by staurosporine or etoposide. Using a microarray approach, we identified caspase 3 (Casp3) as a target of some of these viral miRNAs. Casp3 is a well-known effector caspase (reviewed in [28]) that is critical for apoptosis induction. Using site-directed mutagenesis, we found that KSHV miR-K12-1, K12-3 and K12-4-3p are responsible for Casp3 regulation. Finally, by blocking the function of these miRNAs in infected cells, we showed that both Casp3 levels and apoptosis were increased.

Results

Cell lines expressing KSHV miRNAs are less sensitive to apoptosis

We generated inducible HEK293 cells (FLP-293) expressing the intronic KSHV miRNAs under a doxycycline-inducible CMV promoter. To this end, the sequence spanning the ten intronic miRNAs miR-K12-1 to 9 and miR-K12-11 (K10/12) (Figure 1A) was inserted into the pcDNA5/FRT/TO plasmid, and used to transfect Flp-In T-Rex-293 cells. Stable cell lines were obtained by hygromycin selection, and subsequently named FLP-K10/12. As a negative control, we generated stable cells transfected with a pcDNA5 plasmid with no insert, that we then named FLP-pcDNA. We verified by northern blot analysis that doxycycline treatment readily induced the expression of the miRNAs to level similar to that found in the KSHV-infected BCBL-1 cells [29] (Figure 1B). In all following experiments, we used a final concentration of doxycycline of 1 μg/mL. We also measured by northern blot analysis the level of KSHV miRNAs expression in the induced FLP-K10/12 cells and compared it to KSHV-infected BCBL-1 and BC3 cells [30]. We found that expression of the miRNAs was slightly higher than in BCBL-1, but lower than in BC-3 cells (Figure S1), suggesting expression close to physiological levels. To assess the effect of KSHV miRNA expression on apoptosis, we first grew the FLP-pcDNA and -K10/12 cell lines in the presence of doxycycline to induce expression of the viral miRNAs, and then treated them for 8 h with 2 μM of staurosporine, a well-described inducer of apoptosis [31], or DMSO as a control. To measure the effect of this treatment on apoptosis we used Annexin V binding assay, which allows quantification of the level of phosphatidylserine exposure at the outer membrane side, a well characterized event of early apoptosis [32]. In addition, cells were labeled with propidium iodide (PI), staining both apoptotic and necrotic cells. Statistical analysis of six independent cell-sorting experiments revealed that Annexin V binding levels following staurosporine treatment were not significantly different in the presence or absence of doxycycline for the control FLP cell line (Figure 1C). In contrast, concerning the FLP-K10/12 cell line, a statistically significant decrease in Annexin V levels after staurosporine treatment was observed following doxycycline-induced expression of the microRNAs (Figure 1C). Figure 1D shows one representative experiment of the six biological replicates. In order to get an independent measure of apoptosis, we monitored the activity of effector caspases using a DEVD-aminoluciferin substrate for Casp3 and Casp7 that is measurable by a luciferase assay. As shown in Figure 1E, the luminescent Casp3/7 activity induced by 2 or 5 μM of staurosporine treatment of the stable FLP-K10/12 cells was sharply decreased (2.5 to 3 times) upon doxycycline induction of the KSHV miRNAs expression, while it remained unchanged in the control FLP-pcDNA cells.

To monitor the effect of KSHV miRNAs on apoptosis in a cell line more physiologically relevant for KSHV infection, we used the previously described DG-75-K10/12 cells -a Burkitt lymphoma cell line [33,34] lentivirally transduced with a construct expressing KSHV intronic miRNAs [35]-, and measured the effect of KSHV miRNAs expression on apoptosis in either the DG-75-K10/12 cells or the DG-75-EGFP control cells. Statistical analysis of four independent experiments confirmed that staurosporine treatment readily induced phosphatidylserine exposure in the


A
K12 DR1 DR2
K13 72
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miR-K12-12 10 9 8 7 11 6 5 4 3 2 1
K10/12 (intronic miRNAs)
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miR-K12-1
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Staurosporine
- Dox
+ Dox
FL2-H: PI
FL4-H: Annexin V
E
pcDNA
pcDNA-K10/12
Casp3/7 Activity Fold Induction (Dox/No Dox)
Staurosporine 2 μM
Staurosporine 5 μM

Figure 1. HEK293 cells expressing KSHV miRNAs are less sensitive to apoptosis. A. Schematic representation of KSHV miRNA genomic localization, and of the K10/12 construct that was used for their expression. **B.** Northern blot analysis of inducible FLP-K10/12 cells. Cells were grown for 48h with increasing concentration of doxycycline (0 to 1 μg/mL); a concentration of 1 μg/mL was used in the following experiments. KSHV latently infected BCBL-1 cells were used as a positive control. **C.** Statistical analysis of apoptosis induction measured by Annexin V binding assay in FLP-pcDNA control cell line (left panel) or FLP-K10/12 (right panel) grown continuously in doxycycline-containing medium, and treated with DMSO or staurosporine for 8 h. Error bars represent the standard deviation observed for 6 biological replicates; a significant difference ($p = 0.0306$) of apoptosis induction is observed between the non-treated and doxycycline-treated K10/12 expressing cell lines, but not for the pcDNA cell line. **D.** Dot plot examples of a representative FACS analysis of annexin V and propidium iodide (PI) levels in FLP-pcDNA (left panel) or FLP-K10/12 cells (right panel). **E.** The same cells treated for 8 h with DMSO or 2 and 5 μM staurosporine, were assayed for Casp3/7 activity after addition of a luminescent substrate for the caspases, and normalized to the total protein content. The ratio between doxycycline-treated and non-treated cells is given.
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control cell line, but that this induction was significantly reduced in the K10/12-expressing cells (Figure 2A). A representative experiment of the biological replicates (Figure 2B) shows that the percentage of Annexin V positive cells dropped almost two-fold in DG-75-K10/12 cells *vs.* DG-75-EGFP cells after 8 h of staurosporine treatment. As opposed to the FLP-293 cells, we were unable to induce Casp3/7 activity with staurosporine in the DG75 cells (data not shown). In line with this observation, it has been reported previously that in this particular cell line, the apoptotic protease-activating factor 1 (APAF-1) was sequestered at the plasma membrane, which prevents caspase activation [36].

Microarray analysis of KSHV miRNAs-expressing cell lines

In order to identify putative cellular targets of KSHV miRNAs involved in the miRNA-induced anti-apoptotic phenotype, we used a microarray approach on the two main cell types that are infected *in vivo* by KSHV: endothelial cells and B lymphocytes. In addition to the already described DG-75-K10/12 and DG-75-EGFP cells, we also generated by lentiviral transduction endothelial cells EA.hy926 [37] expressing the K10/12 construct or EGFP as a control. In order to determine the relative expression of KSHV miRNAs were expressed in the DG-75-K10/12 cells, we cloned the small RNA population of these cells and analyzed it by Solexa deep-sequencing. As can be seen in Table S1, KSHV miRNAs represented more than 18% of the total miRNAs in this cell line, which is slightly less than what has been previously described for BCBL1 cells ([38] and data not shown). All intronic miRNAs accumulated to measurable levels with the exception of miR-K12-11, which seemed to be expressed at a low level. We then measured by qRT-PCR the levels of some KSHV miRNAs expression in EA.hy926 and DG-75-K10/12 cells compared to BCBL-1 cells (Table S2). The levels of viral miRNAs expression in both cell lines correlated very well ($r = 0,93$) (Figure S2).

DG-75 and EA.hy926 EGFP control- and miRNA- expressing cell lines were analyzed in triplicate on Affymetrix Human Genome U133 Plus 2.0 microarrays. The clustering of the gene expression profiles primarily correlated with the cell line (DG-75 *vs.* EA.hy926), but also within each cell line with the expression of KSHV miRNAs (Figure S3). In addition, the changes in gene expression levels following the KSHV miRNAs transduction correlated weakly ($r = 0.19$) but significantly ($p<10^{-15}$ at Pearson's test) between the two cell lines (Figure S4).

Target recognition by miRNAs involves a number of determinants, the most important of which appears to be perfect base-pairing of nucleotides 2–7 of the miRNA (the seed), together with either an adenosine opposite miRNA nucleotide 1, or an additional base pair involving the 8th nucleotide of the miRNA [15]. In single miRNA transfection experiments one typically observes that the mRNAs that carry matches to the transfected miRNA are significantly down-regulated in response to transfection compared to mRNAs that do not carry such matches. To determine whether the KSHV miRNAs significantly influenced gene expression levels in a complex experiment such as ours, in which multiple miRNAs are simultaneously induced, we designed the following test. We first computed a KSHV miRNA sensitivity score for each mRNA, defined as the sum over all KSHV miRNAs, the number of matches of the 3′ UTR to the seed of the KSHV miRNA multiplied by the relative abundance of the KSHV miRNA. The relative abundances of the KSHV miRNAs were determined using the DG-75-K10/12 small RNAs deep-sequencing data. The KSHV miRNA sensitivity scores are reported in Dataset S1. We then compared the change in expression level of the 1000 mRNAs with highest KSHV miRNA sensitivity score and of mRNAs with no seed matches to the KSHV miRNAs in the 3′ UTR and found that the KSHV miRNA sensitive mRNAs were significantly down-regulated in both KSHV miRNA expressing DG-75 and EA.hy926 cells ($p<10^{-3}$ and $p<10^{-15}$, respectively in Wilcoxon's rank sum test). We observed however, that the 3′ UTRs of the 1000 mRNAs with highest KSHV sensitivity were on average ten times longer than the 3′ UTRs with no seed matches (Figure S5). To test whether differences in 3′ UTR length alone could account for the down-regulation of the KSHV sensitive mRNAs, we computed the average fold change of 1000 mRNAs sampled in such way that their 3′ UTR length distribution was the same as that of the KSHV sensitive mRNAs (Figure 3A, blue bars). We repeated this procedure 1000 times and found that the set of 1000 KSHV sensitive mRNAs still exhibited a stronger down-regulation compared to mRNAs of similar 3′ UTR length (Figure 3A, red bars) ($p = 0.036$ and 0.002, respectively for the expression changes computed from the DG-75 and EA.hy926 samples). Therefore, the 3′ UTR length alone cannot explain the magnitude of down-regulation of the most KSHV sensitive mRNAs in response to KSHV miRNA expression. These results indicated that KSHV miRNAs exert a detectable effect on mRNA expression in these cell lines and motivated us to proceed with further characterization of candidate direct targets.

As KSHV putative direct targets we extracted transcripts that were significantly down-regulated significantly in the replicate experiments, and which contained at least one seed-match to one of the KSHV miRNAs. We identified 704 putative direct targets in DG-75 cells (Figure 3B), and 980 putative direct targets in EA.hy926 cells (Figure 3C). A complete list of putative direct targets can be found in Dataset S2 for DG-75 cells and in Dataset S3 for EA.hy926 cells. The overlap between the two datasets contained 153 putative direct targets (Dataset S4).

Validation of putative KSHV miRNA targets

In order to validate direct cellular targets of KSHV miRNAs, we turned to classical reporter assays in HEK293 cells (293A cells). We chose, among genes involved in pathways such as cell cycle regulation, DNA damage repair, and apoptosis, a subset of the 3′ UTR sequences identified as putative direct targets by our previous analysis. These candidates were then cloned 3′ to the firefly luciferase gene in the dual-reporter vector psiCHECK-2, also encoding a *Renilla* luciferase as a standard. We cloned and

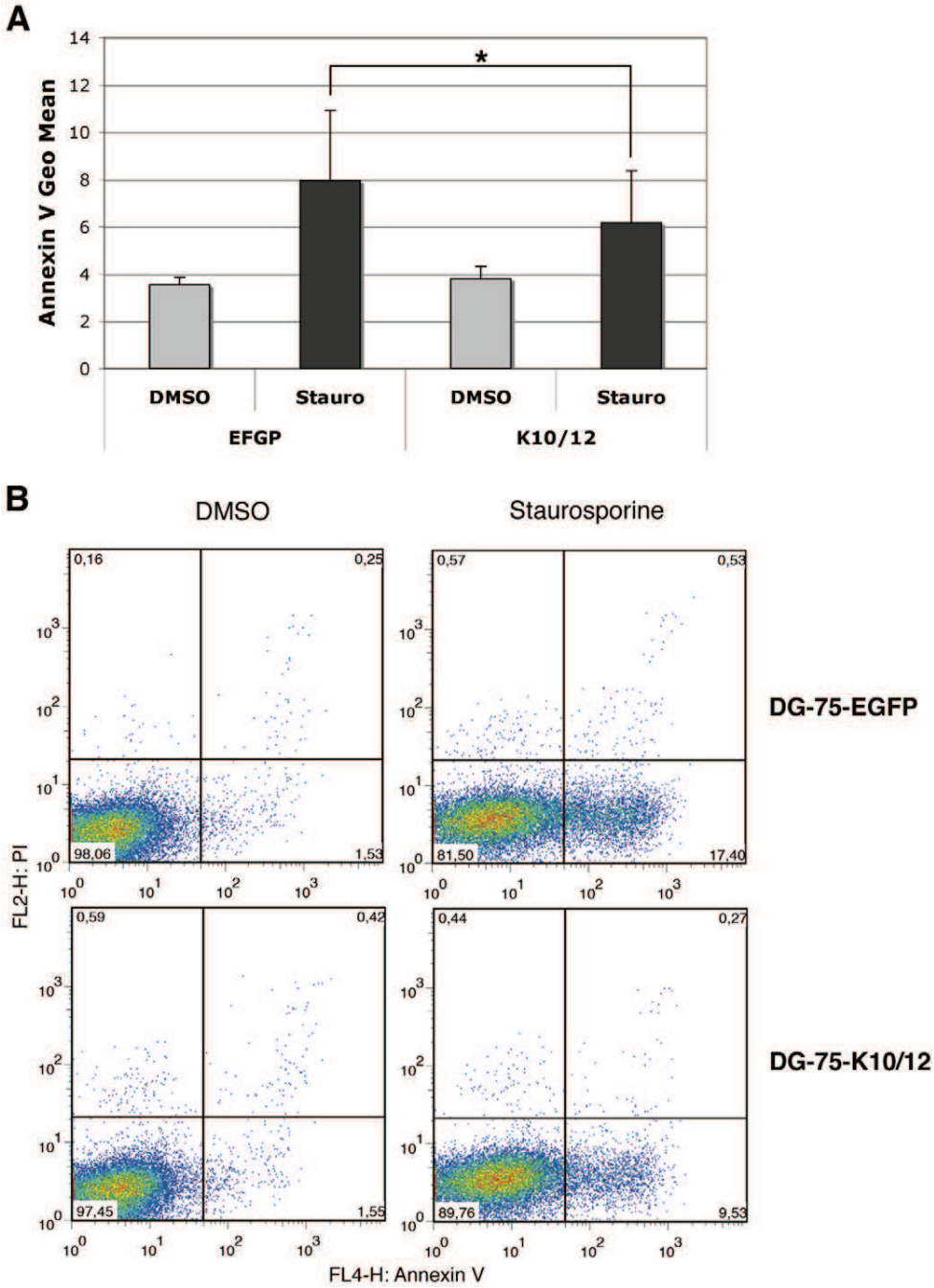

Figure 2. DG-75 cells expressing KSHV miRNAs are less sensitive to apoptosis. A. Statistical analysis of apoptosis induction measured by Annexin V binding assay in stable DG-75-EGFP cells as a control or DG-75-K10/12, treated for 8 h with DMSO or staurosporine. Error bars represent the standard deviation observed for 4 biological replicates; a significant difference of apoptosis induction ($p = 0.0355$) is observed between the EGFP and the K10/12 expressing cell lines. **B.** Dot plot examples of a representative FACS analysis of annexin V and propidium iodide (PI) levels in EGFP (upper panels), or K10/12 DG-75 cells (lower panels).
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tested the full length 3′ UTR of sixteen candidate targets, which were tested in multiple independent assays. We first assessed that the K10/12 construct could repress the activity of luciferase sensors containing bulged complementary sequence (with a bulge at positions 9 to 12) to some of the KSHV miRNAs. For all of the KSHV miRNAs tested, except miR-K12-9, we could show a strong repression in the presence of pcDNA-K10/12 (Figure 4A). The lack of miR-K12-9 activity could relate to its lower expression in the context of the K10/12 construct (Tables S1 and S2). As opposed to what would have been expected based on the DG-75-K10/12 small RNA sequencing data, miR-K12-11 appeared to be functional in the FLP-K10/12, and we confirmed that it accumulated in higher amounts in these cells compared to the DG-75-K10/12 cells (data not shown). As a positive control

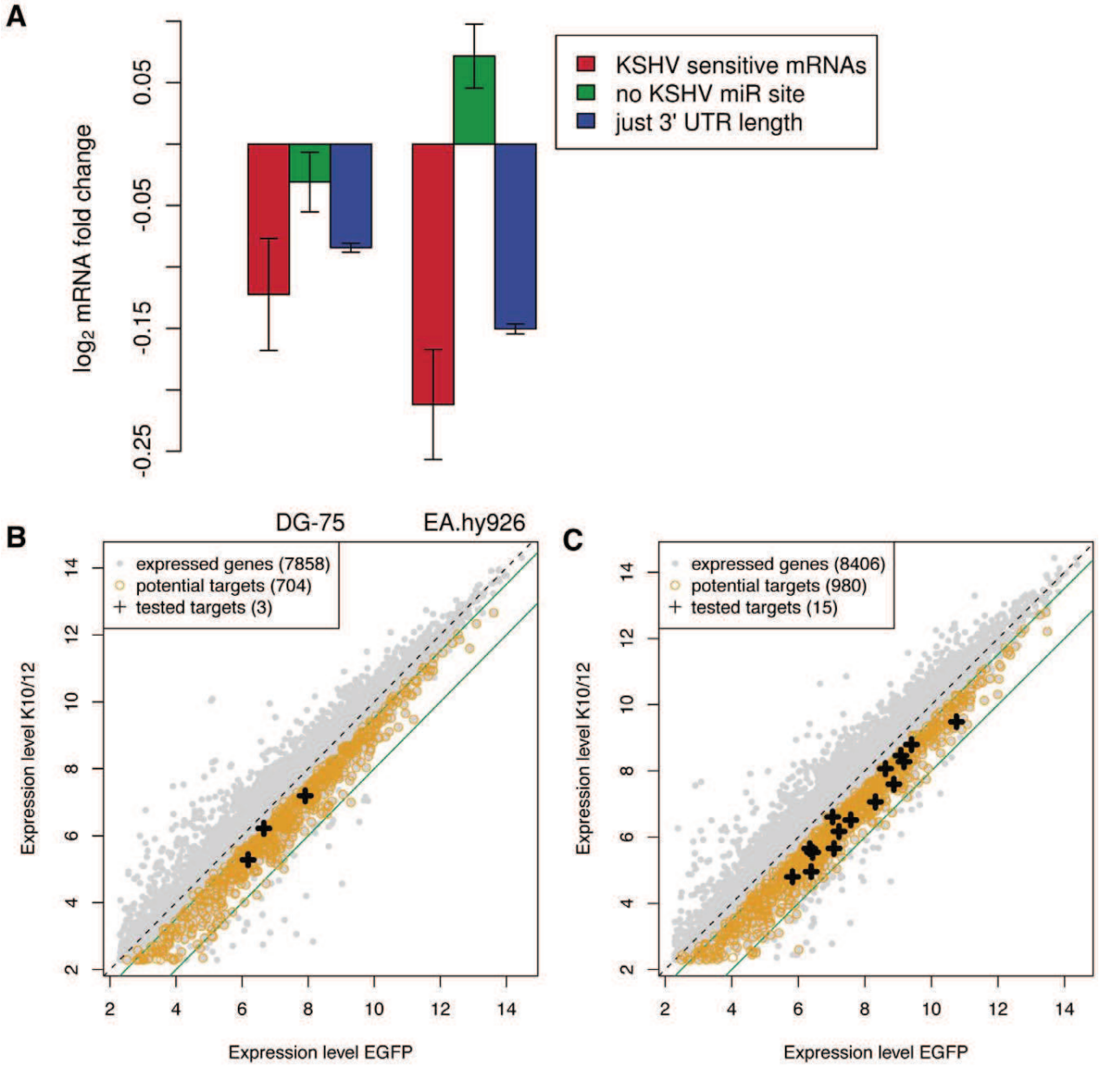

Figure 3. Microarray analysis of KSHV miRNAs expressing cell lines. A. Changes in expression levels of KSHV miRNA sensitive mRNAs, mRNAs without KSHV miRNA seed matches in their 3′ UTR, and randomized sets of genes with the same 3′ UTR length distribution as KSHV miRNA sensitive mRNAs. The analysis was performed separately in DG-75 and EA.hy926 cells. Error bars represent 95% confidence interval on the mean fold change in gene expression upon transducing the KSHV miRNAs. Dot plot representation of the changes in gene expression observed in the DG-75 cells (**B**), and in the Ea.hy926 cells (**C**). The potential targets (black crosses) tested by luciferase assay were selected among the genes down-regulated 1.4 to 4 fold (indicated by the green lines).
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for the luciferase assays with the selected putative targets, we used SPP1, a previously validated target of KSHV miRNAs [39]. The validation assays showed that only a subset of the 3′ UTRs tested resulted in a measurable repression of luciferase activity (Figure 4B). Among all the tested candidates, we observed the most important and reproducible down-regulation for two genes, Rad51AP1, involved in DNA damage repair, and Casp3, one of the main effectors involved in apoptosis induction. The RAD51AP1 reporter showed a down-regulation of 30 to 40% across luciferase experiments, while the Casp3 reporter showed a down-regulation of 40 to 50% (Figure 4B). We thus hypothesized that the anti-apoptotic phenotype of KSHV miRNA-expressing cells could be in part caused by the regulation of Casp3, and decided to continue this study by focusing on this protein.

Targeting of Casp3 by miR-K12-1, K12-3 and K12-4-3p

The initial analysis of Casp3 3′ UTR revealed 8mer or 7mer seed-matches [15] for miR-K12-4-3p (one M8A1 site), miR-K12-1 (two M8 sites), and miR-K12-3 (one A1 site). In addition, 6mer seed-matches to miR-K12-1, miR-K12-2 and miR-K12-10a could

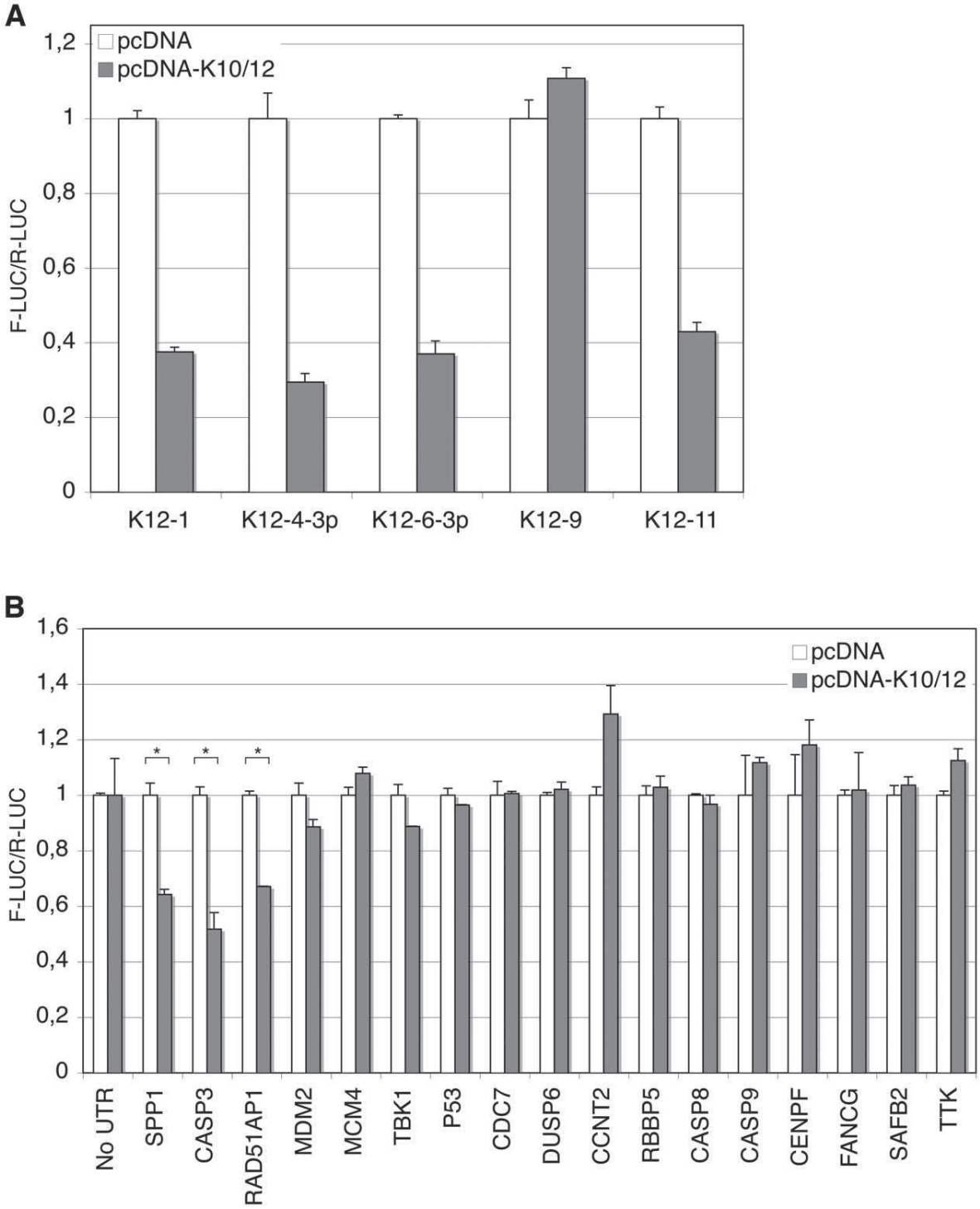

Figure 4. Validation of putative targets of KSHV miRNAs by luciferase assays. Luciferase assays were performed in triplicate 48 h post-transfection. The experiments were repeated at least 3 times, and one representative experiment is shown. **A.** KSHV miRNAs expressed from the pcDNA-K10/12 plasmid, but not miR-K12-9, can regulate the expression of sensor constructs containing complementary sequence to individual KSHV miRNAs. All differences but for miR-K12-9 were statistically significant ($p<0.01$). **B.** Dual-luciferase assay with psiCHECK-2 constructs containing either no UTR, or the indicated 3′ UTR. 293A cells were co-transfected with the luciferase construct and an empty pcDNA, or pcDNA-K10/12 construct. (* $p<0.01$).

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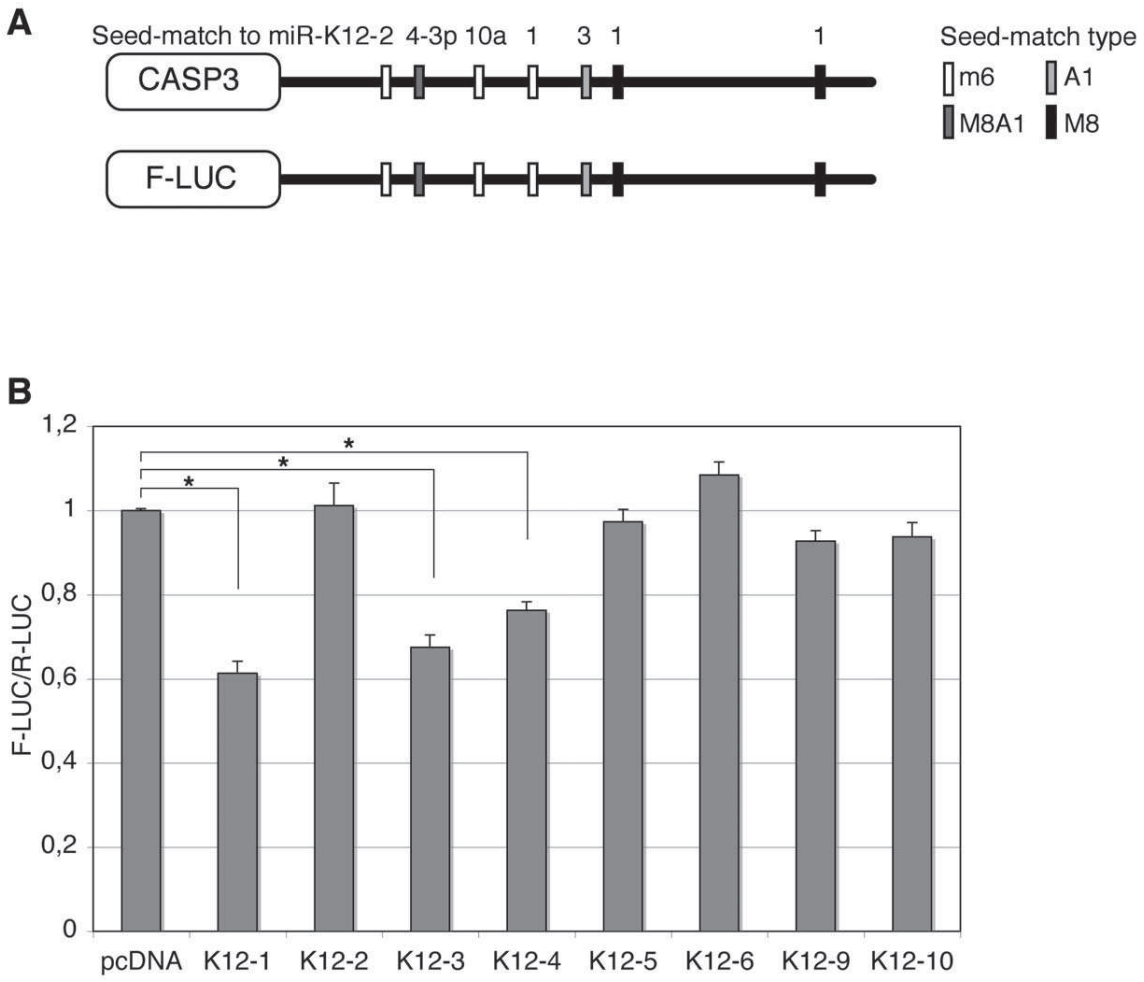

Figure 5. Caspase 3 is targeted by several KSHV miRNAs. Luciferase assays were performed multiple times in triplicate 48 h post-transfection. **A.** Schematic representation of Casp3 3′ UTR showing potential seed-matches for KSHV miRNAs. The seed-match types are described in the text. **B.** Dual luciferase assays performed in 293A cells with the Casp3 luciferase sensor and pcDNA constructs expressing the indicated individual miRNAs. Luciferase ratios relative to empty psiCHECK-2 set to 1 are displayed. (* $p<0.01$).
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be found (Figure 5A). In order to further identify regions of the Casp3 3′ UTR that were susceptible to regulation by KSHV miRNAs, we subdivided the 3′ UTR in three parts and cloned them in the reporter vector. None of the tested fragments showed such a strong repression as the full-length sequence, suggesting that all putative miRNA binding sites are required for efficient repression, or that the binding sites function optimally only in their natural context (Figure S6). We then transfected pcDNA constructs expressing individual miRNAs (miR-K12-1 to -6, K12-9 and K12-10) to identify whether a single, or multiple miRNAs, mediated Casp3 regulation. We found that as suggested by the seed-matches quality, miR-K12-1, K12-3 and K12-4-3p (in decreasing order of repression observed) were able to significantly regulate the expression of the reporter fused to the 3′ UTR of Casp3 (Figure 5B). Expression of miR-K12-2 and K12-10, or of miRNAs with no predicted seed-matches (miR-K12-5, K12-6 and K12-9) had no effect on the Casp3 sensor.

Subsequently, we aimed at determining which of the five putative binding sites for miR-K12-1, K12-3 and K12-4-3p were most important for Casp3 downregulation. To this end, we mutagenized each individual seed-match by introducing three point mutations to disrupt miRNA binding in the luciferase sensor containing Casp3 3′ UTR (Figure 6A). The resulting luciferase reporters were tested with miRNA expression constructs for either the 10 intronic miRNAs, or the individual miR-K12-1, K12-3 and K12-4-3p. As shown in Figure 6B, only the 3′ proximal binding site for miR-K12-1 appears to be functional, as the Casp3 Mut K12-1 3′ luciferase sensor could not be regulated by the pcDNA-K10/12 or the pcDNA-K12-1 constructs. The binding site for miR-K12-3 was also validated, as the mutant luciferase sensor for this miRNA is not regulated by the pcDNA-K10/12 or the pcDNA-K12-3 construct (Figure 6C). Finally, the binding site for miR-K12-4-3p was validated, although it seems to be less potent than the two others in terms of luciferase regulation (Figure 6D). In conclusion, we showed that Casp3 3′ UTR is regulated *via* three binding sites for (from 5′ to 3′) miR-K12-4-3p, K12-3 and K12-1. The positions of these sites explain why the luciferase assay done with the Casp3 3′ UTR fragments (Figure S6) did not reveal obvious differences as each individual fragment contained one of the three validated sites.

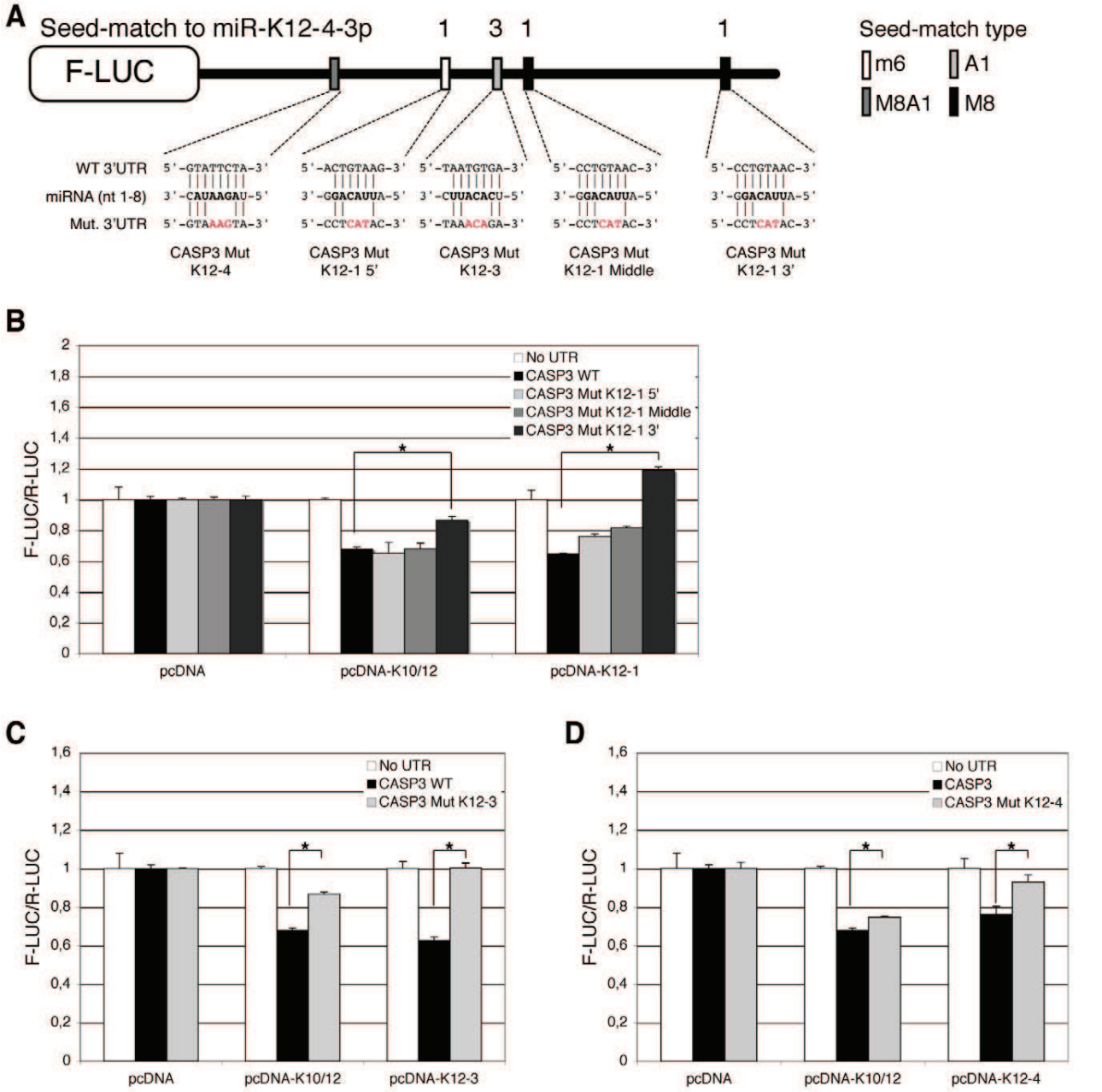

Figure 6. Identification of KSHV miRNAs binding sites in the 3′ UTR of Casp3 transcript by mutational analysis. Luciferase assays were performed multiple times in triplicate, 48 h post-transfection. **A.** Schematic representation of Casp3 luciferase sensor and of the mutagenesis performed within the potential binding sites of miR-K12-1, miR-K12-3 and miR-K12-4-3p. A mutant was generated for each potential miRNA binding site. Dual luciferase assays were performed with the Casp3 luciferase wild type (WT) or mutant sensors and pcDNA constructs expressing either the K10/12 construct or the individual miRNA miR-K12-1 (**B**), K12-3 (**C**) or K12-4-3p (**D**). Luciferase ratios relative to empty psiCHECK-2 set to 1 are displayed. (* $p<0.01$).
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KSHV miRNAs decrease endogenous Casp3 levels

In order to measure the effect of KSHV miRNAs on endogenous Casp3, we first performed real-time quantitative PCR analysis of 293A cells following primary infection and antibiotic selection of rKSHV infected cells [40]. We found that the level of Casp3 transcript decreased two fold following infection (Figure 7A, left panel). We also measured the level of Casp3 mRNA in the doxycycline-inducible FLP cells, and observed a similar down-regulation upon induction in FLP-K10/12 cells, but not in control FLP-pcDNA cells (Figure 7A, right panel). We then measured Casp3 protein levels in FLP-K10/12 and DG-75-K10/12 cells, and observed a significant down-regulation in three independent experiments (average of 0.63-fold, $p = 0.0005$ and 0.69-fold, $p = 0.0046$ respectively) (Figure 7B and C). We then turned to HUVEC endothelial cells, one of the two main cellular types infected *in vivo* by KSHV, and

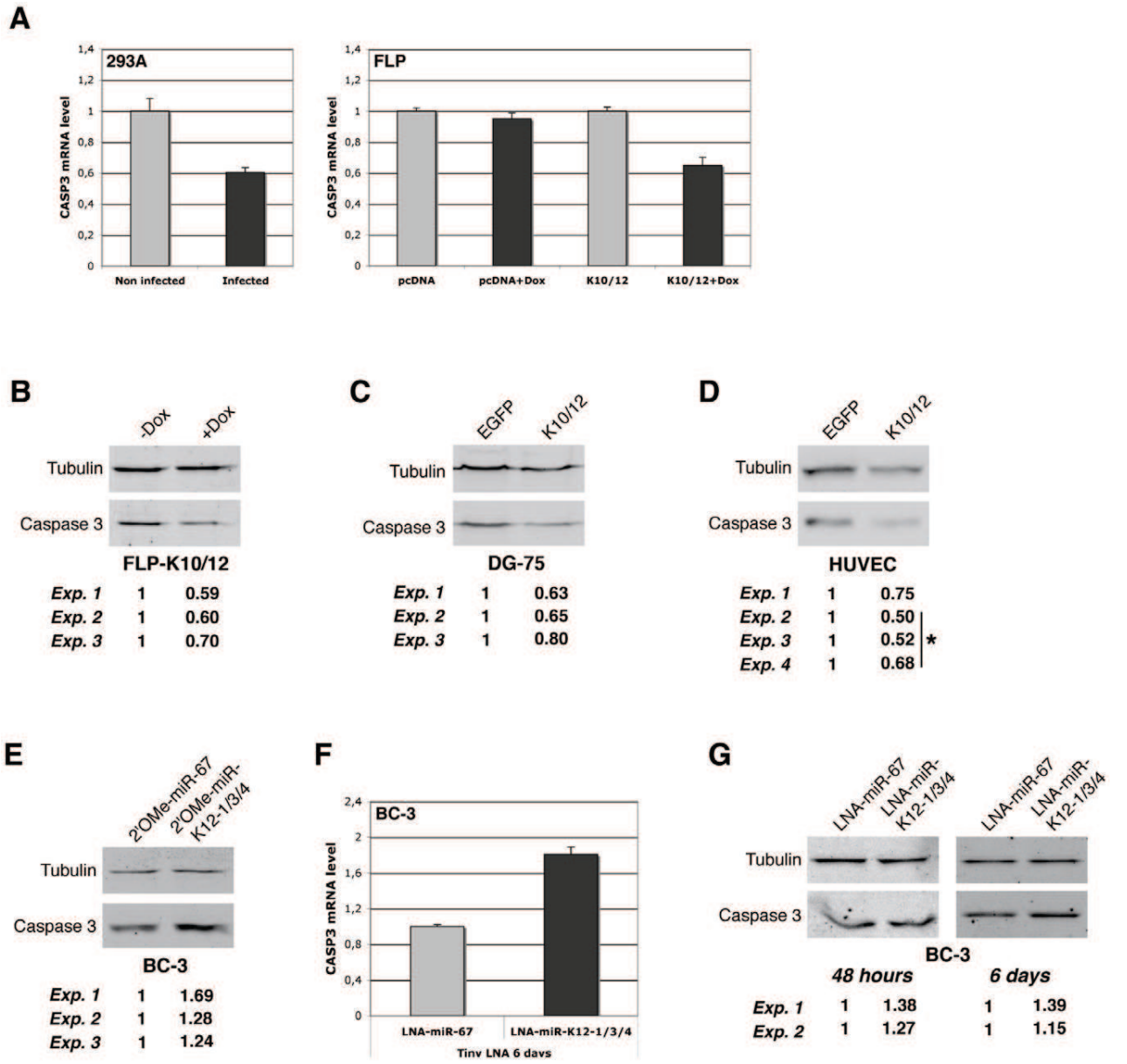

Figure 7. Endogenous Casp3 is regulated by KSHV miRNAs in different cell lines. A. qRT-PCR analysis of Casp3 mRNA expression in non-infected *vs. de novo* KSHV-infected HEK293 cells (left panel), and in inducible FLP-pcDNA or FLP-K10/12 cells (right panel), by comparing the non-treated *vs.* doxycycline-treated conditions. Error bars represent the standard deviation observed for 3 technical replicates. **B.** Western blot analysis and signal quantification from three independent experiments for Casp3 and Tubulin on the inducible FLP-K10/12 cell line, non-doxycycline-treated *vs.* doxycycline-treated conditions, and **C.** DG-75 cells expressing either EGFP or the K10/12 miRNA cluster. **D.** Western blot analysis and signal quantification from four independent experiments for Casp3 and Tubulin on primary or E6/E7 HUVEC cells stably expressing either EGFP or the K10/12 miRNA cluster after lentiviral transduction and antibiotic selection. *indicates experiments done in E6/E7 HUVEC cells. **E.** Western blot analysis and signal quantification from three independent experiments for Casp3 and Tubulin on KSHV miRNA inhibited-BC-3 cells. Cells were transfected with a 2′-O-methylated oligonucleotide antisense to the control cel-miR-67 (2′OMe-miR-67), or with a mix of oligonucleotides antisense to miR-K12-1, K12-3, and K12-4-3p (2′OMe-miR-K12-1/3/4) at the same final concentration, and harvested 48 h later. **F.** qRT-PCR analysis of Casp3 mRNA expression in KSHV miRNAs inhibited-BC-3 cells by tiny LNAs treatment. Cells were incubated for 6 days with 8mer LNA-oligonucleotides antisense to the seed region of the control miR-67 (LNA-miR-67), or with a cocktail of oligonucleotides antisense to miR-K12-1, -3, and 4-3p (LNA-miR-K12-1/3/4) at the same final concentration. **G.** Western blot analysis and signal quantification from two independent experiments for Casp3 and Tubulin on the same cells treated with the indicated tiny LNAs for 48 h (left panel) or 6 days (right panel).
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performed western blot analysis of primary or E6/E7 HUVEC cells stably transduced with either the EGFP, or the K10/12 lentiviral construct. In four independent experiments, the level of Casp3 protein was significantly down-regulated in K10/12 cells compared to the control EGFP cells (average of 0.61-fold, $p = 0.0007$) (Figure 7D).

In order to assess whether the down-regulation of Casp3 in naturally KSHV infected cells was caused by the specific presence of the three previously identified miRNAs, we used an antisense approach to inhibit specifically miR-K12-1, K12-3 and K12-4-3p. We thus employed either classical full-length 2′-O-methylated (2′OMe) antisense oligoribonucleotides [41], or short Locked Nucleic Acid oligonucleotides directed only against the seed of each individual miRNAs (tiny LNAs) [42]. In three independent experiments, transfection of a cocktail of 2′OMe oligonucleotides against miR-K12-1, K12-3 and K12-4-3p (2′OMe-miR-K12-1/3/4) in BC-3 cells resulted in a modest but measurable increase of Casp3 protein level compared to a control 2′OMe oligonucleotide (2′OMe-miR-67) (1.4-fold on average, $p = 0.0486$) (Figure 7E). The advantage of using tiny LNAs to inhibit miRNA function over the 2′OMe oligonucleotides is based on the fact that they do not require transfection to enter the cells. We therefore tested the inhibition efficiency of tiny LNAs on luciferase sensors in HEK293 cells and found that they could readily revert the targeted miRNA regulation (Figure S7). BC-3 cells grown in a medium containing a cocktail of tiny LNAs each directed against one of the three KSHV miRNAs listed above (LNA-miR-K12-1/3/4) also showed an 1.8-fold increase in Casp3 mRNA (Figure 7F) accompanied with a somewhat milder increase in the protein levels compared to control tiny LNA (LNA-miR-67) (1.3-fold on average, $p = 0.0018$) (Figure 7G; left panel for 48 h, and right panel for 6 days). Taking these results altogether, we can definitely conclude that Casp3 is regulated at both mRNA and protein levels by the KSHV-encoded miR-K12-1, K12-3, and K12-4-3p.

Inhibition of KSHV miRNAs reduce apoptosis in infected cells

In order to test the biological relevance of the repression of Casp3 by these KSHV-encoded miRNAs, we decided to look at Casp3 cleavage or its direct and indirect endogenous cleavage substrates, such as respectively Poly[ADP-ribose] polymerase-1 (PARP-1) or genomic DNA. We thus treated BC-3 cells with a cocktail of tiny LNAs directed against the three Casp3-targeting viral miRNAs, and measured PARP-1 cleavage following staurosporine treatment for 8 h. In the absence of staurosporine, inhibition of KSHV miRNAs had no or little effect on PARP-1 levels (Figure S8, left panel). Upon treatment, we found that cells pre-treated with anti-KSHV specific tiny LNAs (LNA-miR-K12-1/3/4), but not with the control tiny LNA (LNA-miR-67), accumulated slightly more of the PARP-1 cleavage product (Figure S8, right panel). We also tested the effect of this inhibition using KSHV-infected immortalized lymphatic endothelial cells (iLECs) by measuring the appearance of cleaved Casp3 and the extent of apoptosis-induced genomic DNA nicks following a 24 h etoposide treatment. iLECs represent one of the most relevant cell types implicated in KSHV pathogenesis [43]. We observed an increase in the number of cleaved Casp3 positive cells (Figure 8A) and TdT-mediated dUTP nick end labeling (TUNEL) positive cells (Figure 8B) over mock-treated (DMSO) controls when miR-K12-1, K12-3 and K12-4-3p were inhibited with the tiny LNA cocktail (LNA-miR-K12-1/3/4), over the control. In three independent experiments, the mean fold induction of etoposide-induced TUNEL positive cells (over the DMSO treated control) following inhibition of miRNAs (LNA-miR-K12-1/3/4) was significantly greater (2.30-fold, $p = 0.041$) than in cells treated with the control tiny LNA (Figure 8C). These data suggests that the KSHV-encoded miR-K12-1, K12-3 and K12-4-3p contribute to protection of etoposide-induced apoptosis in KSHV infected iLECs.

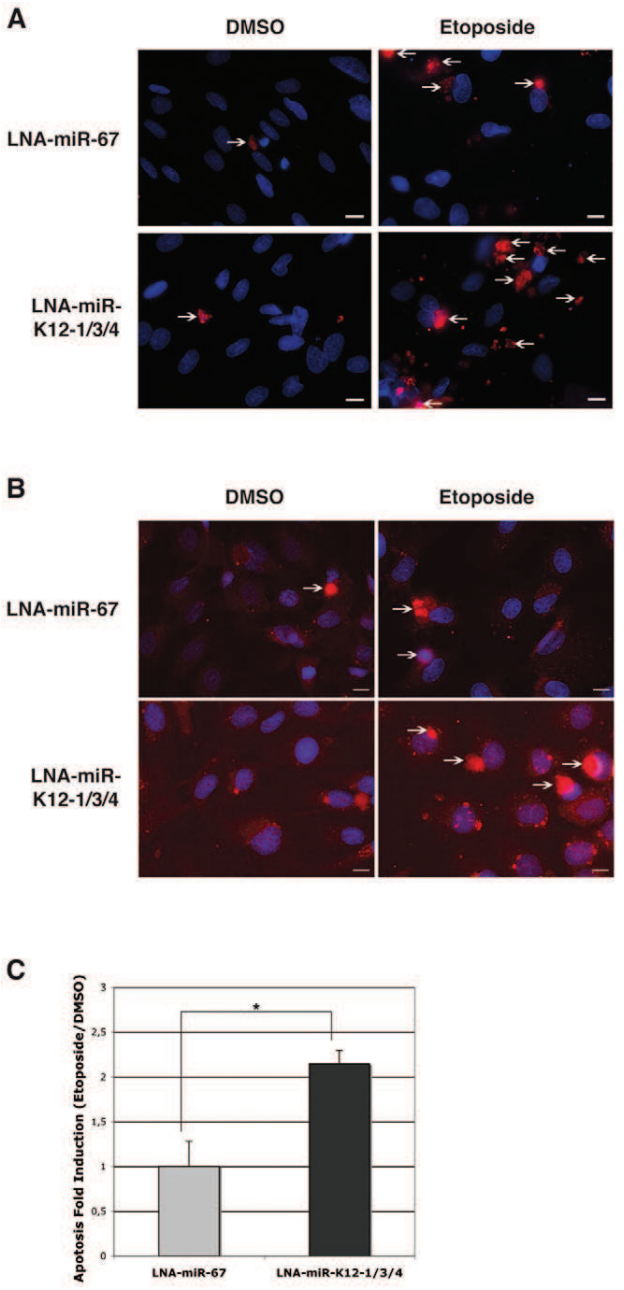

Figure 8. KSHV miRNAs inhibit apoptosis in KSHV-infected endothelial cells. A. Microscopic analysis following DAPI (blue) and cleaved Casp3 (red) staining of K-iLEC cells treated with DMSO or etoposide, and after KSHV miRNA inhibition by tiny LNA (LNA-miR-K12-1/3/4) or the control (LNA-miR-67). Arrows indicate cells that were counted as cleaved Casp3 positive. Bar size is 10 μM. **B.** Microscopic analysis following DAPI (blue) and TUNEL (red) staining of K-iLEC cells treated with DMSO or etoposide, and after KSHV miRNA inhibition by tiny LNA (LNA-miR-K12-1/3/4) or the control (LNA-miR-67). Arrows indicate cells that were counted as TUNEL positive. Bar size is 10 μM. **C.** Mean apoptosis fold induction measured from three independent TUNEL experiments; a significant difference of apoptosis induction ($p = 0.0056$) is observed between the LNA-miR-67 and the LNA-miR-K12-1/3/4 treatment.
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Discussion

Viral miRNAs have only recently attracted attention in studies into viral genetics, and their importance during the course of infection remains to be fully established. Almost all of these miRNAs were found in viruses belonging to the herpesvirus family; viruses that are associated with latency and that suggest long-term disease progression. Like other members of the gammaherpesvirus subfamily, KSHV is associated with a number of neoplastic disorders including Kaposi's sarcoma and B-cell lymphomas [3]. Some cellular targets of KSHV miRNAs have been previously reported. For example, miR-K12-11 has been shown to target a subset of genes that are also targeted by its homologous human miRNA, miR-155, that shares an identical seed region with this miRNA [26,27]. Among the validated targets of miR-K12-11 are two transcription factors, BACH1 and Fos. Although Fos itself provides a potential link between KSHV infection and oncogenesis, the authors did not show that KSHV miRNAs directly participate in cancer progression. The study of Samols and colleagues identified another potential candidate as a KSHV miRNA target that could contribute to cell transformation [39]. Indeed, with a microarray-based approach similar to the one that was used in this study, they found that thrombospondin (THBS1), a gene involved in angiogenesis, is regulated by KSHV miRNA expression. However, the analysis was performed in HEK293 cells, which are not representing cells naturally infected by KSHV. More recently, the Ganem laboratory also reported on the identification of cellular targets of KSHV miRNAs using a transcriptomic-based approach, with the Bcl2-associated factor BCLAF1 as one of the identified targets of several KSHV miRNAs [44]. Other targets of KSHV miRNAs that have been identified very recently are p21, IκBα, TWEAKR and Gemin 8 [35,45–47].

The aim of this study was to define the role played by KSHV miRNAs in apoptosis inhibition. The apoptotic processes can be executed intracellularly by the release of various factors (e.g. cytochrome c or SMAC/DIABLO) from mitochondria, or extracellularly through transmembrane death receptors, which are activated by their ligands. In both the intrinsic and extrinsic pathways, caspases are recruited and activated, and in turn they cleave substrates leading to the execution of apoptosis. In the intrinsic pathway, cytochrome c leaks from mitochondria [48], and binds to the adaptor apoptotic protease activating factor-1 (APAF1) to form the multi-protein structure, coined the apoptosome. The latter recruits Casp9, which in turn activates downstream effector caspases 3, 6 and 7 [49]. In the extrinsic pathway, ligands such as TRAIL and FasL activate specific pro-apoptotic death receptors at the cell surface [50–52], which results in the binding of the intracellular domains of the receptors to the adaptor protein Fas-associated death domain [53]. This leads to the assembly of the death-inducing signaling complex DISC, and to the recruitment of initiator caspases 8 and 10 [54]. Upon stimulation of these two caspases, effector caspases 3, 6 and 7 are activated. Thus, the intrinsic and extrinsic pathways converge at the level of the effector caspases, which highlights Casp3 as a critical factor in the control of apoptosis. In this study, we observed that KSHV miRNAs have a negative effect on apoptosis, as HEK293 cells and DG-75 B lymphocytes expressing these viral miRNAs are partially protected from apoptosis induction by staurosporine. We also measured Casp3 activity in the HEK293 cells, and showed that the presence of KSHV miRNAs resulted in a sharp decrease of Casp3/7 activity upon staurosporine induction. While our data does not rule out that the observed effect in HEK293 cells is due to a decreased activity of Casp7, the evidence available to date indicates that Casp3 activity is predominant over Casp7 activity, and that Casp3 is likely the major executor of apoptosis [55]. However, we were unable to monitor the effect of KSHV miRNA on Casp3/7 activity in DG-75 cells. Indeed, these cells are resistant to caspase activation by the intrinsic pathway [36], and accordingly, we could not induce Casp3/7 cleavage with staurosporine. This result confirms that Annexin V levels do not only measure caspase-dependent apoptosis, and therefore indicates that KSHV miRNAs are regulating both caspase-dependent and -independent apoptosis.

To discover cellular targets of KSHV miRNAs, we used a microarray-based approach to identify transcripts regulated by KSHV miRNAs in both the B lymphocyte DG-75 cell line and the endothelial EA.hy926 cell line. Based on their expression profiles, the samples primarily clustered according to the cell line (DG-75 or EA.hy926), and, within these two clusters, according to the presence of KSHV miRNAs. Using small RNA deep-sequencing data, we determined the relative abundance of each miRNA within the expressed cluster, which enabled us to show that transcripts containing seed-matches to KSHV miRNAs within their 3′UTR were significantly more down-regulated that transcripts without such binding sites. This enabled us to generate a list of putative targets to follow in further functional assays. Our validation rate was relatively low, reflecting presumably the fact that many miRNAs (virus-encoded and endogenous) changed in these experiments, leading to complex secondary effects. We looked for seed-match sites within the coding sequences of down-regulated transcripts and could identify a few (listed in Dataset S1), but the validation of these sites can prove challenging. Nevertheless, we validated two candidate targets that are biologically relevant for KSHV infection, Rad51AP1 and Casp3. Rad51AP1 is a DNA binding protein that participates in RAD51-mediated homologous recombination, and is important for the preservation of genome integrity [56]. Because KSHV has been shown to induce DNA damage response through the expression of v-cyclin [57], the down-regulation of Rad51AP1, which will require further validation, might be important in the context of viral infection. In light of our initial aim to define the role of KSHV miRNAs in apoptosis inhibition, we focused our efforts on the characterization of Casp3 as a target of KSHV miRNAs. We confirmed that a Casp3 3′ UTR luciferase reporter construct is regulated by three KSHV miRNAs, and we identified three miRNAs, miR-K12-1, miR-K12-3 and miR-K12-4-3p, as being responsible for this regulation, as well as their binding sites within Casp3 3′ UTR.

We then showed that endogenous Casp3 was also regulated by KSHV miRNAs, both at the mRNA and protein levels, and in different cell types. We also showed that inhibition of miR-K12-1, K12-3 and K12-4-3p in KSHV-infected cells resulted in an upregulation of Casp3 expression, which in turn translated into an increase in apoptosis, as assessed by cleaved Casp3 quantification and TUNEL assay analysis. These findings are consistent with a report that described the role of KSHV in conferring a survival advantage to endothelial cells [58]. In this report, Wang et al. showed that the level of Casp3 activity was decreased in KSHV-infected HUVEC cells subjected to staurosporine treatment (or other apoptotic insults). The regulation of Casp3 is not the only explanation for KSHV miRNAs-mediated inhibition of apoptosis, especially since we showed that caspase-independent apoptosis was also affected. It is of course highly probable that other factors in the apoptosis pathway are also targeted by KSHV miRNAs. For example, Abend *et al.* recently reported that KSHV miR-K12-10 targeted the TNF-like weak inducer of apoptosis (TWEAK) receptor [47], which indicates another level of regulation of one certain type of apoptosis.

In summary, our findings demonstrate that KSHV miR-K12-1, K12-3 and K12-4-3p target the effector caspase 3. The down-regulation of Casp3 by KSHV miRNAs results in a decrease in apoptosis activity in different cell types including endothelial cells that are biologically relevant for KSHV infection *in vivo*. The specific inhibition of these miRNAs in infected cells increased Casp3 levels and cell death. Apoptosis is frequently inhibited in tumor cells, and our results are in agreement with a recent report that indicates that the active form of Casp3 is detected less frequently in Kaposi sarcoma lesions in patients from Brazil [59]. Our data therefore suggests that apoptosis regulation by the viral miRNAs could contribute to the malignant phenotype triggered by KSHV infection. In the long term, delivery of specific inhibitors of these viral miRNAs in KSHV-infected patients to restore apoptotic clearance of the virus by the immune system could be an interesting novel therapeutic approach.

Material and Methods

Cell lines

DG-75 and BCBL-1 cells (obtained through the NIH AIDS Research and Reference Reagent Program (Cat# 3233 from McGrath and Ganem)) were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 100 UI/mL penicillin, 100 µg/mL streptomycin and 2 mM L-Glutamine. BC-3 cells (ATCC) were grown in the same media with 50 µM ß-Mercaptoethanol. EA.hy926, QBI-HEK 293A (QBiogene), Flp-In T-REx-293 (Invitrogen), and HEK293 cell lines were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin. Primary and E6/E7 HUVEC cells (from Promocell) were cultured in a humidified 5% CO_2 atmosphere at 37°C in endothelial basal medium (Promocell) supplemented with 10% FCS, gentamicin, amphotericin and supplement kit provided with the media. To obtain immortal lymphatic endothelial cells (iLECs) primary human LEC cells (Promocell) were immortalized by the HPV oncogenes E6/E7 as previously described (Moses et al., 1999). iLEC cells were maintained in endothelial basal medium (Promocell) supplemented with 5% human AB serum (HS; Sigma, St. Louis, Mo.).

KSHV infection of iLECs

Wildtype KSHV was produced from BCBL-1 cells induced with 20 ng/mL PMA. The virus-containing supernatant was collected after three days by ultracentrifugation (21,000 rpm at 4°C for 2 h), and resuspended in TNE buffer (150 mM NaCl, 10 mM Tris pH 8, 2 mM EDTA, pH 8). For the KSHV infection iLEC cells were plated in 6-well plates one day before the infection using multiplicity of infection (MOI) 1 in the presence of 8 µg/mL polybrene (Sigma). The infection was performed as spin-infection by centrifugation at 2500 rpm (Heraeus Multifuge 3 S-R; Thermo Scientific) for 30 min at room temperature. Cells were then returned to 37°C, 5% CO_2, and after 4 h of incubation fresh complete media was added. The virus-containing medium was removed the next day, and replaced with fresh complete media. The extent of KSHV infection was monitored by expression of the latent nuclear antigen-1 LANA-1 in the nuclei of KSHV-infected cells (K-iLECs) and detected by immunofluorescence using anti-LANA antibody (13-210-100, Advanced Biotechnologies Inc).

Primary rKSHV infection of HEK293 cells

rKSHV.219 infected HEK293 cells were reactivated by incubating them in DMEM medium containing 1 mM sodium butyrate and 20 ng/mL TPA (tetradecanoyl phorbol acetate) for 24 h, and four more days with media containing sodium butyrate only. The supernatant was collected, filtrated through 0.45 µM filter, and 8 µg/mL polybrene was added before adding the supernatant to QBI-HEK 293A cells seeded one day before. After 4 h, the medium was replaced and the cells grown at 37°C for 2 days. As soon as green fluorescent started to appear, 1 µg/mL puromycin was added to the medium. Cells were harvested for RNA analysis after at least 21 days under puromycin selection.

Generation of stable cell lines expressing KSHV miRNAs

Cell lines stably expressing the ten intronic KSHV miRNAs were generated using the "Virapower" lentiviral transduction system with the vector pLENTI6/V5 (Invitrogen) and Gateway cloning. The miRNA encoding intronic region was amplified by a two-step PCR using cDNA prepared from KSHV infected BCBL-1 cells (PCR primers: KSHV miRK_for and KSHV miRK_rev for the first PCR and attB1_external for and attB2_external rev for the second PCR), cloned into pDONR207 and transferred to pLENTI6/V5-DEST (Invitrogen). PCR primers are provided in Table S3. The control lentiviral vector pLENTI6/V5-EGFP was a kind gift from Oliver Rossmann. In order to generate lentiviruses for transduction of cells with KSHV miRNAs, the ViraPower Lentiviral Gateway Expression System (Invitrogen) was employed according to the manufacturer's instructions. The packaging mix contained plasmids pLP1, pLP2 and pLP/VSVG. Virus-containing medium was cleared with a 0.45 µm filter and added with polybrene (8 µg/mL) to DG-75 (1×10^6 cells/mL) or EA.hy926 (3×10^5 cells/mL) target cells for transduction. In the case of EA.hy926 cells, the plates were centrifugated 30 min at 2500 rpm to increase transduction efficiency. Two days after transduction, when the EGFP signal in the control cells became visible, Blasticidin (1 µg/mL) was added to the medium to select for the transgene and gradually raised to a final concentration of 7.5 µg/mL for DG-75 cells and 3 µg/mL for EA.hy926 cells after six days. Efficiency of selection was determined by analyzing the proportion of EGFP expressing control cells by fluorescence activated cell sorting (FACS). Cell lines were used for experiments when 100% of control cells expressed EGFP.

Primary HUVEC cells were transduced with lentiviruses (pLenti6-vector; Invitrogen) encoding EGFP or 10/12 KSHV miRNA cluster (K10/12) and maintained under blasticidin selection (5 µg/mL) in endothelial basal medium supplemented as above. The cells were replenished with fresh medium every second day and passaged when necessary.

Generation of inducible FLP-293 stable cell lines

The Flip-In stable cell lines were generated using the Flp-In T-REx-293 cell line (Invitrogen) and according to the manufacturer's instructions (Invitrogen). Briefly, cells were seeded one day before at 10^6 cells/well in 6-well plates. Cells were transfected with 3.6 µg and 0.4 µg respectively of pOG44 (Invitrogen) and each pcDNA for each cell line with lipofectamine 2000 (Invitrogen). The media was replaced 24 h after transfection, and cells were passaged into 10 cm dishes 24 h later to achieve a desired confluency of maximum 25% prior selection. Hygromycin (Invivogen) was added at a concentration of 200 µg/mL and then raised 2–3 days later at a concentration of 250 µg/mL. The media was replaced each 3–4 days until 2–3 mm wide foci appeared. Cells were then passaged into 75 cm^2 flasks for amplification. Efficiency of the selection was then assayed by β-galactosidase staining, for the loss of β-galactosidase activity, and/or by northern blot for the detection of the miRNA.

Computational microarray analysis

The microarray data were submitted to the gene expression omnibus database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE18946. We imported the CEL files into the R software (R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org) using the BioConductor affy package [57]. The probe intensities were corrected for optical noise, adjusted for non-specific binding and quantile normalized with the gcRMA algorithm [58].

Per gene log2 fold change was obtained through the following procedure. We first fitted a lowess model of the probe log2 fold change using the probe AU content. We used this model to correct for the technical bias of AU content on probe-level log2 fold change reported by [59]. Subsequently, probe set-level log2 fold changes were defined as the median probe-level log2 fold change. Probe sets with more than half of the probes (6) mapping ambiguously (more than 1 locus) to the genome were discarded, as were probe-sets that mapped to multiple genes. We then collected all remaining probe sets matching a given gene, and averaged their log2 fold changes to obtain an expression change per gene. For sequence analyses, we selected for each gene the RefSeq transcript with median 3′ UTR length corresponding to that gene.

Controls and transductions were performed in triplicates in both cell lines (DG-75, EA.hy926), and we used limma [60] to compute differential regulation p-values. Finally, for each cell line, we only analyzed genes which had at least one probe set that was called present in either all replicates of miRNA transduction or all replicates of the control (or both).

Transcriptome-wide regulatory effect of the KSHV miRNAs. To determine whether transduction of the KSHV miRNAs had the expected effect on mRNA expression, we first computed a KSHV miRNA sensitivity score for each mRNA. This was defined as the sum over all KSHV miRNAs, the number of seed matches to the KSHV miRNA in the 3′ UTR weighted by the relative abundance of the KSHV miRNA. The KSHV miRNA expression was determined in DG-75 cells by Illumina small RNA sequencing and the relative abundance of a miRNA was defined as the number of reads that mapped to this KSHV miRNA divided by the total number of reads that mapped to any KSHV miRNA. Because the relative abundance of KSHV miRNAs was comparable in DG-75 and EA.hy926 cells (Table S1 and Figure S4), we used the same KSHV miRNA sensitivity score to analyze the DG-75 and the EA.hy926 microarrays. The Human Genome U133 Plus 2.0 Affymetrix microarrays used in this study measure the expression of a total of 15678 genes. Of those, we selected the 1000 genes with the highest KSHV miRNA sensitivity score as the most likely targets of KSHV miRNAs. Of those, 611 genes were actually expressed in DG-75 cells and 674 in EA.hy926 cells. We compared the fold change of these genes with those that had no KSHV miRNA seed match in their 3′ UTR (2047 expressed genes in DG-75 and 2137 expressed genes in EA.hy926) using a two-sided Wilcoxon rank sum test. The bar plots in Figure 3A represent the mean and two standard errors around the mean fold change of KSHV sensitive mRNAs and mRNAs with no seed matches in their 3′ UTR.

To test whether the 3′ UTR length could alone explain the downregulation of the KSHV sensitive mRNAs in cells transduced with KSHV miRNAs, we sampled 1000 genes in such way that their 3′ UTR length distribution was identical to that of the KSHV sensitive mRNAs. For each cell line, we then computed the average fold change of the subset of these 1000 genes that were actually expressed. Finally, we repeated this procedure 1000 times. The mean fold change and two standard errors around the mean over these 1000 randomizations are reported on Figure 3A (blue bars).

List of putative direct targets. Transcripts that are direct targets of the intronic KSHV miRNAs would ideally carry at least one seed match to at least one of the intronic KSHV miRNA, and it should be down-regulated in the KSHV miRNAs transduction compared to the EGFP control transduction.

We considered that an mRNA was down-regulated if its log2 fold change was negative and the limma p-value of differential regulation smaller than 0.05. In addition, we only considered genes for which the magnitude of down-regulation was between 40% and 300% (log2 fold M: $-2<M<-0.5$). Through applying these criteria, we generated three lists of putative direct targets: one for the DG-75 cells, one for the EA.hy926 cells, and then intersected these two lists to generate a list of putative direct targets that are common to DG-75 and EA.hy926.

Generation of inducible miRNA expression and luciferase sensor vectors

To generate the pcDNA-K10/12, the KSHV intronic miRNA cluster was PCR-amplified from BAC36 DNA [60] and ligated into the *Bam* HI and *Xho* I sites of the pcDNA5/FRT/TO (Invitrogen). The primer sequences were (sense and antisense primers are indicated in respective order): 5′-ATATGGATCC-GAATGCGTGCTTCTGTTTGA, 5′-ATATCTCGAGTTTA-CCGAAACCACCCAGAG. The empty pcDNA vector was obtained by digesting the pcDNA-K10/12 with *Pme* I, followed by ligation of the plasmid. For KSHV miRNA individual expression vectors, a region of approximately 300 nt surrounding each pre-miRNA (or the miRNA cluster) was PCR-amplified from BAC36 DNA. *att*B1/2 sequences were added by nested PCR and the resulting PCR product were cloned into pDONR207 (Invitrogen) and then recombined in pLenti6/V5-DEST using Gateway technology (Invitrogen). The *att*B1/2 primer sequences are (sense and antisense primers are indicated in respective order): 5′-ACAAGTTTGTACAAAAAAGCAGGCT, 5′-ACCACTTT-GTACAAGAAAGCTGGGT. The specific primers are indicated in Table S3. The individual miRNA expression cassettes were then subcloned *via* PCR amplification from pLenti6/V5-DEST expressing vectors and ligated into the *Xho* I and *Apa* I sites of pcDNA5/FRT/TO, and with the primers indicated in Table S3. To generate luciferase reporter plasmids, psiCHECK-2 (Promega) was modified by inserting the Gateway cassette C.1 (Invitrogen) at the 3′-end of the firefly luciferase gene into the *Xba* I site of psiCHECK-2. *att*B-PCR products were cloned into pDONR/Zeo (Invitrogen) and recombined in the modified psiCHECK-2 vector by Gateway cloning. The 3′ UTR sequence of the different candidates were obtained from the Ensembl database (www.ensembl.org) and were nested PCR-amplified from QBI-HEK 293A cells' genomic DNA with the primers indicated in Table S3 and attB1/2 primers. The imperfect match sensors for KSHV miRNA were obtained by annealing the oligonucleotides indicated in Table S3 and PCR-based addition of the *att*B sequences using *att*B1/2 primers. The resulting PCR product was then cloned by Gateway recombination sequentially in pDONR/Zeo and psi-CHECK-2 plasmids.

Mutagenesis of Casp3 luciferase sensor

Mutagenesis was performed using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions and using the oligonucleotides indicated in Table S3. Briefly, we mutagenised in the Casp3 luciferase reporter construct the nucleotides predicted to pair to position 3 to

5 of the miRNA sequence to prevent pairing of the miRNA seed sequence on Casp3′s predicted target sites.

Luciferase assays

QBI-HEK 293A cells were seeded in 48-well plates at 10^5 cells/well and then incubated a few hours. When cells were adherent, co-transfection of 25 ng of the reporter constructs and 250 ng of the pcDNA-K10/12 (or pcDNA as control vector) were performed using Lipofectamine 2000 (Invitrogen). After 48 h, cells were then washed in PBS and lysed with 65 µL of passive lysis buffer (Promega), and 10 µL were assayed for firefly and *Renilla* luciferase activity, using the dual-luciferase reporter assay system (Promega) and a luminescence module (Glomax, Promega). The relative reporter activity was obtained by first normalizing to the transfection efficiency with the *Renilla* activity, and then, to the firefly activity obtained for the empty control reporter, in presence of the pcDNA-K10/12 or pcDNA, to normalize for the effect of transfection of these expression vectors.

Western blot analysis

For Western blot analysis of HUVEC cells, cells were extracted in ELB lysis buffer (150 mM NaCl; 50 mM HEPES, pH 7.4; 5 mM EDTA and 0.1% NP40) and 30 µg of proteins was separated on 12% SDS-PAGE and transferred on to nitrocellulose membranes according to standard protocols. Primary antibodies used in Western blotting were anti-caspase-3 (MAB4603; Millipore) and anti-γ-tubulin (GTU-88; Sigma-Aldrich). HRP-conjugated anti-mouse (AP308P; Chemicon) immunoglobulin was used as a secondary antibody. Filters were visualized on SuperRX film (Fuji) using the ECL chemiluminescence system (Pierce, Rockford, IL). The intensity of the chemiluminescence signals was quantified with FluoChem 880 imager and software (Alpha Innotech Corporation).

For Western blot analysis of DG-75, BC-3 or FLP-293 cells, cells were extracted in passive lysis buffer (50 mM Tris, 150 mM, NaCl, 5 mM EDTA and 0.5% NP40, 10% Glycerol and 10 µM MG132) and 15 µg or 45 µg of proteins, respectively from BC-3 or FLP-293 cells, was separated on 10% SDS-PAGE for PARP analysis, or on 15% SDS-PAGE for Casp3 analysis, and transferred on to nitrocellulose membranes according to standard protocols. Primary antibodies used in Western blotting were anti-caspase-3 (06-735; UpState), anti-PARP-1 [61] and anti-γ-tubulin (GTU-88; Sigma-Aldrich). IRDye 800CW-conjugated anti-rabbit and anti-mouse (926-32213 and 926-32212; Li-Cor Biosciences) immunoglobulins were used as secondary antibodies. The intensity of the fluorescence signals was quantified with Odyssey Infrared Imaging system and Odyssey v3.0 software (Li-Cor Biosciences).

Northern blot analysis

RNA was extracted using Trizol reagent (Invitrogen) and Northern blotting was performed on 5 to 10 µg of total RNA as described before [23,62]. Probes were 5′ ^{32}P-radiolabelled oligodeoxynucleotides antisense to the miRNA sequence or to part of the U6 snRNA sequence. Blots were analyzed and quantified by phosphorimaging using a FLA5100 scanner (Fuji).

Small RNA cloning and sequencing

Small RNA cloning was conducted from 50 µg of DG-75-K10/12 total RNA as previously described [63]. Small RNA sequencing was performed at the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC, Illkirch, France) using an Illumina Genome Analyzer II with a read length of 36 base pairs (bp).

Processing and annotation of small RNA sequences

An in-house Perl analysis pipeline was used to analyze the data produced by small RNA sequencing. After 3′ adaptor removal and size selection (exclusion of trimmed reads shorter than 15 nt), non-redundant sequences were mapped to the genomes from which they may derive and to other RNAs already annotated, using Nexalign (http://genome.gsc.riken.jp/osc/english/software/src/nexalign-1.3.5.tgz) permitting up to 2 mismatches. The *Homo sapiens* and KSHV genome sequences were respectively downloaded from the UCSC repository (assembly version hg19) and the GenBank database. The following sources of annotated transcripts were used: miRBase v.16 for miRNAs, GenBank v.180 for *Homo sapiens* rRNA, tRNA, sn-snoRNA, scRNA and piRNA, and Repbase v.16.01 for *Homo sapiens* and common ancestral repeats. By doing so, small RNAs that mapped unambiguously to sequences from one single functional category were easily classified, while the other ones were identified by applying this annotation rule based on the abundance of various types of sequences in the cell: rRNA > tRNA > sn-snoRNA > miRNA > piRNA > repeat > pathogen genome > host genome > unknown.

cDNA synthesis and quantitative real-time PCR

miRNAs. Semi-quantitative real-time PCR for KSHV miRNAs was performed using the Light Cycler System (Roche) with a modified protocol from Shi and Chiang [64]. Briefly, total RNA was extracted with Trizol (Invitrogen) and provided with a 3′ poly-A-tail (Poly-A-Tailing Kit, Ambion). After phenol-chloroform extraction and ethanol-acetate precipitation, first strand synthesis with the anchor primer Poly(t)adpt was performed using Superscript II reverse transcriptase (Invitrogen). Quantification of miRNAs was performed using the FastStart DNA MasterPlus SYBR Green I Master Mix (Roche) with specific primer and AdptRev-primer. The PCR program was composed of an initial activation step for the Taq polymerase at 95°C for 10 min followed by 45 cycles of 95°C for 10 sec, 68°C for 5 sec ($\Delta T = 20$°C/sec each) and 72°C for 6 sec ($\Delta T = 5$°C/sec). 5.8S RNA was quantified and the results used for normalization. The changed levels of miRNA transcripts (relative to 100%) were calculated based on the empiric formula "level(%) = 1,8^ΔCt", based on quantification of synthetic miRNAs (data not shown). Primers used are shown in Table S3.

Caspase 3. To monitor the expression of Casp3 mRNA levels, cDNA was prepared from total RNA using Superscript II (Invitrogen). Transcripts were quantified by TaqMan PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems). TaqMan probes were taken from the Universal Probe Library (Roche) and selection of probe-primer combinations was performed using the Assay Design Centre (Roche, www.universalprobelibrary.com). The PCR program was composed of a denaturation step at 94 °C for 12 min followed by 45 cycles of 95°C for 20 sec and 60°C for 1 min 72°C for 6 sec ($\Delta T = 5$°fC/sec). HPRT-transcript was used for normalization of Ct-values. The changed levels of Casp3 transcripts (relative to 100%) were calculated based on the empiric formula "level(%) = 2^ΔCt".

The primers used were: RT_CASP3_for, RT_CASP3_rev and Roche universal probe #68; RT_HPRT_for, RT_HPRT_rev and Roche universal Probe #73 (sequences can be found in Table S3).

BC-3 cells miRNAs inhibition with 2′O-methylated or LNA oligonucleotides

For inhibition of miRNAs, BC-3 cells were cultured in 6-well dishes and transfected with the 2′O-methylated oligonucleotides (provided by G. Meister) against individual KSHV miRNAs using Oligofectamine (Invitrogen). Oligonucleotides were used at a final concentration of 60 nM and transfections were performed according to manufacturer's instructions. Total proteins were extracted for analysis 48 h after transfection.

For inhibition of miRNAs with tiny LNAs, 2×10^6 BC-3 cells were seeded in 6-well plates and incubated with the inhibitors (Table S3) against individual KSHV miRNAs or the control *C. elegans* miR-67. Oligonucleotides were used at a final concentration of 1.5 μM and incubated in the medium for 48 h, or 6 days by replacing twice the medium (day 2 and 5), prior to harvesting the cells.

Annexin V and Casp3/7 activity cell death assays

The effects of the KSHV miRNAs on apoptosis were analysed by both measurement of caspase 3/7 activity and Annexin V/propidium idiode (PI) staining. Cell death was induced by adding 2 to 5 μM staurosporine (Sigma) for 8 h; DMSO was used as a control. For Annexin V binding analysis, 10^5 HEK293 cells were seeded in 12-well plates, incubated overnight prior to addition of staurosporine or DMSO. Cells were harvested by trypsinization, washed in PBS, and resuspended in binding buffer (10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$) containing Annexin V conjugated with Allophycocyanin diluted at 1/100 (BD Biosciences, Le Pont-de-Claix France) and 2 μg/mL PI (Sigma-Aldrich, Lyon, France). The cells were incubated for 15 min in the dark and analyzed with a FacsCalibur flow cytometer (Becton Dickinson, Le Pont-de-Claix, France).

Statistical analysis for Annexin V geo means collected in individual experiments were performed using a Wilcoxon signed-paired rank test, as distribution of measurements in each condition did not fit normality tests. Differences were considered significant when $p<0.05$.

For caspase 3/7 activity assay, 2.5×10^4 cells were seeded in 96-well plates, and staurosporine or DMSO immediately added. Caspase 3/7 activity was then measured using Caspase-Glo 3/7 Assay Kit (Promega) and normalized to the protein concentration determined by DC Protein Assay (Bio-Rad).

Cleaved Casp3 quantification and TUNEL assay

KSHV infected immortalized (by stable expression of HPV16 E6/E7) human Lymphatic Endothelial Cells (K-iLEC) were seeded one day before at 5×10^4 cells/well on 24-well plates. For inhibition of miRNAs, K-iLEC cells were treated with two doses of Tiny LNA oligonucleotides (48 h+48 h) at a final concentration of 1,5 μM. Apoptosis was induced with 500 μM Etoposide (Sigma Aldrich) and DMSO was used as a vehicle control (mock). Cells were fixed with 4% Paraformaldehyde (EMS, Hatfield, PA) 24 h after the treatment with Etoposide or mock. Coverslips were blocked 30 minutes with 5% goat serum and incubated first with 1:800 diluted Cleaved Caspase-3 (Asp175) rabbit monoclonal antibody (Cell signaling) for 1 h at room temperature, then with a 1:1000 dilution of a goat anti-rabbit secondary antibody coupled to Alexa Fluor 594 (Invitrogen). Alternatively, apoptosis was detected with TdT-mediated dUTP nick end labeling (TUNEL) assay according to manufacturer's instructions of the kit (In situ Cell Death Detection Kit, TMR red, Roche, Mannheim, Germany). The fluorochromes were visualized with a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss, Oberkochen, Germany). Images were acquired with a Zeiss Axiocam HRc, using Zeiss AxioVision (version 4.5 SP1) and Adobe Photoshop software (version 7.0; Adobe, San Jose, CA).

Supporting Information

Dataset S1 Microarray analysis summary. Each row in the table represents one of the 15768 genes monitored by the Affymetrix arrays. The "RefSeq mRNA", "Gene Name", "Entrez Gene ID", "gene description" and "mRNA annotation" columns contain the same information as in Dataset S2, S3 and S4 (see below). The "KSHV sensitivity 3′ UTR" and "KSHV sensitivity CDS" columns contain the *KSHV miRNA sensitivity score* described in the methods for the 3′ UTR and the Coding Region, respectively. The "mRNA presence DG-75" and "mRNA presence EA.hy926" columns indicate whether the gene was called present by the Affymetrix arrays in each cell line. The "3′ UTR hits" and "CDS hits" then indicate how many matches to the KSHV miRNAs were found in the 3′ UTR and in the Coding Region. The remaining fields (log2 mRNA fold change, log2 intensity, differential expr. P-value) are defined as in Dataset S2, S3 and S4 (see below).
(XLS)

Dataset S2 Putative direct targets of the KSHV miRNAs in DG75 cells. Each row in the table corresponds to one gene, identified by a representative RefSeq mRNA ID, a set of Affymetrix probes designed to monitor the expression of that gene, the Entrez Gene ID, the gene name and the gene description provided by NCBI RefSeq. The "mRNA annotation" field provides information about the length and the span of the Coding Domain of the representative mRNA used for the analysis. "log2 fold change K10/12 vs EGFP" contains the log2 fold changes in gene expression upon transducing the K12/10 construct compared to the EGFP control, while "log2 intensity" is the average signal intensity on the microarrays. "diff. Expression p-value" is the (uncorrected) P-value of differential expression as computed by the limma algorithm. The "Detectable" field is true whenever the gene could be detected in at least one of the samples from the corresponding cell line. Finally, the two "Seed matches to K10/12 miRNAs" field indicates the KSHV miRNAs for which at least one match to the seed recognition motif could be found in the 3′ UTR of the representative RefSeq mRNA. The procedures we used to build this table are described in the "Methods" section.
(XLS)

Dataset S3 Putative direct targets of the KSHV miRNAs in EA.hy926 cells. For details, please refer to Dataset S2 legend.
(XLS)

Dataset S4 Putative direct targets of the KSHV miRNAs in both cell lines. For details, please refer to Dataset S2 legend.
(XLS)

Figure S1 Northern blot analysis of BCBL-1, BC-3 and FLP-pcDNA and FLP-K10/12 cells grown in doxycycline-containing medium (final concentration of 1 μg/ml). U6 was used as a loading control.
(TIF)

Figure S2 The relative abundance of KSHV miRNAs is similar in K10/12 transduced DG-75 cells and K10/12 EA.hy926 cells. Each dot on the scatter represents one KSHV miRNA whose expression in KSHV-infected BCBL1 cells, DG-75 cells and

EA.hy926 was quantified by qPCR. Plotted are the expression levels of these KSHV miRNAs in DG-75 cells (x-axis) and EA.hy926 relative to their BCBL1 levels.
(TIF)

Figure S3 Clustering of gene expression profiles follows first the cell line (DG-75 vs. EA.hy926), and within each cell line the treatment (transduction of KSHV miRNAs vs EGFP). Shown is the hierarchical clustering of all microarray samples on the Euclidean space of log2 expression levels with Ward linkage, and using all 15,678 genes monitored by the microarrays.
(TIF)

Figure S4 Correlation between changes in gene expression upon transducing the K10/12 vs EGFP constructs in DG-75 vs EA.hy926 cells for all 6916 genes whose expression is detectable in both cell lines.
(TIF)

Figure S5 mRNAs likely to be targeted by KSHV miRNAs are longer than mRNAs with no matches to KSHV miRNAs. The red and green histograms respectively represent the distribution of 3′ UTR length of the 1000 mRNAs with highest *KSHV miRNA sensitivity score* (see methods) and all mRNAs with no matches to KSHV miRNAs.
(TIF)

Figure S6 Caspase 3′UTR fragments are all potentially targeted by KSHV miRNAs. **A.** Schematic representation of Casp3 3′ UTR luciferase reporter and fragments. The seed-match types are described in the text. Either the full length 3′ UTR, or fragments spanning the UTR were cloned downstream of the firefly luciferase in the pSi-Check2 vector. **B.** Dual luciferase assays performed with the constructs depicted in **A**, no fragment of Casp3 UTR showed a stronger repression than the full-length UTR.
(TIF)

Figure S7 Tiny LNAs inhibition effect on KSHV miRNAs and Casp3 luciferase sensors. Dual luciferase assays performed with the indicated sensors co-transfected with the empty pcDNA or pcDNA expressing the K10/12 construct, and incubated with a mix of either control cel-miR-67, or with a mix of oligos antisense to miR-K12-1, -3, and 4-3p, at a final concentration of 1,5 μM. Luciferase ratios relative to empty psiCHECK-2 set to 1 are displayed.
(TIF)

Figure S8 Western blot analysis and signal quantification for PARP-1 and Tubulin on BC-3 cells treated with DMSO (left) or 0.5 μM Staurosporine for 8 h (right), and tiny LNA-oligonucleotides for control miR-67 (LNA-miR-67), or with a cocktail of oligonucleotides antisense to the seed region of miR-K12-1, K12-3, and K12-4-3p (LNA-miR-K12-1/3/4). Arrows and arrowheads indicate the signals corresponding to PARP-1, and cleaved PARP-1 respectively; the asterisk indicates a non-specific band. Though the juxtaposed lanes are not contiguous, all of them are from a single gel (indicated by the dotted line).
(TIF)

Table S1 Repartition of KSHV miRNAs in DG-75-K10/12 cells as assessed by small RNA cloning and Solexa-based sequencing.
(DOC)

Table S2 RT-PCR analysis of KSHV miRNAs expression in DG75 and EA.hy926 cell lines compared to BCBL1. n.d., not determined; mol., molecules
(DOC)

Table S3 Sequences of primers used in this study. Sequences of primers for luciferase miRNA sensors (A), target validation (B) and (C), miRNA expression in pcDNA (D), viral miRNAs qRT-PCR (E), cellular mRNAs qRT-PCR (F), tiny LNAs (G) and for mutagenesis of miRNA binding sites in Casp3 3′UTR (H) are all given 5′ to 3′.
(XLS)

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Author Contributions

Conceived and designed the experiments: PMO JGH SP. Performed the experiments: GS GM JV AF MC TI VB. Analyzed the data: PMO JGH SP GS GM AF MC JH MZ FG OV. Contributed reagents/materials/analysis tools: JH MZ. Wrote the paper: SP.

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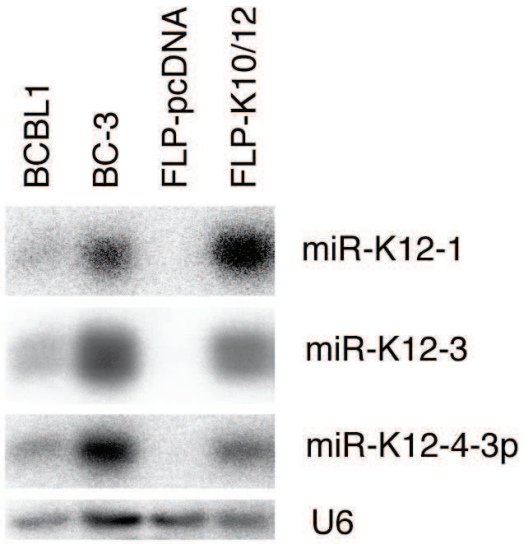
SUPPLEMENTAL DATA



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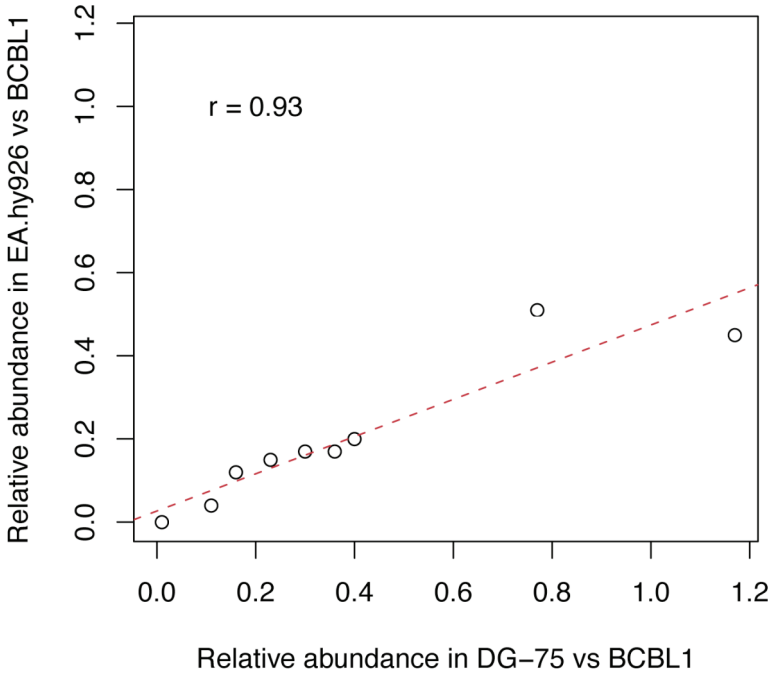

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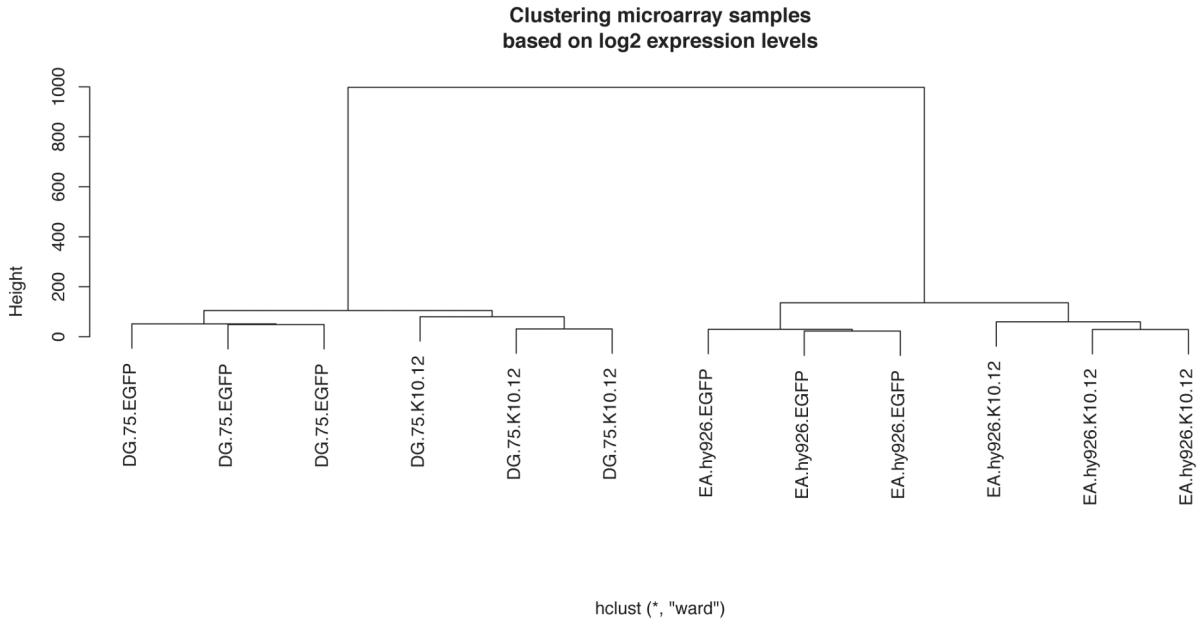

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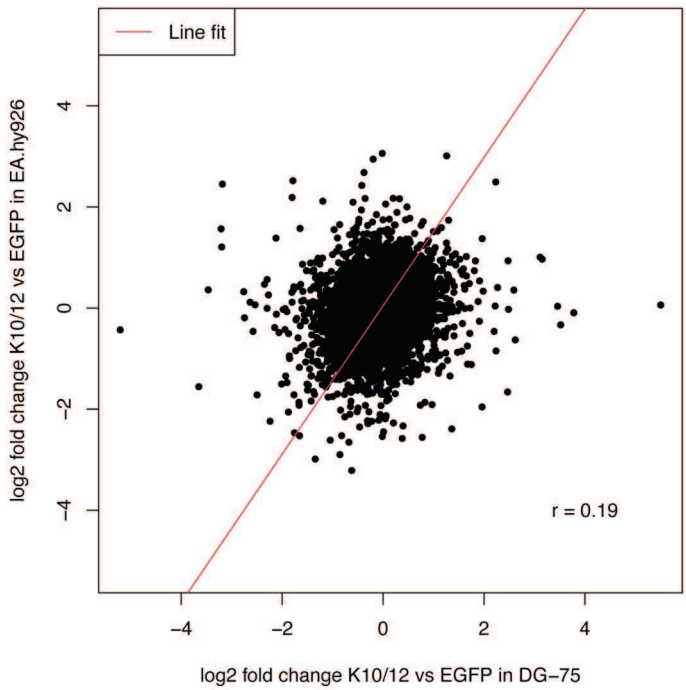

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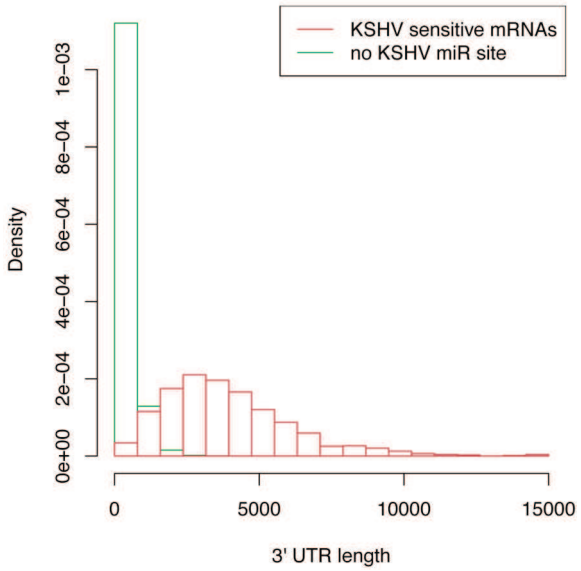

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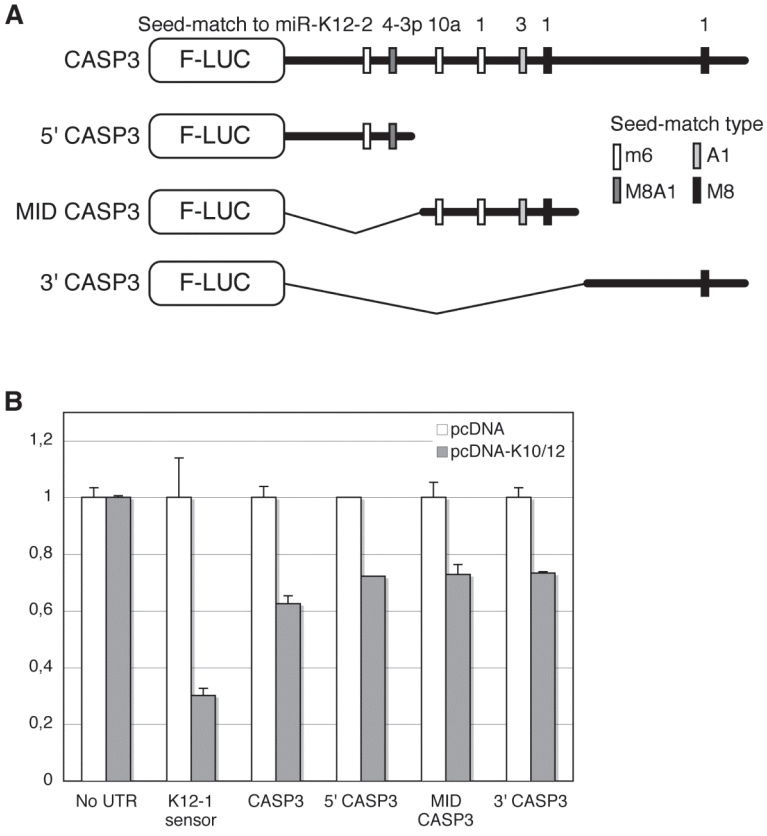

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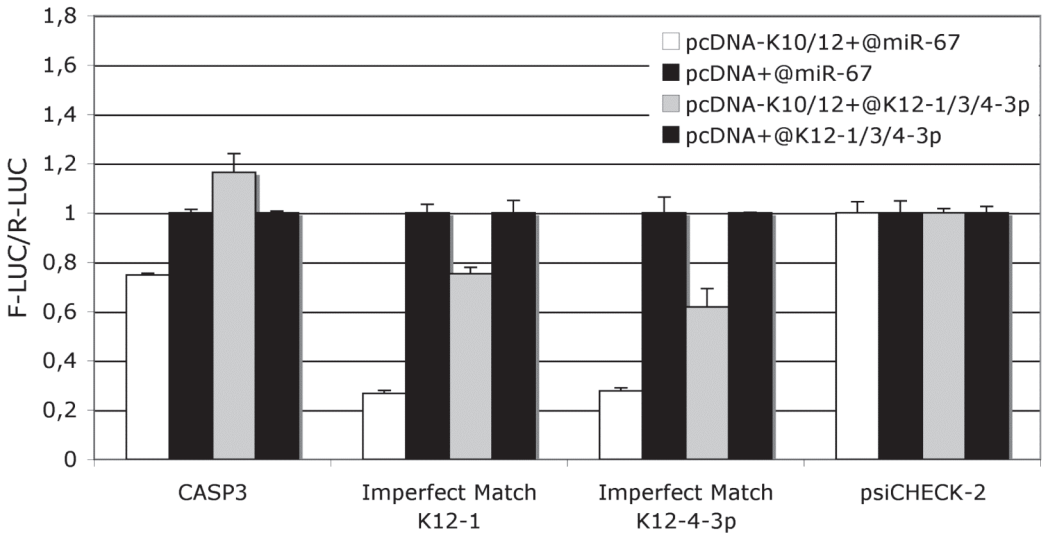

Figure S7: Tiny LNAs inhibition effect on KSHV miRNAs and Casp3 luciferase sensors. Dual luciferase assays performed with the indicated sensors co-transfected with the empty pcDNA or pcDNA expressing the K10/12 construct, and incubated with a mix of either control cel-miR-67, or with a mix of oligos antisense to miR-K12-1, -3, and 4-3p, at a final concentration of 1,5 μM. Luciferase ratios relative to empty psiCHECK-2 set to 1 are displayed.

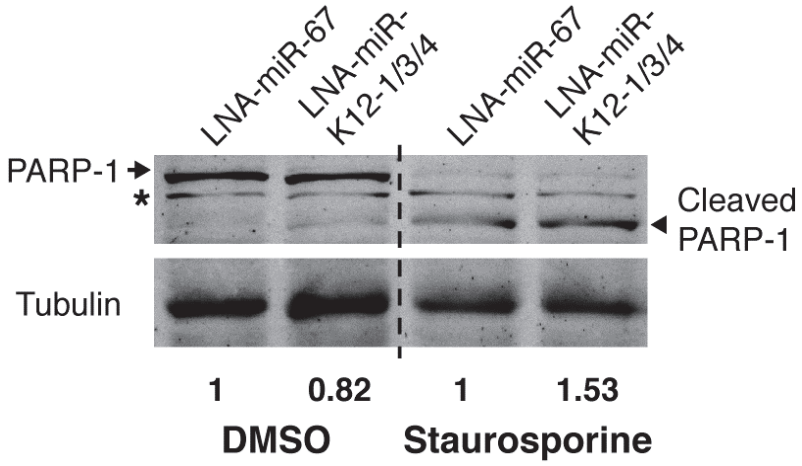

Figure S8: Western blot analysis and signal quantification for PARP-1 and Tubulin on BC-3 cells treated with DMSO (left) or 0.5 μM Staurosporine for 8h (right), and tiny LNA-oligonucleotides for control miR-67 (LNA-miR-67), or with a cocktail of oligonucleotides antisense to the seed region of miR-K12-1, K12-3, and K12-4-3p (LNA-miR-K12-1/3/4). Arrows and arrowheads indicate the signals corresponding to PARP-1, and cleaved PARP-1 respectively; the asterisk indicates a non-specific band. Though the juxtaposed lanes are not contiguous, all of them are from a single gel (indicated by the dotted line).

		DG75-K10/12	
miRNA	**Sequence**	**Seq Number (Total)**	**Seq Ratio (per 100000)**
miR-K12-1	ATTACAGGAAACTGGGTGTAAGC	192140	5555.31
miR-K12-1*	GCAGCACCTGTTTCCTGCAACC	107	3.09
miR-K12-2	AACTGTAGTCCGGGTCGATCTGT	65593	1896.48
miR-K12-2*	GATCTTCCAGGGCTAGAGCTGC	2471	71.44
miR-K12-3	TCACATTCTGAGGACGGCAGCGA	1483	42.88
miR-K12-3*	TCGCGGTCACAGAATGTGACA	24049	695.33
miR-K12-4-3p	TAGAATACTGAGGCCTAGCTGA	825458	23866.34
miR-K12-4-5p	AGCTAAACCGCAGTACTCTAGG	364425	10536.56
miR-K12-5	TAGGATGCCTGGAACTTGCCGGT	81900	2367.96
miR-K12-5*	AGGTAGTCCCTAGTGCCCTAAG	4	0.12
miR-K12-6-3p	TGATGGTTTTCGGGCTGTTGAGC	1191228	34441.79
miR-K12-6-5p	CCAGCAGCACCTAATCCATCGG	41652	1204.28
miR-K12-7	TGATCCCATGTTGCTGGCGCTC	4313	124.70
miR-K12-7*	AGCGCCACCGGACGGGGATTTAT	673	19.46
miR-K12-8	CTAGGCGCGACTGAGAGAGCA	642061	18563.81
miR-K12-8*	ACTCCCTCACTAACGCCCCGCT	884	25.56
miR-K12-9	CTGGGTATACGCAGCTGCGTAA	18352	530.61
miR-K12-9*	ACCCAGCTGCGTAAACCCCGCT	1820	52.62
miR-K12-11	TTAATGCTTAGCCTGTGTCCGA	57	1.65
KSHV miRNA		3458670	100000
Total miRNA		18498293	
Ratio KSHV/Total (%)		18.70	

Table S1: Repartition of KSHV miRNAs in DG-75-K10/12 cells as assessed by small RNA cloning and Solexa-based sequencing.

	DG75 K10/12 (% of BCBL1)	**EA.hy K10/12** (% of BCBL1)	**BCBL1** (mol. per cell)
miR-K12-1	30%	17%	n.d.
miR-K12-2	36%	17%	935
miR-K12-3	77%	51%	51507
miRK-12-4-5p	1%	0%	n.d.
miRK-12-4-3p	11%	4%	9799
miR-K12-5	23%	15%	126
miR-K12-6-5p	117%	45%	13312
miR-K12-6-3p	40%	20%	3543
miR-K12-9-5p	16%	12%	n.d.

Table S2: RT-PCR analysis of KSHV miRNAs expression in DG75 and EA.hy926 cell lines compared to BCBL1. n.d.: not determined; mol.: molecules.

DISCUSSION

1. General considerations

Many cellular processes have been discovered through the study of viruses. Owing to their small size, limited coding capacity, and the relative simplicity with which one can genetically engineer them (compared to their hosts), viruses represent an interesting model to study host-pathogen interactions. Viral infection can be described as a continuous arms race, fought between the virus and the host; as such there is a continual selective pressure placed upon both sides. To counter this pressure, viruses possess certain advantages, including their rapid generation times, and their higher rate of mutation, particularly so for RNA viruses. The host in turn deploys an array of strategies to eradicate viral infections, and as such their evolution can be influenced by the constraints imposed upon them by viruses. Therefore, viruses constitute an important source of genetic innovation, seen at the level of the antiviral mechanisms developed by their hosts, as well as at the level of novel molecular processes they can develop, and that are exclusive to the viral world. Indeed, some of these have been transferred to their hosts, as is the case for certain endogenous retroviral (ERV) sequences (e.g. enhancers). Certain viruses display a level of molecular piracy, capturing cellular genes, which then evolve, and are sometimes eventually transferred back to their hosts. Importantly, viruses display a level of their adaptation to the cellular machinery and to the processes they usurp. In this regard, the study of viruses cannot be dissociated from the study of cellular forms of life, and is essential for a better comprehension of some cellular processes. Indeed, one striking example is that of gene silencing. It is in part through the study of viral infections in plants that the RNAi phenomenon was first characterised and explained (Hamilton and Baulcombe, 1999), and that the study of viral VSRs allowed for a better comprehension of RNAi mechanisms (e.g (Anandalakshmi et al., 1998; Deleris et al., 2006; Schott et al., 2012)).

Animal viruses have also developed non-protein and/or protein factors, that can interact with cellular effectors of the RNA silencing machinery, such as components involved in miRNA biogenesis and its regulation. We are only just beginning to unravel some of the methods used by viruses to modulate the RNA silencing machinery, (e.g. Drosha downregulation upon KSHV lytic-stage infection, Dicer regulation by HIV-1, adenovirus VAI RNA regulation of Exp5 activity). In addition, viruses can act at the level of miRNA turnover - such as the case for HVS and MCMV that use highly abundant RNA transcripts to degrade miR-27. Akin to

the example in plants of the progress made through the study of VSRs, the study of these viral factors, has and will, shed further insight into the molecular mechanisms of the RNA silencing machinery. Identification and study of such factors is often facilitated when working on virus models, for example in the aim to perform genetic screens for their identification, or directed-mutagenesis to study and dissect their functions. The example of miR-27a degradation by the highly abundant putative ncRNAs of HVS and MCMV, provides an interesting model to study the regulation of miRNA stability, and that of natural sponges in general, such as the recently discovered circRNAs mentioned in the section 0 of the introduction.

The non-canonical regulative functions exerted by some virus-encoded miRNAs could eventually represent processes or similar processes that are also used by some cellular miRNAs. For example, a similar type of regulation to the one observed for KSHV KapB protein has been reported for the microprocessor negative feedback loop regulation, even if in this latter case the downregulation does not seem to involve the production of miRNAs (detailed in section 6.3.2 of the introduction). Also, the siRNA-like regulation exerted by viral miRNAs on antisense transcripts, such as HSV-1 miRNAs targeting of ICP0, could potentially be a more general phenomenon. Some short and long ncRNAs which have been characterised as noise, are expressed all over the pervasively transcribed human genome, but do not seem to exert any function (reviewed in (Tisseur et al., 2011)). One can thus speculate that they, or such other yet undiscovered types of ncRNA deprived of any function, could be targeted in an siRNA-like manner by miRNAs expressed antisense to them; these miRNAs would therefore in addition to their canonical function, form part of the RNA surveillance pathway. Indeed, the aim of such targeting would be to initiate/accelerate the degradation of these particular ncRNAs to avoid their undesired accumulation in the cell. Such a mechanism would be difficult to observe, and may appear to be "invisible" when not specifically looking for it. These unstable ncRNAs are effectively hardly detectable under normal conditions because they are very rapidly degraded and/or because their steady-state levels are very low. For example, the promoter upstream transcripts (PROMPTs) have been identified by depletion of components of the human exosome (Preker et al., 2008). MiRNAs responsible for such siRNA-like degradation would thus act in an enzymatic way; it would therefore permit them to avoid being titrated-out by the targeted ncRNAs, and thereby would not alter their activity on their canonical targets. Indeed, only a low absolute number of miRNAs would be needed for such a type of regulation, as in an siRNA-like repression whereby the

small RNA immediately cleaves its target upon recognition, leading to the subsequent and instant release of the miRISC complex. This thus makes these recognition and cleavage processes very short and harder to detect in contrast with miRISC binding of a translationally repressed target. The use of transgenic cells expressing catalytic mutants of the Ago proteins that are capable of siRNA-like cleavage activity (if not lethal for the cells), could perhaps then allow to observe evidence of such hypothetical miRNA function, with a detectable accumulation of some of the already known ncRNAs, or of some new ncRNAs that could be discovered this way. On the other hand, an out-titration of the miRNAs from their canonical function would thus be expected.

As multiple viruses hijack the RNA silencing machinery of their hosts to express their own miRNAs, they thereby provide numerous models of study. Indeed, compared to cellular miRNAs that are generally already expressed in the cell line or tissue used to analyse their role (thus require overexpression or inhibition, which are not always efficient enough to observe a differential effect on the target of interest), viral miRNAs are absent from the uninfected cells, providing an ideal negative control to decipher their functions. In addition, viral miRNAs can be overexpressed alone in the uninfected cells to get rid of the eventual effects of the viral infection/proteins on the analysed targets, or inversely, inhibited in the infected cells, or even more ideally mutated in the virus. The combination of gain and loss of miRNA functions in the appropriate cell types, which are thus easier to achieve with viral miRNAs, provide therefore a real advantage for dissecting miRNA functions compared to cellular miRNAs. Since the first viral miRNAs were identified back in 2004, the study of viral miRNAs has opened-up many novel insights into the RNA silencing machinery, and the ways in which viruses have evolved to exploit it for their own benefit.

2. Critical review of the experimental approach

In our study, we have demonstrated the targeting of the *CASP3* gene product by three KSHV miRNAs, which results in a functional inhibition of drug-induced apoptosis. CASP3 was predicted as a target using a microarray-based approach, in which we observed the alterations resulting from the stable overexpression of the intronic cluster of KSHV miRNAs. In order to get as close as possible to the cellular context of infection we used two cell lines: an endothelial cell line, and a B cell line, which are the two main cell types known to be naturally infected by KSHV, and which are implicated in the pathology associated to disease. Indeed, this was a pertinent choice, as the microarray profiling showed distinct patterns of mRNA differential expression that resulted from expression of the viral miRNAs, thus suggesting cell-specific regulation by the viral miRNAs, with a different impact on the global transcriptome of the cell line used. Where the B-cell line showed many genes involved in the immune response to be downregulated, the endothelial cell line showed mostly genes involved in cell cycle and DNA metabolic processes, as well as some genes in primary metabolic processes to be downregulated. While there was no overlap in the downregulated pathways between the two cell lines, there was some overlap in upregulated pathways, mainly related to translation and mitochondrial activity pathways. Compared to other large-scale approaches, microarray analysis offers the advantage to observe the global effects on the pathways that are affected through the direct targets of miRNAs. It thus can give an overall view of the impact of miRNA expression, and some of the specific pathways in which genes were observed to be downregulated (i.e. immune response in B cells, and cell cycle in endothelial cells) correlated with the cellular pathways that are expected to be affected by viral miRNAs.

When compared to RISC-pulldown-based approaches, it also allows to only look at genes that are effectively repressed, and not at the ones that are bound by the miRISC complex, but without being repressed. However, the targets that are not regulated at the mRNA level cannot be detected, and thus a certain number of them could be missed. Combining microarray analysis and proteomics would have allowed for solving part of this issue, but not completely, as proteomic-based approaches still lack sensitivity and do not allow for detection of low abundance-expressed targets (e.g. targets that arises from leaky transcription) (discussed in section 0 of the introduction). On the other hand, as shown by Baek *et al.*, genes that are repressed by miRNAs by more than one third at the protein level always undergo mRNA destabilisation, and for the more highly repressed genes, mRNA destabilisation appears to be

the major component of repression (Baek et al., 2008). In addition, it has now been shown that miRNA targets are first undergoing translation inhibition followed by a downregulation of the mRNA stability by deadenylation (Bazzini et al., 2012). It is highly plausible that we have missed some functionally relevant targets, as miRNAs exert a subtle regulation on their target genes and thereby a translational repression of less than one third could have a functional role (e.g. protein involved in signal transduction or "switch" targets defined by Bartel and Chen in the micromanager model). However, on the other hand, by looking at the targets repressed at the level of mRNA accumulation, we should have enriched our putative target list in genes that undergo a high level of repression.

It is worthy to note that the expression vector used in this study, in which the ten intronic miRNAs of KSHV were stably integrated into the cells (as a cluster) and then assayed by microarray, lacked the expression of pre-miR-K12-10 and pre-miR-K12-12. Furthermore, no activity was detected for miR-K12-9 (Figure S4 of the article), which is probably due to its very poor level of expression; as indicated by both qRT-PCR and deep sequencing data (Table S1 and S2 of the article). We therefore have not detected the genes that are specifically repressed by only one or more of these KSHV miRNAs. Also, the transcripts that are cooperatively bound by several of the KSHV miRNAs, and of which one or more of these three miRNAs play a major role in their repression, may have also been missed.

To establish our list of putative targets, we applied two bioinformatics filters on the raw microarray data, in order to enrich the predictions with the cellular genes the most likely to be repressed, i.e. through canonical targeting by KSHV miRNAs. The first filter was the extraction of the transcripts that were significantly downregulated by 1.4 to 4-fold, reflecting typical levels of miRNA-mediated repression. It is highly plausible that when applying this filter we have eliminated from our datasets the genes that might undergo low levels, or inversely, unusual high levels of destabilisation of their transcript. However, this allowed us to discard part of the alterations observed in gene expression that are due to natural variations, or on the other hand, part of the genes indirectly repressed by the expression of KSHV miRNAs. The second filter was to only look at the transcripts that contained in their 3'UTR at least one 6-mer seed match to one of the KSHV miRNAs. Even if we introduced that way a bias that potentially excludes certain transcripts, such as those that could be bound by only one of the viral miRNAs through a non-canonical target site (i.e. bulges in the seed region pairing), or those with sites outside of the 3'UTR (CDS or 5'UTR). In this way, we restricted

our analysis in the hope to identify only those putative targets that are the most efficiently repressed.

The cellular specific downregulation seemed to be confirmed regarding our list of putative targets after having applied our filters, with only 21.7% of the B cells' and 15.6% of the endothelial cells' potential targets in common; endothelial cells showed approximately 1.4-fold more predicted targets than B cells. Such cell-specific regulation by KSHV miRNAs may be due to many reasons. The pattern of mRNA expression in each cell type (i.e. cell type-specific expressed genes) could be a first explanation, making observable the repression of solely the targets expressed in each cell type. Similarly, cell-specific expression of alternative transcripts with different shorter 3'UTRs, therefore that miss some, if not all the binding sites, could also explain such a discrepancy. It is worthy to note that if this is effectively one of the explanations, and if this is not simply due to chance, but more so because of a cell-specific selective avoidance of targeting by KSHV miRNAs, it is interesting to thus speculate that this could be the result of the co-evolution between the virus and its host. However, taking into account the lack of conservation of KSHV miRNAs among the other closely related herpesviruses, this interpretation seems intuitively unlikely. Nevertheless, such hypothetic avoidance might be the result of high selection pressures due to strong deleterious consequences of targeting by viral miRNAs, forcing a rapid adaptation of the host-targeted sequences. In effect, such avoidance of detrimental regulation by KSHV miRNAs would also be beneficial for the virus, as its goal is to persist indefinitely in the infected host without threatening its survival. In addition, as KSHV and RRV each express a miRNA with a conserved seed sequence, it could be interesting to further investigate such hypotheses for these potentially ortholgous miRNAs. Combinatorial targeting is an important strategy of regulation employed in animal cells, and some viral miRNAs have converged to target transcripts also bound by cellular miRNAs, as shown for the co-targeting by EBV miRNAs and the cellular miR-17~92 cluster of an important common set of genes (Riley et al., 2012). Furthermore, a case of synergistic targeting of the MICB 3'UTR by one KSHV and one cellular miRNA has also been reported, in addition to its co-targeting by another cellular miRNA (Nachmani et al., 2009). Some of these putative important co-targeting cellular miRNAs (that would be needed for an efficient repression of some of KSHV miRNAs targets and that would be cell specifically expressed), could thus explain our observations if their expression would lack in one or the other cell type. Another possibility would be the need of accessory protein factors for some of the miRNA-repressed targets or inversely the presence

of some protein factors inhibiting miRNA-mediated repression, and that would be cell-specifically expressed. Finally, according to Seitz's model, an important part of the predicted targets are pseudotargets, within both the common and the cell-specific sets of putative targets. Therefore, a last interesting explanation to mention would be the patterns of expression of pseudotargets that would substantially differ in each cell line, giving rise to different efficiencies of repression of the functional targets in each cell line.

Apart from the possibility of protein factors altering some of the miRNA-mediated regulations (and which are not really currently well known), most of these hypotheses could be tested using bioinformatics. For example, through combining the data of our seed-match analysis to that of the growing amount of data from recent research in public databases obtained from large-scale approaches such as PAR-CLiP. Ideally, this would be matched to the particular cell type in question, and linked to cell-specific mRNA and miRNA expression, what can also be found in public databases. In particular we would need to look at alternative 3'UTRs, and perform an analysis of our data on the phenomenon of cellular and viral miRNAs that potentially co-target the same transcripts. An exception has to be made for the pseudotarget hypothesis, which, if tested by bioinformatics, would probably require a complex algorithm taking in to account many parameters, and that would have to be developed at the bioinformatic level. The issues of cell-specific mRNA and alternative 3'UTR expression patterns should have been alleviated when screening the candidates by luciferase assays, as we force the expression of the longest form of the candidate's 3'UTR, as described in the Ensembl database. However, it is highly probable that the reporter assays have resulted in some false negatives due to a potentially different set of pseudotargets or to the lack of expression of some cellular miRNA in HEK293 cells that were used. Ideally, to solve these issues, the luciferase assays could have been performed in B cells and endothelial cells. However, for convenience they were realised in HEK293 cells; a cell line that is frequently used for such type of assays, due to the ease of achieving high rates of transfection efficiency, as well as the ease of co-transfection. Indeed, efficiencies of transfection for such cell types are around 100%. By contrast endothelial cell lines, such as HUVECs are harder to culture, difficult to transfect, and more susceptible to the toxic effects of reagents. B cells lines are also difficult to transfect, in addition that they require electroporation or nucleofection, which are more laborious methods, especially when considering reporter assay screening.

In the end, after the screening by luciferase assays of our chosen candidates from our prediction list, we unfortunately obtained a low rate of validation. Only two of the candidate genes assayed showed an interesting significant repression: CASP3 and RAD51AP1. Out of the 16 candidates assayed, this represents a validation rate of only 12.5%. Nevertheless, 3 other candidates showed very reproducible but low downregulation: MDM2, TBK1 (approximately 10% for both) and p53 (approximately 5%) (Figure 4B of the article). Although the p-values have not been tested for these genes, the repressions observed seem effectively significant given their very low standard deviation. Whether postulating that these were significant downregulations, this would then lead to a validation rate of approximately 31%, which is better, but still low, and which could be subject of discussion regarding the relevance of such low repression (discussed in the next section). The low rate of validation we obtained can be explained by a certain number of flaws in our approach, of which some of them are discussed above. These may have resulted in the predictions of targets that in fact are genes which are definitely not directly regulated by the viral miRNAs, and whose expression are indirectly altered upon expression of the viral miRNAs; we also have to consider the fact that some of the non-validated genes could represent false negative results, i.e. genes targeted by KSHV miRNAs, but for which our experimental conditions failed to observe any regulation. The fact that the genes indirectly regulated by KSHV miRNAs contain seed-matches is not a sufficient condition to allow for their binding and repression. As discussed in section 3.2.1.2 of the introduction, the pairing of a miRNA to its binding site is not only sequence dependent, but depends on other factors. It is now accepted, that a key consideration in miRNA/target interaction is the notion of accessibility, imparted by the secondary structure of the RNA. In addition, the miRNA can also eventually bind to a target site, but without inducing the repression of the transcript; this has been observed in some PAR-CLiP experimental data. Furthermore, a 6-mer seed-match is not the best seed-match determinant. Indeed, when looking at the binding sites we validated for CASP3 targeting by KSHV miRNAs, they all contained a 7-mer seed-match, which is a better determinant for miRNA target sites, referred to as M8 in Figure 6 of the article (described in section 3.2.1.1 of the introduction). However, the risk of applying to one of our filters a more stringent 7-mer seed-match research, would have been to exclude a certain number of real targets, even if it would have perhaps considerably enriched our list of putative targets, and thus increased our validation rate. Concerning the false negatives, they could result from the considerations discussed above concerning the cell type context used to perform the reporter assays, or the fact that some of our putative candidates could have harboured important binding sites for an

effective repression in their CDS or 5'UTR, as shown by an Ago2-IP-based approach for some KHSV miRNAs validated targets (Dölken et al., 2010a). In effect, on closer inspection of some of the PAR-CLiP data obtained on KSHV-infected PEL cells (Gottwein et al., 2011), putative binding sites of both viral and cellular miRNAs were identified in the 3'UTRs of MDM2, TBK1, CCNT2 and CASP9; in our study, the two firsts, as mentioned above, did show very low downregulation, and the two others showed no downregulation (Figure 4B of the article). Although using our validation approach, we were unable to verify that a viral miRNA alone (or a combination thereof) could lead to their downregulation, it is interesting to speculate that as Gottwein *et al.* also identified these candidates, that in our study they may indeed be false negatives. For example they could be regulated in a cooperative manner with the cellular miRNAs predicted to target them by PAR-CliP, and that could possibly, be not expressed in HEK293. Of particular interest is CCNT2, for which Gottwein *et al.* data show numerous putative binding sites for several KSHV miRNAs.

The recently developed large-scale RISC-IP-based approaches, and more particularly CLIP-based approaches (detailed in section 4.1.3 of the introduction), offer a powerful alternative to candidate-based approaches. Indeed, they represent reliable methods with much higher validation rates, and result in a global overview of the genes targeted without the inherent bias of candidate approaches where the gene of interest is chosen subjectively. A striking example of their efficiency is the PAR-CLiP conducted by Gottwein *et al.*, in which they predicted CASP3 as a target of KSHV miRNAs. Importantly, they were able to identify the same three binding sites that we validated (Gottwein et al., 2011). As such, this represents an interesting example of how both a candidate approach and a biochemical approach can arrive at the same conclusion for a given gene. However, despite the power of these genome-wide methods, the identified binding sites do not assess the functionality of the predicted target interactions. In summary, such methods definitely represent the best current approaches to identify putative targets of both viral and cellular miRNAs.

3. Insights from our results

During my thesis, we have demonstrated the ability of three KSHV miRNAs to target and efficiently repress the expression of the CASP3 gene, resulting in a biologically relevant inhibition of CASP3-dependent drug-induced apoptosis in KSHV-infected cells. It is worthy to note that this correlates with a recent study indicating that the active form of CASP3 is less frequently detected in Kaposi's sarcoma lesions in patients from Brazil (Ramos da Silva et al., 2007). Our data therefore suggests that apoptosis regulation by the viral miRNAs could contribute to the malignant phenotype triggered by KSHV infection.

In addition to CASP3, we have validated by luciferase assay the repression of RAD51AP1 gene by KSHV miRNAs. Consistently with our results, RAD51AP1 is also found to be predicted as a KSHV miRNAs target in Gottwein *et al.* PAR-Clip data. RAD51AP1 is a DNA binding protein that participates in RAD51-mediated homologous recombination (Modesti et al., 2007). The repression of RAD51AP1 will require further validation, as it might play a role in the genomic instability in which vCyc has been implicated in the context of viral infection (Koopal et al., 2007). The reproducible downregulations observed by luciferase assays of MDM2, TBK1 and p53, but weak (approximately 5% to 10%), raise the question about their functional relevance. These genes could be pseudotargets of KSHV miRNAs, taking in account their low repression. On the other hand, due to their role in the respective signaling pathways in which they are involved, such low downregulation could be amplified by the downstream proteins which they activate in addition to the fact that these pathways are already targeted by KSHV proteins and could thus be seen as a redundancy operated by KSHV miRNAs to insure a good control by the virus of these pathways. Indeed, MDM2 oncogene and p53 tumour suppressor are intimately linked together, and their downregulation has major implications in cancer development; p53 is mutated in 50% of all cancers. The pathway in which they are involved is targeted at many different steps by KSHV proteins, as detailed in section 5.6 of the introduction. TBK1 is also an interesting gene to consider. In effect, TKB1 is an IκB kinase (IKK)-related kinase, such as IKBKE, the latter which plays a similar role (reviewed in (Shen and Hahn, 2011)). IKBKE has been reported to be a target of KSHV miRNAs and thereby to promote viral latency (mentioned in section 6.3.3 of the introduction); the putative targeting of TBK1 could thus have the same function. It remains also possible that the cellular context of the HEK293 was not favourable to the regulation of these genes by KSHV miRNAs, and that in the cells naturally infected by the virus, they would have undergone a stronger downregulation, that would thus have appeared more

relevant; another hypothesis could be that these genes are currently in the course of acquisition as KSHV miRNAs targets. Some further investigations of their downregulation by KSHV miRNAs could thus be of interest, to check if they could, or not, be functionally relevant targets.

Concerning CASP3 targeting, it is worthy to note that during our experiments, we were not able to detect any downregulation of the protein or decrease of its activity upon transient expression of the miRNAs, but only in stable cell lines, or after a long induction of several days of KSHV miRNAs expression in our Flip-In system. Furthermore, when looking in the literature, there is no study that could perform any knock-down of CASP3 protein upon transient experiments, but only by the stable expression of small haipins RNAs (shRNAs). This led us to hypothesise that CASP3 is a very stable protein with a very long half-life and not subjected to any turn-over (which seems consistent with its key role in the induction of apoptosis), and that the miRNAs could only exert an effect on the protein after a long period of expression. As such the levels of CASP3 would reduce in consequence of its dilution following cell division and through the alteration of its *de novo* synthesis by the miRNAs. This would thus imply that during *de novo* infection, the viral miRNAs would not play any role through the targeting of CASP3, and that this repression would only exert its function in controlling apoptosis once the latent infection is well established. Indeed, during lytic infection, many of the KSHV proteins are known to play a role in the inhibition of apoptosis, and therefore the miRNAs' role could be negligible in this function. The viral miRNAs would then act as a relay of the viral proteins functions, after the shut off of their expression induced by the establishment of the latency. This is in agreement with the hypothesis that viral miRNAs do not play any role (or if so a marginal role) during acute lytic infection, and that one of their principal functions is the control, during latency, of cellular genes (and naturally such cellular pathways) in a non-immunogenic manner, which would allow for a beneficial environment for the virus maintenance, permitting minimal viral protein expression and going "undetected" by the cell (detailed in sections 6.2 and 6.3 of the introduction).

Repressing CASP3 expression makes perfect sense for the virus when considering the key effector role of this protein. Activation of CASP3 is the convergent step of all the major pathways of apoptosis induction, and which when activated, induces the events leading to programmed cell death in an irreversible manner. Nevertheless, as discussed in section 4 of the results, two other pro-apoptotic genes have been reported to be targets of KSHV miRNAs:

TWEAKR and BclAF1. While it remains unclear if BclAF1 plays a role in the inhibition of apoptosis during KSHV infection, the role of TWEAKR in the control of IFN-γ-induced cell death has clearly been established. In addition, as detailed in section 5.6 of the introduction, the viral proteins expressed during KSHV latency have been shown for most of them (LANA, LANA-2, vCyc and vFLIP) to play a direct or indirect role in inhibiting programmed cell death at different levels. Furthermore, when studying the role of KSHV miRNAs during apoptosis, we have also observed through Annexin V assays on the staurosporine-treated DG-75 cell line, an inhibition of caspase-independent apoptosis. This means that the viral miRNAs target at least another additional cellular factor, which is involved in the caspase-independent induction of apoptosis. Therefore, it is highly probable that KSHV miRNAs have evolved to target several cellular genes involved in programmed cell death to ensure the good control of its inhibition. In conclusion, as with other viral mechanisms of host interactions, KSHV miRNAs display redundancy or more so a combinatorial activity, by acting in concert with the viral proteins expressed during latency, in order to target cellular apoptotic pathways.

As a perspective, as most KSHV miRNAs bear viral-specific seed sequences with no homology to their cellular counterparts (such as for the three miRNAs that we have demonstrated to target CASP3 transcript), one can thus envisage as a novel therapeutic approach the delivery of specific inhibitors of these viral miRNAs in KSHV-infected patients, with no or minimal adverse effects, as the cellular miRNAs would thus not be affected. Indeed, very promising results have been obtained *in vivo* and in preliminary clinical studies when inhibiting miR-122 in HCV-positive patients, effectively blocking viral replication and cure the patients from the infection (Haussecker and Kay, 2010; Lanford et al., 2010). We can thus expect that the specific inhibition in KSHV-patients of both the viral miRNAs targeting CASP3 and TWEAKR, could permit to restore apoptotic clearance induced by both the inner cellular mechanisms triggered by the viral infection and through the stimulation of the extrinsic pathway by the immune system.

4. A potential inhibition of CASP3-mediated cleavage of Dicer by viral miRNAs

It would be interesting to expand upon our field of investigation concerning the regulation of CASP3 by the miRNAs of KSHV in respect to the recent evidence that CASP3 is a substrate for cleavage by Dicer, and to determine whether the targeting of CASP3 by viral miRNAs would be also in the aim to ensure their proper production during infection.

In effect, it was shown by Matskevich and Moelling in a human cell line, that during the induction of apoptosis, Dicer undergoes cleavage by CASP3, liberating two fragments (185 kDa, and 50 kDa). The authors observed that the cleavage resulted in a loss of the catalytic activity of the RNAse III domain of Dicer. Interestingly, they observed during HIV infection that the gp120-induced apoptosis led to a cleavage of Dicer (Matskevich and Moelling, 2008). An independent study by Ghodgaonkar *et al*., arrived at the same conclusion concerning Dicer cleavage by CASP3 during the initiation of apoptosis, but with the difference that the cleavage site was different (being located in the middle of the N-terminal RNAse III domain), and resulting in cleavage fragments of approximately equivalent size. The authors also investigated the consequences of Dicer cleavage resulting from the induction of apoptosis on the stability of cellular miRNAs, revealing a rapid degradation of 3 miRNAs they quantified (including miR-21 and let-7), starting to be observable 6 hours post induction (hpi) and that showed 40% to 60% of degradation 18-24 hpi (Ghodgaonkar et al., 2009). Lastly, a third study by Nakagawa *et al*. in *C. elegans* demonstrated that the mechanism of CASP3-mediated Dicer cleavage is conserved for the respective homologues (CED-3, DCR1). In this report, the identified cleavage site correlated with Ghodgaonkar *et al*. results. More interestingly, the smaller DCR1 cleavage fragment, which contains an intact RNAse III domain, was shown to be converted into a deoxyribonuclease (DNAse). The DNAse activity of this fragment was implicated in the fragmentation of genomic DNA, conferring an essential role for DCR-1 in the successful implementation of apoptotic processes in this species (Nakagawa et al., 2010).

As such, we would like to test whether Dicer is cleaved in our model of study, and secondly study the expression of KSHV miRNAs, to test whether they have an impact on Dicer cleavage, by reducing the level of cleaved Dicer resulting from miRNA-mediated repression of CASP3 in infected cells. This indirect mechanism induced by the viral targeting of CASP3 could be of importance in maintaining the proper level of expression of the viral miRNAs to ensure that their functions are not compromised. To date, no study has been conducted looking at the general impact of CASP3-mediated cleavage of Dicer on the overall pool of

miRNAs in a given cell. It can be envisaged to use small RNA cloning techniques, and high-throughput sequencing technologies to be able to study the impact of a potential loss of Dicer function on the expression levels of KSHV miRNAs, and equally to broaden this to the impact upon cellular miRNAs. Equally, it would be interesting to determine whether the cleavage of Dicer by CASP3 results in a conversion of its enzymatic activity to that of a DNAse, as observed in *C. elegans*. To date, this has not been shown for any mammalian system, as such it would be of particular interest to investigate this further and look at the role of DNA fragmentation during apoptosis in infected cells.

Finally, using Ago2-IP coupled to bioinformatics predictions, we have identified with our collaborators CASP3 as a potential target of two miRNAs from EBV. The predicted miRNAs do not display any homology with the three KSHV miRNAs that we determined, and they do not target the same 3'UTR sites. As such, this could possibly represent a case of convergent evolution of their miRNAs in the inhibition of apoptosis for ensuring the continued survival of the viruses within the infected cell. Furthermore, taking in account the fact that co-infections of EBV and KSHV are frequently observed, a co-targeting of CASP3 in co-infected cells could probably result in a stronger inhibition of CASP3 expression, and would thus establish an example of two viruses cooperating to repress with a stronger efficiency a given gene. To validate the EBV miRNAs potentially responsible for this regulation, we can use the same techniques as were used to identify CASP3 as a target of KSHV miRNAs, notably tiny LNAs directed against the EBV miRNAs predicted to target CASP3, as well as reporter based assays. In conclusion, another very interesting aspect of this potential convergent evolution to investigate, which would thus explain the reason and the necessity of targeting in particular CASP3 -knowing that other proteins involved in apoptosis have already been identified as targets of KSHV and EBV miRNAs-, is the fact that these viruses would need to inhibit the cleavage of Dicer by CASP3 to ensure the proper expression of their own miRNAs.

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ANNEXES

Systematic analysis of viral and cellular microRNA targets in cells latently infected with human gamma-herpesviruses by RISC immunoprecipitation assay.
Dölken, L., Malterer, G., Erhard, F., Kothe, S., Friedel, C.C., Suffert, G., Marcinowski, L., Motsch, N., Barth, S., Beitzinger, M., et al.

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Systematic Analysis of Viral and Cellular MicroRNA Targets in Cells Latently Infected with Human γ-Herpesviruses by RISC Immunoprecipitation Assay

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SUMMARY

The mRNA targets of microRNAs (miRNAs) can be identified by immunoprecipitation of Argonaute (Ago) protein-containing RNA-induced silencing complexes (RISCs) followed by microarray analysis (RIP-Chip). Here we used Ago2-based RIP-Chip to identify transcripts targeted by Kaposi's sarcoma-associated herpesvirus (KSHV) miRNAs (n = 114), Epstein-Barr virus (EBV) miRNAs (n = 44), and cellular miRNAs (n = 2337) in six latently infected or stably transduced human B cell lines. Of the six KSHV miRNA targets chosen for validation, four showed regulation via their 3′UTR, while two showed regulation via binding sites within coding sequences. Two genes governing cellular transport processes (TOMM22 and IPO7) were confirmed to be targeted by EBV miRNAs. A significant number of viral miRNA targets were upregulated in infected cells, suggesting that viral miRNAs preferentially target cellular genes induced upon infection. Transcript half-life both of cellular and viral miRNA targets negatively correlated with recruitment to RISC complexes, indicating that RIP-Chip offers a quantitative estimate of miRNA function.

INTRODUCTION

Herpesviruses are large DNA viruses which after primary infection persist for life, leaving the infected individual at risk for reactivation and subsequent disease. They are divided into three subfamilies based on sequence homologies and unique biological features (α-, β-, and γ-herpesviruses). Humans are infected with two members of the γ-herpesvirus family, namely Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). Both infect B cells and can induce proliferative diseases in humans (Barozzi et al., 2007). KSHV is involved in the development of several human tumors, including Kaposi's sarcoma, primary effusion lymphoma (PEL), and multicentric Castleman disease (reviewed in Dourmishev et al., 2003; Schulz, 2006). PEL tumor cells are thought to originate from postgerminal center B cells due to the presence of hypermutated immunoglobulin genes (Gaidano et al., 1997) and display an intermediate immunophenotype between immunoblasts and plasma cells (Carbone et al., 1996; Nador et al., 1996). The cell line body cavity-based lymphoma-1 (BCBL-1) was established from a malignant effusion (Renne et al., 1996) and serves as a model cell line for PEL. Four groups independently reported on 12 miRNAs expressed during latent infection in PEL cells (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005), which are remarkably conserved among isolates from many different clinical sources (Marshall et al., 2007).

EBV has been linked to various human malignancies including Burkitt's and Hodgkin's lymphoma, NK/T cell and peripheral T cell lymphoma, posttransplant lymphoma, and nasopharyngeal and gastric carcinoma and is responsible for posttransplant lymphoproliferative disease (PTLD) (Delecluse et al., 2007). EBV readily transforms primary human B-lymphocytes which are the in vitro correlate of EBV-associated PTLD arising under severe immunosuppression (Carbone et al., 2008). EBV encodes for at least 25 miRNAs derived from three separate miRNA clusters (Cai et al., 2006; Grundhoff et al., 2006; Pfeffer et al., 2004; Zhu et al., 2009).

Since viral miRNAs were first described in 2004 (Pfeffer et al., 2004), only very few targets have been identified. For KSHV these include targets involved in angiogenesis, proliferation, immune evasion, or repression of apoptosis (Gottwein et al., 2007; Nachmani et al., 2009; Samols et al., 2007; Skalsky

et al., 2007; Ziegelbauer et al., 2009). In addition, EBV miRNAs promote cell survival (Choy et al., 2008) and target cellular chemokines (CXCL-11) (Xia et al., 2008). Similar to human cytomegalovirus miR-UL112-1, both kshv-miR-K12-7 and ebv-miR-BART2 target MICB, an activating stress-induced NK cell ligand involved in immune recognition of infected cells (Nachmani et al., 2009; Stern-Ginossar et al., 2007).

Recently, immunoprecipitation of RISCs followed by microarray analysis of the RISC-bound miRNA targets (RIP-Chip) was shown to permit the identification of hundreds of cellular miRNA targets with high specificity (Baroni et al., 2008; Beitzinger et al., 2007; Easow et al., 2007; Hendrickson et al., 2008; Karginov et al., 2007; Keene et al., 2006). Here we applied Argonaute 2 (Ago2)-RIP-Chip to identify both cellular and viral miRNA targets in six human B cell lines. We report on the identification of 2337 putative targets of cellular and 158 targets of viral miRNAs in human B cells. Thus, we provide a comprehensive atlas of cellular miRNA targets in human B cells and extend the list of cellular targets of all viral miRNA targets identified so far by > 5-fold.

RESULTS

Study Design

Cellular targets of human γ-herpesvirus miRNAs in B cells were identified by immunoprecipitation (IP) of RISCs followed by microarray analysis of the coimmunoprecipitated mRNAs (RIP-Chip). To identify KSHV miRNA targets, we used the KSHV-positive cell line BCBL-1 and the KSHV- and EBV-negative cell line DG75 transduced by a lentiviral vector to stably express the ten intronic KSHV miRNAs (DG75-10/12). DG75 transduced with eGFP (DG75-eGFP) served as control (Wang et al., 2004). To identify viral miRNA targets of EBV, the prototype B95.8 strain of EBV (BL41 B95.8) and its parental Burkitt's lymphoma cell line (BL41) were used. As B95.8 has lost more than half of the viral miRNAs (Cai et al., 2006), the Jijoye cell line featuring a nondeleted strain, where all known EBV miRNAs are present (Grundhoff et al., 2006), was included.

Identification of Cellular miRNA Targets in Human B Cells

We established a protocol for RISC IP in human B cells using the recently described monoclonal antibody to human Ago2 (α-hAgo2; 11A9) (Rudel et al., 2008). A monoclonal antibody against bromodesoxyuridine (BrdU) served as control. In all experiments the efficiency of the IP was analyzed by quantitative PCR. Usually, an ~1000-fold (670- to 3300-fold) enrichment of cellular Let7a levels was observed when comparing the Ago2-IP with the BrdU-IP samples. A consistent recovery rate of ~50% of Let7a was observed, indicating that Ago2-bound miRNAs comprise at least half of the cellular miRNA pool in human B cells (see Figure S1 available online). Enrichment of cyclin E1 mRNA (CCNE1), a cellular miRNA target of both hsa-miR-16 and hsa-miR-15a (Bandi et al., 2009; Liu et al., 2008), served as a positive control. It was typically enriched by ~8-fold.

Next, microarray analyses of two independent biological replicates for each cell line were performed using Affymetrix human Gene ST 1.0 arrays. For DG75-eGFP, DG75-10/12, and BCBL-1, both the α-Ago2-IP and the α-BrdU-IP samples were analyzed. For both BL41 and BL41 B95.8, the amounts of RNA obtained by the α-BrdU-IPs were too small (50–100 ng) for microarray analysis using the same experimental setting and microarray platform. As the control IP is thought to merely represent total RNA levels in the cell and total RNA has been successfully used as control for the Ago2-IP (Weinmann et al., 2009), we analyzed total RNA instead of the α-BrdU-IP sample for BL41, BL41 B95.8, and Jijoye. Enrichment of transcripts in the Ago2-IP was highly concordant for all six human B cell lines irrespective of whether total RNA or the α-BrdU-IP sample was used (Pearson's correlation coefficient between 0.34 and 0.96 for all combinations, $p < 2.2 \times 10^{-16}$). The complete set of data is provided in Table S1.

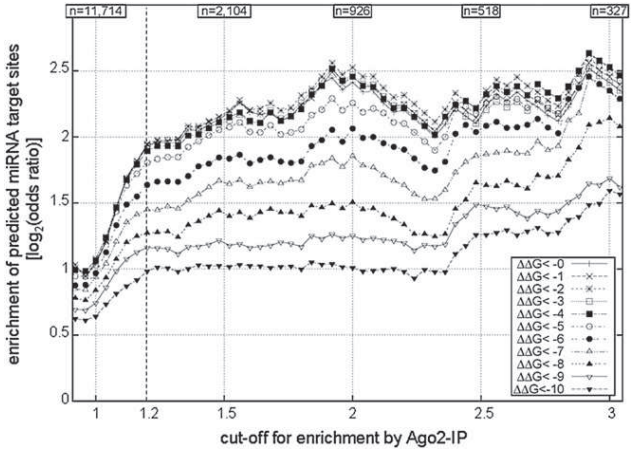


Figure 1. Overrepresentation of Predicted Binding Sites for Cellular miRNAs

Binding sites for these 44 cellular miRNAs expressed in human B cells (Landgraf et al., 2007) were predicted using PITA software and different cutoffs for free binding energies (ΔΔG) ranging from 0 to −10 kcal/mol. Odds ratios for predicted miRNA-binding sites are shown for all genes with a mean enrichment in the Ago2-IP versus control across all six cell lines greater than the indicated value. For each value, the number of transcripts with a mean enrichment greater than this cutoff is shown.

To test for overrepresentation of predicted miRNA-binding sites of cellular miRNAs among the enriched transcripts, we obtained 44 cellular miRNAs expressed in BL41, BL41 B95.8, and DG75 from the miRNA expression atlas (Landgraf et al., 2007) (Table S2A). We used the miRNA target prediction program PITA (Kertesz et al., 2007) and different ΔΔG cutoff values ranging from 0 to −10 kCal/mol to test for overrepresentation of favorable target sites of any of these miRNAs in target 3′UTRs (Figure 1). Overrepresentation was highly significant down to very low mean enrichments ($p < 10^{-58}$ for a mean enrichment of 1.2-fold). Based on statistical analysis, we identified 2337 transcripts significantly enriched across all six B cell lines ($p < 0.05$, one-sided paired t test) with a mean enrichment > 1.2-fold. Enrichment of their transcripts in the six cell lines is shown in Figure 2A, and a complete list is provided in Table S2B. For these genes, predicted binding sites were significantly overrepresented within 5′UTRs ($p = 8.59 \times 10^{-3}$), coding sequences ($p = 2.02 \times 10^{-27}$), and 3′UTRs ($p = 6.19 \times 10^{-63}$, Fisher's exact test and ΔΔG < −4 kCal/mol, see also Table S3A).

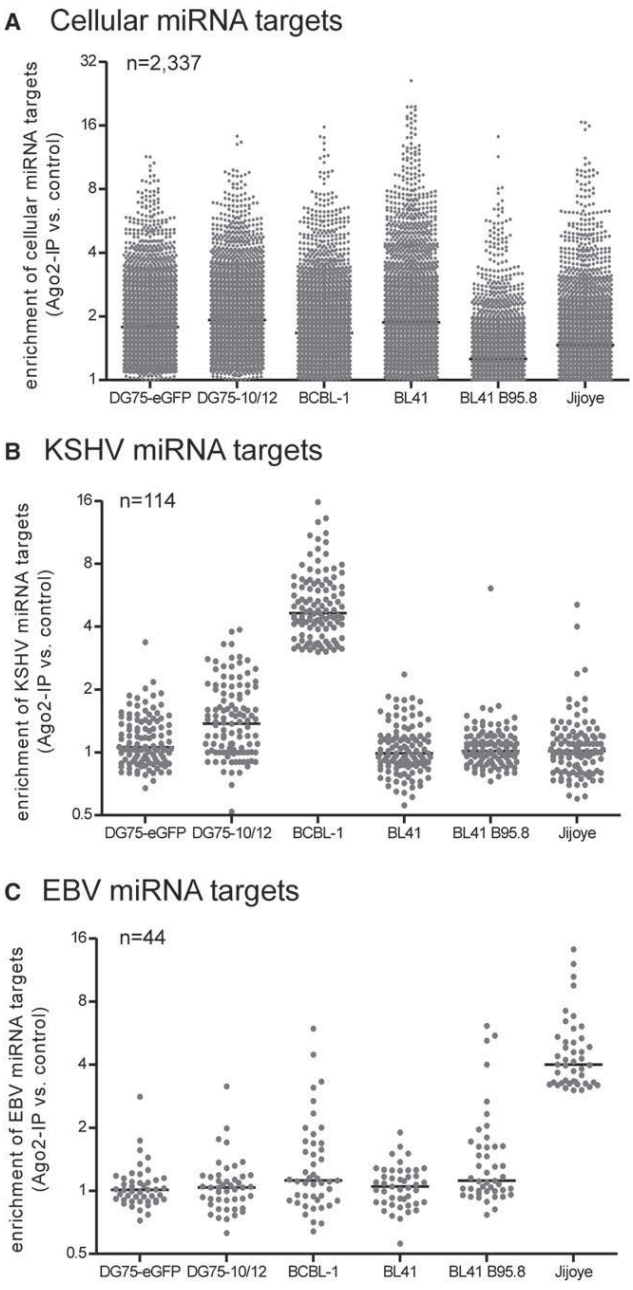


Figure 2. Enrichment of miRNA Targets by RISC Immunoprecipitation

Enrichment of cellular (A, n = 2337), KSHV (B, n = 114), and EBV (C, n = 44) miRNA targets in the Ago2-IPs of the six cell lines under study is shown.

Identification of Viral miRNA Targets

Targets of viral miRNAs may either be exclusively targeted by viral miRNAs or also be targeted by cellular miRNAs. In the first case, their transcripts should only be enriched in the cell lines expressing the viral miRNAs, but not in the two control cell lines DG75-eGFP and BL41. For transcripts targeted by both cellular and viral miRNAs, enrichment in BCBL-1 or Jijoye should be substantially greater than in the two control cell lines. In order to exclude false positives, we defined stringent criteria for a transcript to be considered a KSHV or EBV miRNA target. Enrichment in either BCBL-1 or Jijoye had to be > 3.0 and enrichment in both DG75-eGFP and BL41 < 1.2. For targets of cellular miRNAs (enriched > 1.2-fold in DG75-eGFP and/or BL41), a > 3-fold greater enrichment in BCBL-1 or Jijoye than in both DG75-eGFP and BL41 was required. Enrichment of the 114 KSHV and the 44 EBV putative miRNA targets which fulfilled these criteria are shown in Figures 2B and 2C, respectively. Interestingly, no transcripts were enriched > 3-fold in either DG75-10/12 or BL41 B95.8 and < 1.2-fold in DG75-eGFP and BL41, which were not also enriched > 3-fold in BCBL-1 or Jijoye, respectively. This most likely reflects the reduced levels of viral miRNAs in DG75-10/12 (see Figures S2A and S2B) and BL41 B95.8.

False-positive viral miRNA targets may result from both cellular miRNAs and cellular transcripts induced upon infection. On the one hand, cellular miRNAs induced upon infection may mimic the effect of the viral miRNAs. While little is known about the effect of KSHV infection on cellular miRNA expression, EBV infection has been shown to result in induction of a number of cellular miRNAs, including hsa-miR-155, hsa-miR-146a, and hsa-miR-21 (Gatto et al., 2008; Mrazek et al., 2007). Out of 17 confirmed targets of hsa miR-155 (Gottwein et al., 2007; Skalsky et al., 2007; Vigorito et al., 2007), we noted six (BACH1, FOS, IKBKE, RFK, RPS6KA3 [Gottwein et al., 2007] and SPI1 [Vigorito et al., 2007]) to be enriched between 1.4- and 2.7-fold in Jijoye (Table S3B). This is consistent with significant repression of hsa-miR-155 targets upon EBV infection (Gatto et al., 2008). Still, none of them fulfilled our more stringent criteria. In addition, none of the 12 targets of hsa-miR-21 identified by Yang et al. (Yang et al., 2009b) and none of the four targets of hsa-miR146a (Pauley et al., 2008; Tang et al., 2009) were enriched > 1.5-fold in any of the six cell lines. Thus, we believe that induction of cellular miRNAs did not significantly corrupt our list of EBV miRNA targets.

On the other hand, virus infection may result in the induction of genes that are then targeted by cellular miRNAs. If expression levels are below the detection limit of the microarrays in uninfected B cells, this would present as a selective enrichment of their transcripts in the Ago2-IP in the infected cells; i.e., they would be misinterpreted to be viral miRNA targets. Therefore, we distinguished noninduced targets (KSHV, n = 72; EBV, n = 26; see Tables S4A and S4B) from targets that showed >1.5-fold higher expression levels in the BrdU-IPs of BCBL-1 compared to DG75-eGFP or in total RNA levels of Jijoye when compared to BL41 (KSHV, n = 42; EBV, n = 18; see Tables S4C and S4D). To investigate whether these genes are indeed targeted by viral miRNAs, we studied the overrepresentation of predicted viral miRNA target sites among both the noninduced and induced targets using the PITA algorithm. Indeed, predicted target sites of viral miRNAs were significantly overrepresented in the 3′UTRs and, to a lesser extent, in the 5′UTRs among both the induced and noninduced KSHV and EBV miRNA targets (Tables S3A, S5A, and S5B). Therefore, we concluded that also the majority of the genes induced > 1.5-fold in either BCBL-1 or Jijoye are indeed targets of viral miRNAs.

Validation of Viral miRNA Targets by TaqMan PCR

We representatively validated miRNA targets identified by RIP-Chip analysis using quantitative TaqMan PCR (qPCR). We

randomly chose 11 KSHV miRNA targets that were also enriched > 1.5-fold in DG75-10/12. In addition, we chose eight EBV miRNA targets as well as four well-described targets of cellular miRNAs. Three of the eight EBV miRNA targets (IPO7, TRIM32, and FBXO9) were enriched > 1.5-fold in BL41 B95.8. The rest (n = 5) were not enriched in BL41 B95.8 (enrichment = 0.77–0.96). New RISC-IPs were performed for all six cell lines using two independent biological replicates. Quantitative PCR was performed, and expression levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT), a commonly used housekeeping gene (Fu et al., 2009), found not to be enriched in our Ago2-IPs. The noncoding RNA H19 (Weinmann et al., 2009) and cyclin E1 mRNA (CCNE1) served as quantitative positive controls (Figures 3A and 3B). Enrichment was significant for 9 of the 11 KSHV miRNA targets in both DG75-10/12 and BCBL-1. ZNF684 was significantly enriched only in BCBL-1. All EBV miRNA targets (8/8 = 100%) were significantly enriched in Jijoye. Significant enrichment in BL41 B95.8 was also observed for IPO7 and TRIM32. Recently, the activating NK cell ligand MICB was found to be targeted by both ebv-miR-BART2-5p, kshv-miR-K12-7 (Nachmani et al., 2009) and hcmv-miR-UL112-1 (Stern-Ginossar et al., 2007). In contrast, MICA, a closely related NK cell ligand, is predominantly targeted by cellular miRNAs (Stern-Ginossar et al., 2008). MICB was enriched significantly stronger in both BL41 B95.8 and Jijoye than in BL41, consistent with ebv-miR-BART2-5p being one of the EBV miRNAs still expressed in BL41 B95.8. Enhanced enrichment of MICB in BCBL-1 was less pronounced but nevertheless detectable. In summary, 18 of 19 (95%) of the viral miRNA targets we tested by qPCR were confirmed.

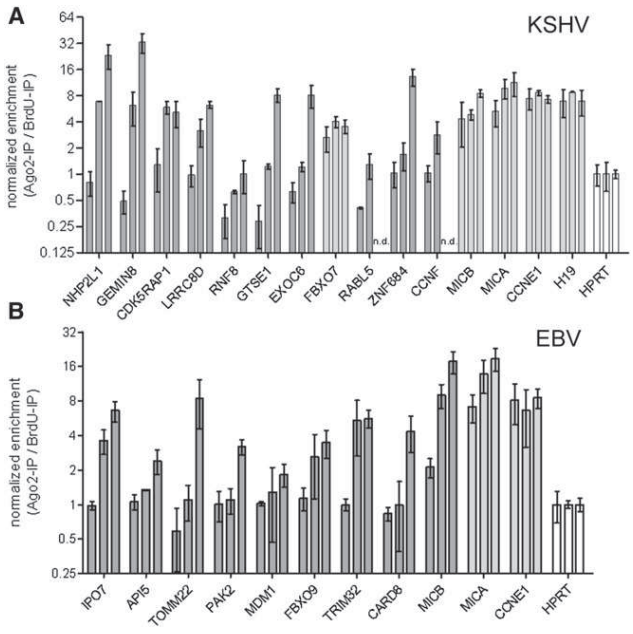


Figure 3. Validation of KSHV and EBV miRNA Targets by qPCR

(A) For each gene, mRNA enrichment (Ago2-IP versus control-IP) normalized for HPRT expression levels is shown for DG75-eGFP (left), DG75-10/12 (middle), and BCBL-1 (right).

(B) For each gene, mRNA enrichment (Ago2-IP versus control-IP) normalized for HPRT expression levels is shown for BL41 (left), BL41 B95.8 (middle), and Jijoye (right). Transcripts with significant enrichment in cells expressing viral miRNAs are indicated in dark gray and cellular miRNA targets in light gray. MICA, CCNE1, and H19, which are known to be targeted by cellular miRNAs, were used as controls. Values represent the mean ± SD of two independent experiments performed in duplicate.

Validation of KSHV miRNA Target Candidates by Dual-Luciferase Assays

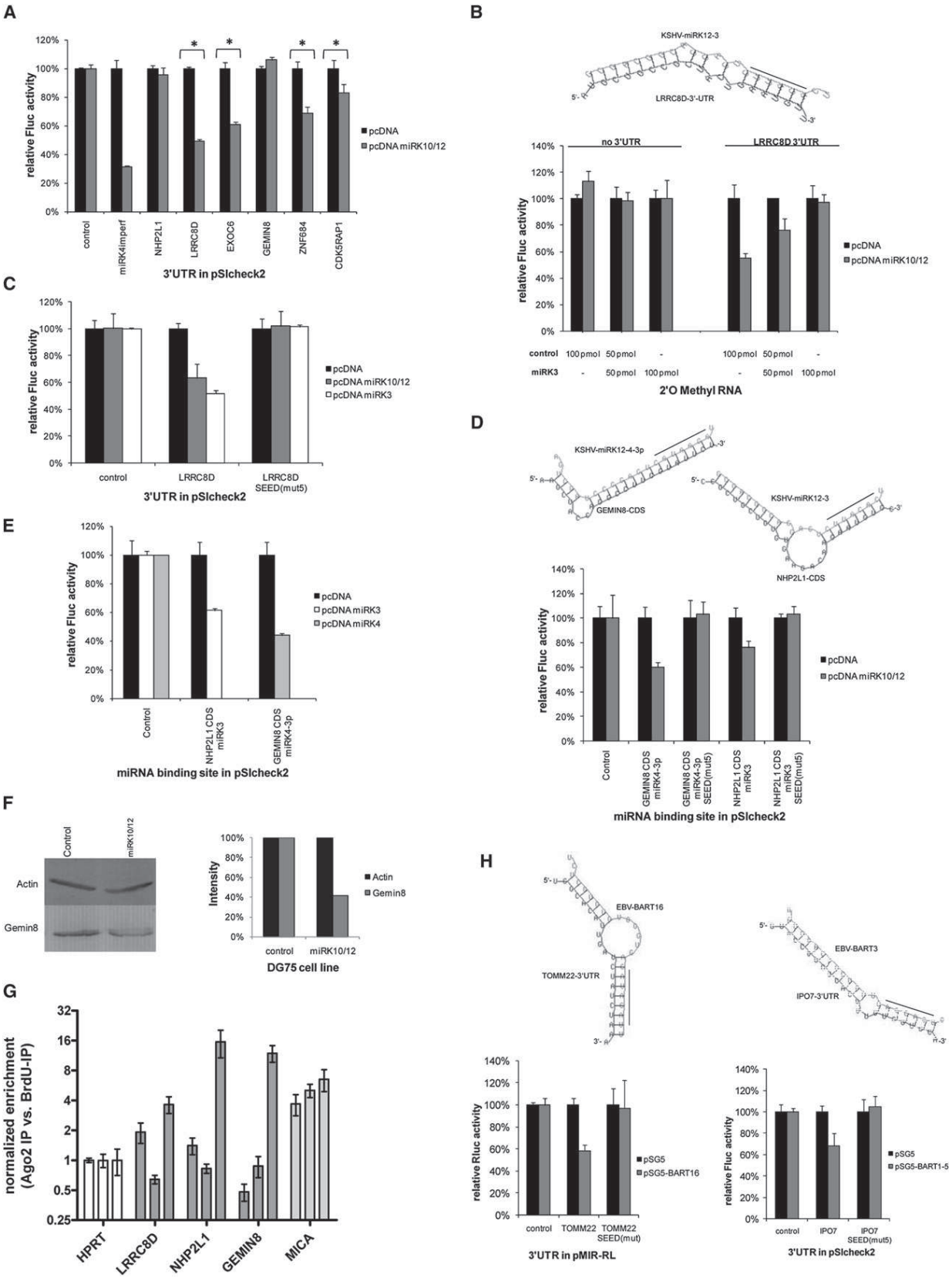
In order to experimentally validate viral miRNA targets identified by RIP-Chip and confirmed by qPCR, we cloned the full-length 3′UTRs of 6 of the 11 putative KSHV miRNA targets into a GATEWAY-compatible derivative of the dual-luciferase vector pSIcheck. This vector constitutively expresses renilla and firefly luciferase. Expression of the latter is regulated by the subcloned 3′UTR. KSHV miRNAs were expressed by cotransfection of pcDNAmiR10/12, a vector encoding the ten intronic KSHV miRNAs with the reporter constructs, and luciferase assays were performed 16 hr later. While no effect of KSHV miRNA expression was detectable for the 3′UTR reporter constructs of NHP2L1 and GEMIN8, significant suppression of firefly luciferase expression was detectable for the other four candidates in three independent experiments (Figure 4A). The strongest inhibition was exerted on the LRRC8D reporter (~50%) construct, while the effect on CDK5RAP1 was rather small (~15%) but nevertheless reproducible and significant.

LRRC8D Is Regulated by kshv-miR-K12-3 through a Single Target Site in Its 3′UTR

LRRC8D is a leucin-rich type III transmembrane protein thought to be involved in proliferation and activation of lymphocytes and macrophages. Modeling of possible hybrids between the LRRC8D 3′UTR and the KSHV-encoded miRNAs suggested a strong interaction of the 3′UTR with kshv-miR-K12-3 (Figure 4B, Table S6A). To test whether expression of kshv-miR-K12-3 alone was responsible for the downregulation of luciferase activity in the LRRC8D 3′UTR reporter construct, we performed derepression assays by using a 2′-O-methyl RNA complementary to kshv-miR-K12-3. The inhibition of kshv-miR-K12-3 resulted in a concentration-dependent reversion of luciferase repression, indicating that kshv-miR-K12-3 is selectively capable of causing the named effect (Figure 4B). To test whether the predicted binding site on the LRRC8D mRNA is the true and sole site of interaction with kshv-miR-K12-3, we mutated the seed region of the putative binding site of kshv-miR-K12-3 by inserting five point mutations according to Ziegelbauer et al. (Ziegelbauer et al., 2009) (Figure S3B). When the vector carrying this mutation was cotransfected with the vector expressing the ten intronic KSHV miRNAs or miR-K12-3 alone, the miRNA effect was no longer detectable (Figure 4C). These findings demonstrate that binding of kshv-miR-K12-3 to this single site in the 3′UTR of LRRC8D transcripts is necessary and sufficient to exert the inhibitory effect.

GEMIN8 and NHP2L1 Are Both Regulated through a KSHV miRNA-Binding Site within Their Coding Regions

Two KSHV miRNA targets, GEMIN8 and NHP2L1, could not be validated by 3′UTR dual-luciferase assays. Using RNAhybrid, we tested whether they might be regulated by binding sites located within their coding sequences or 5′UTRs. We identified


A
relative Fluc activity
pcDNA
pcDNA miRK10/12
control
miRK4imperf
NHP2L1
LRRC8D
EXOC6
GEMIN8
ZNF684
CDK5RAP1
3'UTR in pSIcheck2
B
KSHV-miRK12-3
LRRC8D-3'-UTR
no 3'UTR
LRRC8D 3'UTR
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KSHV-miRK12-4-3p
GEMIN8-CDS
KSHV-miRK12-3
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Control
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GEMIN8 CDS miRK4-3p SEED(mut5)
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miRNA binding site in pSIcheck2
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relative Fluc activity
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Control
NHP2L1 CDS miRK3
GEMIN8 CDS miRK4-3p
miRNA binding site in pSIcheck2
F
Control
miRK10/12
Actin
Gemin8
Intensity
Actin
Gemin8
control
miRK10/12
DG75 cell line
G
normalized enrichment (Ago2 IP vs. BrdU-IP)
HPRT
LRRC8D
NHP2L1
GEMIN8
MICA
H
EBV-BART16
TOMM22-3'UTR
EBV-BART3
IPO7-3'UTR
relative Rluc activity
pSG5
pSG5-BART16
control
TOMM22
TOMM22 SEED(mut)
3'UTR in pMIR-RL
relative Fluc activity
pSG5
pSG5-BART1-5
control
IPO7
IPO7 SEED(mut5)
3'UTR in pSIcheck2

a high confidence interaction site for kshv-miR-K12-4-3p in the coding sequence of GEMIN8 and for kshv-miR-K12-3 in the coding sequence of NHP2L1 (Figure 4D, Tables S6B and S6C). Reporter constructs were created containing the putative binding sites (128 nt for GEMIN8 and 146 nt for NHP2L1), and dual-luciferase assays were performed. In both cases, expression of the intronic KSHV miRNAs as well as the predicted individual KSHV miRNAs resulted in a significant repression of firefly luciferase activity, indicating that these two putative miRNA-binding sites can indeed be targeted by the respective KSHV miRNAs. In both cases, binding sites were confirmed by mutating the seed region of the predicted binding site by introducing five point mutations (Figure 4D, Figures S3B and S3C). In addition, significant repression of the luciferase signal was also observed upon cotransfection with the respective single KSHV-miRNA-expressing vectors (Figure 4E). To test whether regulation by KSHV miRNAs had any effect on endogenous protein levels, we performed quantitative Western blot analysis using antibodies to GEMIN8 and NHP2L1. While no specific signal could be obtained for NHP2L1 (data not shown), a 2.5-fold downregulation of GEMIN8 protein levels was observed for GEMIN8 in DG75-10/12 compared to DG75-eGFP (Figure 4F).

To test whether isolated expression of kshv-miR-K12-3 or -K12-4 was sufficient to efficiently recruit LRRC8D, NHP2L1, and GEMIN8 transcripts to Ago2 complexes, we performed RISC pull-downs in 293 cells expressing doxycyclin (dox)-inducible kshv-miR-K12-3 (293-miR-K3) or -K12-4 (293-miR-K4). Consistent with regulation of LRRC8D and NHP2L1 by kshv-miR-K12-3, qPCR revealed significant enrichment of their transcripts in the Ago2-IPs of 293-miR-K3 cells following dox induction, but not in 293-miR-K4 cells (Figure 4G). A weak enrichment of GEMIN8 in dox-induced 293-K12-4 cells was also detectable, but this did not quite reach significance. Therefore, additional miRNAs may be involved in recruiting NHP2L1 and GEMIN8 to Ago2 complexes.

Finally, we also cloned 3′UTRs of three putative EBV miRNA targets, namely TOMM22 (mitochondrial import receptor subunit TOM22 homolog), IPO7 (importin 7), and RAB13 (RAB13, member RAS oncogene family) into dual-luciferase vectors. Potential binding sites for EBV miRNAs were predicted using RNAhybrid (Rehmsmeier et al., 2004). In both cases, the predicted binding sites (for ebv-miR-BART-16 and ebv-miR-BART-3, respectively) were validated by luciferase assays by cotransfection of expression vectors for the respective miRNAs (Figure 4H). Mutagenesis of the seed regions of the putative binding sites resulted in complete loss of inhibition. No inhibition of luciferase expression by EBV miRNAs was detectable for the RAB13 reporter construct (data not shown).

Characteristics of Cellular and Viral miRNA Targets

It has been a long-standing matter of debate to what extent miRNAs influence the stability of their targets. Therefore, we compared transcript half-lives of the cellular miRNA targets with those of ~8300 genes expressed in BL41 taken from our recently published atlas of transcript half-lives in human B cells (Friedel et al., 2009). This atlas contained precise transcript half-lives for ~70% of the cellular miRNA targets. With a median half-life of 4.3 hr, transcript half-lives of the targets of cellular miRNAs were significantly shorter (Kolmogorov-Smirnov test, $p < 2.2 \times 10^{-16}$) than transcript half-lives of the other genes expressed in human B cells (median $t_{1/2} = 5.4$ hr) (Figure 5A). In contrast, the median half-life of transcripts identified as viral miRNA targets was not significantly shorter than the overall median RNA half-life, indicating that cellular transcripts with short half-lives are not preferentially targeted by viral miRNAs. Next, we looked at the correlation of the enrichment in the Ago2-IP in BL41

Figure 4. Validation of KSHV miRNA Targets by Dual-Luciferase Assays

Indicator vectors carrying no additional sequences, or candidate 3′UTR sequences inserted 3′ to the firefly luciferase gene, were cotransfected into HEK293 either with empty pcDNA or pcDNA expressing the ten intronic KSHV miRNAs (pcDNAmiRK10/12), kshv-miR-K12-3 (pcDNAmiRK3), or kshv-miR-K12-4 (pcDNA-miRK4). A vector containing an imperfect match for kshv-miR-K12-4 was used as positive control. Dual-luciferase assays were performed 16 hr later. Firefly to renilla luciferase ratios were normalized to the empty pSIcheck2 vector as described (Gottwein et al., 2007). All dual-luciferase experiments were performed at least three times in triplicate. Values represent the mean ± SD of representative experiments.

(A) Six candidate 3′UTRs of putative KSHV miRNA targets were tested, four of which reproducibly caused significant downregulation of firefly luciferase expression (indicated by asterisks).

(B) The interaction between kshv-miRK12-3 and the 3′UTR of LRRC8D was modeled using RNA Hybrid (Rehmsmeier et al., 2004) (top). The black line indicates the seed region of kshv-miR-K12-3. Luciferase derepression analysis of LRRC8D 3′UTR with a sequence-specific AntagomiR (2′O-methyl RNA) demonstrated a concentration-dependent derepression indicating that LRRC8D is indeed targeted by kshv-miR-K12-3 (bottom).

(C) KSHV miRNA mediated repression of luciferase activity via the LRRC8D 3′UTR after coexpression of the ten intronic KSHV miRNAs or miR-K12-3 alone was lost upon deletion of the putative SEED match for kshv-miR-K12-3.

(D) Putative target sites for KSHV miRNAs located in the coding sequence of GEMIN8 and NHP2L1 (top) were cloned into pSIcheck2 in the 3′UTR of the firefly luciferase gene. Repression of firefly luciferase activity occurred when KSHV miRNAs were cotransfected. This effect was abolished by the introduction of five point mutations into the putative SEED-matching regions (bottom).

(E) The sensor vectors carrying putative miRNA target sites from the coding sequence of NHP2L1 and GEMIN8 were cotransfected with the respective individual constructs expressing miR-K12-3 or miR-K12-4 resulting in significant repression of luciferase activity.

(F) Proteins were labeled by using Alexa488-conjugated secondary antibodies and scanned in the Fluoimager. Reduction of GEMIN8 level was quantified by employing the AIDA software.

(G) RISC-IPs were performed from 293 cells expressing either kshv-miR-K12-3 (293-miR-K3) or -miR-K12-4 (293-miR-K4) following induction with doxycyclin. BCBL-1 served as control. For each gene, mRNA enrichment (qPCR of Ago2-IP versus control-IP) normalized for HPRT expression levels is shown for 293-miR-K3 (left), 293-miR-K4 (middle), and BCBL-1 (right). Enrichment of LRRC8D ($p = 0.011$) and NHP2L1 ($p = 0.037$) was significant in 293-miR-K3 compared with 293-miR-K4 (unpaired t test). Enrichment of GEMIN8 did not quite reach significance ($p = 0.062$). Means ± SDs represent combined data from three independent RISC-IPs per condition.

(H) Binding sites for ebv-miR-BART16 and ebv-miR-BART3 in the 3′UTR of TOMM22 and IPO7, respectively, are shown (top). Luciferase assays with sensor vectors carrying the 3′UTRs of TOMM22 and IPO7 demonstrated significant repression by ebv-miR-BART16 and ebv-miR-BART3, respectively. These effects were completely reversed when the seed regions of the predicted binding sites (RNAhybrid) were mutated (bottom).

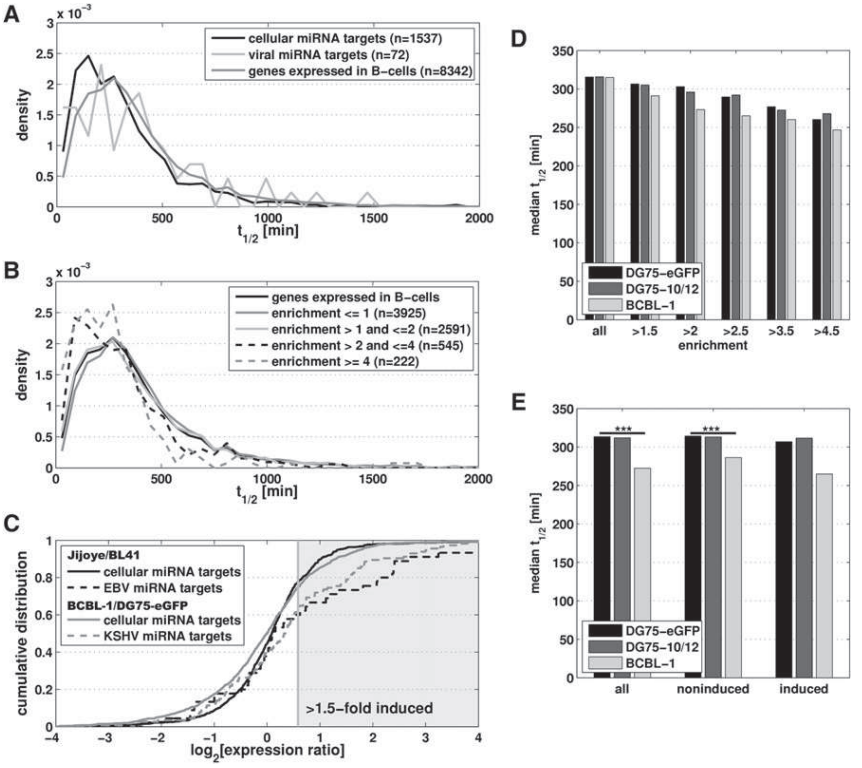

Figure 5. Effect of miRNAs on Target RNA Stability

(A) Distribution of the transcript half-life of the targets of cellular and viral miRNAs was compared with the transcript half-life of all genes for which RNA half-lives were available from our recently published atlas of RNA half-lives in human B cells (BL41; n = 8,342). The density distribution was estimated by binning genes according to transcript half-lives in intervals of 60 min, counting the number of genes in each bin, and then normalizing the counts such that the area under the curve is 1. Transcript half-life (*x* axis) is plotted against the estimated density (*y* axis). RNA half-lives of cellular miRNA targets were significantly lower (Kolmogorov-Smirnov test, $p < 2.2 \times 10^{-16}$) than for all other cellular transcripts expressed in human B cells for which RNA half-lives were available (Friedel et al., 2009). For viral miRNA targets, no significant difference was observed. Please note that no viral miRNAs are expressed in BL41 for which the RNA half-lives were obtained.

(B) The extent of recruitment to Ago2 complexes (enrichment in the Ago2-IP) negatively correlated with transcript half-life. The distribution of the half-life of transcripts enriched either $\geq$ 4-fold, 2- to 4-fold, 1- to 2-fold, and less than 1-fold as well as of all 8342 cellular transcripts was compared. A highly significant, negative correlation in between transcript half-life and recruitment to Ago2 complexes was observed (Spearman correlation coefficient, −0.13; p value $< 10^{-16}$).

(C) Effect of viral miRNAs on target mRNA levels. The cumulative distribution of alterations in expression levels of cellular, KSHV, and EBV miRNA targets represented either by the BrdU-IP (for DG75-eGFP versus BCBL-1) or total RNA (for BL41 versus Jijoye) is shown. Genes induced either in BCBL-1 or Jijoye are represented by a $\log_2$ expression ratio > 1.0; genes downregulated in these cells compared to either DG75-eGFP or BL41 by a ratio < 1.0. Genes induced > 1.5-fold in infected cells (right of gray vertical line) are provided in Tables S4C and S4D.

(D) Correlation of enrichment in the Ago2-IP with transcript half-life. The median half-life of transcripts which were enriched more than the indicated value in the Ago2-RIP-Chip in each of the indicated cell lines (DG75-eGFP, DG75-10/12, and BCBL-1) are shown.

(E) KSHV miRNAs destabilize their targets. RNA half-lives of the KSHV miRNA targets identified by Ago2-RIP-Chip were determined in DG75-eGFP, DG75-10/12, and BCBL-1 based on nascent / total RNA ratios (n = 3; for details, see Table S7B). Their RNA half-lives were significantly shorter in BCBL-1 (Friedman test, $p = 6.74 \times 10^{-6}$) than in both DG75-eGFP and DG75-10/12. This was seen for both the induced and noninduced KSHV miRNA targets but was only significant for the noninduced ($p = 5.25 \times 10^{-6}$), presumably due to the smaller number of induced (n = 35) versus noninduced (n = 70) KSHV miRNA targets for which RNA half-lives could be determined.

with transcript half-life (Figure 5B). We observed a small but highly significant negative correlation in between transcript half-life and recruitment to Ago2 complexes (Spearman correlation coefficient, −0.13; p value $< 10^{-16}$). Thus, these data support previous studies demonstrating that cellular miRNAs have a small but measurable impact on target RNA stability (Baek et al., 2008; Selbach et al., 2008). Our data now extend these findings, showing that the effect on RNA stability correlates with the extent of target recruitment to Ago2 complexes.

To investigate the effect of viral miRNAs on the transcript levels of their targets, we first compared expression levels in DG75-eGFP and BCBL-1 (for KSHV miRNA targets) as well as in BL41 and Jijoye (for EBV miRNA targets). As a reference, the changes in expression levels of the 2337 targets of the cellular miRNAs were used (Figure 5C). For both KSHV and EBV miRNA targets, the extent and distribution of downregulation was very similar to that observed for the cellular miRNA targets. Interestingly, we noted that a substantial number of both KSHV and EBV miRNA targets were expressed at higher levels in BCBL-1 and Jijoye, respectively. In order to assess whether KSHV miRNAs preferentially target transcripts transcribed at higher rates in infected than in uninfected B cells or whether they are directly responsible for the increased total RNA levels by enhancing RNA stability, we determined RNA half-lives for DG75-eGFP, DG75-10/12, and BCBL-1 by metabolic tagging of nascent RNA with 4-thiouridine (Friedel et al., 2009). Following separation of total RNA into nascent and untagged pre-existing RNA, three biological replicates of all three RNA fractions were analyzed on Affymetrix Gene ST 1.0 arrays. RNA half-lives of cellular and viral miRNA targets are provided in Tables S7A and S7B, respectively. Again, we observed a significant, linear correlation of recruitment of transcripts to Ago2 complexes with RNA half-life for all three cell lines (Figure 5D). In DG75-eGFP and DG75-10/12, half-life distribution and median RNA half-life of all KSHV miRNA targets were indistinguishable from other cellular transcripts. In contrast, RNA half-life of KSHV miRNA targets was significantly shorter in BCBL-1 ($t_{1/2m}$ = 272 min, Friedman test $p = 6.74 \times 10^{-6}$) than in both DG75-eGFP ($t_{1/2m}$ = 314 min) and DG75-10/12 ($t_{1/2m}$ = 312 min, Figure 5E). These data are consistent with the substantially greater mean

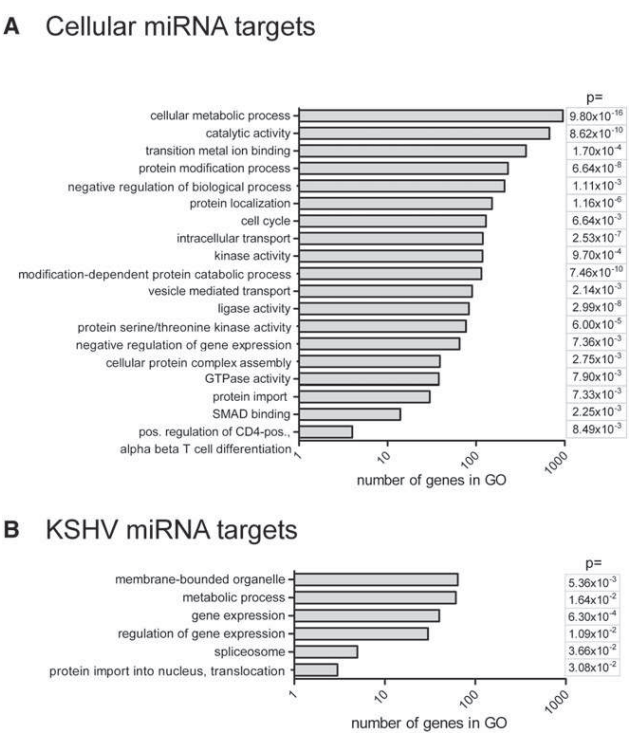

Figure 6. Transcripts Targeted by miRNAs Are Enriched for GO-Annotated Functional Classes

Selected GO functional classes of transcripts targeted by cellular (A) or KSHV (B) miRNAs are shown. The number of transcripts in each class is indicated on a logarithmic scale, along with the respective p value.

enrichment of the 114 KSHV miRNA targets in the Ago2-IP of BCBL-1 (~5.5-fold) than of DG75-10/12 (~1.5-fold) and DG75-eGFP (~1.2-fold). The half-lives of induced and noninduced target transcripts were very similar in BCBL-1, indicating that the increased levels of miRNA target transcripts in BCBL-1 are not due to enhanced RNA stability but rather an increase in transcriptional rate that is counteracted by the viral miRNAs.

To identify functional categories enriched among both cellular and viral miRNA targets, we performed a Gene Ontology (GO) overrepresentation analysis. For targets of cellular miRNAs, a large number of GO terms were significantly overrepresented, consistent with cellular miRNAs being involved in a broad spectrum of cellular processes (Table S8A). A selected list of these is shown in Figure 6A. KSHV miRNA targets were preferentially involved in gene expression and its regulation as well as the spliceosome and protein import into the nucleus (Figure 6B, Table S8B). No functional categories were overrepresented among the EBV miRNA targets (Table S8C).

DISCUSSION

Since the identification of virally encoded miRNAs, research on them has been hampered by the lack of high-confidence targets. Here we provide a large number of such targets for the two human γ-herpesviruses, KSHV (n = 114) and EBV (n = 44), as well as cellular miRNAs (n = 2337). Predicted miRNA-binding sites of cellular miRNAs were highly overrepresented in coding sequences ($p = 2.02 \times 10^{-27}$) and 3′UTRs ($p = 6.19 \times 10^{-63}$), but not in 5′UTRs ($p = 8.59 \times 10^{-3}$). For the viral miRNA targets we identified, predicted binding sites were predominantly overrepresented in target 3′UTRs. We used dual-luciferase assays to validate six KSHV miRNA targets. For four of them we observed significant regulation by KSHV miRNAs via their 3′UTRs. The two remaining genes (GEMIN8 and NHP2L1) are regulated by binding sites located within their coding regions. For GEMIN8 this resulted in a significant reduction of protein levels.

Despite the much larger number of EBV miRNAs potentially being responsible for regulation of transcripts selectively enriched in EBV-infected cells, we could validate TOMM22 and IPO7 as targets of ebv-miR-BART16 and ebv-miR-BART3, respectively. These two proteins are involved in import of proteins from the cytosol into the mitochondria (TOMM22) (Saeki et al., 2000; Yano et al., 2000) and the nucleus (IPO7) (Gorlich et al., 1997). Antisense knockdown of TOMM22 has been shown to inhibit the association of the proapoptotic protein BAX with mitochondria and thus prevent BAX-induced apoptosis (Bellot et al., 2007). Recently, IPO7 has also been implicated in innate immunity. Antisense knockdown of IPO7 in macrophages resulted in reduced production of the proinflammatory cytokine IL-6 upon LPS challenge (Yang et al., 2009a). This study also identified FBXO9 as one of four factors required for efficient production of IL-6 in J774A.1 macrophages. While the function of FBXO9 is unknown, it is possible to be involved in phosphorylation-dependent ubiquitination of IκB or another important signaling molecule in the TLR4 pathway or another pathway that is activated in response to LPS (Yang et al., 2009a). Interestingly, FBXO9 is also in our list of 44 EBV miRNA targets and was validated by qPCR. Thus, it is intriguing to speculate that EBV taps the proinflammatory signaling networks by recruitment of both IPO7 and FBXO9 transcripts to Ago2 complexes. In summary, this is indicative of EBV miRNAs modulating cellular trafficking and protein localization to counteract innate immunity and apoptosis.

In the last few years a number of transcripts have been identified to be targeted by KSHV and EBV miRNAs. The majority of KSHV targets were identified based on the finding that KSHV encodes an ortholog of hsa-miR-155 (kshv-miR-K12-11) (Gottwein et al., 2007; Skalsky et al., 2007). Gottwein et al. identified 12 genes to be targeted by both kshv-miR-K12-11 and hsa-miR-155. We found 3 of 12 (25%) of these genes to be enriched > 2-fold in BCBL-1, but not in DG75-eGFP or BL41. Of these, SLA (Src-like-adaptor, transcript variant 1) was the most strongly enriched transcript in BCBL-1 (18.3-fold). Interestingly, it appeared to be induced > 4-fold in BCBL-1. An additional 4 of 12 genes (33%) were enriched > 1.5-fold in BCBL-1. Finally, 5 genes (42%) were enriched > 1.5-fold stronger in Jijoye or BL41 B95.8 than in both control cell lines, consistent with their regulation by hsa-miR-155 induced upon EBV infection (Gatto et al., 2008). In summary, only 1 of the 12 genes (BCLAF1) was not enriched > 1.5-fold in BCBL-1, BL41 B95.8, or Jijoye. We could confirm the differential regulation of MICA and MICB by both KSHV and EBV as well as cellular miRNAs (Stern-Ginossar et al., 2007, 2008). During the revision of this paper, Lei et al. identified kshv-miR-K12-1 to target the NFκB inhibitor IκBα (NFKBIA) via two binding sites in its 3′UTR

(Lei et al., 2010). By reducing IκBα protein levels the NFκB pathway is activated, mediating vFLIP inhibition of KSHV lytic replication (Ye et al., 2008). Our data nicely confirm this regulation by revealing a selective 7.3-fold enrichment of NFKBIA transcripts in the Ago2-IP of BCBL-1 and to a lesser extent (2.5-fold) in DG75-10/12. In total, about 60% of the known KSHV and EBV miRNA targets showed detectable enrichment in our assay (summarized in Table S3B). The analysis of other published miRNA targets exemplifies an important aspect of using RIP-Chip to identify miRNA targets. In contrast to other methods, this approach is based on positive selection of viral miRNA targets. In consequence, a lack of enrichment does not exclude a transcript to be targeted by miRNAs due to the following reasons: First, some of the transcripts may be expressed at very low levels and thus be below the detection limit of the microarrays. Second, an enrichment of as little as 1.2-fold still resulted in a highly significant overrepresentation of predicted miRNA-binding sites for cellular miRNA targets ($p < 10^{-62}$). Thus, it may still be consistent with regulation by a viral miRNA. As we restricted our analysis to strongly enriched targets (>3-fold), we excluded a large number of viral miRNA targets. Finally, expression levels of both miRNA and its targets define whether a miRNA is able to exert a significant effect. Expression of both is influenced by the cell line and assay employed; e.g., luciferase assays in HEK293 cells with transfected miRNA expression vectors and reporter constructs.

We observed a significantly overrepresented number of KSHV and EBV miRNA targets to be expressed at higher levels in BCBL-1 and Jijoye, respectively, than in the uninfected cells. By measuring RNA half-lives in DG75-eGFP, DG75-10/12, and BCBL-1 using a new approach we recently developed (Dölken et al., 2008; Friedel et al., 2009), we found that, despite higher total RNA levels, the RNA half-life of the induced KSHV miRNA targets was significantly shorter in BCBL-1 than in DG75-10/12, DG75-eGFP, and BL41. We conclude that KSHV miRNAs counteract the increased transcription rates of these genes in the infected cells and hypothesize that KSHV (and also EBV) miRNAs preferentially target transcripts induced in the infected cells.

Another important finding of our study is the linear correlation between recruitment of transcripts to Ago2-complexes, i.e., enrichment in the Ago2-IP, and transcript half-life. This indicates that this approach allows a quantitative estimate on the true extent of miRNA mediated regulation. It is tempting to speculate that more strongly regulated targets are more likely to be biologically relevant. Therefore, this approach may help to decide which of the large number of viral miRNA targets to pursue in further studies. This concept is supported by the strong enrichment we observed for both MICB and NFKBIA, both of which were not only confirmed by luciferase assay but were also shown to exert a significant biological effect upon downregulation. In this respect, we provide a large number of high-confidence viral miRNA targets. In addition, we provide data for numerous other transcripts predominantly enriched in either BCBL-1 or Jijoye that did not quite fulfill our stringent criteria but which most likely are also targeted by the viral miRNAs. Thus, these data provide an important basis for further studies aimed to characterize the viral miRNA targets relevant for the interaction of the virus with its human host.

EXPERIMENTAL PROCEDURES

Generation of Cell Lines Expressing KSHV miRNAs

Human B cells (DG75-eGFP and DG75-10/12) stably expressing either eGFP or the ten intronic KSHV miRNAs were generated using the "Virapower" lentiviral transduction system (Invitrogen) and recombinational cloning following the manufacturer's instructions. HEK293 cell lines expressing either doxycyclin-inducible kshv-miR-K12-3 or -K12-4 (293-K3 and 293-K4) were generated using the Flp-In T-REx-293 cell line (Invitrogen) according to the manufacturer's instructions. To induce KSHV miRNA expression, 1 μg/mL doxycycline was added for 48 hr.

Immunoprecipitation of Human Argonaute 2 Complexes

For the RISC-IPs of human B cells, 5×10^8 cells were taken for each replicate and washed twice in PBS before lysis in 10 ml lysis buffer containing 25 mM Tris HCl (pH 7.5), 150 mM KCl, 2 mM EDTA, 0.5% NP-40, 0.5 mM DTT, and protease inhibitor Complete (Roche). DTT and protease inhibitors were always prepared freshly and added immediately before use. Lysates were incubated for 30 min at 4°C and cleared by centrifugation at 20,000 g for 30 min at 4°C. Total RNA was prepared from 100 μl of cell lysates using the miRNeasy kit (QIAGEN) following the manufacturer's instructions. RISC-IPs were performed with a few modifications to the previously described protocol (Beitzinger et al., 2007). In short, 6 μg of purified monoclonal hAgo2 antibody (α-hAgo2; 11A9) or monoclonal BrdU-antibody (Abcam; used as control) was added to 5 ml of RPMI medium and incubated with 60 μl of protein G Sepharose beads (GE Healthcare) in Pierce centrifuge columns (Thermo Scientific) under constant rotation at 4°C overnight. Columns were drained by gravity flow and washed once with the lysis buffer. Beads were subsequently incubated with 5 ml of cell lysates for 2.5 hr under constant rotation at 4°C. After incubation, the beads were washed four times with IP wash buffer (300 mM NaCl, 50 mM Tris HCl [pH 7.5], 5 mM $MgCl_2$, 0.1% NP-40, 1 mM NaF) and once with PBS to remove residual detergents. RNA was recovered from the beads by adding 700 μl of Qiazol to the columns. After 5 min, the Qiazol lysates were collected from the columns. This step was repeated once, and the Qiazol lysates were combined. RNA was prepared using the miRNeasy kit (QIAGEN) according to the manufacturer's instructions. RNA samples were eluted in 30 μl H_2O. For RISC-IPs from 293 cells (expressing single KSHV-miRNAs upon 48 hr induction by doxycyclin), lysates prepared from a single 15 cm dish (~80% confluent) were used, and the IP assay was scaled down by 2-fold. Lysates from BCBL-1 served as control.

Microarray Sample Labeling, Hybridization, and Preprocessing

For the microarray analysis, 200 ng RNA of each sample was amplified and labeled using the Affymetrix Whole-Transcript (WT) Sense Target Labeling Protocol without rRNA reduction. Affymetrix GeneChip Human Gene 1.0 ST arrays were hybridized, washed, stained, and scanned according to the protocol described in WT Sense Target Labeling Assay Manual. Microarray data were assessed for quality and normalized with RMA. All microarray data are available at Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lnelhkuucokacjo&acc=GSE17180.

cDNA Synthesis and Quantitative Real-Time PCR

3′-polyadenylation and cDNA synthesis were performed for Ago2-IP, BrdU-IP, and total RNA samples in a single-step reaction using the miScript Reverse Transcription kit (QIAGEN). The efficiency of every RISC-IP was monitored by Light Cycler qRT-PCR for cellular miRNA Let7a using the let7a-specific primer 5′-TGAGGTAGTAGGTTGTATAGTT-3′. Microarray data of 24 transcripts were validated by TaqMan PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems). TaqMan probes were taken from the Universal Probe Library (Roche), and selection of probe-primer combinations was performed using the Assay Design Centre (Roche, http://www.universalprobelibrary.com). A list of the utilized PCR primers and probes is provided in Table S9.

Luciferase Repression and 2′O-Methyl-RNA Derepression Assay

To analyze 3′UTRs of genes of interest in terms of their ability to interact with miRNAs, we generated the vector pSIcheck-XE-DEST-sense (reporter plasmid, see Table S9 for details). For 6 of the 11 KSHV miRNA targets

confirmed by quantitative PCR, we were able to amplify their full-length 3′UTRs by PCR using DNA prepared from DG75. To validate putative EBV miRNA targets, we amplified the full-length 3′UTRs of TOMM22, IPO7, and RAB13 by PCR. TOMM22 and RAB13 were inserted into pMIR-RL (Zhu et al., 2009) by conventional cloning, and IPO7 was inserted into pSIcheck-XE-DEST by recombinatorial cloning. For dual-luciferase assays, 10 ng of the reporter plasmid was cotransfected into HEK293 cells in 24-well plates with either the respective miRNA-expressing pcDNA vector (200 ng) or the empty pcDNA vector as negative control. Transfection was carried out using FuGene 6 (Roche) according to the manufacturer's instructions. Sixteen hours after transfection, luciferase activity was measured employing the Dual-Luciferase Reporter Assay (Promega) and the luminometer Fluostar Optima (BMG-Labtech) as recommended by the manufacturers. For derepression analysis, HEK293 cells were seeded on 24-well plates as for the luciferase repression assay and transfected with 100 pmol 2′O-methyl-RNA using Lipofectamine 2000 (Invitrogen). Six hours after transfection, the medium was changed and reporter and miRNA expression plasmids were cotransfected. Sixteen hours later, luciferase activity was measured.

Overrepresentation of Predicted miRNA-Binding Sites

Cellular miRNAs expressed in all six cell lines were obtained from the miRNA expression atlas (Landgraf et al., 2007). Their sequences as well as the sequences of the KSHV and EBV miRNAs were obtained from miRBase v.12 (http://microrna.sanger.ac.uk/sequences/). The 3′UTRs, coding sequences, and 5′UTRs for all cellular genes were downloaded from Ensembl (http://www.ensembl.org/). For all of these, miRNA target sites were predicted using PITA (Kertesz et al., 2007). Overrepresentation was determined using Fisher's exact test.

Metabolic Tagging of Nascent RNA Using 4-Thiouridine

Nascent RNA was tagged in DG75-eGFP, DG75-10/12, and BCBL-1 for 1 hr by adding 100 μM 4-thiouridine (4sU) to cell culture medium. Biotinylation of 4sU-tagged RNA and separation of total RNA into nascent and untagged pre-existing RNA were performed as described (Dölken et al., 2008; Friedel et al., 2009). RNA half-lives were determined based on nascent/total RNA ratios. Median RNA half-life was normalized to 315 min as obtained for BL41.

Further experimental details are provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and nine tables and can be found with this article at doi:10.1016/j.chom.2010.03.008.

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Bruton's Tyrosine Kinase Is Involved in miR-346-Related Regulation of IL-18 Release by Lipopolysaccharide-Activated Rheumatoid Fibroblast-Like Synoviocytes[1]

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MicroRNAs (miRNAs) have emerged as key players in the regulation of expression of target mRNAs expression. They have been associated with diverse biological processes, and recent studies have demonstrated that miRNAs play a role in inflammatory responses. We reported previously that LPS-activated fibroblast-like synoviocytes (FLS) isolated from rheumatoid arthritis (RA) patients express IL-18 mRNA but they do not release IL-18. Based on the observation that this inhibition was due to a rapid degradation of IL-18 mRNA, our group has conducted a study to identify miRNAs that could play a role in the "antiinflammatory" response of LPS-activated RA FLS. LPS challenge modulated the expression of 63 miRNAs as assessed by microarray analysis. Fifteen miRNAs were up-regulated, including miR-346, for which overexpression upon LPS treatment was validated by quantitative RT-PCR. We then transfected FLS with an antisense oligonucleotide targeting miR-346 and found that, in these conditions, IL-18 release could be measured upon LPS activation of FLS. Moreover, we also demonstrated that miR-346 indirectly regulated IL-18 release by indirectly inhibiting LPS-induced Bruton's tyrosine kinase expression in LPS-activated RA FLS. These findings suggest that miRNAs function as regulators that help to fine-tune the inflammatory response in RA. *The Journal of Immunology,* **2009, 182: 5088–5097.**

In rheumatoid arthritis (RA),[5] stimulation of innate immune cells, and especially organ-specific resident cells such as fibroblast-like synoviocytes (FLS), seems to be the primary fundamental event leading to the inflammatory response and to the development of organ-specific autoimmunity (1, 2).

Activation of FLS may be linked either to the cytokine environment, to cell-to-cell contacts, or to interactions between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) (3). FLS exert a proinflammatory activity, essentially by synthesizing cytokines, chemokines, prostanoids, and NO (4, 5). However, they also produce proangiogenic factors that help with the appearance of neovessels, thereby allowing the intrasynovial recruitment and migration of immune cells with the help of release of chemokines. FLS have a key role in osteoarticular destruction and take also part in the differentiation and activation of osteoclasts by the receptor activator of NF-κB (RANK)-RANK ligand pathway through the release of prostaglandin E_2 and IL-6 (6).

Conversely, FLS secrete little or no TNF-α and neither IL-1 nor IL-18, which are major cytokines implicated in the inflammatory response in RA. This "antiinflammatory" activity, which is specific to FLS, suggests the existence in these organ-specific resident cells of selective and complex mechanisms of regulation. We previously reported that LPS and protein I/II, a ligand of integrin $\alpha_5\beta_1$, induced IL-18 mRNA expression in both THP-1 monocytic cell line and in RA FLS, but in contrast to THP-1 cells, FLS did not release mature IL-18. Furthermore, our studies revealed that the lack of IL-18 release by RA FLS was due to a defect of translation of IL-18 mRNA into pro-IL-18 because of a rapid degradation of the IL-18 mRNA (7).

MicroRNAs (miRNAs) are an evolutionarily conserved class of endogenous small noncoding RNAs. Since their discovery, miRNAs have emerged as key players in the regulation of translation and degradation of target mRNAs (8–11). They provide an additional posttranscriptional mechanism by which protein expression can be regulated (12–14). The expression of ~10,000 genes, or 30% of the human genome, could potentially be regulated by miRNAs (15, 16). In mammals, miRNAs have been associated with diverse biological processes such as cell differentiation, cancer, regulation of insulin secretion, and viral infection (17, 18). Cells of the immune system must employ a multilayered control system to keep innate immunity and inflammation in check, and

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[5] Abbreviations used in this paper: RA, rheumatoid arthritis; Btk, Bruton's tyrosine kinase; FLS, fibroblast-like synoviocytes; LFM, leflunomide metabolite analog; miRNA, microRNA; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; UTR, untranslated region.

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recently a role for miRNAs has been proposed in the regulation of the inflammatory response in monocytes/macrophages (19). Taganov et al. (20), by using microarray studies, found an increased expression of three miRNAs (miR-132, miR-146, and miR-155) in response to LPS in THP-1 cells. They identified IL-1R-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6) as the key targets of miR-146 and proposed a model by which this miRNA regulates TLR signaling in response to various PAMPs. Tili et al. (21) later demonstrated that miR-155 plays an important role in innate immunity and is involved in the negative regulation of immune cells in response to LPS.

Based on these observations, our group has conducted a study to identify miRNAs that could play a role in the antiinflammatory response of LPS-activated RA FLS. To monitor the expression profile of miRNAs in RA FLS activated with LPS, we employed a microarray approach that detects 409 miRNAs of human origin. Fifteen miRNAs were overexpressed >2-fold in LPS-activated FLS, and among them we identified one miRNA, miR-346, that had partial sequence complementarity within the 3′ untranslated region (3′-UTR) region of the IL-18 mRNA. The up-regulation of miR-346 expression was validated by quantitative RT-PCR analysis. We then used antisense oligonucleotide molecules to block miR-346 activity; transfection of the inhibitor targeting miR-346 reestablished IL-18 release by LPS-activated FLS. Moreover, we also showed that transient transfection of miR-346 in THP-1 cells inhibited IL-18 secretion by these cells in response to LPS. Using a luciferase assay, we showed that miR-346 does not directly target IL-18 mRNA. In our search for another target that could explain the effect of miR-346 on IL-18, we also demonstrated that miR-346 inhibited indirectly Bruton's tyrosine kinase (Btk) expression at the transcriptional level in LPS-activated RA FLS and that indirect inhibition of Btk expression negatively regulated IL-18 release. These results suggest an important role of miR-346 in the control of IL-18 synthesis by LPS-activated FLS and indicate that its expression may be critical to prevent an excessive inflammatory response.

Materials and Methods

Reagents

Cell culture media (RPMI 1640, M199, and DMEM), FCS, L-glutamine, penicillin, streptomycin, amphotericin B, and TRIzol reagent were from Invitrogen. LPS from *Salmonella abortus equi* and type XI collagenase, leflunomide metabolite analog (LFM-A13), and anti-β-actin mouse IgG mAbs were obtained from Sigma-Aldrich. The LightCycler FastStart DNA Master SYBR Green I was from Roche Applied Science. The miScript system was obtained from Qiagen. Clear-MiR miRNA inhibitors were from Eurogentec. MiRIDIAN miR-346 mimic and miRIDIAN miRNA mimic negative control were supplied by Dharmacon/Perbio Science. Human dermal fibroblast Nucleofector kit and cell line Nucleofector kit V were from Amaxa. The enzyme immunoassay kits for human IL-18 detection was from MBL and for human IL-6 detection was from R&D Systems. Throughout this study, buffers were prepared with apyrogenic water obtained from Braun Medical. Anti-Btk mouse IgG mAbs were from BD Transduction Laboratories.

Cell culture

Human FLS were isolated from synovial tissues from four different RA patients at the time of knee joint arthroscopic synovectomy as described previously after informed consent was obtained from patients (22). The diagnosis was conformed to the revised criteria of the American College of Rheumatology (23). FLS cultures were done as previously described (24). Experiments were performed between the third and the ninth passage. During that time, cultures were constituted of a homogeneous population of fibroblastic cells, negative for CD16 as determined by FACS analysis. Cell number and cell viability were checked by the MTT test as described elsewhere (25). THP-1 cells (no. 88081201; European Collection of Cell Cultures) were cultured as described previously (26). HEK293 cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 μM L-glutamine, 40 U/ml penicillin, and 50 μg/ml streptomycin.

Stimulation of cells for total RNA extraction

For miRNA studies, FLS and THP-1 cells (10^6 cells) were stimulated with 2 ml of medium alone or medium containing LPS (1 μg/ml). After a 3- or 6-h incubation period, total RNA was extracted using TRIzol according to the manufacturer's instructions. For IL-18 studies, cells were stimulated as previously described (6), and total RNA was extracted using TRIzol according to the manufacturer's instructions.

MiRNA microarray analysis

MiRNA microarray analyses were performed by Eurogentec using the MiRanalyser microarray, which contains 409 calibrated human miRNA oligonucleotides from the Sanger miRBase that wereHPLC purified and spotted in duplicate into two separate fields on 25 × 75-mm high-quality glass slides. The microarray data were submitted to the Minimum Information About a Microarray Experiment (MIAME) database (www.ebi.ac.uk/) with the accession no. E-MEXP-1970.

Luciferase reporter constructs

To generate luciferase reporters plasmids, psiCHECK-2 (Promega) was modified by inserting the Gateway cassette C.1 (Invitrogen) at the 3′ end of the firefly luciferase gene (*f-luc*) into the *Xba*I site of psiCHECK-2. The 3′-UTR sequence of IL-18 was amplified from HEK293 cell genomic DNA and after addition of attB1 and attB2 sequences, the resulting PCR products were cloned into pDONR/Zeo and then recombined in the modified psiCHECK-2 vector using Gateway technology (Invitrogen). A similar construct was generated for the 3′-UTR sequence of Btk.

The primer sequences were (sense and antisense primers are indicated in respective order, and the anchor sequence used for the nested PCR is underlined): IL-18, 5′-AGAAAGCTGGGTCACATTATGAATTTTTTATTTGTTTA, 5′-AAAAAGCAGGCTCTATTAAAATTTCATGCCGGGC; attB1/2, 5′-ACAAGTTTGTACAAAAAAGCAGGCT, 5′-ACCACTTTGTACAAGAAAGCTGGGT; Btk, 5′-AAAAAGCAGGCTTGTCATGGATGAAGAATCCTG, 5′-AGAAAGCTGGGTCCTCCCCTCCCATCTTTATG.

Real-time quantitative RT-PCR

Total RNA isolated from FLS was reverse transcribed using the FirstStrand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). Real-time quantitative RT-PCR was performed in a total volume of 20 μl using the LightCycler FastStart DNA Master SYBR Green I and gene specific primers: *IL-18*, 5′-ATCAGGATCCTTTGGCAAGCTTGAATCTAAATTATC-3′ and 5′-ATAGGTCGACTTCGTTTTGAACAGTGAACATTATAG-3′; *GAPDH*, 5′-GGTGAAGGTCGGAGTCAACGGA-3′ and 5′-GAGGGATCTCGCTCGCTCCTGGAAGA-3′; *Btk*, 5′-GAAGCTGGTGCAGTTGTATG-3′ and 5′-TATACCCTCTCTGATGCCAG-3.

After an initial denaturing at 96°C for 10 min, the temperatures used were: 95°C for 10 s, 60°C for 15 s, and 72°C for 25 s for IL-18; 95°C for 15 s, 57°C for 10 s, and 72°C for 10 s for GAPDH; and 95°C for 10 s, 60°C for 30 s, and 72°C for 25 s for Btk using the LightCycler instrument (Roche Applied Science). Amplification products were detected as an increased fluorescent signal of SYBR Green during the amplification cycles. Results were obtained using SDS Software (PerkinElmer) and evaluated using Microsoft Excel. Melting curve analysis was performed to assess the specificity of PCR products.

Quantitative RT-PCR analyses for miRNAs were performed using the miScript system and the primers (Qiagen), and RNA concentrations were determined with a NanoDrop instrument (NanoDrop Technologies). One microgram of RNA per sample was used for the assays. Reverse transcriptase reactions and real-time quantitative PCR were performed according to the manufacturer's protocols. A U6 endogenous control was used for normalization. All reactions were run in triplicate in a LightCycler Instrument (Roche Applied Science). Relative expression was calculated using the comparative threshold cycle (*Ct*) method.

Northern blot analysis

Twenty micrograms of RNA per sample was resolved on a 15% urea-PAGE gel and transferred to a nylon membrane (Hybond NX; Amersham Biosciences). After UV cross-linking, the membrane was prehybridized in PerfectHyb Plus buffer (Sigma-Aldrich) at 42°C for 60 min and then hybridized with ^{32}P-labeled probes at 42°C overnight. After being washed, the membrane was exposed on a phosphorimager plate and the signal was quantified after scanning the plate on a Fuji FLA7000 phosphorimager. The membrane was stripped and rehybridized twice.

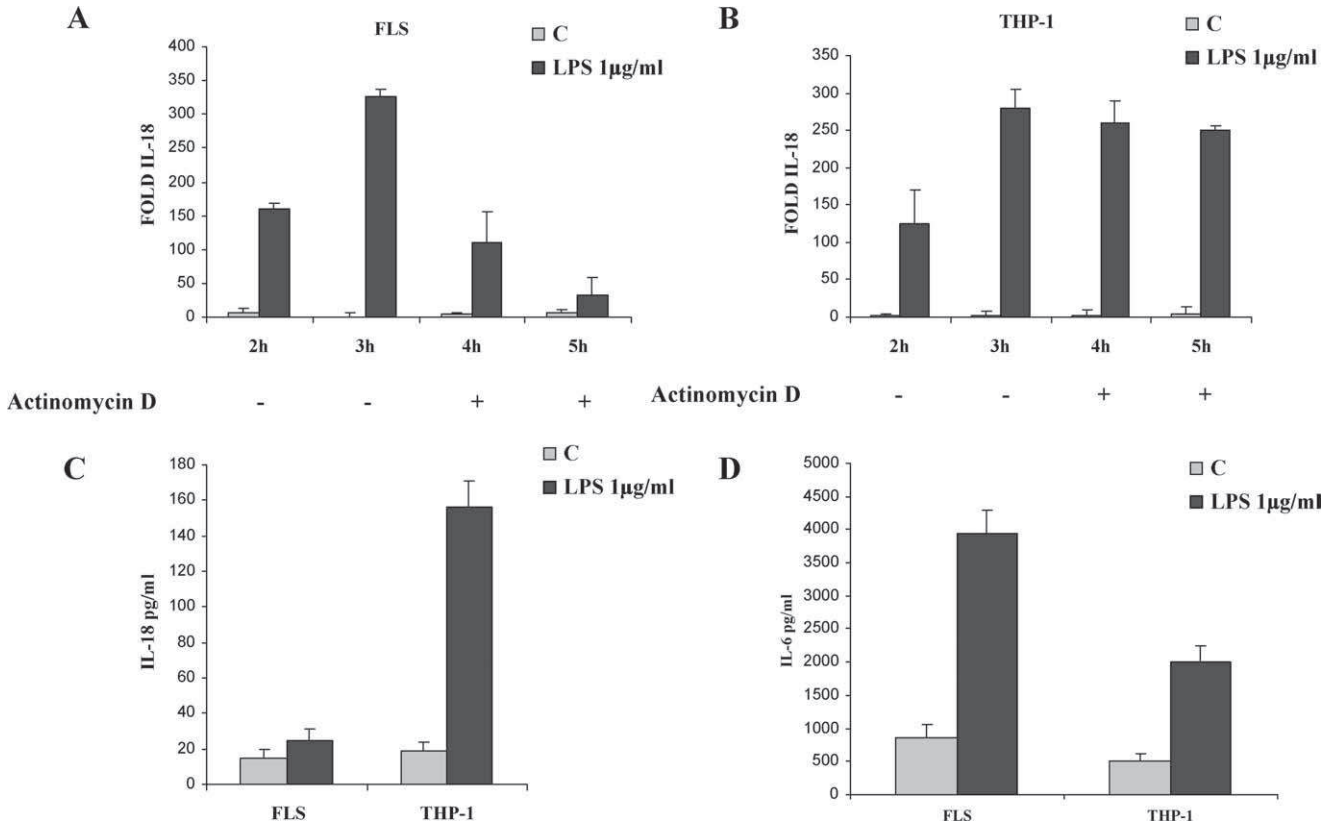

FIGURE 1. Effect of LPS on IL-18 mRNA expression and release by RA FLS and THP-1 cells. *A* and *B*, IL-18 mRNA levels were determined using quantitative RT-PCR in RA FLS (*A*) and THP-1 cells (*B*) activated with LPS (1 μg/ml) for 2 and 3 h, or incubated for another 1–2 h with actinomycin D. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *C*, IL-18 release by activated RA FLS and THP-1 cells was determined by ELISA in culture supernatants, harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). *D*, IL-6 release was determined by ELISA in culture supernatants harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). Data are expressed as the mean of triplicate samples $\pm$ SD and are representative of three independent experiments.

Transfections and luciferase assay

The Clear-MiR anti-miR-346 used in our study was designed to inhibit efficiently the activity of miR-346. It consists of a sequence of 21 nucleotides complementary to the miRNA and was supplied by Eurogentec. The miR-346 mimic was supplied by Dharmacon.

Transient transfection of FLS with Clear-MiR miRNA inhibitor (200 nM) and reporter constructs (200 ng/ml) was performed using the human dermal fibroblast Nucleofector kit from Amaxa as previously described (27). Transient transfection of THP-1 cells with miR-346 mimic (200 nM) was performed using the cell line Nucleofector kit V from Amaxa. FLS and THP-1 cells were then plated in 24-well plates (2×10^5 cells/well and 10^6 cells/well, respectively). All assays were performed 24 h after transfection. Controls were conducted with the Clear-MiR negative control or with the mimic miRNA negative control. Transfection efficiency was evaluated with the pmaxGFP vector.

Transfection of HEK293 cells plated in 24-well plates (2×10^5 cells/well) with reporter constructs and miR-346 mimic (200 nM) was performed using Lipofectamine 2000 (Invitrogen). After 48 h cells were washed and lysed with passive lysis buffer (Promega), and *f-luc* and *Renilla* luciferase (*r-luc*) activities were determined using the dual-luciferase reporter assay system (Promega) and a luminometer (Glo-Max; Promega). The relative reporter activity was obtained by normalization to *r-luc* activity.

Cell numbers and cell viability were assessed using the MTT test. IL-18 release was measured in culture supernatants by a heterologous two-site sandwich ELISA according to the manufacturer's instructions.

Western blot

Cells (10^6; FLS and THP1) were incubated for various times (24 h) with LPS (1 μg/ml). Controls were performed with cells maintained in medium with 5% heat-inactivated FCS for 6 h. After stimulation, cells were centrifuged and the pellets were suspended for 20 min on ice in 300 μl of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 130 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitors). Lysates were centrifuged for 10 min at 14,000 $\times$ *g* at 4°C, and supernatants were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked using 1% BSA in TBS (20 mM Tris (pH 7.5), 150 mM NaCl) for 1 h at 25°C. The blots were incubated with anti-Btk mouse IgG mAbs (BD Transduction Laboratories) for 2 h at 25°C followed by incubation with HRP-conjugated goat anti-mouse IgG mAbs (1 h at 25°C) and detected by ECL (SuperSignal West Femto Maximum Sensitivity substrate; Pierce) according to the manufacturer's instructions. To confirm the presence of equal amounts of proteins, bound Abs were removed from the membrane by incubation in 62.5 mM Tris (pH 6.7), 100 mM 2-ME, and 2% SDS for 30 min at 50°C and reprobed again with anti-β-actin (clone AC-74; Sigma-Aldrich) mouse mAbs.

Statistical analysis

Statistical analysis was performed using Student's *t* test. Values were compared between different groups in the experiment. A p value of <0.05 was considered statistically significant.

Results

LPS induced synthesis of IL-18 mRNA in RA FLS

In this study, we first verified the capacity of LPS from *S. abortus equi* to stimulate IL-18 mRNA expression in RA FLS. Quantitative RT-PCR was performed with RNA isolated from control and activated FLS. Stimulation with LPS at a concentration of 1 μg/ml resulted in an increasing amount of IL-18 transcript, which was detectable within 2 h and increased up to 3 h (the mean increases were 150-fold for 2 h and 300-fold for 3 h) (Fig. 1*A*). No constitutive expression of IL-18 mRNA was detectable in control cells. We verified also that this cytokine was not released in activated

cell supernatants: although LPS activation induced IL-6 secretion, activated FLS did not release any detectable amount of mature IL-18 in cell culture supernatants (Fig. 1, *C* and *D*). THP-1 cells were used as a control: upon LPS treatment, the level of IL-18 transcript was strongly up-regulated, which resulted in a high accumulation of IL-18 in the supernatant (Fig. 1, *B–D*). Moreover, using actinomycin D (cells were incubated with LPS for 3 h and then for another 1 and 2 h with actinomycin D at a concentration of 5 μg/ml), we observed a rapid decay of IL-18 mRNA (330-fold to 45-fold) in FLS cells but not in LPS-activated THP-1 cells (300-fold to 275-fold), which confirms the IL-18 mRNA instability in LPS-activated RA FLS (Fig. 1, *A* and *B*).

LPS induced synthesis of miRNAs targeting IL-18 mRNA in RA FLS

To examine the potential involvement of miRNAs in the regulation of IL-18 mRNA instability, we used two strategies. We first used a bioinformatic approach to identify potential miRNAs targeting IL-18 mRNA. An online search of the miRBase Target database (microrna.sanger.ac.uk/) demonstrated that 49 miRNAs have been predicted to potentially target the 3′-UTR region of the human IL-18 mRNA. In this approach, each miRNA is given a score, reflecting the local complementarity between miRNA seed sequences and the 3′-UTR region of the IL-18 mRNA. For this study, we focused on miRNA miR-197, as it showed the best score among all candidate miRNAs targeting IL-18 mRNA and it was predicted to form a thermodynamically stable complex with the considered mRNA (free energy = −25.35 kcal/mol).

We stimulated RA FLS with LPS (1 μg/ml) or medium and measured miR-197 levels by quantitative RT-PCR. We found that LPS did not induce miR-197 expression in RA FLS after 6 h of stimulation as compared with unstimulated control RA FLS (Fig. 2*A*).

In parallel, we analyzed the global expression profile of miRNAs using a microarray-based approach after exposure of RA FLS to LPS to identify LPS-induced miRNAs. FLS were stimulated with LPS (1 μg/ml) or medium for 6 h and the extracted RNAs were compared using a DNA microarray containing 409 oligonucleotide probes complementary to miRNAs of human origin. Fourteen miRNAs predicted to be able to target IL-18 mRNA were present on the array.

After LPS challenge, this initial screening identified 63 miRNAs, the expression levels of which were increased or attenuated in response to LPS. Data are presented on a scatter plot showing $\log_{10}$-transformed signal intensities for each probe on both channels for the Cy3-labeled media controls and Cy5-labeled samples stimulated with LPS (Fig. 2*B*).

Among the miRNAs showing an up-regulation following LPS treatment, 15 were up-regulated >2-fold. Within these 15 up-regulated miRNAs, 1 miRNA potentially targeting IL-18 mRNA showed a strong expression level in LPS-treated cells compared with control cells: miR-346 (>7-fold). The expression of miR-197, which was included in the array, was not modified after activation with LPS, confirming preceding results.

After LPS challenge, the array revealed also a strong up-regulation in the expression of miR-30d, miR-10a, and miR-125b. Interestingly, these miRNAs are predicted to target the 3′-UTR of IL-1β and TNF-α. The expression of 42 miRNAs was down-regulated, especially miR-633, miR-320, and let-7a (<0.5). The main results are presented in Table I.

To confirm the validity of miR-346 induction as assessed by the microarray analysis, quantitative RT-PCR was performed using primers that recognized their respective mature forms. Consistent with the microarray findings, miR-346 was strongly induced by

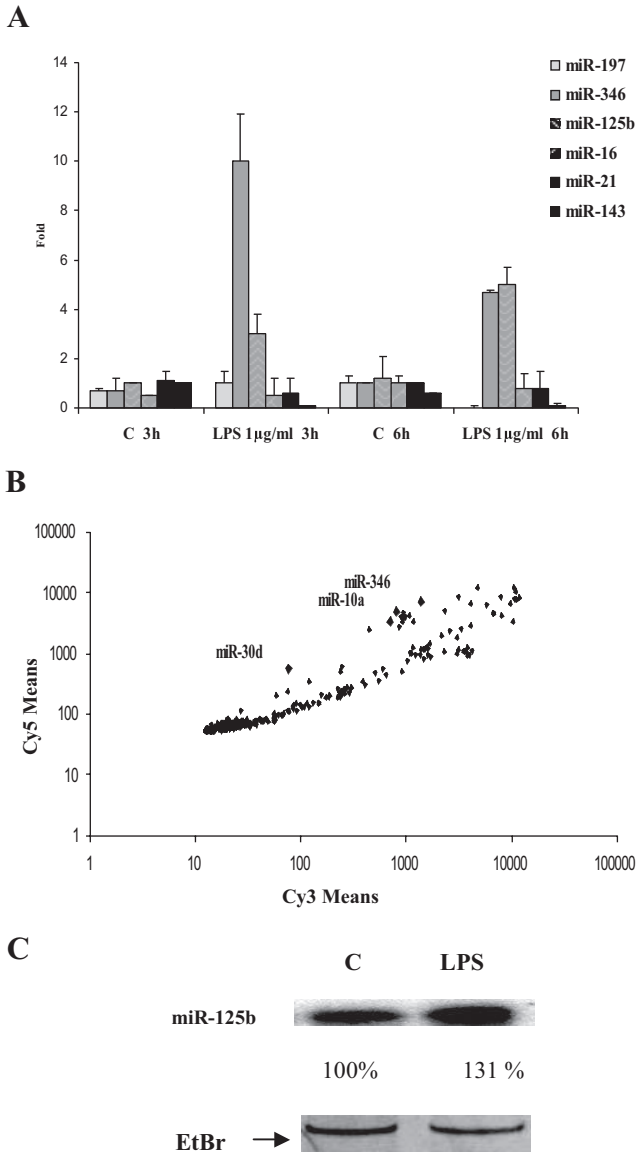

FIGURE 2. Microarray and quantitative RT-PCR analysis of miRNA expression in LPS-activated RA FLS. *A*, MiR-346, miR-125b, miR-197, miR-16, miR-21, and miR-143 levels were determined by quantitative RT-PCR in RA FLS stimulated with LPS (1 μg/ml) for 3 and 6 h. U6 small nuclear RNA was used as endogenous control for data normalization. The control (C) corresponded to untreated cells. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. *B*, RA FLS were stimulated with medium or LPS (1 μg/ml) for 6 h. RNA was extracted and used in a microarray assay to determine the expression levels of 409 human miRNAs. Data are presented on a scatter plot showing $\log_{10}$-transformed signal intensities for each probe on both the channel for the Cy3-labeled control and the Cy5-labeled 6-h LPS-treated samples and represent the mean values. Each dot represents one miRNA probe. Main results are summarized in Table I. *C*, Northern blot analysis of miR-125b expression was performed under the same conditions with locked nucleic acid-modified oligodeoxynucleotides complementary to the indicated miRNAs. RNA was used as a loading control. The results are representative of three different experiments.

LPS (the mean increases were 11-fold for 3 h and 5-fold for 6 h) (Fig. 2*A*); however, we were unable to confirm this result by Northern blot analysis (data not shown). MiR-125b was detected in activated cells either by Northern blot analysis or quantitative RT-PCR and the observed changes were confirmed by either means (Fig. 2, *A* and *C*). The validity of the results was also confirmed by

Table I. *MiRNA differentialy expressed between nonactivated RA FLS and LPS-activated RA FLS*[a]

Name	Intensity Ratio of Cy5/Cy3
hsa-miR-346	7.01
hsa-miR-10a	6.38
hsa-miR-10b	4.94
hsa-miR-30d	5.99
hsa-miR-30c-2, hsa-miR-30c-1	3.41
hsa-miR-487b	3.33
hsa-miR-596	3.07
hsa-miR-100	2.88
hsa-miR-30b	2.84
hsa-miR-125b	2.70
hsa-miR-508	2.67
hsa-miR-585	2.44
hsa-miR-563	2.36
hsa-miR-411	2.12
has-miR-155	1.7
hsa-let-7a	0.34
hsa-miR-320	0.32
hsa-miR-633	0.48

[a] Data are presented on intensity ratio of the Cy3-labeled media controls and Cy5-labeled samples stimulated with LPS.

quantitative RT-PCR using miR-16, miR-21, and miR-143, the expression of which was not modified by LPS in the array (Fig. 2*A*).

IL-18 synthesis is repressed posttranscriptionally by miR-346 in LPS-activated RA FLS

To study the functional consequence of miR-346 induction on IL-18-release by LPS-activated RA FLS, we performed miRNA inhibition experiments. We used an antisense oligonucleotide complementary to miR-346 to block its activity, and the Clear-MiR was used as a negative control. Oligonucleotide*s* were transfected in FLS at a concentration of 200 nM. A plasmid encoding GFP was cotransfected to evaluate transfection efficiency.

Transfection of miR-346 antisense molecules impaired endogenous miRNA expression by a factor of 2 as compared with miR-346 expression in activated cells transfected with an anti-miR control as assessed by quantitative RT-PCR analysis (Fig. 3*A*).

We also performed experiments demonstrating the fate of the IL-18 mRNA in RA FLS transfected with miR-346 antisense molecules or anti-miRNA control and incubated with LPS for 3 h and then for another 2 h with actinomycin D. As compared with the control, we observed a stabilization of IL-18 mRNA (Fig. 3*B*).

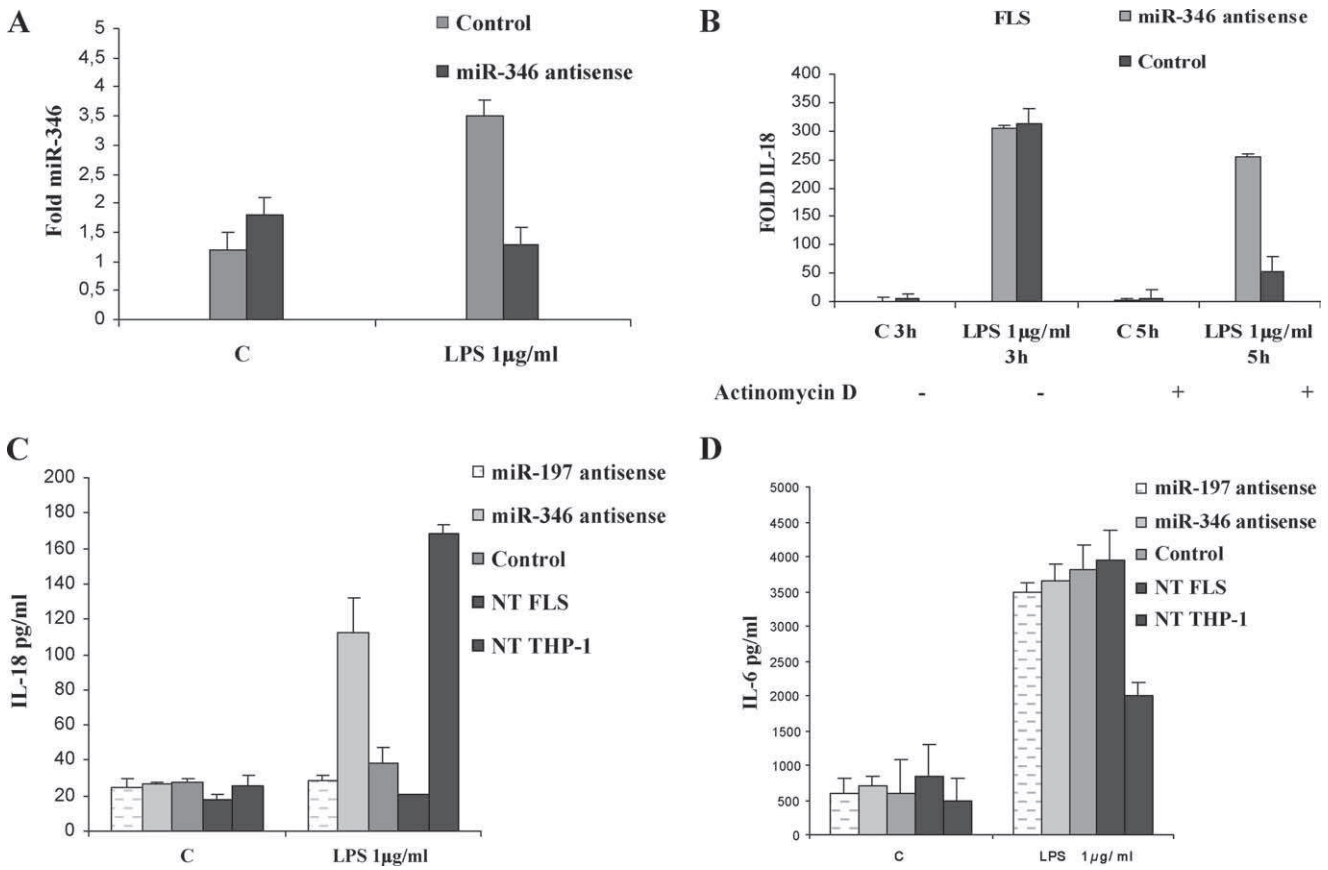

FIGURE 3. Effect of transfection of miRNA antisense molecules on IL-18 synthesis by RA FLS. *A*, RA FLS were transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected RA FLS was performed 24 h posttransfection for 6 h. MiR-346 expression was determined by quantitative RT-PCR. U6 small nuclear RNA was used as endogenous control for data normalization. *B*, IL-18 mRNA levels were determined using quantitative RT-PCR in RA FLS transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected cells was performed 24 h posttransfection for 3 h, and cells were then incubated for another 2 h with actinomycin D. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *C* and *D*, RA FLS were transfected with miR-346, miR-197 antisense molecules, or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected RA FLS was performed 24 h posttransfection. Nontransfected RA FLS and THP-1 cells were used as negative and positive controls (NT). IL-18 and IL-6 release was determined by ELISA in culture supernatants harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments.

We next tested whether alteration of miR-346 cellular levels affected IL-18 protein release by LPS-stimulated FLS. We transfected cells with antisense molecules for 24 h and then measured IL-18 release by FLS stimulated with LPS (1 μg/ml) for 24 h at 37°C. Supernatants were tested for IL-18 secretion levels by ELISA measurements. As illustrated in Fig. 3*C*, treatment with LPS significantly induced IL-18 release by activated RA FLS transfected with antisense oligonucleotides targeting miR-346 (120 vs 25 pg/ml) as compared with activated FLS transfected with control oligonucleotides or with oligonucleotides targeting another miRNA (miR-197). A similar increase in IL-18 secretion was obtained in nontransfected LPS-activated THP-1 cells used as positive control. No IL-18 release was observed in nontransfected but LPS-activated RA FLS (Fig. 3*C*). The effect of transfection on cell viability was also determined by the MTT test, with no significant difference being observed for cells transfected with targeting and nontargeting antisense oligonucleotides (data not shown).

The transfection of antisense oligonucleotides did not impair FLS activation since nontransfected FLS as well as FLS transfected with targeting and nontargeting miRNA inhibitors produced IL-6 after 24 h of activation with LPS (Fig. 3*D*).

Taken together, these data demonstrate that miR-346 is implicated in the negative regulation of IL-18 synthesis in LPS-activated FLS.

MiR-346 repressed IL-18 synthesis in LPS-activated THP-1 cells

We next tested by transient transfection whether the presence of miR-346 affected IL-18 mRNA expression and IL-18 release in LPS-activated THP-1 cells. THP-1 cells did not express miR-346 when activated with LPS for 3 and 6 h as assessed by quantitative RT-PCR (Fig. 4*A*). We transfected THP-1 cells with miR-346 mimic for 24 h and incubated cells with LPS for 3 h and then for another 2 h with actinomycin D. As compared with the control, we observed that transfection of miR-346 mimic in THP-1 cells induces a degradation of IL-18 mRNA (Fig. 4*B*). We also measured IL-18 release by transfected cells stimulated with LPS (1 μg/ml) for 24 h at 37°C. As illustrated in Fig. 4*C*, cells transfected with miR-346 mimic showed a significant decrease of IL-18 release as compared with IL-18 secretion by activated cells transfected with the miRNA mimic negative control or nontransfected LPS-activated THP1 cells used as positive control. Collectively, these results confirm that miR-346 plays a role in the control of IL-18 release in a cell type-independent manner.

MiR-346 did not directly regulate the expression of IL-18

Computer analysis predicted that miR-346 might directly target the 3′-UTR of human IL-18 transcript (Fig. 5*A*), suggesting that this miRNA could play a direct role in IL-18 posttranscriptional regulation. To test the hypothesis of a direct regulation by miR-346, we cotransfected HEK293 cells with a luciferase reporter fused to IL-18 3′-UTR along with miR-346 mimic. FLS were also transfected with the same construct and then activated with LPS. Transfections in both kinds of cells did not result in a measurable change in luciferase activity (Fig. 5, *B* and *C*). These results indicate that miR-346 does not repress directly the expression of IL-18.

MiR-346 repressed Btk expression in LPS-activated RA FLS

Btk is a nonreceptor tyrosine kinase belonging to the Tec family of protein tyrosine kinases, and recent studies have shown that Btk is involved in the stabilization of various cytokine mRNAs such as TNF-α (28–30). FLS express Etk, which is implicated in IL-6 and IL-8 release by activated FLS, but they fail to express constitutively or after LPS activation either Btk mRNA or the mature protein. Surprisingly, we found by quantitative RT-PCR that transfection of FLS with the antisense oligonucleotides targeting miR-346 led to the induction of Btk mRNA expression after activation with LPS compared with activated cells transfected with the negative control (Fig. 6*A*). These results point to a putative effect at the transcriptional level. We also measured Btk expression by Western

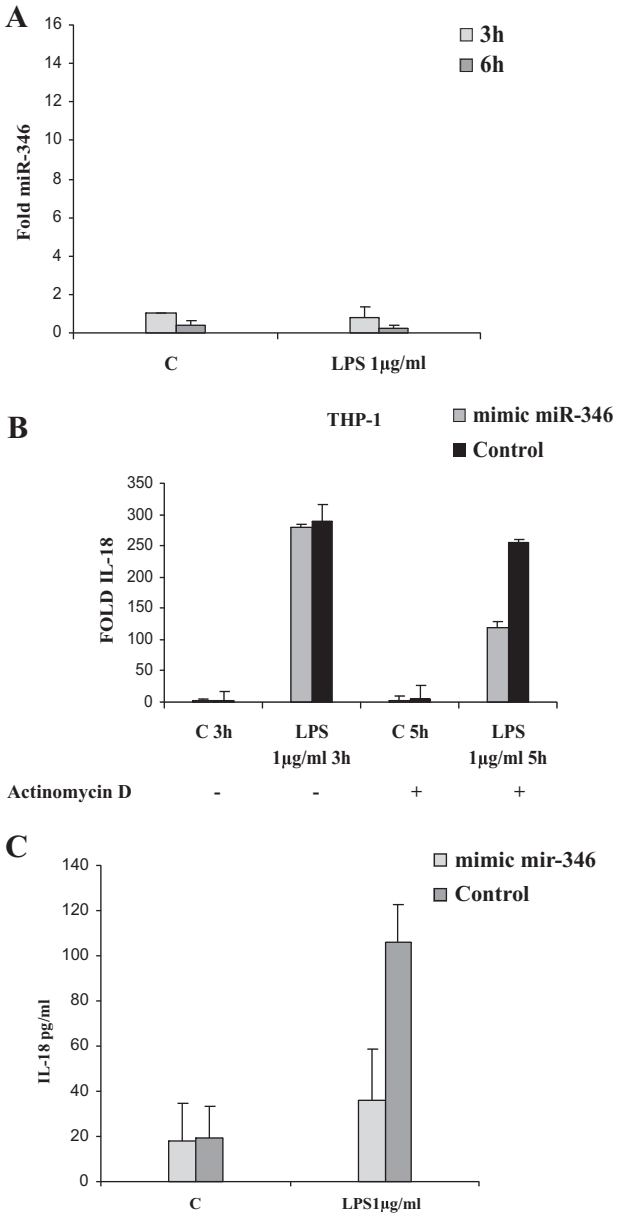

FIGURE 4. Effect of transfection of miR-346 mimic on IL-18 synthesis by THP-1 cells. *A*, MiR-346 expression was analyzed in THP-1 cells activated for 3 and 6 h with LPS (1 μg/ml) by quantitative RT-PCR. U6 small nuclear RNA was used as endogenous control for data normalization. *B*, IL-18 mRNA levels were determined using quantitative RT-PCR in THP-1 cells transfected with miR-346 mimic or with the miRNA mimic negative control (Control). Cells were activated with LPS (1 μg/ml) 24 h posttransfection for 3 h and incubated for another 2 h with actinomycin D. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *C*, THP-1 cells were transfected with miR-346 mimic or with the miRNA mimic negative control (Control) and activated with LPS (1 μg/ml) 24 h posttransfection. IL-18 release was determined by ELISA in culture supernatants harvested 24 h after stimulation with LPS (1 μg/ml) or medium (C). Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments.

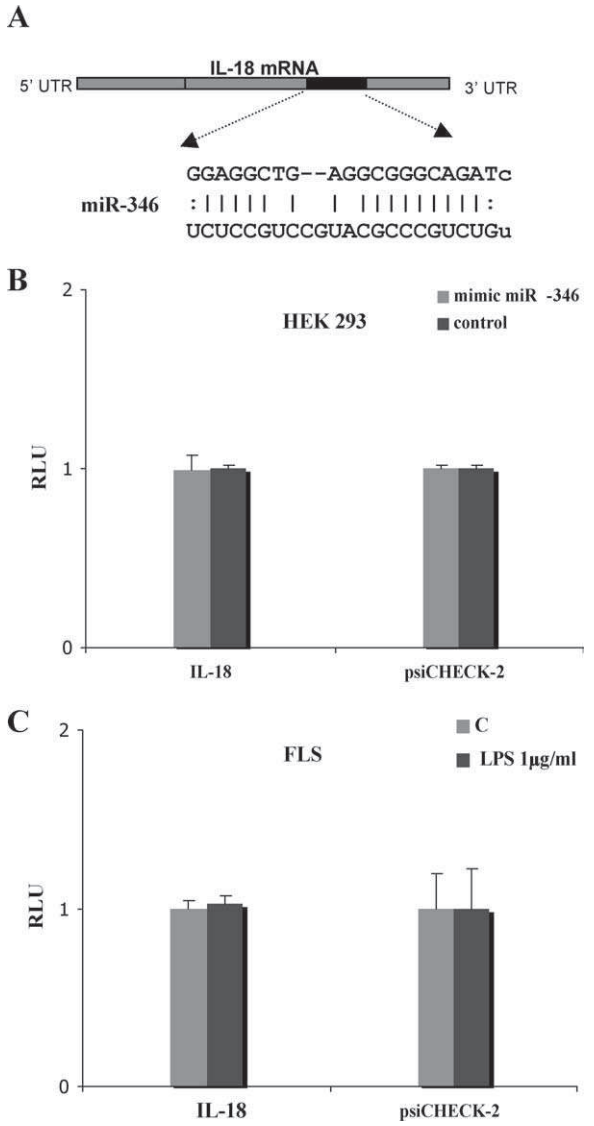

FIGURE 5. MiR-346 does not regulate the expression of IL-18 directly. *A*, Sequence alignment of miR-346 and its target sites in the 3′-UTR of IL-18 mRNA. *B* and *C*, Targeting of miR-346 to the 3′-UTR of IL-18 mRNA. A reporter construct with the potential binding site for miR-346 in the 3′-UTR of IL-18 was generated. HEK293 cells were transiently cotransfected with the reporter construct and either miR-346 mimic or miRNA mimic negative control (Control) (*B*); RA FLS were transfected with the reporter construct and activated with LPS (1 μg/ml) (*C*). Luciferase activities were measured for 48 h and normalized to the control psiCHECK-2 luciferase level. Bars represent the mean ± SD from three independent experiments.

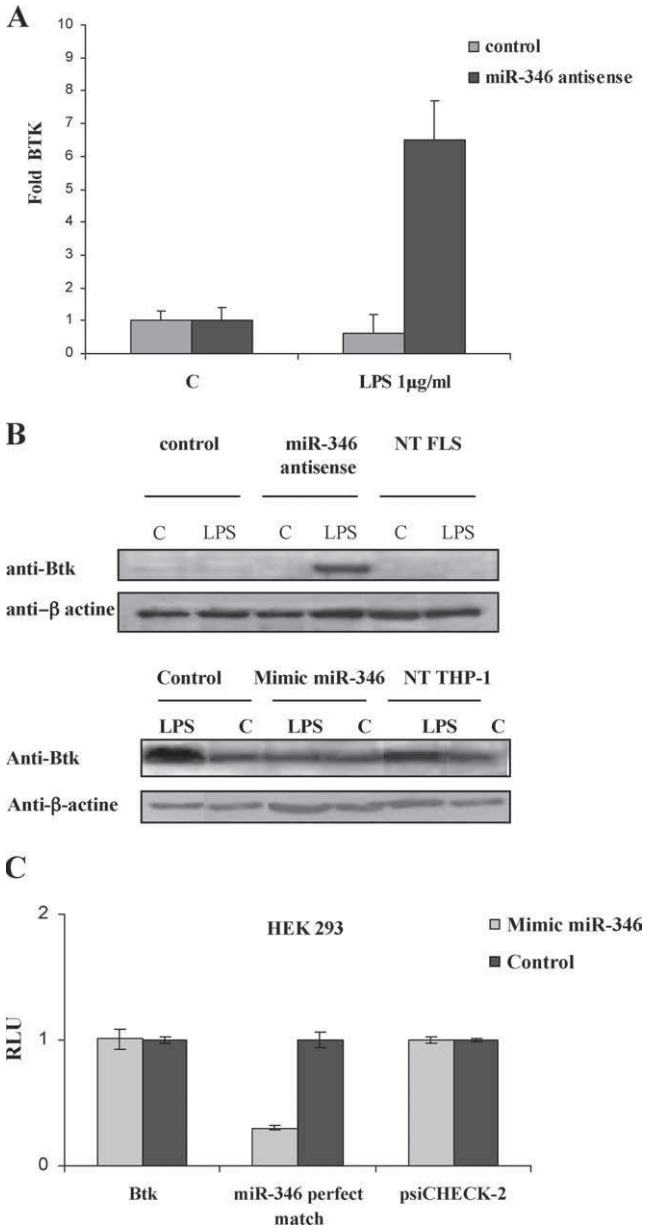

FIGURE 6. MiR-346 regulates the expression of Btk in activated RA FLS and THP-1 cells. *A*, Btk mRNA levels were determined using quantitative RT-PCR in RA FLS transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control). LPS (1 μg/ml) activation of transfected cells was performed 24 h posttransfection for 6 h. Results were normalized to GAPDH and expressed as the fold change compared with samples from cells incubated in medium (C). *B*, Btk expression was determined by Western blotting with anti-Btk mouse mAbs in FLS transfected with miR-346 antisense molecules or with the Clear-MiR negative control or in nontransfected FLS. Twenty-four hours posttransfection, FLS were either incubated in 5% FCS medium (C) or activated with LPS for 24 h. Similar experiments were performed with THP-1 cells transfected with the miR-346 mimic or the miRNA mimic negative control. For protein loading control, membranes were reprobed with anti-β-actin mouse mAbs. The results shown are representative of three separate experiments. *C*, MiR-346 does not regulate the expression of Btk directly. A reporter construct with the 3′-UTR of Btk or with the miR-346 perfect match were generated. HEK293 cells were transiently cotransfected with the reporter constructs and with either miR-346 mimic or miRNA mimic negative control (Control). Luciferase activities were measured for 48 h and normalized to the control psiCHECK-2 luciferase level. Bars represent the mean ± SD from three independent experiments.

blotting analysis of FLS stimulated with LPS for 24 h at 37°C. As shown in Fig. 6*B*, inhibition of miR-346 led also to a strong expression of the protein. Thus, these data collectively indicate that miR-346 inhibits Btk expression in LPS-activated RA FLS.

We also found that in THP-1 cells transfected with miR-346 mimic, Btk expression was down-regulated (Fig. 6*B*) and that miR-346 did not repress Btk expression directly, as transfection of HEK293 cells with a luciferase reporter fused to Btk 3′-UTR along with miR-346 mimic did not result in a measurable change in luciferase activity (Fig. 6*C*).

Btk regulates IL-18 release in LPS-activated RA FLS

We therefore hypothesized that miR-346 inhibits Btk expression in RA FLS, which could result in the subsequent inhibition of IL-18.

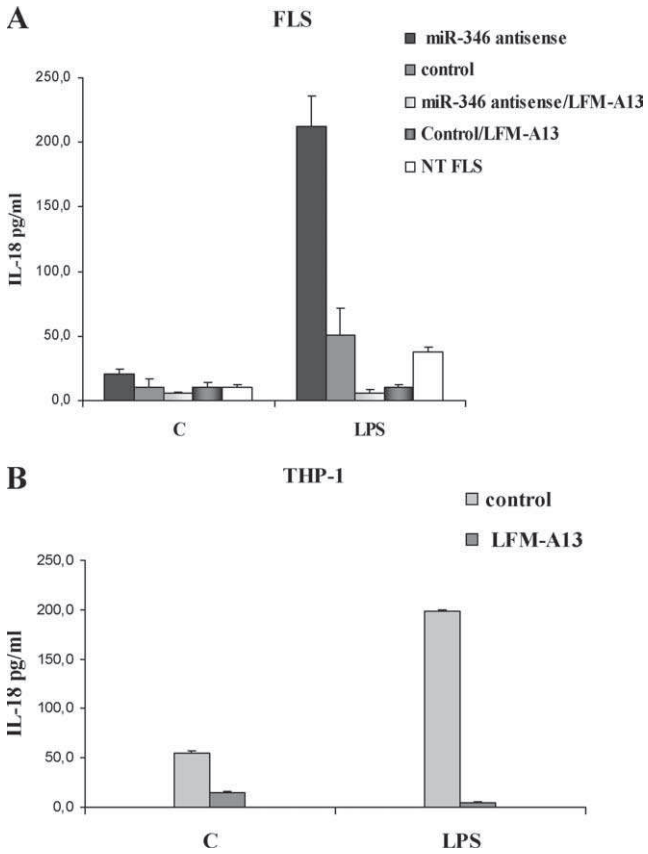

FIGURE 7. Btk regulates IL-18 expression. *A*, RA FLS were transfected with miR-346 antisense molecules or with the Clear-MiR negative control (Control) for 24 h. IL-18 release by FLS preincubated with LFM-A13 and then stimulated with LPS for 24 h was evaluated by ELISA. Nontransfected RA FLS were used as negative controls (NT). *B*, THP-1 cells was pretreated with LFM-A13 for 1 h before activation with LPS (1 μg/ml) for 24 h.

We transfected FLS with antisense molecules targeting miR-346 for 24 h and then examined IL-18 release by FLS pretreated with LFM-A13, a Btk inhibitor, before LPS stimulation. In this case, IL-18 production induced by LPS was impaired in cells treated with LFM-A13 when compared with cells not treated with the inhibitor (Fig. 7*A*). Similarly, in THP-1 cells activated by LPS in the presence of LFM-A13, IL-18 release was strongly inhibited (Fig. 7*B*). These results demonstrate that IL-18 release is Btk-dependent. Taken together, our data indicate an important role for miR-346 and Btk in the regulation of IL-18 release by RA FLS.

Discussion

The key findings in this study are that LPS induces the expression of miR-346 in PRR-activated synoviocytes from RA patients and that this miRNA negatively regulates the IL-18 response of FLS by inhibiting the transcription of the Btk gene. One important observation obtained from previous results was that PRRs-activated FLS, in contrast to other resident cells from the synovial membrane such as macrophages, have a more restricted cytokine profile: they up-regulate, for example, IL-18 mRNA, but release of this cytokine is impaired via translational suppression, and similar results were obtained with TNF-α and IL-1 (data not shown). IL-18 is normally produced from macrophage-like cells, but some epithelial cells such as gastric epithelial cells release this cytokine in response to *Helicobacter pilori* infection (31). These data suggest that IL-18 protein expression is tightly regulated depending of the origin of the cell. This study was designed to test the possibility that PAMPs might induce cellular miRNAs that target IL-18 transcripts in FLS and inhibit its release.

An initial microarray screen identified 63 miRNAs, the expression of which was increased or attenuated in FLS in response to LPS. This approach revealed a promising result insofar as one miRNA predicted to target IL-18 transcript was considerably up-regulated: miR-346. This was suggested by an online search of the miRBase Target database maintained by the Sanger Institute, which predicts potential target sequences of miRNAs. The change in expression of miR-346 was then validated by quantitative RT-PCR but not by Northern blot. In fact, some mature miRNAs are produced at low levels and are not detected by Northern blot, but they are still functional, suggesting that high levels of mature miRNAs are not necessary for their efficient inhibition (32). A small variation in one component that is upstream of a transduction cascade can have strong effects in the end.

When comparing our microarray data with previous studies from others, we found similarities as well as differences. Recently, Taganov et al. analyzed miRNA expression in a monocytic cell line treated with LPS (20). They found that miR-146a, miR-155, and miR-132 were endotoxin-responsive genes. Similar results were obtained by Stanczyk et al. (33) in RA FLS activated with either TNF-α or TLR ligands such as LPS, bacterial lipopeptide, and poly(I:C). They also observed an up-regulation of miR-146a and miR-155. Similarly, we also found that miR-155 is up-regulated in response to LPS, but more faintly than miR-346. These results confirm the important role of miR-155 in innate immune response (34). However, the arrays of Stanczyk et al. (33) never revealed the up-regulation of miR-346, which was one of the most overexpressed miRNAs in RA FLS activated with LPS. A simple possible explanation for this discrepancy could be that their screen did not include the oligonucleotide probe for miR-346: only 200 sequences complementary to mammalian miRNAs were tested in the Taganov et al. (20) studies. It is also possible that miR-346 was not detected in cells treated for 8 h with LPS. In fact, quantitative RT-PCR analysis indicated that miR-346 reached its highest level by 3 h and was still present but at reduced levels by 6 h. To assess the reproducibility of the data, the array was performed twice with FLS isolated from different patients, and we found in every instance that miR-346 was overexpressed. Of note, we could not detect miR-146a/b in LPS-activated RA FLS, but it was demonstrated that this miRNA family, which is implicated in TLR signaling and in endotoxin tolerance, has a rather complicated mode of regulation of expression (35). Weber et al. (36) also reported the up-regulation of miR-346, as well as miR-197, in follicular thyroid carcinoma compared with follicular adenoma. They linked the up-regulation of these two miRNAs to cellular proliferation. We did not observe a significant effect of miR-346 on cell viability as assessed by MTT assay.

IL-18 is present in significantly elevated levels in the synovium of RA patients and is mainly produced by macrophages and dendritic cells. Its expression was demonstrated to correlate with the inflammatory phase of the disease. Studies with RA synovial cell culture have shown increased production of NO as well as TNF-α, IFN-γ, and IL-6 after stimulation with IL-18. This suggests that IL-18 is a pre-TNF-α cytokine (37–39). The expression of the IL-18 gene is regulated at different levels, such as control of promoter activity, degradation of mRNA, and posttranslational regulation through processing of the inactive precursor pro-IL-18 to an active form via activation of caspase 1 (40). Two different promoters are controlling the expression of IL-18, one constitutively active and one inducible by LPS. Additionally, IL-18 mRNA does not contain an AU-rich element within the 3′-UTR region, which

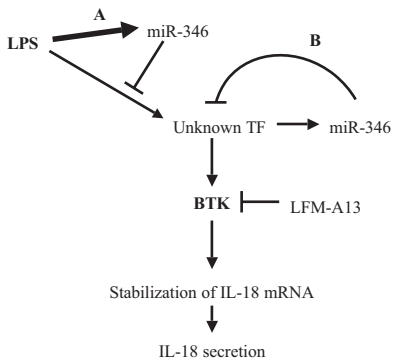

FIGURE 8. Model of action for miR-346 and Btk in IL-18 secretion regulation. See text for details. TF indicates transcription factor.

is a motif known to promote rapid mRNA degradation, and this implies that IL-18 mRNA may have a longer half-life than ARE-containing transcripts (41, 42). However, we demonstrated that IL-18 mRNA is very instable in LPS-activated RA FLS and that this instability negatively regulates IL-18 release (6).

It was thus important to investigate whether the expression levels of miR-346 could modulate IL-18 expression. RA FLS were transfected with the antisense miR-346 inhibitor. For the first time, we demonstrated that inhibition of endogenously expressed miR-346 by an anti-miR-346 oligoribonucleotide markedly increased IL-18 expression. Further evidence of miR-346 inhibitory effect on IL-18 release was obtained by transient transfection of miR-346 mimic in LPS-activated THP-1 cells. Taken together, these data clearly suggest that one of the functions of miR-346 is to turn off IL-18 expression in response to LPS.

We also demonstrated, using luciferase reporter constructs containing the 3′-UTR of the human IL-18 gene, that miR-346 does not directly regulate the expression of IL-18. A general principle regarding miRNAs is that each miRNA can potentially regulate a wide spectrum of protein-coding genes. This suggests that miRNA can be involved in the regulation of multiple independent physiological processes. Thus, it was tempting to speculate that miR-346 may use different mechanisms to down-regulate at a posttranscriptional level the expression of IL-18. Several reports have established that Btk is implicated in TLR signaling and regulates TNF-α synthesis by stabilizing its mRNA, without having any effect on IL-6 or IL-12 release (28). FLS do not express either Btk mRNA or the mature protein but Etk, another Tec kinase, which was demonstrated to be implicated in IL-6 and IL-8 release in response to integrin $\alpha_5\beta_1$ and TLR4 stimulation (27). Results obtained here demonstrated that transient transfection of miR-346 antisense induced a strong expression of Btk in FLS and reestablished IL-18 release. Moreover, inhibition of induced Btk by LFM-A13 led to the impairment of IL-18 secretion by RA FLS. These data demonstrated the implication of miR-346 in Btk expression that results in IL-18 release.

Interestingly, as in THP-1 cells transfection of miR-346 mimic diminished protein expression, we investigated whether miR-346 was capable of a direct inhibition of Btk by using a luciferase reporter assay. A direct inhibition was not observed. Thus, as previously demonstrated with FLS, miR-346 inhibits IL-18 secretion by an indirect mechanism involving the regulation of Btk. The interaction between miR-346 and Btk must involve other yet-undefined factors acting probably at the transcriptional level.

We propose a model in which LPS would induce expression of both Btk and miR-346 through two distinct transcription factors (Fig. 8*A*) or through a common transcription factor (Fig. 8*B*). The induced Btk would then act on stabilizing IL-18 mRNA, which would ultimately result in its secretion. Blocking Btk either chemically (with the inhibitor LFM-A13) or by overexpressing miR-346 (in THP-1 cells) would therefore result in the inhibition of IL-18 secretion. The action of miR-346 could be upstream of the transcription factor inducing Btk expression (Fig. 8*A*) or, alternatively, miR-346 could regulate its own transcription factor in a feedback loop manner (Fig. 8*B*). The latter model would explain why miR-346 is only transiently detected after LPS challenge of FLS cells and seems to gradually disappear.

In conclusion, our findings provide evidence that miRNAs, which are induced by bacterial ligands, can act as potential negative regulators of inflammation and may be novel targets for immunomodulating inflammatory responses in humans. These data also demonstrated that cytokine secretion by resident cells of target organs of autoimmunity can be negatively regulated at the post-transcriptional level by miRNAs. Understanding these complex controls has important implications for the development of future therapeutic applications.

Disclosures

The authors have no financial conflicts of interest.

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miR-346 Controls Release of TNF-α Protein and Stability of Its mRNA in Rheumatoid Arthritis via Tristetraprolin Stabilization

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Abstract

TNF-α is a major cytokine implicated in rheumatoid arthritis. Its expression is regulated both at the transcriptional and posttranscriptional levels and recent data demonstrated that miRNAs are implicated in TNF-α response in macrophages. LPS-activated FLS isolated from RA patients express TNF-α mRNA but not the mature protein. This prompted us to look for miRNAs which could be implicated in this anti-inflammatory effect. Using a microarray, we found two miRNAs, miR-125b and miR-939 predicted to target the 3′-UTR of TNF-α mRNA, to be up-regulated in RA FLS in response to LPS, but their repression did not restore mature TNF-α expression in FLS. We showed previously that miR-346, which is upregulated in LPS-activated FLS, inhibited Btk expression that stabilized TNF-α mRNA. Blocking miR-346 reestablished TNF-α expression in activated FLS. Interestingly, transfection of miR-346 in LPS-activated THP-1 cells inhibited TNF-α secretion. We also demonstrated that TTP, a RNA binding protein which inhibited TNF-α synthesis, is overexpressed in activated FLS and that inhibition of miR-346 decreases its expression. Conversely, transfection of miR-346 in LPS-activated THP-1 cells increased TTP mRNA expression and inhibited TNF-α release. These results indicate that miR-346 controls TNF-α synthesis by regulating TTP expression.

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Introduction

One of the key players in rheumatoid arthritis (RA) is TNF-α and therapies targeting this cytokine have already proved beneficial [1]. In RA, TNF-α is produced by many cell types, mainly by macrophages and dendritic cells in response to interactions between pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and pattern-recognition receptors (PRRs) or to the cytokine environment [2].

The resident cells of the joint space, the fibroblast-like synoviocytes (FLS,) play a crucial role in RA [3,4,5]. They are implicated in the inflammatory response essentially by synthesizing cytokines, chemokines, prostanoids, nitric oxyde (NO), and pro-angiogenic factors FLS play a key role in osteoarticular destruction and take also part in the differentiation and activation of osteoclasts by the RANK-RANK ligand pathway, and through the release of PGE-2 and IL-6 [6,7,8]. However RA FLS secrete no TNF-α, a major cytokine implicated in RA [9], but intriguingly they express TNF-α mRNA in response to LPS. This was also observed with osteoarthritis (OA) and trauma FLS (unpublished data). It was therefore of interest to identify the molecular basis of this anti-inflammatory mechanism. Post-transcriptional regulation of TNF-α expression depends on AU-rich elements (ARE) located in the 3′-untranslated region (UTR) of TNF-α mRNA. RNA binding proteins such as tristetraprolin (TTP), T-cell intracellular antigen (TIA-1) and T-cell intracellular antigen-related protein (TIAR) can bind to ARE of TNF-α mRNA inducing mRNA lability or inhibition of translation [10,11]. Mice lacking TTP develop a systemic inflammatory syndrome characterized by cachexia, dermatitis, erosive arthritis and myeloid hyperplasia [12]. Besides the direct destabilization of mRNA by regulatory proteins, another mode of regulation involves miRNAs [13]. MiRNAs are an evolutionarily conserved class of endogenous small non-coding RNAs. They are processed from long primary transcripts by the ribonuclease Drosha in association with DGCR8. After being transported into the cytoplasm, the pre-miRNA is further processed by Dicer and its cofactor TRBP. One strand is then assembled in the RISC complex which always contains a member of the Argonaute family. The miRNA then

guides the RISC complex to its target 3′-UTR leading to a decrease of mRNA stability or inhibition of translation [14,15]. Regulation of miRNAs expression is controlled at the level of transcription, processing and subcellular localization and several recent studies have indicated that these processes can be influenced by factors such as inflammation [16,17]. Since their discovery, miRNAs have been implicated in a wide array of cellular and developmental processes and emerging data have identified an important contribution of miRNAs to the development and control of the inflammatory response [18–21]. For example, Tili et al. [22] demonstrated that up-regulation of miR-155 in LPS-activated macrophages resulted in an enhanced translation of TNF-α mRNA. Mice overexpressing miR-155 produce more TNF-α when challenged with LPS. By contrast, they found miR-125 to be implicated in the posttranscriptional repression of TNF-α mRNA, hence the need for its down-regulation for TNF-α production. A negative correlation between miR-146a expression and TNF-α release was also demonstrated in THP-1 cells [23,24]. These data indicate that miRNAs can exert both negative as well as positive effects in inflammatory pathways and this prompted us to look for miRNAs which could be implicated in the anti-inflammatory effect of LPS in RA FLS. We found two miRNAs, miR-125b and miR-939, which were predicted to target the 3′-UTR of TNF-α mRNA, to be up-regulated in RA FLS in response to LPS, but their repression did not restore either intracellular expression of TNF-α or its release by LPS-activated FLS. We further demonstrated that inhibition of miR-346, a negative regulator of Bruton's tyrosine kinase (Btk) expression, reestablished mature TNF-α expression in LPS-activated FLS by blocking TTP expression. In parallel, over-expression of miR-346 in LPS-activated THP-1 cells increased the expression of TTP which resulted in inhibition of TNF-α synthesis. These results indicate that miR-346 controls TNF-α synthesis by regulating TTP expression and can therefore act as a negative regulator of inflammation.

Results

LPS does not induce TNF-α production by RA FLS

We first confirmed that TNF-α was not released by FLS in response to LPS. FLS (5. 10^5 cells) were activated with LPS from *Salmonella abortus equi* (1 μg/ml) for 3 h, 6 h and 24 h and activated-cells supernatants were analyzed by ELISA. Although LPS stimulation induced IL-6 secretion (Figure 1A), activated FLS did not release any detectable amount of mature TNF-α in cell culture supernatants (Figure 1B). THP-1 cells were used as a positive control: upon LPS treatment, a high accumulation of TNF-α was found in cell culture supernatants with values reaching 2400 pg/ml (6 h) representing a five-fold increase compared to control (C) (Figure 1C). Finally, we demonstrated by western blotting that non-activated or LPS-activated FLS did not express the mature protein (Figure 1D).

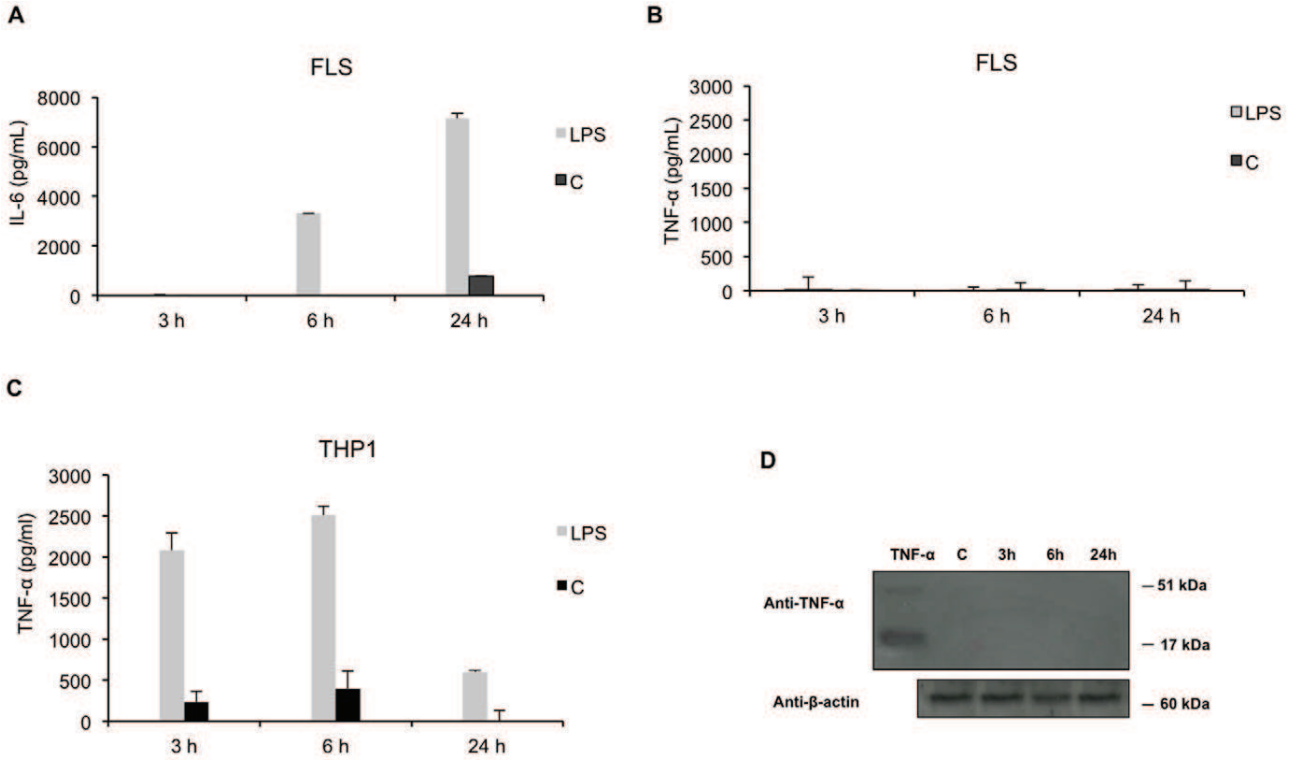

Figure 1. Effect of LPS on TNF-α release by RA FLS and THP-1 cells. A. IL-6 release was determined by ELISA in culture supernatants harvested 3 h, 6 h and 24 h after stimulation with LPS (1 μg/ml) or medium (C). **B, C**. TNF-α release by RA FLS and THP-1 cells was determined by ELISA in culture supernatants harvested 3 h, 6 h and 24 h after stimulation with LPS or medium (C). Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. **D**. TNF-α expression was determined by western-blotting with anti-TNF-α antibodies in RA FLS, 3 h, 6 h and 24 h after stimulation with LPS or medium (C). Recombinant TNF-α was used as control. For protein loading control, membranes were reprobed with anti-β-actin antibodies. Data are expressed as the mean of triplicate samples +/− SD of three independent experiments for each patient.
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LPS induced synthesis of TNF-α mRNA in RA FLS

We then examined the effect of LPS treatment on TNF-α mRNA accumulation in FLS. RT-PCR was performed with RNA isolated from FLS either non-activated or activated with 1 μg/ml LPS for 2 h, 4 h and 6 h. No constitutive expression of TNF-α mRNA was detectable in control cells, whereas LPS treatment of FLS resulted in a detectable accumulation of TNF-α mRNA within 2 h (Figure 2A). Expression of TNF-α mRNA in FLS was similar to that in THP-1 cells, where TNF-α is normally expressed after LPS treatment (Figure 2B). To investigate the mechanisms responsible for the lack of TNF-α protein synthesis in LPS-activated FLS, cells were incubated with LPS for 3 h and then for another 1 to 4 h with 5 μg/ml actinomycin D. After 1 h of actinomycin D treatment, TNF-α mRNA became undetectable in LPS-activated FLS (Figure 2C) but not in LPS-activated THP-1 cells, where the mRNA accumulation decreased but was still detectable after 1 and 2 h of actinomycin D treatment (Figure 2D). These results indicate that *de novo* synthesized TNF-α mRNA is unstable in LPS-activated FLS.

TNF-α synthesis is not repressed by miR-125b and miR-939 in LPS-activated RA FLS

Using a DNA microarray, we identified two upregulated miRNAs in LPS-treated cells compared to control cells: miR-125b (2.3 fold) and miR-939 (6.1 fold). We confirmed the induction of miR-125b and miR-939 expression by quantitative RT-PCR analysis. The mean increase was 2 and 7 times for miR-125b and miR-939 respectively, after 6 h of LPS-activation (Figure 3A). Similar experiments were performed in THP-1 cells as control, and no changes in the expression of these miRNAs were observed. Since these two miRNAs were predicted to target TNF-α mRNA, we studied the functional consequence of their inhibition by antisense oligonucleotides on TNF-α release by LPS-activated RA FLS. As shown in Figure 3B, transfection of miR-125b and miR-939 antisense molecules impaired endogenous miRNAs expression as compared to miR-125b and mir-939 expression in activated cells transfected with a control antisense oligonucleotide. We next tested whether inhibition of miR-125b and miR-939 affected TNF-α protein release in LPS-activated FLS. We transfected FLS for 24 h with antisense oligonucleotides and then measured TNF-α release by LPS-stimulated FLS. As illustrated in Figure 3C, treatment with LPS did not restore TNF-α release by activated RA FLS transfected with antisense oligonucleotides targeting miR-125b, miR-939 or with a combination of these two inhibitors (Figure 3C). No significant effect of antisense oligonucleotides transfection on cell viability was observed, as determined by MTT assay (data not shown). In addition, the transfection of antisense oligonucleotides did not impair FLS activation since FLS transfected with targeting and non-targeting miRNA inhibitors still produced IL-6 after 6 h of activation with LPS (Figure 3D).

Finally, transfection of antisense oligonucleotides did not restore intracellular or membrane bound TNF-α (Figure 3E). Taken together these data demonstrate that miR-125b and miR-939 are not implicated in the down-regulation of TNF-α synthesis in LPS-activated FLS.

Inhibition of miR-346 stabilizes TNF-α mRNA but does not induce TNF-α release from LPS-activated RA FLS

We previously showed that miR-346 which is overexpressed in LPS-activated FLS, inhibited Btk expression [25]. As Btk is implicated in TNF-α mRNA stabilization, we therefore hypothesized that the inhibition of Btk expression in RA FLS by miR-346 could result in the subsequent instability of TNF-α mRNA and therefore lack of TNF-α release.

We first confirmed that miR-346 is overexpressed in FLS but not in THP-1 cells in response to LPS (Figure 4). We then

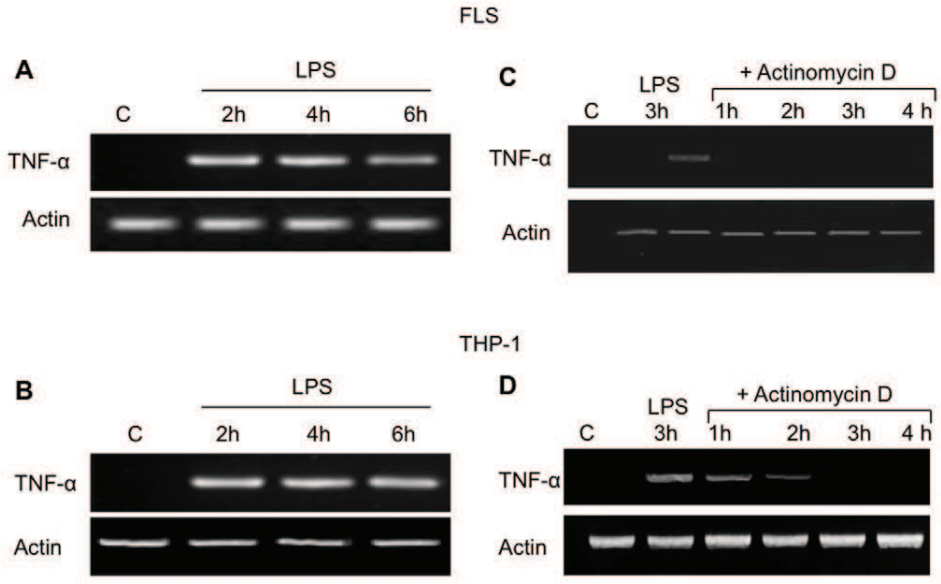

Figure 2. Effect of LPS on TNF-αmRNA expression in RA FLS and THP-1 cells. A, B. TNF-α mRNA expression was determined by RT-PCR in RA FLS (**A**) and THP-1 cells (**B**) stimulated with LPS (1 μg/ml) for 2 h, 4 h and 6 h. Control cells were incubated for 4 h with medium (C). **C, D.** RA FLS and THP-1 cells were stimulated for 3 h with LPS and then incubated for another 1, 2, 3 and 4 h with actinomycin D (5 μg/ml). Control cells were incubated for 3 h with medium. TNF-α mRNA expression was determined by RT-PCR. The results are representative of three different experiments for each patient.
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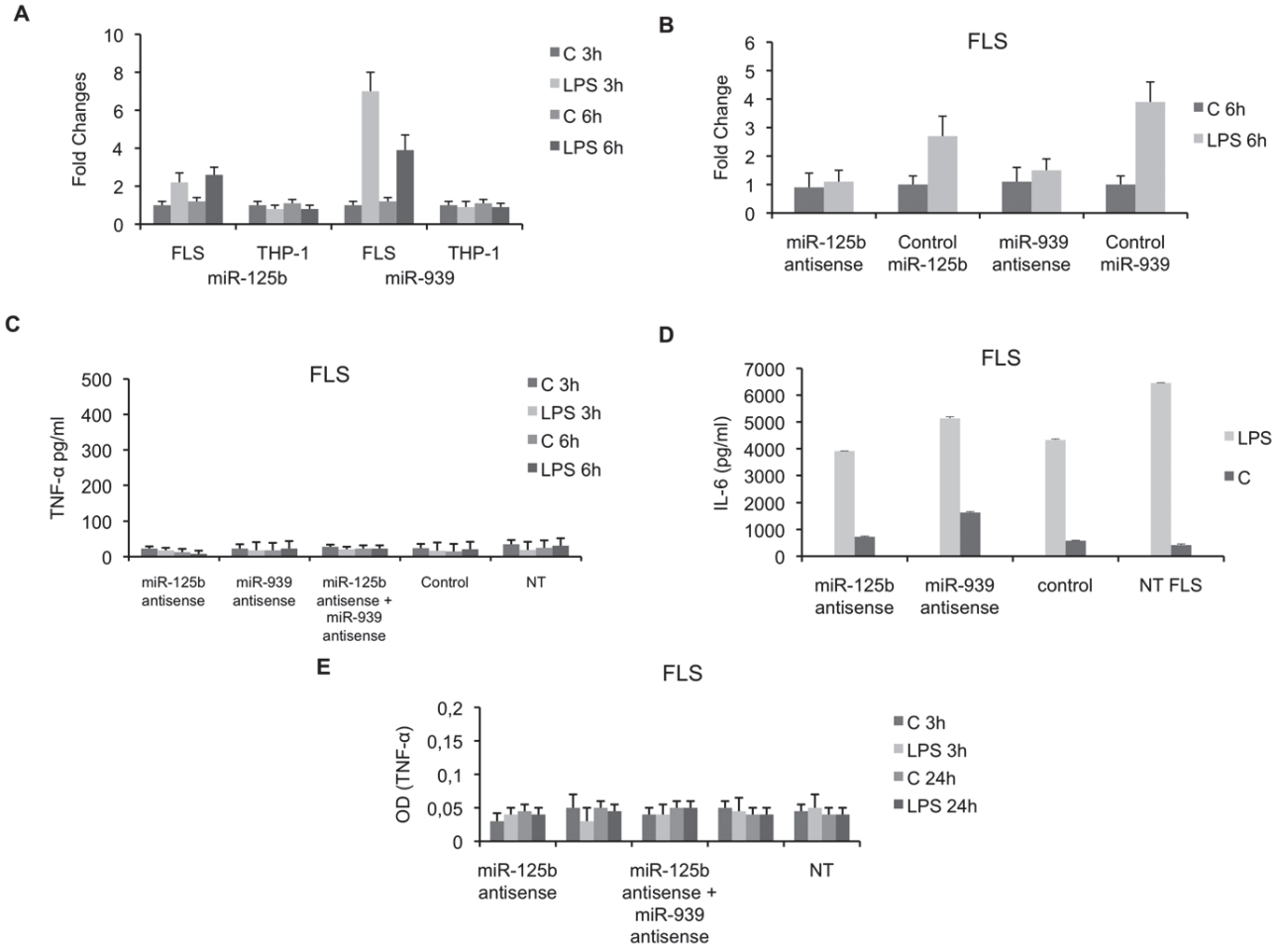

Figure 3. Effect of transfection of miRNA antisense molecules on TNF-αsynthesis by RA FLS. A, B. MiR-125b and miR-939 levels were determined by quantitative RT-PCR in RA FLS and THP-1 cells stimulated with LPS for 3 h and 6 h. RNAU6 was used as endogenous control for data normalization. The control (C) corresponded to untreated cells. RA FLS were transfected with either miR-125b or miR-939 antisense molecules or in combination or with the Clear-miR™ negative control (control). LPS or medium (C) activation of transfected cells was performed 24 h post-transfection, for 3 h and 6 h. Non transfected RA FLS were used as negative controls (NT). **C, D.** TNF-α and IL-6 release were determined by ELISA in culture supernatants harvested 6 h after stimulation with LPS or medium (C). **E.** Intracellular TNF-α expression was determined in transfected FLS and activated with LPS for 24 h. Data are expressed as the mean of triplicate samples +/− SD of three independent experiments for each patient.
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monitored the fate of the TNF-α mRNA in RA FLS transfected with miR-346 antisense oligonucleotides or control antisense oligonucleotides and treated first with LPS for 3 h and then with actinomycin D for another 1, 2, 3 and 4 h. As opposed to the control, we observed a stabilization of TNF-α mRNA after actinomycin D treatment in LPS-activated FLS transfected with miR-346 antisense molecules (Figure 5A). These results indicate that miR-346 plays a role in the control of the stability of TNF-α mRNA. Moreover, we found, using intracellular Elisa, that transfection of FLS with antisense oligonucleotides targeting miR-346, led to the accumulation of intracellular TNF-α after activation with LPS for 6 h. No effect was observed in cells that were not transfected or transfected with a control oligonucleotide (Figure 5B). This result was confirmed by western blotting analysis using anti-TNF-α antibodies (Figure 5C). Nevertheless, treatment with LPS surprisingly did not restore TNF-α release (Figure 5D) or TNF-α membrane expression (data not shown) in activated RA FLS transfected with antisense oligonucleotides targeting miR-346.

miR-346 and Btk regulate TNF-α release in LPS-activated THP-1 cells

We next tested whether miR-346, which inhibits Btk expression in THP-1 cells [25], could impair TNF-α release from THP-1 cells and act as an anti-inflammatory agent in these cells. We thus examined TNF-α release by THP-1 cells stimulated with LPS and pretreated with Leflunomide metabolite analog (LFM-A13), a known inhibitor of Btk. In these conditions, LPS-induced TNF-α production was strongly inhibited when compared with cells not treated with the inhibitor (Figure 6A). These results demonstrate that TNF-α release is Btk-dependent in THP-1 cells.

We next tested whether the transient expression of miR-346 affected TNF-α release in LPS-activated THP-1 cells. We transfected THP-1 cells with miR-346 mimic and, 24 h later, measured TNF-α release after 3 and 6 h of LPS stimulation. As illustrated in Figure 6B, cells transfected with miR-346 mimic showed a significant decrease of TNF-α release compared to cells transfected with the control miRNA mimic. Altogether, these

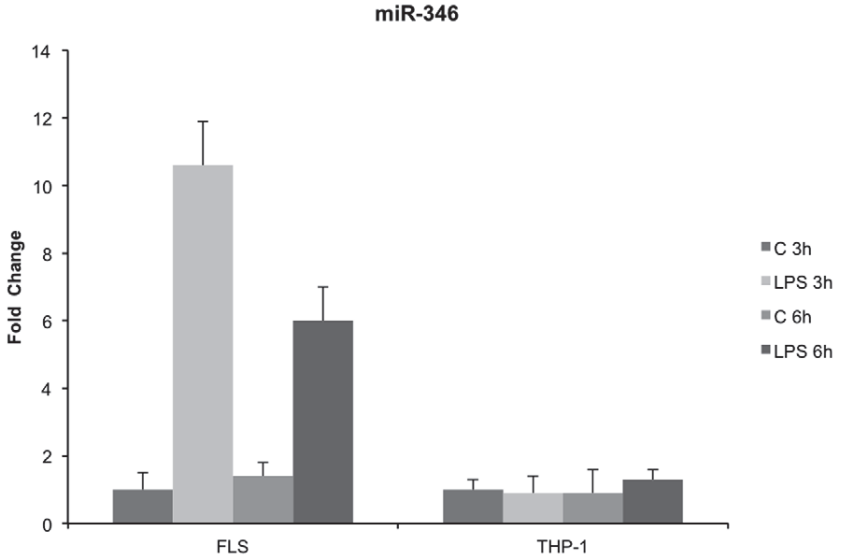

Figure 4. Quantitative RT-PCR analysis of miR-346 expression in LPS-activated RA FLS and THP-1 cells. MiR-346 level was determined by quantitative RT-PCR in RA FLS and THP-1 cells stimulated with LPS (1 μg/ml) for 3 h and 6 h. U6 small nuclear RNA (RNAU6) was used as endogenous control for data normalization. The control (C) corresponded to untreated cells. Data are expressed as the mean of triplicate samples +/− SD of three independent experiments for each patient.
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results confirm that miR-346 participates in the control of TNF-α release in both FLS and THP-1 cells.

Btk regulates TNF-α synthesis and release in LPS-activated FLS and THP-1 cells by controlling TTP expression

Given the prominent role played by TTP in mRNA decay, we looked into the involvement of Btk on the control of TTP expression. To that end, we transfected THP-1 cells with miR-346 mimic for 24 h and measured TTP mRNA expression after stimulation with LPS for 2 and 4 h. As illustrated in Figure 7A, cells transfected with miR-346 mimic showed a significant increase in TTP expression compared to cells transfected with the control miRNA mimic. We also examined TTP mRNA expression by THP-1 cells pretreated with LFM-A13 before LPS stimulation. In this condition, TTP production by LPS-treated cells was also strongly upregulated compared with non-treated cells (Figure 7B).

We next tested whether inhibition of miR-346 affected TTP expression in LPS-activated RA FLS. We transfected miR-346 specific or control antisense oligonucleotides in RA FLS and treated the cells 48 h later with LPS for 2 and 4 h. We observed a decrease of TTP mRNA expression in the presence of miR-346 antisense oligonucleotides but not with control oligonucleotides (Figure 7C).

To further confirm that miR-346 regulated TNF-α synthesis by controlling TTP expression, we co-transfected THP-1 cells with miR-346 mimic and a siRNA targeting TTP for 48 h and measured TNF-α mRNA expression and cytokine release by transfected cells stimulated with LPS. As illustrated in Figure 7 (D, E), treatment of THP-1 cells with a siRNA targeting TTP stabilized TNF-α mRNA and restored TNF-α release by cells co-transfected with miR-346 mimic as compared to cells transfected with miR-346 mimic alone.

Taken together, these results demonstrate that miR-346 and Btk are implicated in the regulation of LPS-induced TTP expression, which in turn controls TNF-α mRNA stability and TNF-α release.

Discussion

As opposed to other cells from the synovial membrane, PRRs-activated FLS fail to release TNF-α. This study was aimed at identifying miRNAs that might be involved in the control of TNF-α release. Our results establish that miR-346 can act as a negative regulator of TNF-α release in RA FLS in response to LPS, by inhibiting transcription of the Btk gene. Moreover, since transfection of miR-346 in macrophages also inhibits TNF-α release induced by LPS, these data are a strong indication that miR-346 plays a global role in the control of the inflammatory response.

In an initial attempt to identify miRNAs involved in the control of TNF-α release, we performed a miRNAs microarray analysis of LPS-activated FLS. miR-125b and miR-939 were strongly induced after LPS treatment, and both were predicted to target TNF-α. This finding correlated well with results from Tili et al. [22], who showed that LPS activation of mouse Raw 264.7 macrophages down regulated miR-125b, which allowed TNF-α synthesis by activated macrophages. This result was also in agreement with the fact that overexpression of miR-125b leads to a significant inhibition of the ERK 1/2 pathway without affecting ERK 1/2 levels consistent with the idea that this miRNA acts upstream of ERK [26]. However, inhibition of both miRNAs with antisense nucleotides was not able to restore either TNF-α intracellular expression or release by LPS-activated FLS. Our results suggest that the inhibition of these miRNAs is not sufficient to override the negative regulatory effect induced by LPS signaling on TNF-α release in FLS. Another non-exclusive possibility would be that they may act in FLS in cooperation with other factors.

Previous studies from our laboratory [25], showed that LPS also induces the expression of miR-346 in LPS-activated RA FLS and that this miRNA regulates negatively IL-18 release by inhibiting Btk expression. Btk is involved in the stabilization of various cytokines mRNAs, including the TNF-α mRNA [27]. Moreover TLR2-mediated stimulation of XLA mononuclear cells resulted in impaired production of TNF-α and IL-1β while IL-6, IL-8 and IL-

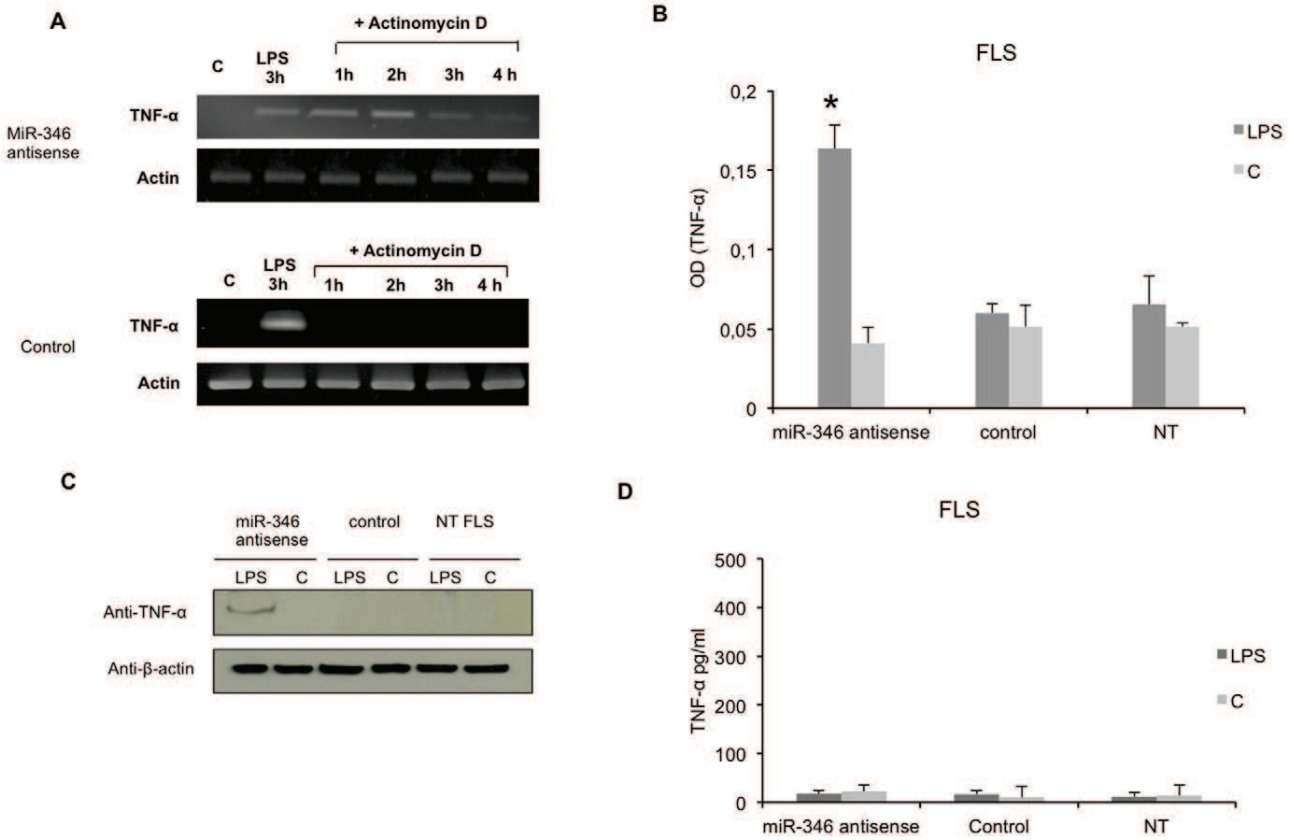

Figure 5. Effect of transfection of miR-346 antagomirs on TNF-α mRNA stability and release in RA FLS. A. FLS were transfected with miR-346 antisense molecules or with the Clear-miR^{TM} negative control (control), activated with LPS 24 h post-transfection for 3 h and incubated for another 1, 2, 3 and 4 h with actinomycin D. Control cells were incubated for 3 h with medium (C). NT: non transfected cells. **B, C**: TNF-α expression was detected using cellular ELISA or western blotting with anti-TNF-α antibodies, in FLS transfected with miR-346 antisense molecules or with the Clear-miR^{TM} negative control (control) or in non transfected FLS (NT). 24 h post-transfection, FLS were either incubated in medium (C) or activated with LPS for 6 h. The results are representative of three different experiments for each patient. **D.** TNF-α release was determined by ELISA in culture supernatants after stimulation with LPS or medium (C). Data are expressed as the mean of triplicate samples +/− SD of three independent experiments for each patient. $p<0.01$.
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10 remained unaffected through a pathway involving the p38 MAPK [28]. Similar results were observed with dendritic cells from the same patients; stimulation with TLR2, TLR4, TLR7/8 and TLR3 ligands results in lower release of TNF-α IL-6 and IL-12 production was unaffected [29]. On the contrary, in murine bone marrow-derived mast cells, Btk is dispensable in LPS- or lipopeptide-induced secretion of IL-6 and TNF-α [30]. FLS express the epithelial and endothelial tyrosine kinase (Etk), which is implicated in IL-6 and IL-8 release [31], but they fail to express either Btk mRNA or the mature protein constitutively or after LPS activation. Our results established that in FLS, inhibition of miR-346 expression resulted i) in stabilization of TNF-α mRNA and ii) intracellular expression of the mature protein. However this was not sufficient to override completely the inhibition of TNF-α release in cell supernatants. The fact that TNF-α is neither expressed at the cell surface nor secreted indicates that additional mechanisms exist to regulate TNF-α release from activated FLS. There are multiple transport pathways to the cell surface, which depend on the individual cargo. Recently, Lieu et al. showed that secretion of TNF-α in LPS-activated macrophages is controlled by a trans-Golgi network golgin, p230 which is mobilized in response to LPS [32]. It is possible that in RA FLS this mechanism is compromised and additional experiments are now required to resolve this possibility.

Further investigations revealed that LFMA-13, a Btk inhibitor, inhibited TNF-α secretion in THP-1 cells. This result was consistent with other reports, which demonstrated that LFMA-13 inhibited the activation of NF-κB by LPS in THP-1 cells [33]. Moreover, we also demonstrated that transfection of miR-346 mimic in THP-1 cells strongly diminished TNF-α release. As we previously showed that miR-346 inhibits Btk expression in THP-1 cells by an indirect mechanism probably involving yet undefined transcription factors, we propose that in THP-1 cells, the control of TNF-α release by miR-346 involves the inhibition of Btk expression. At present the details of the mechanism that mediate the effect of Btk on TNF-α mRNA stability are unclear. Arsivatham et al. indicated that the components of the ARE-mediated decay pathway are heavily targeted by miRNAs [34]. It appears that ARE control of TNF-α expression may operate at multiple post-transcriptional levels including the miRNAs regulation of ARE-binding proteins.

Among the many ARE-binding proteins that target TNF-α, TTP has emerged as a primary player as it was shown to promote its mRNA lability [11]. As a matter of fact, TTP knocked-out mice

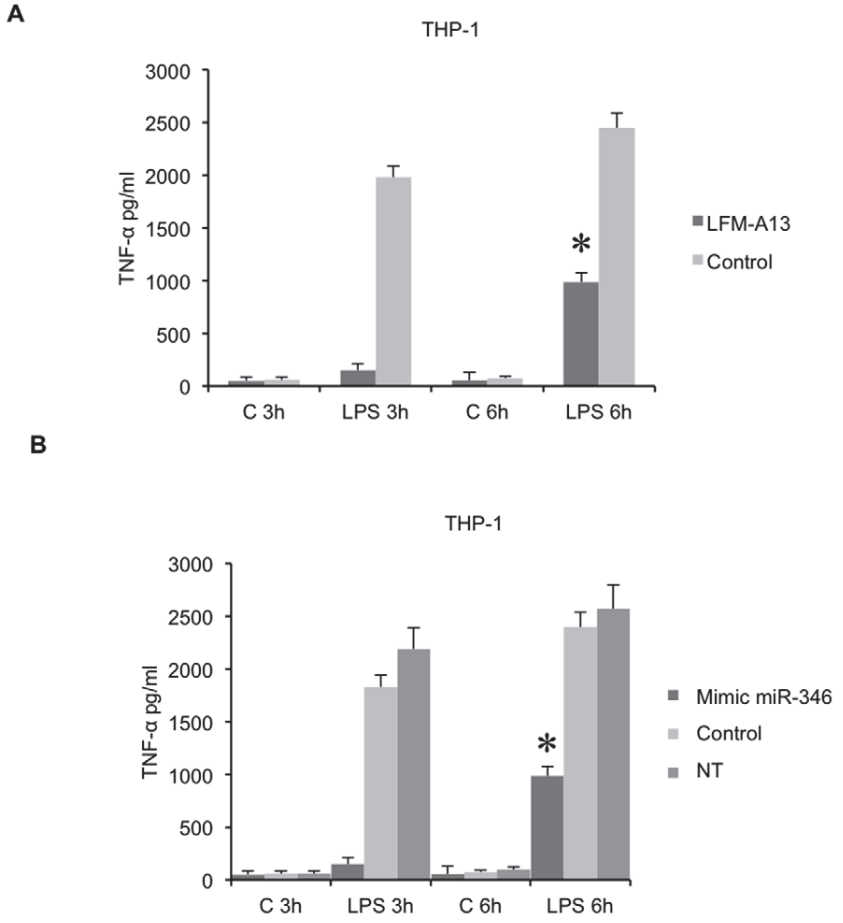

Figure 6. Effect of transfection of miR-346 mimic on TNF-α release by THP-1 cells. A. TNF-α release by THP-1 cells preincubated or not (control) with LFMA-13 (172 mM) for 1 h and then stimulated with LPS or medium (C) for 3 and 6 h, was evaluated by ELISA. **B.** THP-1 cells were transfected with miR-346 mimic or with the miRNA mimic negative control (control) and activated 24 h post-transfection with either LPS or medium (C) for 3 and 6 h. TNF-α release was evaluated by ELISA. Data are expressed as the mean of triplicate samples +/− SD of three independent experiments for each patient. $p<0.01$.
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show elevated levels of TNF-α production in response to LPS [12]. The question naturally arose whether Btk would be implicated in TTP expression. We showed that transfection of miR-346 antisense oligonucleotides decreased TTP mRNA expression in LPS-activated RA FLS. Furthermore, we also demonstrated that Btk inhibition by either LFM-A13 or miR-346 mimic transfection increased TTP mRNA accumulation in LPS-activated THP-1 cells. These results suggest that stabilization of TNF-α mRNA by Btk is mediated by down-regulation of TTP.

We therefore propose a model of regulation of TNF-α release in which LPS induces TNF-α mRNA, miR-346 and TTP expression in FLS. Inhibition of Btk expression by miR-346 would then enable stabilization of TTP expression and would therefore result in the rapid decay of TNF-α mRNA. Blocking Btk either chemically (with the inhibitor LFM-A13), or by overexpressing miR-346 (in THP-1 cells), would therefore result in the inhibition of TNF-α secretion (Figure 8A, B). Taken together, our data indicate an important role for miR-346 in the regulation of TNF-α release by controlling Btk which in turn regulates TTP expression.

In conclusion, our findings provide clear evidence that miR-346 which is induced by bacterial ligands can act as a negative regulator of inflammation in human.

Materials and Methods

Reagents

Cell culture media (RPMI 1640, M199 and DMEM), fetal calf serum (FCS), L-glutamine, penicillin, streptomycin, amphotericin B, TRIzol reagent were from Invitrogen (Cergy-Pontoise, France). LPS from *Salmonella abortus equi* and type XI collagenase, actinomycin D, LFMA-13 and anti-β-actin mouse IgG monoclonal antibodies were obtained from Sigma (Saint-Quentin-Fallavier, France). The Lightcycler Faststart DNA Master SYBR Green I was from Roche Applied Science (Penzberg, Germany). The miScript System was obtained from Qiagen (Courtabeuf, France). Clear-miR^{TM}miRNA inhibitors were from Eurogentec (Seraing, Belgium). MiRIDIAN® miR-346 mimic and miRIDIAN miRNA mimic negative control and siRNATTP were supplied by Dharmacon (Brebieres, France). Human Dermal Fibroblast Nucleofector^{TM} kit and Cell Line Nucleofector Kit V were from Amaxa (Cologne, Germany). The enzyme immunoassay kits for human TNF-á detection and for human IL-6 detection were from R&D (Lille, France). Anti-Btk mouse IgG monoclonal antibodies were from BD Transduction Laboratories (Le Pont de Claix, France) and anti-TNF-α mouse monoclonal antibodies were from

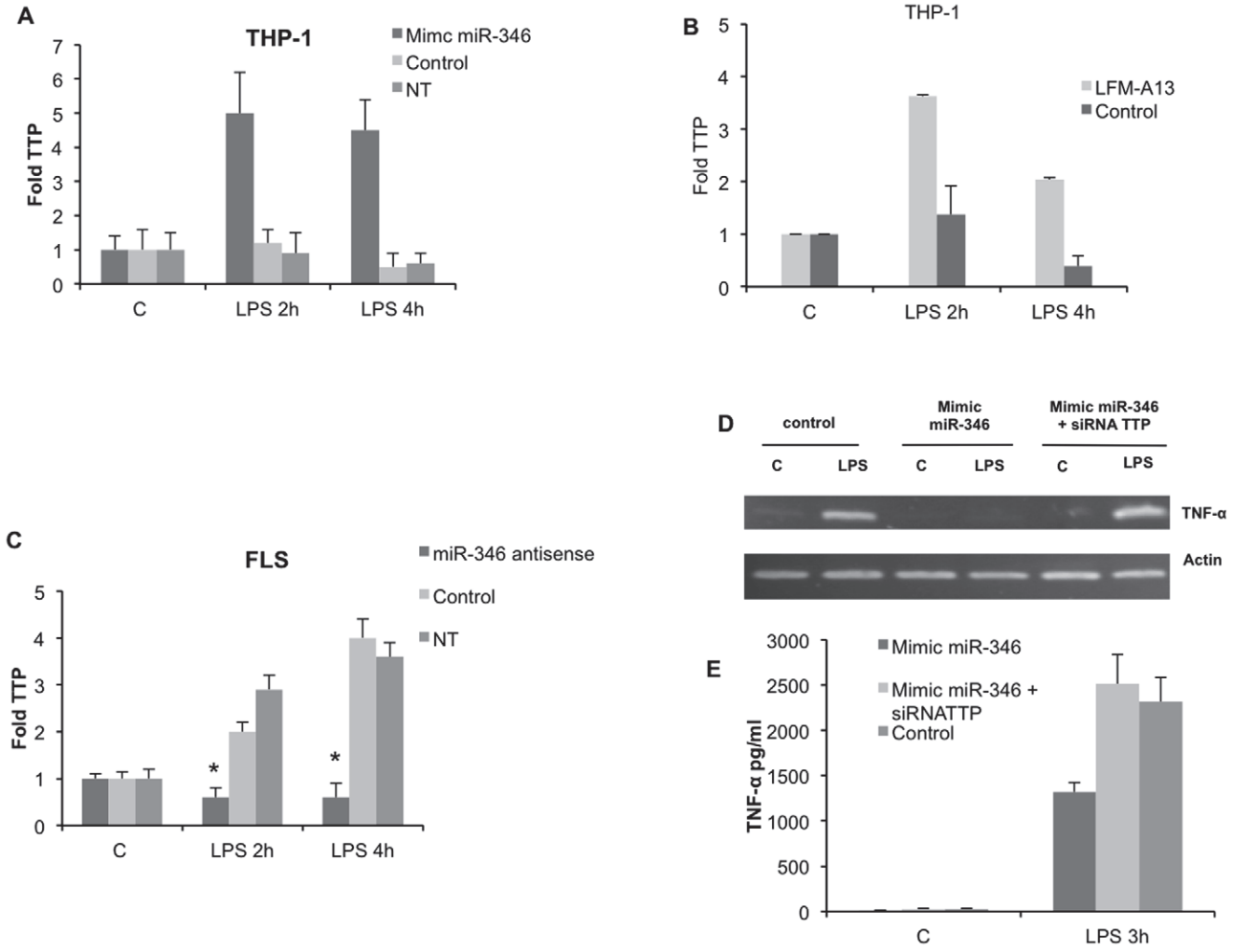

Figure 7. MiR-346 regulates the expression,of TTP in activated RA FLS and THP-1 cells. A, B: TTP mRNA levels were determined using quantitative RT-PCR in LPS-activated THP-1 cells transfected with miR-346 antisense molecules or preincubated for 1 h with LFMA-13. **C:** TTP mRNA levels were determined using quantitative RT-PCR in LPS-activated RA-FLS transfected with miR-346 antisense molecules Results were normalized to GAPDH and expressed as fold change compared with samples from cells incubated in medium (C). **D, E:** THP-1 cells were transfected with miR-346 mimic, with miR-346 mimic and siRNATTP or with a negative control (control) and activated 48 h post-transfection with either LPS or medium (C). TNF-α mRNA levels were determined using RT-PCR and TNF-α release was evaluated by ELISA. Data are expressed as the mean of triplicate samples +/− SD of three independent experiments for each patient.
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Santa Cruz Biotechnology (Heidelberg, Germany). Throughout this study, buffers were prepared with apyrogenic water obtained from Braun Medical (Boulogne, France). The microarray data were submitted to the Minimum Information About Microarray Experiment (MIAME) database with the accession number E-MEXP-1970.

Cell culture

Human FLS were isolated from synovial tissues from four different RA patients at the time of knee joint arthroscopic synovectomy as described previously after informed consent was obtained from patients [35]. The diagnosis was conformed to the revised criteria of the American College of Rheumatology [36]. Informed consent was provided according to the Declaration of Helsinki and obtained from all patients. Approval by the ethical committee of the Hopitaux Universitaires de Strasbourg was obtained. FLS cultures were done as previously described [37]. Experiments were performed between the 3rd and the 9th passage. During that time, cultures were constituted of a homogeneous population of fibroblastic cells, negative for CD16 as determined by FACS analysis. Cell number and cell viability were checked by the MTT test (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test) as described elsewhere [38]. THP-1 cells (no. 88081201, European collection of cell cultures, Salisbury, UK) were cultured as described previously [39].

Cell activation

FLS (5.10^5 cells) and THP-1 cells (10^7 cells) cells were stimulated with 2 ml of medium alone or medium containing LPS (1 μg/ml) for 3 h, 6 h and 24 h. After stimulation, supernatants were harvested and assayed for cytokine contents using commercially available ELISA tests for human IL-6 and TNF-α.

Stimulation of cells for total extraction

Total RNA was extracted from human FLS (10^6 cells) or THP-1 cells (10^7 cells) incubated for 2 h, 4 h and 6 h with 2 ml of

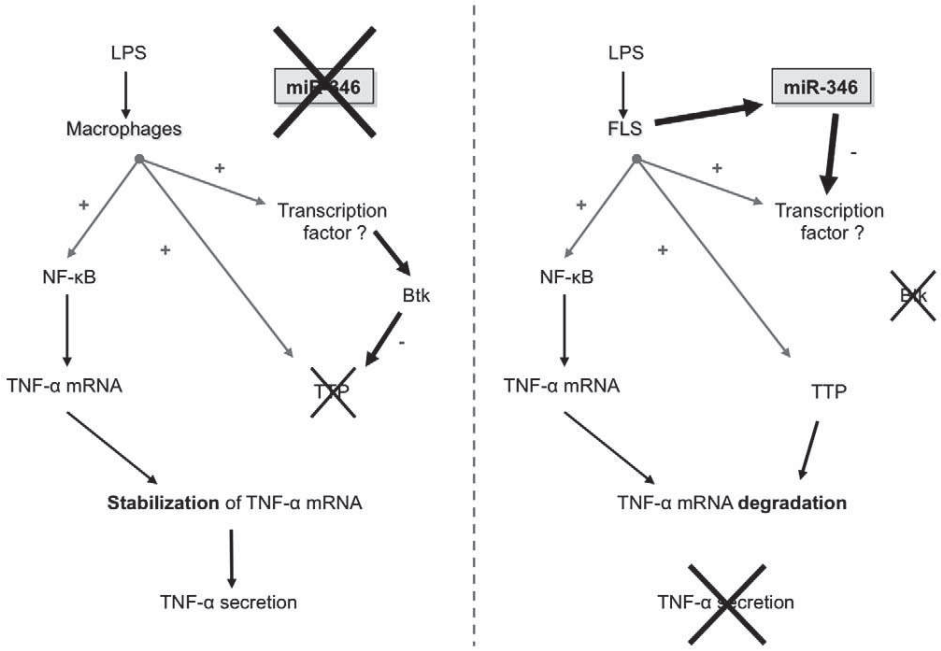

Figure 8. Model of action for miR-346 and Btk in the regulation of TNF-α secretion. See text for details.
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medium alone or medium containing LPS (1 μg ml) using TRIzol according to the manufacturer's instructions.

Total RNA isolated from FLS and THP-1 cells was reverse transcribed using the First-Strand cDNA Synthesis Kit according to the manufacturer's instructions (In Vitrogen) and amplified. For TNF-α, after an initial denaturing at 95°C for 5 min, the temperatures used were 95°C for 30 s, 57°C for 30 s and 72°C for 2 min followed by an extension of 5 min at 72°C. For β-actine, after an initial denaturing at 94°C for 5 min, the temperatures used were 94°C for 1 min, 56°C for 45 s and 72°C for 1 min followed by an extension of 5 min at 72°C. PCR products were separated in 2% agarose gels and visualized with ethidium bromide. The primers used were: (i) TNF-α: 5′- AGC-ACT-GAA-AGC-ATG-ATC-CGG-3′ and 5′- CAT-GGG-CTA-CAG-GCT-TGT-CAC-3′; and (ii) β-actin: 5′-CCA-ACC-GCG-AGA-AGA-TGA-CC-3′and 5′-GAT-CTT-CAT-GAG-GTA-GTC-AGT-3′.

Real-time quantitative RT-PCR

Quantitative RT-PCR analyses for miRNAs were performed using the miScript System and the primers (Qiagen). RNA concentrations were determined with a NanoDrop instrument (NanoDrop Technologies). Reverse transcriptase reactions and quantitative RT-PCR were performed according to the manufacturer's protocols. A U6 endogenous control was used for normalization. All reactions were run in triplicate in a Lightcycler Instrument (Roche Applied Science). Relative expression was calculated using the comparative threeshold cycle (Ct) method. All reactions were run in triplicate in a using *a Rotor-Gene™ 6000 real-time PCR machine (Corbett Life Science®, Sydney, Australia)*. Relative expression was calculated using the comparative threeshold cycle (Ct) method.

Total RNA isolated from FLS was reverse transcribed using the First Strand cDNA Synthesis Kit according to the manufacturer's instructions (In Vitrogen). Real-time quantitative RT-PCR was performed in a total volume of 20 μl using a SensiMix Plus SYBR® (Quantace, Corbett Life Science®, Sydney, Australia), and gene specific primers:

GAPDH: 5′-GGTGAAGGTCGGAGTCAACGGA-3′
and 5′-GAGGGATCTCGCTCGCTCCTGGAAGA-3′
TTP: 5′-TCGGGACCCTGGAGCCTGAG-3′.
and 5′ -AGCCAGCGGTGCGAAGCC-3′

TTP and GAPDH were reverse transcribed and amplified. Amplification products were detected as an increased fluorescent signal of SYBR®Green during the amplification cycles. Results were obtained using SDS Software (Perkin Elmer) and evaluated using Excel (Microsoft). Melting-curve analysis was performed to assess the specificity of PCR products.

Transfections

The Clear-miR anti-miR-125b, anti-miR-939 and anti-miR-346 used in our study were designed to inhibit efficiently the activity of miR-125b, miR-939 and miR-346. They consist of a sequence of 21 nucleotides complementary to the miRNA.

Transient transfection of FLS with Clear-miR™ miRNA inhibitors (200 nM) was performed using the Human Dermal Fibroblast Nucleofector™ kit from Amaxa as previously described [26]. Transient transfection of THP-1 cells with the miR-346 mimic (200 nM) or with miR-346 mimic and siRNATTP was performed using the Cell Line Nucleofector Kit V from Amaxa. FLS and THP-1 cells were then plated in 24-well plates (2.10^5 cells per well and 10^6 cells per well respectively). All assays were performed 24 h for miR-346 mimic and 48 h for siRNATTP, post-transfection. Controls were carried out with the Clear-miR™ negative control or with the mimic miRNA negative control. Transfection efficiency was evaluated with the PmaxGFP vector.

Cell numbers and cell viability were assessed using the MTT test. TNF-α release was measured in culture supernatants by a

heterologous two-site sandwich ELISA according to the manufacturer's instructions.

Western Blot

10^6 cells (FLS) were incubated for various times (3 h, 6 h and 24 h) with LPS (1 μg/ml). Controls were performed with cells maintained in medium with 5% heat inactivated FCS for 6 h. After stimulation, cells were centrifuged and the pellets were suspended 20 min on ice in 300 μL of ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl pH 8.0, 130 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). Lysates were centrifuged for 10 min at 14,000 *g* at 4°C and supernatants were subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked using 1% bovine serum albumin in TBS (20 mM Tris, pH 7.5, 150 mM NaCl) for 1 h at 25°C. The blots were incubated with mouse anti-TNF-α IgG monoclonal antibodies for 2 h at 25°C followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG monoclonal antibodies (1 h at 25°C) and detected by enhanced chemiluminescence (Super Signal West Femto Maximum Sensitivity Substrate, Pierce) according to the manufacturer's instructions. To confirm the presence of equal amounts of proteins, bound antibodies were removed from the membrane by incubation in 62.5 mM Tris, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS for 30 min at 50°C and reprobed again with anti β-actin (clone AC-74, Sigma) mouse monoclonal antibodies.

Detection of cellular TNF-α

Transfected FLS (2.10^4 cells) with miR-125b, miR-939 and miR-346 antisense oligonucleotides, were seeded into 96-well plates and then incubated 24 h post-transfection in 200 μl of complete medium containing LPS, (1 μg/ml) for 6 h. Cells were fixed with 4% paraformaldehyde in PBS pH 7.4 for 20 min. Free aldehyde groups were quenched with NH_4Cl 50 mM in PBS pH 7.4 for 20 min. Non specific binding was blocked by incubation in PBS containing either 0,2% bovine serum albumin or 0,2% bovine serum albumin and 0.05% saponin for 30 min at 37°C. The cells were then incubated with biotinylated anti-TNF-α antibodies for 2 h. Absorbance was measured at 450 nm.

Statistical analysis

Statistical analysis was performed using Student's t-test. Values were compared between different groups in the experiment. P values less than 0.05 were considered statistically significant.

Author Contributions

Conceived and designed the experiments: DW SP JS JEG. Performed the experiments: NS LF GA GS. Analyzed the data: GA DW SP. Contributed reagents/materials/analysis tools: JS JEG. Wrote the paper: DW SP.

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TLR2 Expression Is Regulated by MicroRNA miR-19 in Rheumatoid Fibroblast-like Synoviocytes

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TLR2 Expression Is Regulated by MicroRNA miR-19 in Rheumatoid Fibroblast-like Synoviocytes

Lucas Philippe,[*,†] Ghada Alsaleh,[*,†] Guillaume Suffert,[‡] Alain Meyer,[*,†] Philippe Georgel,[§] Jean Sibilia,[*,†] Dominique Wachsmann,[*,†] and Sébastien Pfeffer[‡]

Resident cells, such as fibroblast-like synoviocytes (FLS), play a crucial role in rheumatoid arthritis (RA). They are implicated in the inflammatory response and play a key role in osteoarticular destruction. Moreover, RA FLS spread RA to unaffected joints. Pathogen-associated molecular patterns and damage-associated molecular patterns have been found to activate RA FLS by interacting with pattern recognition receptors, such as TLR. RA FLS express a large number of TLR, and TLR2 was demonstrated to be involved in RA inflammation. Because microRNA have emerged as important controllers of TLR expression and signaling, the aim of this study was to evaluate their potential involvement in the control of TLR2 expression by RA FLS. We first showed that *Tlr2* expression is strongly upregulated in RA FLS in response to TLR2 ligands. Using a microRNA microarray analysis, we identified one miRNA in activated RA FLS, miR-19b, which was downregulated and predicted to target Tlr2 mRNA. Downregulation of miR-19b and miR-19a, which belongs to the same cluster, was confirmed by real-time quantitative PCR. Transfection of RA FLS with miR-19a/b mimics decreased TLR2 protein expression. In parallel, we found that both IL-6 and matrix metalloproteinase 3 secretion was significantly downregulated in activated FLS transfected with either mimic. Moreover, using a luciferase assay, we showed that miR-19a/b directly target Tlr2 mRNA. Taken together, our data point toward an important role for miR-19a/b in the regulation of IL-6 and matrix metalloproteinase 3 release by controlling TLR2 expression, as well as provide evidence that miR-19a/b can act as negative regulators of inflammation in humans. *The Journal of Immunology*, 2012, 188: 454–461.

Rheumatoid arthritis (RA) is a systemic inflammatory disease that predominantly affects multiple peripheral joints. It is characterized by synovial inflammation, as well as bone and cartilage destruction. The development of RA is thought to result from interactions between genetic and environmental factors. Molecules of microbial origin have been found in the joints of patients (1, 2), where they trigger inflammation by activating various pattern recognition receptors. In addition, these pattern recognition receptors can be activated by noninfectious molecular patterns or damage-associated molecular pattern molecules, which are generated upon tissue injury. These include cellular components released from necrotic cells (HMGB1, microparticles), extracellular molecules upregulated upon injury (Hsps, ATP, S100 proteins), or extracellular matrix fragments, such as tenascin (3).

The TLR family is one of the best-characterized groups of innate immune receptors in term of described ligands, signaling pathways, and functional relevance. TLRs are expressed both by immune cells and by resident cells of the joint, such as fibroblast-like synoviocytes (FLS), which play a crucial role in RA. FLS are implicated in the inflammatory response essentially by synthesizing cytokines, chemokines, prostanoids, NO, and proangiogenic factors (4–6). FLS also play a key role in osteoarticular destruction and take part in the differentiation and activation of osteoclasts by the RANK-RANK ligand pathway and through the release of PGE_2 and IL-6 (7). Moreover, synovial fibroblasts spread RA to unaffected joints (8, 9). RA FLS express a large number of TLRs, such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR7. In basal conditions, RA FLS express Tlr3 and Tlr4, as well as Tlr2 and Tlr6 mRNAs (10).

With the assistance of the scavenger receptor CD36, TLR2 is implicated in cytokine and matrix metalloproteinase (MMP) release in response to ligands from $Gram^+$ bacteria, such as glycolipids, lipopeptides, or GPI-anchored structures. Interestingly, TLR2 is the most significantly induced TLR in FLS treated with cytokines, such as TNF-α and IL-1β, or with TLR ligands, such as polyinosinic-polycytidylic acid, bacterial lipoprotein (BLP), and LPS (11). In addition, TLR2 levels are higher in macrophages isolated from RA synovium compared with macrophages isolated from control synovium (12). Further evidence for the involvement of TLR2 in RA was obtained in a mouse model of arthritis induced by streptococcal cell wall, in which animals deficient for *Tlr2* were shown to develop reduced symptoms (13). Recent data

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Abbreviations used in this article: BLP, bacterial lipoprotein; FLS, fibroblast-like synoviocyte; miRNA, microRNA; MMP, matrix metalloproteinase; RA, rheumatoid arthritis; RT-qPCR, real-time quantitative PCR; siRNA, small interfering RNA; UTR, untranslated region.

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suggested a potential role for TLR2 as a therapeutic target. Indeed, Rabeximod suppresses arthritis downstream of TLR2 stimulation by preventing the activation of inflammatory cells, most likely macrophages, in a time-dependent fashion (14). OPN301, a TLR2-specific mAb inhibits TLR2-mediated proinflammatory cytokine production (15). AP177, a molecule selected by the systematic evolution of ligands by exponential enrichment method, was demonstrated to antagonize TLR2 signaling by binding directly to TLR2 and to suppress IL-6 and IL-8 release by activated cells (16).

It appears that the level of TLR2 needs to be tightly controlled to avoid a scenario where its deregulated expression will amplify the deleterious effect of inflammation on cell integrity. The modulation of gene expression can take place at several levels, among which regulation by microRNAs (miRNA) has gained increased interest in the recent years. miRNA are an evolutionarily conserved class of endogenous small noncoding RNAs. They are produced from long precursor molecules by the consecutive action of the RNase III enzymes Drosha and Dicer, before being loaded on an Argonaute protein within the RNA-induced–silencing complex. The mature miRNA acts a guide for RNA-induced–silencing complex to mediate destabilization and/or translational repression of target mRNAs (17, 18). The regulation of miRNA expression is itself controlled at various levels, such as transcription, processing, or stability (19), and can be influenced by various stress factors, including inflammation (20). In addition, emerging data have identified an important contribution of miRNA to the development and control of the inflammatory response that position these small noncoding RNAs at the heart of feedback and feed-forward loops controlling the inflammation process in both immune and nonimmune cells. Thus, inflammatory stimuli in pulmonary cells induce expression of miR-146a, which, in turn, promotes translational silencing of IL-8 and RANTES (21). Overexpression of miR-9 by activation of NF-κB leads to a feedback control of NF-κB–dependent acute inflammatory response (22). miR-147, which is induced by multiple TLR stimulation, attenuates TLR-induced inflammatory response in macrophages (23).

These data indicated that miRNA can exert negative effects in inflammatory pathways and this prompted us to look for miRNA directly targeting TLR2 and controlling cytokine release in response to TLR2 stimulation. In this article, we show that stimulation of RA FLS with BLP and LPS induces a decrease in expression of miR-19a and -19b and that this decrease is associated with upregulation of TLR2. Furthermore, we demonstrate that TLR2 is a direct target of miR-19a and -19b and that the downregulation of miR-19b in BLP-activated RA FLS is associated with an increase in IL-6 and MMP3 release.

Materials and Methods

Reagents

Cell culture media (RPMI 1640, M199, and DMEM), FCS, L-glutamine, penicillin, streptomycin, amphotericin B, and TRIzol reagent were from Invitrogen (Cergy-Pontoise, France). LPS from *Salmonella abortus equi* was obtained from Sigma (Saint-Quentin-Fallavier, France). Synthetic bacterial lipopeptide Pam_3CSK_4 (BLP) was obtained from EMC Microcollections (Tübingen, Germany). The Lightcycler Faststart DNA Master SYBR Green and the miScript System, miRNA mimics, and AllStars negative control small interfering RNA (siRNA) were obtained from Qiagen (Courtabeuf, France). Human Dermal Fibroblast Nucleofector kit was from Amaxa (Cologne, Germany). The enzyme immunoassay kits for human IL-6 and MMP3 detection were from R&D (Lille, France). Throughout this study, buffers were prepared with apyrogenic water obtained from Braun Medical (Boulogne, France). Anti-TLR2 mouse IgG mAbs were from Imgenex, and anti-GAPDH mouse IgG mAbs were from Millipore (Molsheim, France).

Cell culture

Human FLS were isolated from synovial tissues from four RA patients at the time of knee joint arthroscopic synovectomy, as described previously, after informed consent was obtained (24). The diagnosis conformed to the revised criteria of the American College of Rheumatology (25). FLS cultures were done, as previously described (26). Experiments were performed between passages three and nine. During that time, cultures consisted of a homogeneous population of fibroblastic cells, negative for CD16 as determined by FACS analysis. HEK293 cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 40 U/ml penicillin, and 50 mg/ml streptomycin. Cell number and cell viability were checked by the MTT assay.

Stimulation of cells for total RNA extraction

FLS (10^6 cells) were stimulated with 2 ml medium alone or medium containing LPS (1 μg/ml) and BLP (1 μg/ml). After a 6- and 24-h incubation period, total RNA was extracted using TRIzol reagent, according to the manufacturer's instruction.

Luciferase reporter constructs

To generate luciferase-based reporter plasmids, psiCHECK-2 (Promega) was modified by inserting the Gateway cassette C.1 (Invitrogen) at the 3′ end of the firefly luciferase gene (*f-luc*) into the Xba I site of psiCHECK-2. The 3′ untranslated region (UTR) sequence of Tlr2 was amplified from HEK293 cell genomic DNA; after addition of attB1 and 2 sequences, the resulting PCR products were cloned into pDONR/Zeo and then recombined in the modified psiCHECK-2 vector using Gateway technology (Invitrogen). The following primer sequences were used (sense and antisense primers are indicated in this respective order, and the anchor sequence used for the nested PCR is underlined): TLR2: 5′-AAAAAGCAGGCTTCCCATATTTAAGACCAGTCTTTGT-3′ and 5′-AGAAAGCTGGGTTGTAAAGTTTAATAGGAAATACACAGC-3′ and attB1/2: 5′-ACAAGTTTGTACAAAAAAGCAGGCT-3′ and 5′-ACCACTTTGTACAAGAAAGCTGGGT-3′.

The target seed sequence was modified using the QuickChange II Site-Directed Mutagenesis kit (Stratagene) and the following primers: 5′-GATCAAGTCCCTTATAAGAGTGGCATAGTAAAACGTAATAACCTGTGTACATTCT-3′ and 5′-AGAATGTACACAGGTTATTACGTTTTACTATGCCACTCTTATAAGGGACTTGATC-3′.

Real-time quantitative PCR

Total RNA isolated from FLS was reverse transcribed using the First-Strand cDNA Synthesis Kit, according to the manufacturer's instructions (Invitrogen). Real-time quantitative PCR (RT-qPCR) was performed in a total volume of 20 μl using SensiMix Plus SYBR kit (Quantace; Corbett Life Science) and gene-specific primers: *Tlr2*, 5′-GGCCAGCAAATTACCTGTGT-3′ and 5′-CTCCAGCTCCTGGACCATAA-3′; *Gapdh*, 5′-GGTGAAGGTCGGAGTCAACGGA-3′ and 5′-GAGGGATCTCGCTCCTGGAAGA-3′; *pri-miRNA 17– 92*, 5′-TGGTTTATAGTTGTTAGAGTTTGAGG-3′ and 5′-TCAGTTTTACAAGGTGATGTTCTCTT-3′; *Tlr4*, 5′-GGTGGGAATGCTTTTTCAGA-3′ and 5′-AATTGCCAGCCATTTTCAAG-3′; and *Il-6*, 5′-TCACTGGTCTTTTGGAGTTTGA-3′ and 5′-AGAGCCCTCAGGCTGGACT-3′.

After an initial denaturing at 96°C for 10 min, the temperatures used were 95°C for 10 s, 60°C for 15 s, and 72°C for 25 s for Gapdh and Tlr2 using a Rotor-Gene 6000 real-time PCR machine (Corbett Life Science). Amplification products were detected as an increased fluorescent signal of SYBR Green during the amplification cycles. Results were obtained using SDS Software (Perkin Elmer) and evaluated using Excel (Microsoft). Melting-curve analysis was performed to assess the specificity of PCR products.

RT-qPCR analyses for miRNA were performed using the miScript System and the primers (Qiagen). RNA concentrations were determined with a NanoDrop instrument (NanoDrop Technologies). Five hundred nanograms of RNA/sample was used for the assays. Reverse-transcriptase reactions and RT-qPCR were performed according to the manufacturer's protocols. Expression of endogenous U6snRNA was used for normalization. Relative expression was calculated using the comparative threshold cycle method.

Transfections and luciferase assay

Transient transfection of FLS with miR-19a and miR-19b mimics and Dicer siRNA (20 pM/sample) or with the AllStars negative-control siRNA was performed using the Human Dermal Fibroblast Nucleofector kit

from Amaxa, as previously described (27). FLS were then plated in 24-well plates (2×10^5 cells per well). All assays were performed at 48 h post-transfection. Transfection efficiency was evaluated with the PmaxGFP vector.

Transfection of HEK293 cells plated in 24-well plates (2×10^5 cells per well) with reporter constructs and miR-19b/a mimics (200 nM) was performed using Lipofectamine 2000 (Invitrogen). After 48 h, cells were washed and lysed with passive lysis buffer (Promega), and firefly luciferase (*f-luc*) and *Renilla* luciferase (*r-luc*) activities were determined using the dual-luciferase reporter assay system (Promega) and a luminometer (Glomax; Promega). The relative reporter activity was obtained by normalization to *r-luc* activity.

IL-6 and MMP3 release was measured in culture supernatants by a heterologous two-site sandwich ELISA, according to the manufacturer's instructions.

Western blot

A total of 10^6 cells (FLS) was incubated for 24 h with BLP (1 μg/ml). Controls were performed with cells maintained in medium with 5% heat-inactivated FCS for 24 h. After stimulation, cells were centrifuged, and the pellets were suspended for 20 min on ice in 300 ml ice-cold lysis buffer (1% Triton X-100, 20 mM Tris-HCl [pH 8], 130 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF, and protease inhibitors). Lysates were centrifuged for 10 min at 14,000 × *g* at 4°C, and supernatants were subjected to SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were blocked using 1% BSA in TBS (20 mM Tris [pH 7.5], 150 mM NaCl) for 1 h at 25°C. The blots were incubated with anti-TLR2 mouse IgG1 mAbs (IMG-319, clone 1030A5.138; Imgenex) for 2 h at 25°C, followed by incubation with HRP-conjugated goat anti-mouse IgG mAbs (1 h at 25°C) and detection by ECL (Amersham ECL Plus Western blotting Detection Reagents), according to the manufacturer's instructions. To confirm the presence of equal amounts of proteins, bound Abs were removed from the membrane by incubation in 0.2 M glycine [pH 2.8] 0.5 M NaCl for 10 min at room temperature and reprobed with anti-GAPDH mouse mAbs (clone 6C5; Millipore).

Statistical analysis was performed using the Student *t* test. Values were compared between different groups in the experiment. The p values < 0.05 were considered statistically significant.

Results

LPS and BLP treatments increase expression of TLR2 in RA FLS

We first showed that the expression of Tlr2 mRNA is significantly enhanced in RA FLS treated with LPS and BLP. RA FLS were stimulated for 6 h with LPS from *S. abortus equi* (1 μg/ml) or with BLP (1 μg/ml), and RT-qPCR was performed on RNA isolated from both control and activated cells. We observed a 17–20-fold increase in Tlr2 transcripts in response to LPS and BLP treatment (Fig. 1*A*). In accordance with previous observations from other investigators (11), Tlr4 mRNA expression was only slightly affected by LPS or BLP treatment (Fig. 1*B*). To determine whether increased Tlr2 mRNA expression correlated with enhanced TLR2 protein expression, we performed Western blotting experiments. As shown in Fig. 1*C*, stimulation of RA FLS with either LPS or BLP led to an increased expression of TLR2. As expected from the moderate modification of Tlr4 mRNA expression after LPS and BLP activation, TLR4 protein expression was not modified (data not shown).

Role of miRNA in the regulation of TLR2 expression in RA FLS

To investigate the importance of miRNA in the control of TLR2 expression in RA FLS, we first used a global approach by evaluating the role of Dicer. Dicer is a cytoplasmic RNase III type endonuclease, required for the generation of most miRNA (28). RA FLS were transfected with an siRNA targeting Dicer at a concentration of 100 nM. A plasmid encoding the GFP protein was used to evaluate transfection efficiency. As assessed by RT-qPCR, Dicer mRNA expression level was efficiently downregulated upon transfection of Dicer-specific siRNA (Fig. 2*A*). Transfection of Dicer siRNA, but not of a nontargeting control siRNA, upregulated Tlr2 mRNA expression in RA FLS (Fig. 2*B*). Interestingly, Dicer knockdown did not impact Tlr4 mRNA (Fig. 2*C*).

miR-19b is downregulated in LPS- and BLP-activated RA FLS

Next, we searched for miRNA exhibiting misregulated expression in activated versus nonactivated RA FLS. For this, we analyzed the miRNA-expression profile of LPS-activated RA FLS using a DNA microarray containing 409 nucleotide probes complementary to miRNA of human origin, which was performed previously in our laboratory. The microarray data were submitted to the Minimum Information About Microarray Experiment database under accession number E-MEXP-1970 (http://www.ebi.ac.uk/microarray-as/aer/login) (27). After LPS challenge, this initial screening identified 17 downregulated miRNA, which are listed in Table I. An online search of the miRNA target database microCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5) predicted several miRNA candidates involved in Tlr2 targeting. miR-19b (indicated in gray in Table I) was the only miRNA predicted to target Tlr2 and downregulated on the microarray analysis. Interestingly, miR-19b, together with miR-19a, which differs by only one nucleotide, was among the top 10 predictions of the algorithm.

We first performed an RT-qPCR analysis to confirm the downregulation of miR-19b observed with the microarray analysis. As

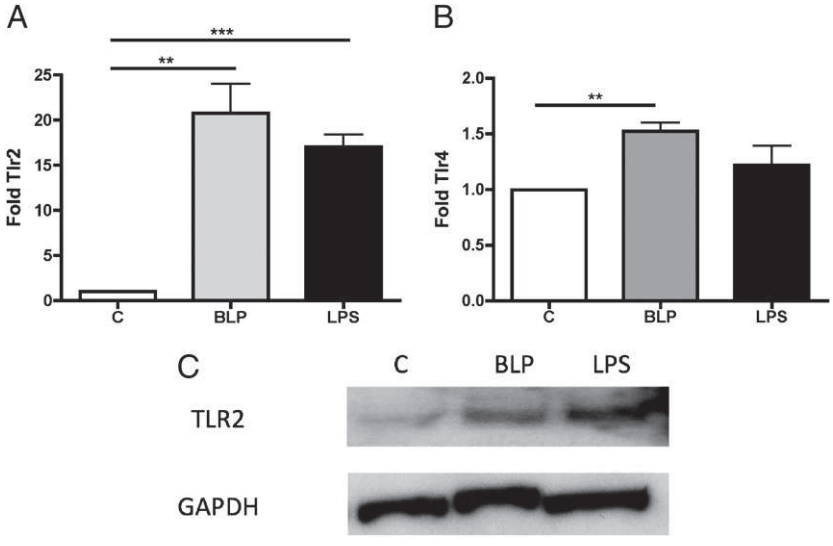

FIGURE 1. Effect of BLP and LPS on TLR2 and TLR4 expression by RA FLS. Tlr2 mRNA (*A*) and Tlr4 mRNA (*B*) expression was determined by quantitative RT-PCR in RA FLS stimulated with BLP (1 μg/ml), LPS (1 μg/ml), and medium (C) for 6 h. Results were normalized to Gapdh and expressed as fold change compared with samples from cells incubated in medium (C). *C*, TLR2 and TLR4 expression in RA FLS, 24 h after stimulation with BLP, LPS, and medium (C), was determined by Western blotting with anti-TLR2 Abs. For protein loading control, membranes were reprobed with anti-GAPDH Abs. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

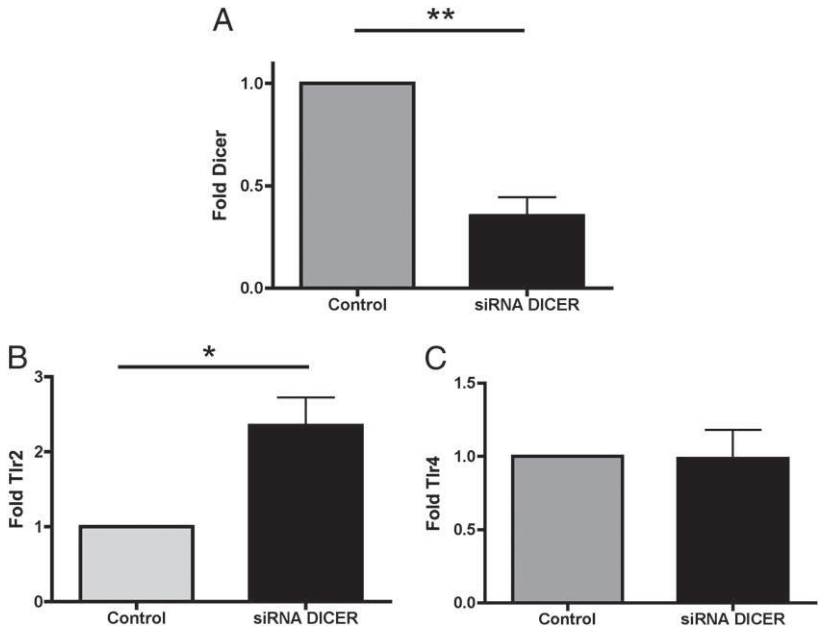

FIGURE 2. miRNA regulate Tlr2 expression in RA FLS. RA FLS were transfected with siRNA Dicer or siRNA control and then incubated 48 h post-transfection with medium for 6 h. *A*, Dicer mRNA levels were determined using RT-qPCR. Tlr2 (*B*) and Tlr4 (*C*) mRNA expression was determined by RT-qPCR in RA FLS transfected with siRNA Dicer or siRNA control. Results were normalized to Gapdh and expressed as fold change compared with samples from cells incubated in medium. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$.

shown in Fig. 3*A*, miR-19b was strongly downregulated in response to both LPS and BLP (Fig. 3*A*). Given the high sequence homology between miR-19a and miR-19b, it is difficult to estimate which of the two was detected by PCR. The primary transcript containing miR-19b derives from two genomic loci, which give rise to two miRNA clusters: mir-17–92 on chromosome 13 and mir-106a–363 on chromosome X (Fig. 3*B*). To confirm the specific downregulation of miR-19b, we measured the expression of its pre-miRNA, pre–miR-19b-1, and found that it was also significantly downregulated upon BLP or LPS treatment (Fig. 3*C*). To assess whether the regulation of miR-19b was occurring at a transcriptional level, we used RT-qPCR to measure the expression level of pri-miRNA 17–92 in RA FLS and found that it was downregulated in response to BLP and LPS (Fig. 3*D*). This result also indicated that both miR-19a and -19b must be downregulated after LPS and BLP treatment. We failed to detect the cluster 106a–363 or the pre–miR-19b-2 (data not shown), which indicated that only the cluster on chromosome 13 is expressed in FLS cells.

In oral keratinocytes stimulated with heat-inactivated *Porphyromonas gingivalis*, miR-105 inversely regulates Tlr2 expression; this was demonstrated to occur through binding to the 3′-UTR of Tlr2 mRNA, thereby inhibiting its translation (29). miR-105 was not expressed in unstimulated RA FLS, as assessed by RT-qPCR, and activation with LPS or BLP did not modulate its expression (data not shown).

Table I. Downregulated miRNA following LPS activation of RA FLS

Name	Mean
hsa-mir-494	0.679
hsa-mir-335	0.556
hsa-mir-633	0.467
hsa-mir-29b	0.645
hsa-let-7a	0.374
hsa-mir-99a	0.695
hsa-mir-22	0.684
hsa-mir-21	0.657
hsa-mir-299	0.719
hsa-mir-24	0.714
hsa-mir-19b	0.675
hsa-mir-203	0.609
hsa-mir-222	0.690
hsa-mir-656	0.517
hsa-let-7e	0.760
hsa-mir-620	0.549
hsa-mir-320	0.325

Mean expression was obtained from microarray data collected previously (38).

miR-19a and miR-19b modulate TLR2 expression in BLP-activated RA FLS

miRNA mediate posttranscriptional regulation via either mRNA destabilization or translation inhibition. To test whether miR-19b and miR-19a have an effect on the level of Tlr2 mRNA, we measured its expression by RT-qPCR in RA FLS transfected with miR-19b or miR-19a mimics. A plasmid encoding GFP was cotransfected to evaluate transfection efficiency. Forty-eight hours after transfection, cells were stimulated with BLP for 24 h. No significant difference in Tlr2 mRNA expression was found in cells transfected with the miR-19a or -19b mimics compared with cells transfected with the control mimics (Fig. 4*A*). The upregulation of Tlr2 mRNA after BLP treatment was not affected by the overexpression of miR-19a or -19b (Fig. 4*A*). We also measured the stability of Tlr2 mRNA by incubating BLP-treated cells for 2–4 h with 5 μg/ml actinomycin D. After 4 h of actinomycin D treatment, Tlr2 mRNA was still detectable in BLP-activated FLS treated with either control or miR-19b mimics (Fig. 4*B*).

We next tested whether expression of miR-19b and miR-19a affected TLR2 protein levels in RA FLS. We transfected control or miR-19b mimics in RA FLS, activated them with BLP or LPS, and measured TLR2 protein expression by Western blotting. As can be seen in Fig. 4*C*, we found that overexpression of miR-19b led to a global decrease in TLR2 protein in protein extracts from BLP- and LPS-activated cells (2- and 3-fold, respectively, as determined by densitometry analysis). These results indicated that miR-19a and miR-19b likely regulate the expression of TLR2 at the translational level.

miR-19a and miR-19b repress IL-6 and MMP3 production in BLP-activated RA FLS

To examine the consequence of Tlr2 regulation by miR-19a and -19b, we next tested whether their overexpression could affect Il-6 mRNA expression and IL-6 release in BLP-activated RA FLS. IL-6 is a cytokine with widespread activities, is one of the central

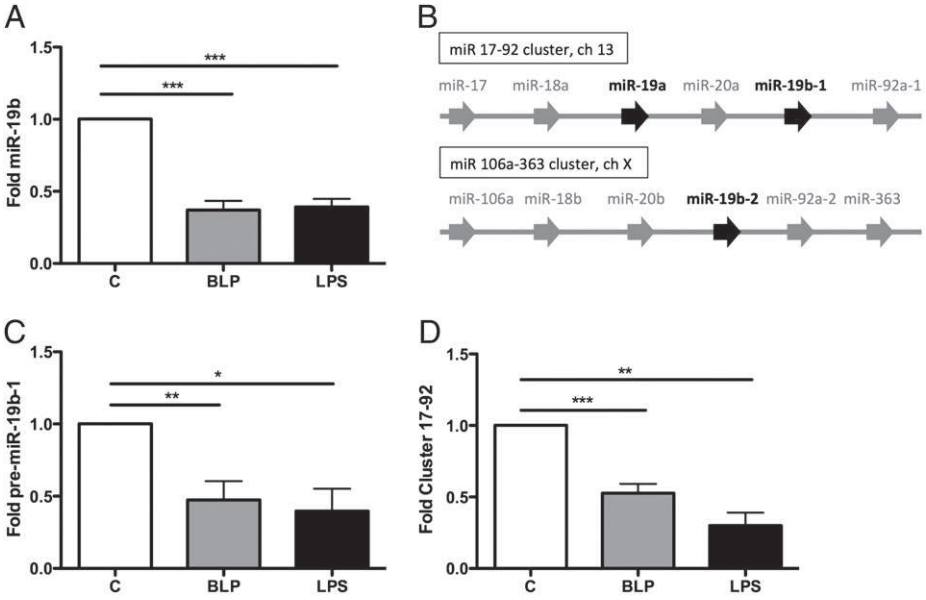

FIGURE 3. miR-19b is downregulated in LPS- and BLP-activated RA FLS. *A*, MiR-19b expression was determined by RT-qPCR in RA FLS stimulated with BLP (1 μg/ml) and LPS (1 μg/ml) for 6 h. U6snRNA was used as endogenous control for data normalization. The control (C) corresponded to untreated cells. *B*, Schematic representation of mir-17–92 cluster on chromosome 13 and mir-106a–363 cluster located on chromosome X. pre–miR-19b1 (*C*) and mir-17–92 cluster (*D*) expression was determined by RT-qPCR in RA FLS stimulated with BLP (1 μg/ml), LPS (1 μg/ml), or medium (C) for 6 h. Results were normalized to U6snRNA and expressed as fold change compared with samples from cells incubated in medium. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

mediators of inflammation, plays a major role in RA, and is an important therapeutic target (30). We measured Il-6 mRNA levels in RA FLS transfected with either miR-19a or miR-19b mimics for 48 h and then activated with BLP for 6 h. Compared with the control, we observed that transfection of miR-19a and miR-19b mimics impaired the induction of Il-6 mRNA expression in response to BLP (Fig. 5*A*). A similar experiment was performed with LPS instead of BLP; however, in this case, cells transfected with the miR-19a and -19b mimics showed no alteration in Il-6 mRNA expression in response to LPS (Fig. 5*B*). In parallel, we measured the secretion of IL-6 by ELISA and found that it was significantly downregulated in BLP-activated FLS transfected with miR-19a and -19b mimics (Fig. 5*C*) but was not modified in LPS-activated FLS (Fig. 5*D*). We also measured the secretion of MMP3, which is one of the major MMPs involved in invasion and cartilage destruction in RA. We found that, as for IL-6, MMP3 secretion was downregulated in BLP-activated FLS transfected with miR-19a and -19b mimics (Fig. 5*E*) but was not modified in LPS-activated FLS (Fig. 5*F*). Taken together, these data demonstrated that the Tlr2 regulation by miR-19a and -19b has an effect on IL-6 and MMP3 release by BLP-activated RA FLS.

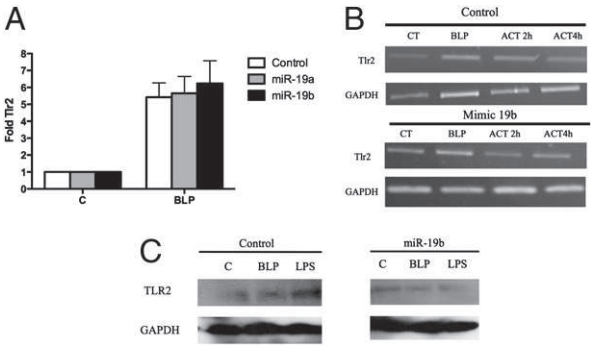

FIGURE 4. miR-19a and -19b transfection affects Tlr2 mRNA translation in RA FLS. *A*, FLS were transfected with miR-19a and -19b mimics or with a negative control (control). Forty-eight hours later, cells were activated with BLP (1 μg/ml) for 24 h. Control cells were incubated for 24 h with medium (C). Tlr2 mRNA expression was determined by RT-qPCR. Results were normalized to Gapdh and expressed as fold change compared with samples from cells incubated in medium. *B*, Tlr2 mRNA expression was determined by RT-qPCR in RA FLS transfected with miR-19b mimics or with a negative control. Transfected cells were activated with BLP 48 h posttransfection for 6 h and then incubated for another 2 and 4 h with actinomycin D. *C*, TLR2 expression was detected using Western blotting with anti-TLR2 Abs in FLS transfected with miR-19a and -19b mimics or with an AllStars negative control siRNA (control). Forty-eight hours posttransfection, FLS were either incubated in medium (C) or activated with BLP (1 μg/ml) or LPS (1 μg/ml) for 24 h. For protein-loading control, membranes were reprobed with anti-GAPDH Abs. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments.

miR-19a and miR-19b directly regulate TLR2 expression

To verify whether the predicted binding site for miR-19a or -19b within Tlr2 mRNA was functional (Fig. 6*A*), we generated luciferase reporter constructs (psiCHECK-2) that contain the firefly luciferase gene fused to the entire 3′-UTR of Tlr2 and the *Renilla* luciferase for normalization. We also generated luciferase reporter constructs in which we inserted a mutant version of Tlr2 3′-UTR to disrupt the predicted binding site for miR-19a or -19b. Before testing these constructs in HEK293 cells, we measured the expression levels of miR-19a and -19b in these cells. As can be seen in Fig. 6*B*, HEK293 cells express much higher levels of both miRNA than RA FLS. Therefore, we cotransfected the psiCHECK-2 constructs with control or miR-19a and -19b antisense 2′*O*-methylated oligoribonucleotides. In the presence of specific antisense oligonucleotides, we observed an upregulation of the Tlr2 3′-UTR–controlled luciferase sensor but not of the luciferase sensor fused to the mutated Tlr2 3′-UTR (Fig. 6*C*). Altogether, these data suggested that Tlr2 mRNA is a direct target for posttranscriptional regulation by miR-19a and miR-19b.

Discussion

We and other investigators reported that in RA FLS, TLR2 signaling was increased in response to LPS and BLP and that this could be involved in the inflammatory response in RA (31). Because the most obvious point to control this pathway is at the level of receptor expression, this study was aimed at identifying whether miRNAs might be involved in the control of Tlr2 expression. Our results established that stimulation of RA FLS with BLP and LPS decreases miR-19a and -19b expression, which correlates with upregulation of Tlr2. Furthermore, our results in-

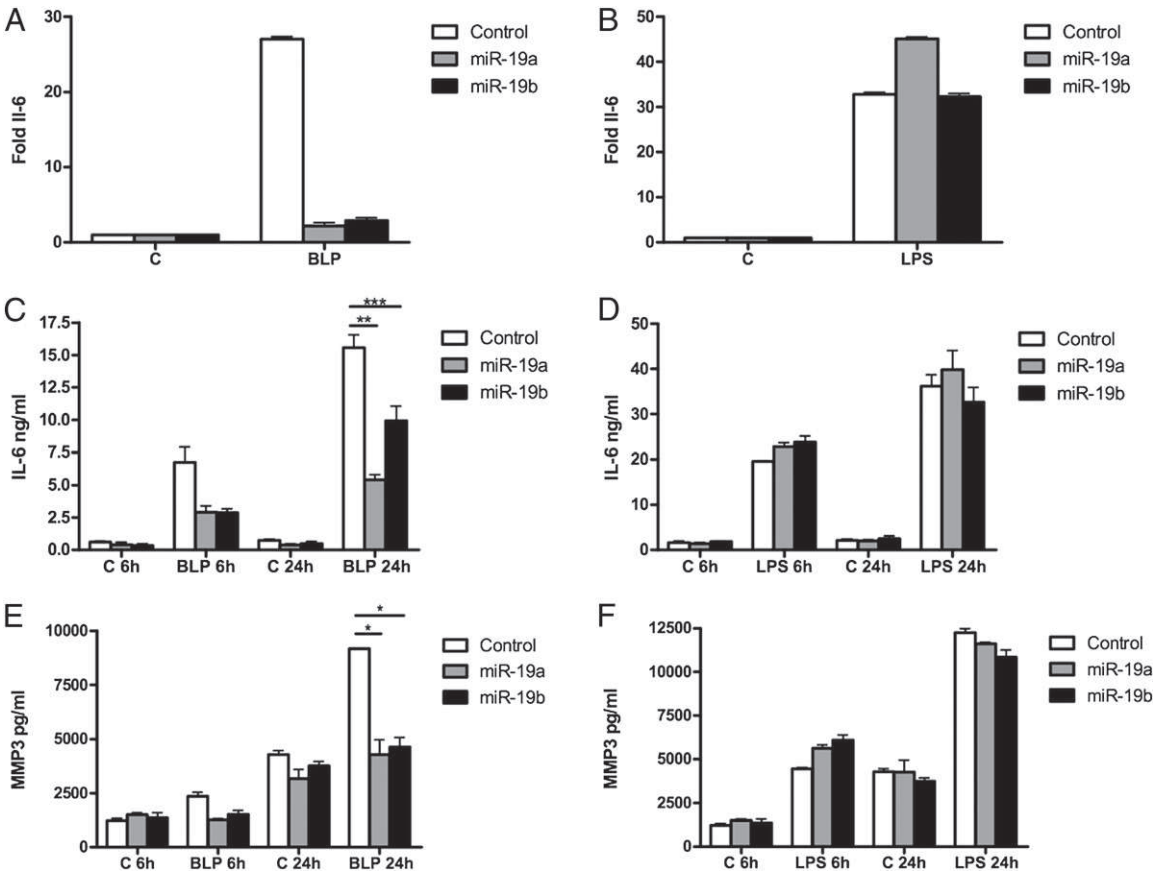

FIGURE 5. miR-19a/b repress IL-6 and MMP3 production in BLP-activated RA FLS. Il-6 mRNA expression was determined by RT-qPCR in RA FLS transfected with miR-19a and -19b mimics or with an AllStars negative control siRNA (control) and then activated (for 6 and 24 h) with BLP (1 μg/ml) (*A*) or LPS (1 μg/ml) (*B*) for 48 h posttransfection. Control cells were incubated with medium (C). Results were normalized to Gapdh and expressed as fold change compared with samples from cells incubated in medium (C). *C* and *D*, IL-6 release was determined by ELISA in culture supernatants after stimulation of RA FLS in the same conditions as in *A* and *B*. *E* and *F*, MMP3 release was determined by ELISA in culture supernatants after stimulation of RA FLS in the same conditions as in *A* and *B*. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

dicated that suppression or induction of miR-19a and -19b caused reciprocal alterations in IL-6 release induced by BLP-mediated TLR2 activation.

Many studies showed that TLR stimulation can modulate miRNA expression. Usually, miRNA are upregulated in response to TLR2 and TLR4 activation, and this is the case for miR-155,

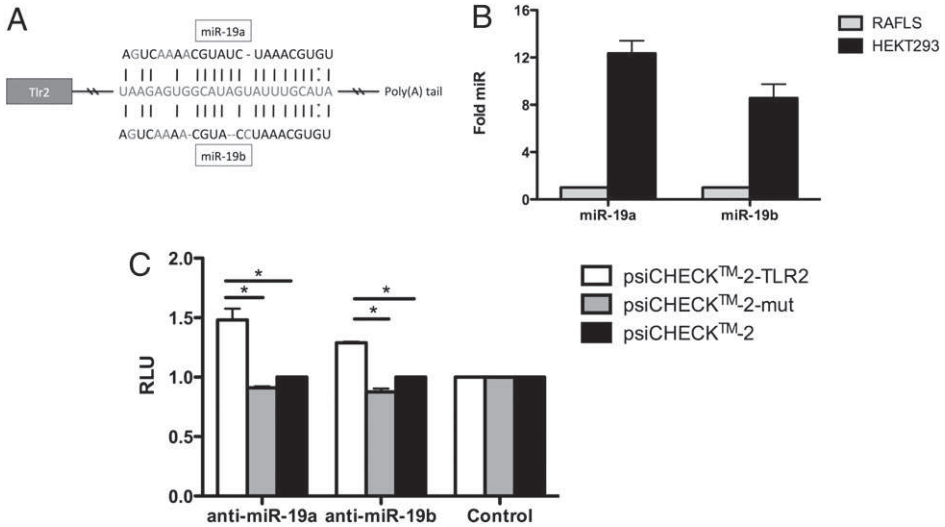

FIGURE 6. miR-19a and -19b directly regulate TLR2 expression. *A*, Sequence alignment of miR-19a and -19b and their target sites in the 3′-UTR of Tlr2 mRNA. *B*, miR-19a and -19b relative expression levels were determined by RT-qPCR in RA FLS and HEK293 cells. U6snRNA was used as endogenous control for data normalization. *C*, miR-19a and -19b directly target the 3′-UTR of Tlr2 mRNA. Luciferase reporter constructs with wild-type or mutated sequence (for miR-19a and -19b binding sites) were generated. HEK293 cells were transiently cotransfected with reporter constructs and either miR-19a or -19b antisense molecules. Luciferase activities were measured 48 h after transfection and normalized to luciferase expressed by the control psiCHECK-2 vector devoid of 3′-UTR sequences. Data are expressed as the mean of triplicate samples ± SD and are representative of three independent experiments. $*p < 0.05$.

miR-146, miR-147, and miR-9 (32). Moreover, we previously demonstrated that miR-346 is upregulated in RA FLS in response to LPS and that this upregulation participated in the control of Il-18 and Tnf-α mRNA stability and cytokine release (27, 33). Conversely, we found in this study that activation of TLR2 and TLR4 by their respective ligands led to a downregulation of miRNA, including miR-19a and miR-19b, by a currently unidentified mechanism. This regulation has a proinflammatory effect because it allows an increase in TLR2 expression. Such a decrease was also observed for other miRNA, such as let-7i, miR-125b, and miR-98, in response to TLR4 stimulation (32).

An important observation in this study is that the effect of miR-19a and -19b on TLR2 expression occurs at a translational level in RA FLS. Although transfection of miR-19a and -19b mimics had no significant impact on Tlr2 mRNA levels, it caused a decrease at the protein level, as shown by Western blotting. In addition, inhibition of miR-19a and -19b with antisense oligonucleotides significantly increased the activity of a luciferase reporter containing the 3′-UTR of Tlr2. A mutation in the binding sequence completely abrogated the regulation of the luciferase reporter by miR-19a and -19b. These results indicated that, in BLP-activated RA FLS, miR-19a and miR-19b play a role in the control of TLR2 expression by regulating mRNA translation.

There is only limited evidence that TLR are directly regulated by miRNA (32). Nevertheless, it was shown that Tlr4 mRNA is regulated by members of the let-7 miRNA family. let-7i was downregulated in cholangiocytes in response to *Cryptosporidium parvum*, which resulted in upregulation of TLR4 expression (34). The mRNA-encoding TLR4 can also be targeted by others isoforms of the let-7 family, such as let-7e (35).

Modulation of TLR2 expression in human gingival epithelial cells by miR-105 was reported recently (29). The investigators showed that regulation occurs via binding to the 3′-UTR of Tlr2 mRNA, leading to translational inhibition. They also found that miR-19b was differentially expressed but to a lesser extent than miR-105. In contrast to this study, we could not detect expression of miR-105 in nonactivated or activated RA FLS. Tlr2 3′-UTR has overlapping binding sites for miR-19 and miR-105 (miR-19: nt 335–358 and miR-105: nt 331–355), indicating that this binding site is accessible in vivo and plays a critical role in the post-transcriptional regulation of Tlr2 mRNA. Why is miR-105 not expressed in FLS? miR-105 is located on Xq28 in the intronic region of *Gabra3A* gene. Lee et al. (36) reported that miR-105 belongs to a group of miRNA that are detectable in their mature form only in certain cell types and tissues, whereas their precursors are expressed in all or most cell/tissues. This could explain the differential expression of miR-105 between epithelial cells and FLS.

miR-19 belongs to the cluster mir-17–92, which is located within the nonprotein-coding gene *C13orf25* at 13q31.3. Essential roles for this cluster in the development of heart, lungs, and B cells were recently established. Mouse embryos lacking this cluster are characterized by a deficiency of pre-B cells but not earlier B cell progenitors, whereas other hematopoietic cells are largely unaffected (37). In humans, the cluster mir-17–92 is amplified in several types of lymphoma, solid tumors, and neuroblastoma and plays a key role in the control of cell cycle and cell death (38). In particular, miR-17 and miR-20a target p21 and the proapoptotic factor Bim (39), and recent data from Mestdagh et al. (40) indicated that this cluster is a potent inhibitor of TGF-β signaling by acting on TGFBR2, SMAD2, and SMAD4.

In this study, we found that the expression of the mir-17–92 cluster was downregulated in BLP-activated RA FLS and that the expression of one of its paralogs, the mir-106a–363 cluster, is undetectable. Unlike the mir-17–92 cluster, this cluster is most often undetectable or expressed at trace levels in various cell types, which indicates that it might have more cell-specific functions. This also suggests that the mir-106a–363 cluster plays no role in regulating the expression of TLR2 in RA FLS. It is also worth noting that the mir-17–92 cluster contains multiple miRNA of the miR-19 family; according to in silico predictions, these miRNA should have a redundant activity. Indeed, both miR-19a and miR-19b acted on TLR2 protein expression, as observed in our experiments. This raises the possibility that the expression of Tlr2 mRNA may be subjected to a regulation by multiple miRNA of a same cluster, which might increase the efficiency of this regulation.

The role of the mir-17–92 cluster in inflammation is largely unknown. miR-19a, which is overexpressed in esophageal squamous cell carcinoma, was recently shown to directly target Tnf-α mRNA (41). Results obtained in our study demonstrated that miR-19a and miR-19b negatively regulate the synthesis of IL-6, because their overexpression leads to a strong reduction in IL-6 synthesis in BLP-activated RA FLS. These data indicated that these two miRNAs are anti-inflammatory and that their downregulation in response to TLR2 stimulation is crucial for the development of an inflammatory response. This effect is exclusively dependent of TLR2 regulation, because the transfection of miR-19a and -19b in FLS stimulated with LPS (which triggers TLR4 signaling) had no effect on IL-6 synthesis. IL-6 is a major cytokine implicated in RA and a current therapeutic target (30). This cytokine was recently shown to be directly targeted by two miRNA: let-7 and miR-365 (42–44). IL-6 can also be indirectly regulated, as demonstrated by Stanczyk et al. (45), who linked the expression levels of IL-6 to that of miR-203. Moreover, we also showed that miR-19a and miR-19b regulate the expression of MMP3 and that their downregulation will promote cartilage invasion and destruction. Our results demonstrated a new way of controlling the synthesis of IL-6 and MMP3 by modulating the expression of TLR2.

Taken together, our data point toward an important role for miR-19a and miR-19b in the regulation of IL-6 and MMP3 release by controlling TLR2 expression. Our findings provide clear evidence that miR-19a and miR-19b, which are induced by bacterial ligands, can act as negative regulators of inflammation in humans.

Disclosures

The authors have no financial conflicts of interest.

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Guillaume SUFFERT

Régulation de l'apoptose par les microARN du virus associé au sarcome de Kaposi

Le virus associé au sarcome de Kaposi (KSHV) code pour un cluster de 12 précurseurs de micro (mi)ARN abondamment exprimés pendant les phases lytiques et latentes de l'infection. Des études précédentes ont rapporté que KSHV est capable d'inhiber l'apoptose pendant l'infection latente ; nous avons donc testé si les miARN du virus étaient impliqués dans ce processus. Nous avons trouvé que des cellules HEK293 et DG-75 exprimant de manière stable les miARN de KSHV étaient protégées de l'apoptose. Les cibles cellulaires potentielles qui étaient significativement négativement régulées lors de l'expression des miARNs de KSHV ont été identifiées par analyse transcriptomique par microarray. Parmi celles-ci, nous avons validé par tests rapporteurs luciférase, PCR quantitative, et western blot, Caspase 3 (CASP3), un facteur jouant un rôle critique dans le contrôle de l'apoptose. Via le biais de mutagenèse dirigée, nous avons montré que trois miARN de KSHV, miR-12-1, 3 et 4-3p, étaient responsables du ciblage de CASP3. L'inhibition spécifique de ces miARN dans des cellules infectées par KSHV a résulté en une augmentation des niveaux d'expression de CASP3 endogène, et en une apoptose plus accrue. Vus dans leur ensemble, nos résultats suggèrent que les miARN de KSHV participent directement à l'inhibition précédemment rapportée de l'apoptose par le virus, et donc qu'ils jouent probablement un rôle dans l'oncogenèse induite par KSHV.

Mots-clefs : KSHV, miARN, cibles cellulaires, CASP3, apoptose, oncogenèse.

Kaposi's sarcoma herpesvirus (KSHV) encodes a cluster of twelve micro (mi)RNA precursors, which are abundantly expressed during both latent and lytic infection. Previous studies reported that KSHV is able to inhibit apoptosis; we thus tested the involvement of viral miRNAs in this process. We found that both HEK293 epithelial cells and DG-75 cells stably expressing KSHV miRNAs were protected from apoptosis. Potential cellular targets that were significantly down-regulated upon KSHV miRNAs expression were identified by microarray profiling. Among them, we validated by luciferase reporter assays, quantitative PCR and western blotting Caspase 3 (CASP3), a critical factor for the control of apoptosis. Using site-directed mutagenesis, we found that three KSHV miRNAs, miR-K12-1, 3 and 4-3p, were responsible for the targeting of CASP3. Specific inhibition of these miRNAs in KSHV-infected cells resulted in increased expression levels of endogenous CASP3 and enhanced apoptosis. Altogether, our results suggest that KSHV miRNAs directly participate to the previously reported inhibition of apoptosis by the virus, and are thus likely to play a role in KSHV-induced oncogenesis.

Keywords : KSHV, miRNA, cellular targets, CASP3, apoptosis, oncogenesis.