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EMHEMMED Fathi

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Evaluation des effets anticarcinogéniques de la flavagline synthétique FL3 sur les cellules souches cancéreuses; caractérisation des mécanismes moléculaires mis en jeu

Rapporteurs externes

Prof. **Jean-Yves JOUZEAU** Biopôle de l'Université de Lorraine (Nancy-France)

Dr. **Céline HUSELSTEIN** Biopôle de l'Université de Lorraine (Nancy-France)

Rapporteur interne

Prof. **Jean-Marie REIMUND** INSERM UMR_S1113, Université de Strasbourg

Directeur de thèse

Dr. **Christian MULLER** UMR 7200 CNRS, Université de Strasbourg

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Co-Encadrant de thèse / Membre invité

Dr. **Guy FUHRMANN** UMR 7213 CNRS, Université de Strasbourg

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Abbreviations

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APC: adenomatous polyposis coli

AML: acute myeloid leukemia

AXIN: Axis inhibition protein

ATP: ATP-binding cassette transporters

Apaf-1: Apoptotic Protease-Activating Fctor-1

AP-1: Activator Protein-1

APO-1/Fas: Apoptosis Antigen1/FAS

ATRA: All-trans retinoic acid

AIF: Apoptosis Inducing Factor

AhR: aryl hydrocarbon receptor

Akt: kinase protein kinase B

B

Bmi-1: Polycomb complex protein

BAK1: BCL2-Antagonist/Killer 1

Bad: Bcl-2-associated death promoter

Bax: Bcl-2–associated X protein

Bid: BH3 interacting-domain death

Bcl-xL: B-cell lymphoma-extra large

C

CSCs: Cancer stem cells

CK1α: casein-kinase 1α

Cdk1: cycline-dependent kinase1

Cdx2: Caudal type homeobox

ChIP- on chip: Chromatin immunoprecipitation with DNA microarray

CSL: CBF1, Suppressor of Hairless, Lag-1

CaMKIIγ: Ca2+/calmodulin-dependent protein kinase IIγ

D

Dnmt1: DNA methyltransferase 1

DDR: DNA damage response

Dsh: disheveled

Dhh: Desert Hedgehog

E

eIF4: eukaryotic initiation translation factor-4

ECCs: Embryonal carcinoma cells

ERK: Extracellular-Signal-Regulated Kinases

EMT: Epithelial to mesenchymal transition

ESCs: embryonic stem cells

F

FOXD3: forkhead box 3

FADD: Fas-associated death domain

FLIP: FLICE-like inhibitory protein

G

GLI: Glioma-associated oncogene homolog

GSK3β: Glycogen synthase kinase

GADD: Growth arrest and DNA damage

GPCRs: G protein-coupled receptors

GCNF: Germ Cell Nuclear Factor

H

HDACs: Histone deacetylases)

HIF-1α: Hypoxia inducible factor-1 alpha

HIF-2α: Hypoxia inducible factor-2 alpha

HSP27: Heat shock protein- 27

hECCs: Human embryonal carcinoma cells

I

Ihh: Indian Hedgehog

iPSCs : induced pluripotent stem cells

ITE: 2-(1′ H-indole-3′ -carbonyl)-thiazole- 4-carboxylic acid methyl ester

J

JAK: Janus Kinase

JNK: c-Jun N-terminal Kinases

K

KIF17: Kinesin Family Member 17

L

Lef: lymphoid enhancer-binding factor

LRH-1: Liver Receptor Homolog-1

LRP: low-density lipoprotein receptor- related protein

M

MAML: co-factor Mastermind-like

mTOR: mammalian target of rapamycin

mRNA: Messenger ribonucleic acid

miRNAs: microRNAs

Mcl-1: myeloid cell leukemia 1

Myc :v-Myc myelocytomatosis viral

N

NICD: Notch Intercellular Domain

NF-kB: Nuclear factor –Kappa B

NADPH: Nicotinamide adenine dinucleotide phosphate

O

Oct4: Octamer-binding transcription factor

POU: Pituitary-specific, Octamer and neural Unc-86 transcription factors

Oct-4pg1: Octamer-binding transcription factor Pseudogene, 1

P

RTKs: receptor tyrosine kinases

PHBs: prohibitins

PTCH 1: patched protein 1

RT-PCR: Reverse transcription polymerase chain reaction

PI3: phosphatidylinositol-3

PARP: ADP-ribose polymerase

p38 MAPK: p38 mitogen-activated Protein kinase

R

RARs: the retinoic acid receptor

S

SCs: stem cells

Shh: Sonic Hedgehog

SMO: smoothened

SUFU: Suppressor of Fused

SAHA: Suberoylanilide hydroxamic acid

SUMO-1: Small Ubiquitin-related Modifier, 1

SF-1: Steroidogenic Factor 1

STAT3: Signal Transducer and Activator of Transcription

Sox2: SRY (SEX determining Region Y) –box2

SSEA-3: Stage-Specific Embryonic Antigen 3

Smac /DIABLO: Second Mitochondria- derived Activation of Caspase /direct IAP binding protein with low pI

T

Tcf: T-cell-specific transcription factor

TNF: Tumor necrosis factor

TRAIL: TNF-Related Apoptosis-Inducing Ligand

TNFRI: TNF receptor 1

V

VEGF: Vascular Epithelial Growth Factor

W

Wwp2: ww domain-containing protein 2

Thesis abstract in english

The aim of my thesis is based on the evaluation of the anticancer properties of flavaglines on cancer-initiating cells also known as cancer stem cells (CSCs).

Flavaglines are a family of natural cyclopenta [b] benzofurans compounds extracted from the plant genus *Aglaia.* The anticancer activity of lead bioactive compound, rocaglamide has been well characterized. More than 100 flavaglines are now identified, including rocaglaol and silvestrol. The team of Dr Laurent DESAUBRY had recently synthetized the flavagline derivative FL3 with enhanced cytotoxicity against several cancer cell lines, Our purpose was then to examine the anticancer activity of this FL3 on CSCs and to identify the underlying mechanisms involved.

Accordingly, CSCs have been defined by different molecular markers, which allow to isolate them from various tissues or organs. These cells are highly suspected to be responsible for tumor recurrence and resistance to both chemo- and radio-therapy. We are focusing our study on highly aggressive and poorly differentiated CSCs, which are known to express the stemness marker Oct4. This transcription factor is the key regulator of the self-renewal of a highly pluripotent state of normal stem cells (NSCs). Recent studies demonstrated that Oct4 is expressed in many cancers and contributes to tumor growth and inhibition of apoptosis. Downregulation or suppression of Oct4 expression, following administration of specific pharmacological agents, then assumed that the cells undergo apoptosis or differentiation. Such differentiation therapy represents a novel attractive tool for the reduction of CSC aggressiveness.

The first study aimed to examine whether there is a selective cytotoxic effect of FL3 on CSCs.The work was conducted on several cancer stem-like cell models, namely the human pluripotent embryonal carcinoma stem cell line NT2/D1 and NCCIT or the normal fibroblastic cell line BJ which have limited lineage competences. We found that FL3 selectively inhibited cell proliferation and cell viability of NT2/D1and NCCIT cells as measured by MTS or by trypan blue exclusion assays respectively. Cell growth inhibition was accompanied by cell cycle arrest at G1 phase, as measured by flow cytometry, and this arrest was associated with a downregulation of G1-phase marker cyclin D1, as detected by western

blot. Furthermore, we showed that the cytotoxic activity of FL3 involved a proapoptotic process, as shown by both TUNEL and annexin V-FITC/propidium iodide staining assays. By using immunoblot analysis, we found that FL3 triggered apoptosis in Oct4-expressing CSCs via a pathway dependent on the activation of p38 MAPK and caspase 3, followed by a significant decrease in the expression of the stemness regulator. Indeed use of pan caspase inhibitor (z-VAD-fmk), as well as inhibitor of p38MAPK activity by (SB203580) or expression by (siRNA) reduced FL3-induced cell death,this effect was accompanied by a significant accumulation of Oct4. Finally, these results revealed that FL3 is a strong anticancer drug that kills cancer stem-like cells.

Since the first study showed that, synthetic flavagline FL3 had no effect on NSCs, we aimed to identify the underlying molecular mechanisms involved in the protection of these cells against the cytotoxic effects of the drug. First we provided additional evidence for the absence of cytotoxic effect of FL3 on the normal fibroblasts by using annexin V-FITC and mitochondrial membrane potential $\Delta\Psi_m$ assays. We found that FL3 failed to induce apoptosis in NSCs and was unable to induce mitochondrial depolarization and appearance of cytosolic cytochrome C. Most importantly, FL3 selectively phosphorylated Akt (Ser473), which is known to promote cell survival, FL3 also phosphorylated Bad at Ser112 and Ser136 in normal stem-like cells but not in cancer stem-like cells. The fact that its phosphorylated form serves as antiapoptotic protein could explain the resistance of NSCs. Indeed, forced inhibition of p-Akt by the specific inhibitor LY-294002 or gene knockdown of Bad by siRNA sensitized normal stem-like cells to FL3, by undergoing a caspase-3 dependent proapoptotic process, as measured by annexin V-FITC and western blot. These findings, therefore, provide new insights into the signaling pathway that could underly the resistance of normal stem-like cells against the potent anticancer agent FL3.

It is believed that the subpopulations of CSCs which reside within the bulk tumor, are responsible for tumor development and malignancy due to the deregulation of their self-renewal process. As a consequence it expected that pharmacological agents which are able to differentiate these cells, will lead to a loss of their self-renewal capacities and aggressiveness. We therefore aimed in the third study to evaluate whether FL3 administration at low concentration and for long period could be able to trigger cell differentiation of cancer stem - like cells. Our results indicated that FL3 downregulated in teratocarcinomal cell the expression of the stemness factor Oct4, at both the transcriptional and translational levels, as

early as 4 days of treatment. This effect coincided with a reduction of tumorsphere growth. The significant upregulation of the expression of specific neural markers such as GFAP (Glial fibrillary-acidic protein) and βTUBB3 (Tubulin beta-3 chain) further supported the evidence of the differentiation capacity of FL3. Interestingly, decreased expression levels of Oct4 was correlated with the appearance of cleaved caspase-3, followed by increased expression levels of GCNF (Germ cell-nuclear factor). This suggests that the degradation and gene repression of Oct4 could involve interconnected unknown mechanisms. Finally all these results showed that FL3 has similar effects than the common differentiating agent retinoic acid. Taken as a whole, my thesis has clearly demonstrated that the synthetic flavagline FL3 is a powerful anticancer compound, since it acts as a selective proapoptotic and pro-differentiating agent on cancer stem-like cells, without having any effect on normal stem-like cells.

Résumé de la thése de doctorant en français

L'objectif de ma thèse concerne l'évaluation des propriétés anticancéreuses des flavaglines sur les cellules initiatrices du cancer, plus connues sous le nom de cellules souches cancéreuses (CSCs).

Les flavaglines appartiennent à une famille de composés cyclopenta-benzofuranes naturels extraits de la plante du genre *Aglaia.* Le chef de file de cette famille, la rocaglamide, avait été identifié dès 1982 pour sa forte activité anticancéreuse. Depuis, plus de 100 flavaglines ont été décrites, dont le rocaglaol et le silvestrol. L'équipe du Dr. Laurent Désaubry avait récemment synthétisée la flavagline FL3 qui présentait une cytotoxicité accrue vis-à-vis de plusieurs lignées de cellules cancéreuses. Notre but était donc d'examiner l'activité anticancéreuse de la flavagline synthétique FL3 sur les CSCs et d'en caractériser les mécanismes moléculaires qui la sous-tendent.

Les CSCs se définissent par different marqueurs moléculaires qui permettent leur isolement de nombreux tissus ou organes. Ces cellules sont suspectées d'être responsables de la résurgence du processus cancéreux, ainsi que de la résistance à la chimio- et la radio-thérapie. Nous avons centré nos efforts sur des modèles de CSCs hautement agressives et peu différenciées qui sont connues pour exprimer un facteur central de la souchitude, à savoir Oct4. Ce facteur de transcription est un régulateur clé de l'auto-renouvellement de la pluripotence à spectre large des cellules souches normales (CSNs). Des données récentes ont montré qu'Oct4 est exprimé dans de nombreux cancers et contribue au développement tumoral et à l'inhibition de l'apoptose. Une répression de l'expression d'Oct4, suite à l'administration d'agents pharmacologiques, laisse donc présager que les cellules concernées rentrent en apoptose ou se différencient. Une telle thérapie de différenciation représente depuis très récemment une nouvelle arme pour réduire l'agressivité des CSCs.

La première étude que nous avons entreprise cherchait à examiner si FL3 a une activité cytotoxique sélectivement dirigée contre les CSCs. Ce travail a été conduit sur plusieurs lignées de cellules pluripotentes tératocarcinomales simili-souches NT2/D1 et NCCIT, et sur une lignée de cellules souches fibroblastiques qui répresente un modèle de CSNs ayant des potentialités restreintes de différenciation. Nous avons constaté, grâce aux tests de MTS et du trypan bleu respectivement, que FL3 inhibe sélectivement la prolifération et la viabilité des cellules NT2/D1 et

NCCIT. Cette inhibition de la croissance cellulaire s'accompagne d'un arrêt du cycle cellulaire en G1 et d'une répression de l'expression de la cycline D, une protéine majeure de régulation de la phase G1. De plus, l'activité cytotoxique de FL3 implique une induction d'un processus ayant les caractéristiques de l'apoptose, comme le montrent les tests de marquage par effet TUNEL et annexine V-FITC/propidium d'iodure. Par des analyses d'immuno-empreinte, nous avons observé que FL3 induit l'apoptose dans les CSCs exprimant Oct4, via une activation de la voie de signalisation impliquant la MAPK p38 et la caspase-3; Ceci conduit d'ailleurs à une chute significative des taux d'expression du régulateur de la souchitude. En confirmation de nos résultats, l'utilisation d'un inhibiteur universel de la caspase (z-VAD-fmk), ainsi que celle d'un inhibiteur de l'activité ou de l'expression de la MAPK p38 (respectivement par l'agent pharmacologique SB203580 ou des ARN interférents dirigés) a permis de contrecarrer les effets pro-apoptotiques de FL3 et de provoquer une accumulation significative d'Oct4 dans les cellules simili-souches cancéreuses. Finalement cette première étude a démontré que FL3 est un puissant agent anticancéreux qui tue spécifiquement les CSCs.

Comme nous avions démontré que la flavagline synthétique FL3 n'avait pas d'effets cytotoxiques sur les CSNs, nous avons cherché à identifier les mécanismes moléculaires qui pourraient rendre compte de cette résistance. Dans un premier temps, des expériences complémentaires ont permis de confirmer l'absence d'une quelconque activité délétère de FL3 sur les fibroblastes. En effet, la drogue ne modifie pas le pourcentage de cellules annexine V positives et n'induit pas de dépolarisation membranaire mitochondriale et de relargage du cytochrome C nucléaire dans le cytoplasme. De plus, FL3 phosphoryle la protéine de survie cellulaire Akt (au niveau de sa sérine 473) et la protéine régulatrice de l'apoptose Bad (au niveau de la sérine 112 et 136) dans les CSNs, et pas dans les CSCs. En fait, Bad, dans sa forme phosphorylée, assure une fonction anti-apoptotique; ceci expliquerait l'insensibilité des cellules fibroblastiques lorsqu'elles sont mises en présence de l'agent anticancéreux FL3. En effet, une inhibition forcée de la phosphorylation d'Akt (par un traitement avec le composé LY-294002), ainsi qu'une invalidation de l'expression génique de Bad (par un traitement avec des ARNs interférents), déclenche un processus pro-apoptotique caspase-3 dépendant dans les CSNs, lorsque celles-ci sont traitées par FL3. Ces résultats mettent donc en avant les principaux mécanismes moléculaires qui rendent compte de la résistance des cellules saines au puissant agent anticancéreux FL3.

Il est maintenant reconnu que la sous-population de CSCs, qui réside dans la masse tumorale, est responsable du développement tumoral, car celle-ci ne peut réguler son activité

d'auto-renouvellement. Il est dès lors postulé que les agents pharmacologiques qui sont capables d'induire la différenciation, vont affecter les capacités d'auto-renouvellement et donc l'aggressivité des CSCs. Aussi avons-nous cherché à évaluer, dans une troisième étude, si un traitement par FL3, à doses faibles et pendant une longue période, est susceptible d'entraîner une différenciation des cellules souches tératocarcinomales. Nos résultats indiquent que l'agent pharmacologique réprimait, après 4 jours d'administration, l'expression d'Oct4, tant au niveau transcriptionnel que traductionnel. Cet effet s'accompagne d'une réduction de la croissance de sphères tumorales. Une augmentation significative de l'expression de marqueurs neuraux, telle la GFAP ("Glial fibrillary-acidic protein") et la βTUBB3 ("Tubulin beta-3 chain") conforte l'hypothèse que FL3 pourrait agir, dans certaines conditions de traitement, comme un agent pharmacologique de différenciation cellulaire. Il est intéressant de noter également que la répression de l'expression d'Oct4 est corrélable avec l'apparition de la caspase-3 clivée, suivie d'une augmentation significative de l'expression du répresseur transcriptionnel d'Oct4, à savoir GCNF ("Germ cell-nuclear factor"). Ceci suggère que la dégradation d'Oct4, ainsi que sa répression génique, implique des mécanismes interconnectés qu'il s'agira ultérieurement d'identifier. Finalement nos résultats démontrent que FL3 présente des propriétés identiques à l'acide rétinoïque tout-trans qui est à ce jour un des plus puissants agents pharmacologiques de la différenciation cellulaire.

En conclusion, ma thèse a clairement mis en évidence que la flavagline synthétique FL3 est un puissant composé anticancéreux qui induit sélectivement l'apoptose et la différenciation des CSCs, tout en épargnant les CSNs.

Introduction

Introduction

1. Cancer

Cancer disease has been identified as an anarchic cell proliferation resulting from gene mutations and epigenetic modifications in a single cell or subset of cells that will impair the ability to control its division. These mutations occur by chance during cell division or are induced by specific intrinsic factors (*i.e.* metabolite products) or exogenous agents (*i.e.* chemicals). In general, mutated genes in cancer are classified into two categories: genes with gain-of-function mutation promote cell toward a cancer phenotype; this category corresponds to oncogenes. The second class includes genes with loss-of-function mutation which block cell to progress and form tumor; this category corresponds to tumor suppressors.(Hanahan and Weinberg 2000) (Knudson 2001).

So far, there are at least 100 different human cancers and according to the world health organization, death correlated to cancer is estimated of about 8.2 million people per year. In the next two decades, the expected new cases will be estimated to be 22 million per year (world health organization).

At the beginning of the 20th century, the overall cause of cancer had been identified and was mainly related to chemical agents, but the molecular mechanisms and cellular targets remain unknown. At the end of the 20th century, due to different new technologies and tools improvements, many molecular pathways that promote carcinogenesis have been discovered (Loeb and Harris 2008). However, cancer cure remains a big challenge facing the world today. Currently, a great advance has been achieved by understanding the origin of the carcinogenesis process and the resistance properties of tumor cells to existing therapies. The concept of stem cells (SCs) and the theory of cancer stem cells (CSCs) has recently attracted most attention in cancer research to design a new therapeutics that selectively target some aspects of the cancer process.

2. Stem cell biology

2.1. Normal stem cells

SCs are known to give rise to all the specific cell types of the body with distinct biological properties. It is expected that the acquirement of multiple genetic mutations in these cells will activate the carcinogenesis process. This has led to the hypothesis of the existence of CSC. In this point of view, it is worth to briefly recall the biology of the SC.

2.1.1. Definition and classification

SCs are responsible for the maintenance of homeostasis and the repair of injured organs. These cells have been identified in nearly all tissues (e.g., skeletal muscle tissue, nervous tissue, skin's tissue ,etc). SCs are characterized by their ability to long-term self-renew, a process by which one SC divides and copies itself, remaining in an undifferentiated state. This therefore maintains their number and thus their persistence throughout life. The second characteristic of SCs is their ability to differentiate and to give rise to one or more specialized cell types with specific function (Beck and Blanpain 2013).

The first stem cell is initiated from the fertilization of an egg by sperm. This unique cell is a totipotent stem cell, which is able to give rise to the precursor cells of all the embryonic and extra-embryonic tissues. After several divisions, the totipotent cells themselves will lose their high proliferative potential and begin to specialize into pluripotent stem cells.

Pluripotent stem cells are capable of generating all the tissue types from the three germ layers (endoderm, mesoderm, ectoderm) necessary for the embryogenesis. Divided pluripotent stem cells give rise to adult stem cells (defined as pluripotent stem cells with restricted capacities, also called multipotent stem cells), able to generate various stem cell types and later on colony forming units and monopotent stem cells. Finally these cells will give rise to mature cells which are in the terminal stage of differentiation and have lost their self-renewal properties. As an example, the multipotent hematopoietic stem cell will produce either common myeloid or lymphoid progenitors, which will then produce colony forming

units of the myeloid or lymphoid lineage and finally all the different blood cell types (erythrocytes, platelets and white blood cells) (Kuldip S. Sidhu 2012).

We thus observe different degrees of plasticity or differentiation potential of normal SCs, as shown in Fig. 1. Alba de Luca.(2015,July 6).

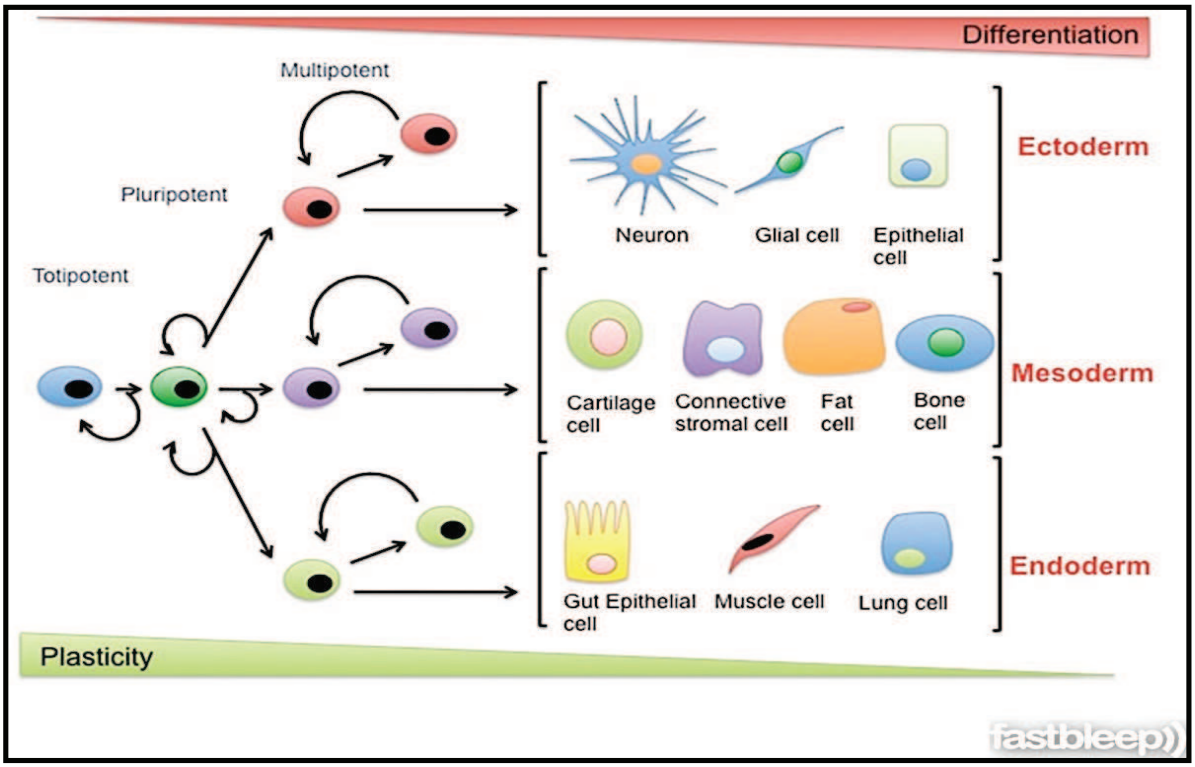

Fig. 1. Differentiation potential and classes of stem cells . (Retrieved from http://www.fastbleep.com/biology- notes/32/158/852 .)

2.1.2. Stem cell organization and niche concept

SCs reside and are protected in a specific place called “niche”. This is a specialized microenvironment which includes SCs themselves, neighboring cells and extracellular matrix (see Fig. 2). The niche provides various molecular signals enabling the SCs 1) to undergo self-renew, 2) to differentiate to repair damaged tissues or 3) to go in a quiescent state in terms of cell cycle in order to maintain their potential throughout life (Frank, Schatton et al. 2010, Rezza, Sennett et al. 2014).

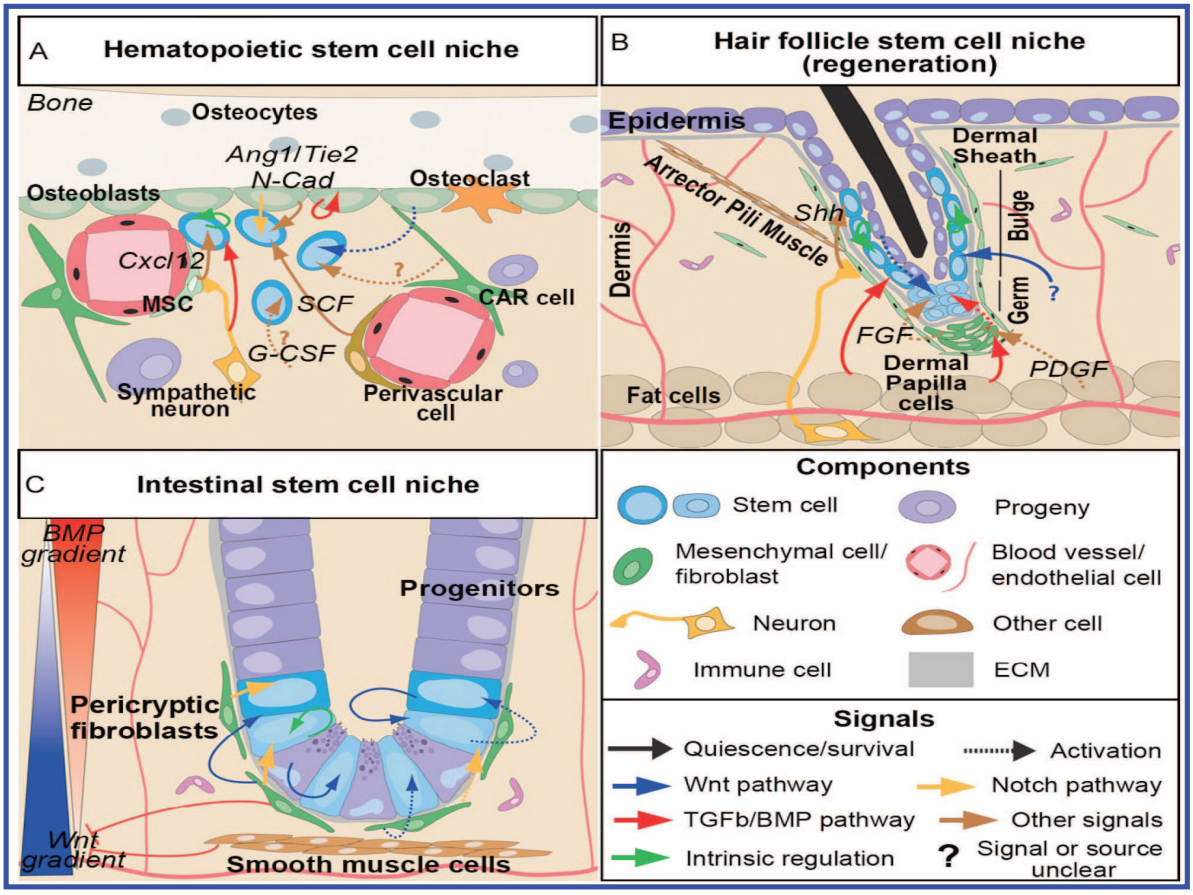

Fig. 2. Cellular and molecular components of three fast-cycling adult stem cell niches. (Rezza, Sennett et al. 2014).

2.1.3. The mode of division

SCs have the ability to divide by two different mechanisms. By symmetrical division, the SC gives rise to two identical daughter cells having the same properties as their mother; this allows to expand the pool of SCs (self-renew), as found during the embryonic development. The asymmetric division allows to generate one daughter cell identical to the mother cell (self-renew), while the other daughter cell will be differentiated into a more specialized cell. The balance between asymmetric and symmetric divisions are controlled by the niche that ensures the necessary balance between undifferentiated and differentiated cells (Morrison and Kimble 2006).

2.2. Cancer stem cells

2.2.1.Generalities

2.2.1.1. Historical overview

The earliest proof of the existence of the CSC is announced in 1855. Actually it was suspected that the cancer arises from embryonic residues present in adults. This hypothesis is based on the similarities between teratocarcinoma and embryo development and can be summarized as follows: “The existence of teratocarcinomas which contain cells of all germ layers and afflict young adults along midline migration pathways between gonads to brain endorses this embryonal rest theory” . This theory then developed and postulated that adult tissues could include embryonic remnants that are quiescent with the possibility to become cancerous if stimulated. The residues of SCs or their descendants in tissues may therefore acquire cancer features after exposure to specific carcinogenic agents (R.Cogle 2011) .

2.2.1.2. Evidence of the existence of CSCs

After improvements in cell sorting techniques and increasing knowledge in immunology, the first conclusive evidence of the existence of CSCs could be published in 1997 by the group of John Dick (Bonnet and Dick 1997). They isolated and identified a subpopulation of human acute myeloid leukemia (AML) cells that express the specific surface marker CD34, but do not express the surface marker CD38. These $CD34^{+}/CD38^{-}$ cells showed two properties that define normal SCs, namely their ability to proliferate and differentiate after multiple transplantation in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice). Furthermore, Dick’s team showed a hierarchical organization of the blast population of AML cells, having a $CD34^{+}/CD38^{-}$ phenotype similar to normal hematopoietic stem cells. It was therefore hypothesized that mutations of these SCs could have been the cause of the appearance of cancer-initiating cells that were responsible for the emergence of the disease.

The hypothesis of leukemic stem cells encourages the research to investigate whether CSCs exist in solid tumors. This research however progressed slowly because the knowledge of the biology of other normal stem cells, the identification of specific markers and their link

with the occurrence of cancer was much less advanced compared to that related to hematopoietic stem cell and leukemia. Again cell-sorting techniques allow the identification of specific cell surface markers that characterize specific CSCs in variety of solid tumors. The first CSCs were identified in breast cancers (Al-Hajj, Wicha et al. 2003), brain tumors (Singh, Hawkins et al. 2004), prostate cancers (Collins, Berry et al. 2005), colon cancers (O'Brien, Pollett et al. 2007), pancreatic cancers (Li, Heidt et al. 2007) , liver cancers (Ma, Chan et al. 2007), ovarian cancers (Zhang, Balch et al. 2008), Melanoma (Schatton, Murphy et al. 2008) and lung cancers (Eramo, Lotti et al. 2008). In all these cases, the "tumor stem cells" share features of those present in normal SCs (immature phenotype, self-renewal capacity and differentiation potential). As an example, patient-derived brain tumor cells can form spheres having the same phenotypic heterogeneity than that found in the parental tumor.

2.2.1.3. Origin of the tumor heterogeneity

Since 150 years, it has been postulated that the genetic background of cells forming the bulk tumor is multi-faceted, which explains the heterogeneity of a tumor (Paget 1989). These variations include differences in proliferation and differentiation potential, in cell surface markers, in invasive capacity, and in responses to therapy (Pietras 2011). Regarding to this heterogeneity, two models for cellular origin of cancer have been proposed: the stochastic and the hierarchy models (Dick 2008).

2.2.1.3.1. Stochastic model

In the stochastic model (Fig. 3), it is predicted that tumor cells are biologically homogenous and have equal likelihood for their contribution in the tumor growth, with a possibility to become tumor-initiating cells (via a dedifferentiation process); this will generate a heterogeneous tumor, due to the influence of intrinsic signals (*i.e.* transcription factors) or extrinsic signals (*i.e.* interactive proteins from the microenvironment) (Nowell 1976) (Chandler and Lagasse 2010) .

2.2.1.3.2. Hierarchy model

The hierarchy model (Fig. 3) postulates that the tumor is hierarchically organized and consists of heterogeneous cells. However only subpopulations of cells have the tumorigenic capacity and contribute to long-term tumor growth. These cells originate from transformed SCs and are considered as cancer-initiating cells, namely also CSCs. In the hierarchical model, CSCs reside at the top, the proliferating progenitors with a limited proliferation and differentiation capacities in the middle and the terminally differentiated cancer cells at the bottom (Nowell 1976). Recent observations revealed that both models can exist together depending on the microenvironment signals. For instance, tumor-initiating cells can originate from both transformed SCs or more differentiated cells which are affected by additional mutations or are epigenetically reprogrammed (Campbell and Polyak 2007).

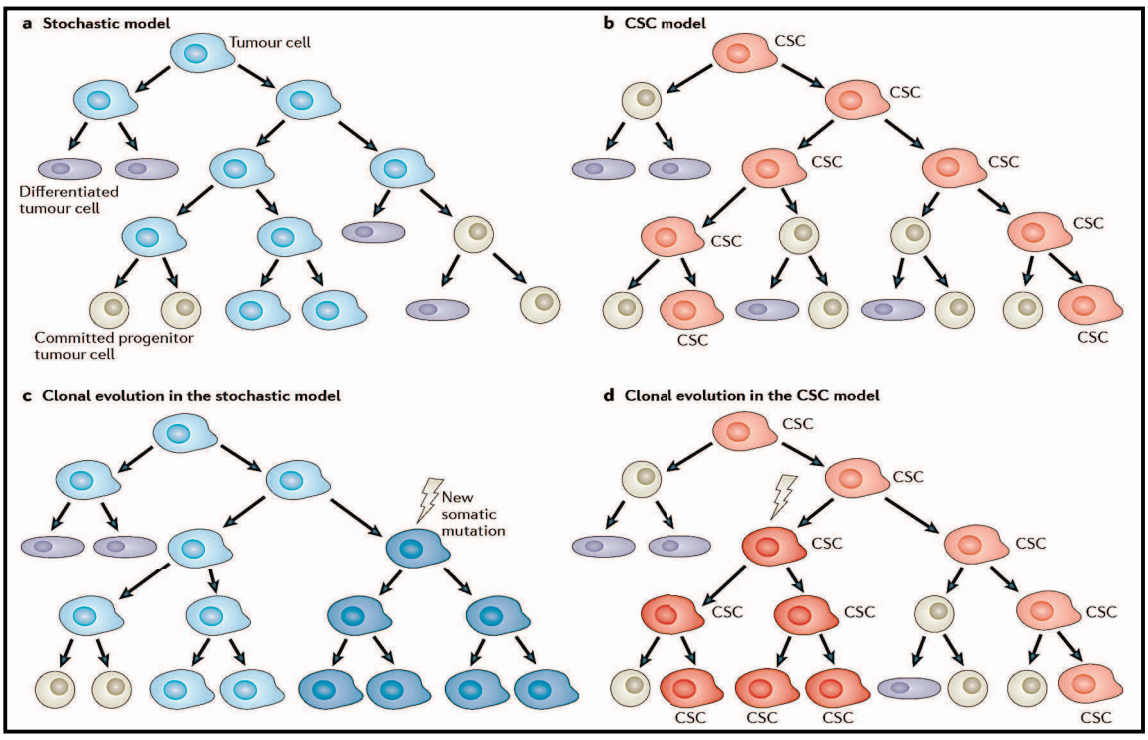

Fig. 3. Models of tumor heterogeneity a) Tumor cells are stochastically self-renewed or differentiated and form an heterogeneous tumor. **b**) Only subset CSCs give hierarchically rise to committed progenitors with limited proliferation and differentiation potentials. **c, d**) In both models, new somatic mutations can increase tumor heterogeneity (Beck and Blanpain 2013).

2.2.2. Carcinogenesis and its consequence

It is well known that tumorigensis is a multistep process initiated by a single normal cell or SC that is affected by irreversible mutations, epigenetic alterations, errors in DNA repair or inappropriate signals from its microenvironment (Brenda Loaiza 2012). In solid tumors, twenty mutations seem be required to induce a cancer phenotype. Actually, to gain this number of mutations, cells should have long lifespan; therefore SCs, which possess this property, are suitable for the initiation of the carcinogenesis process (Jilkine and Gutenkunst 2014). When the initiated cells escape from apoptosis or become undetected by the immune system, they can expand in number and finally can produce malignant tumors. The fact that differentiated descendants might acquire CSC properties by the dedifferentiation process due to additional mutations, can increase the malignity (Trosko, Chang et al. 2004) (Frank, Schatton et al. 2010) (Fig. 4).

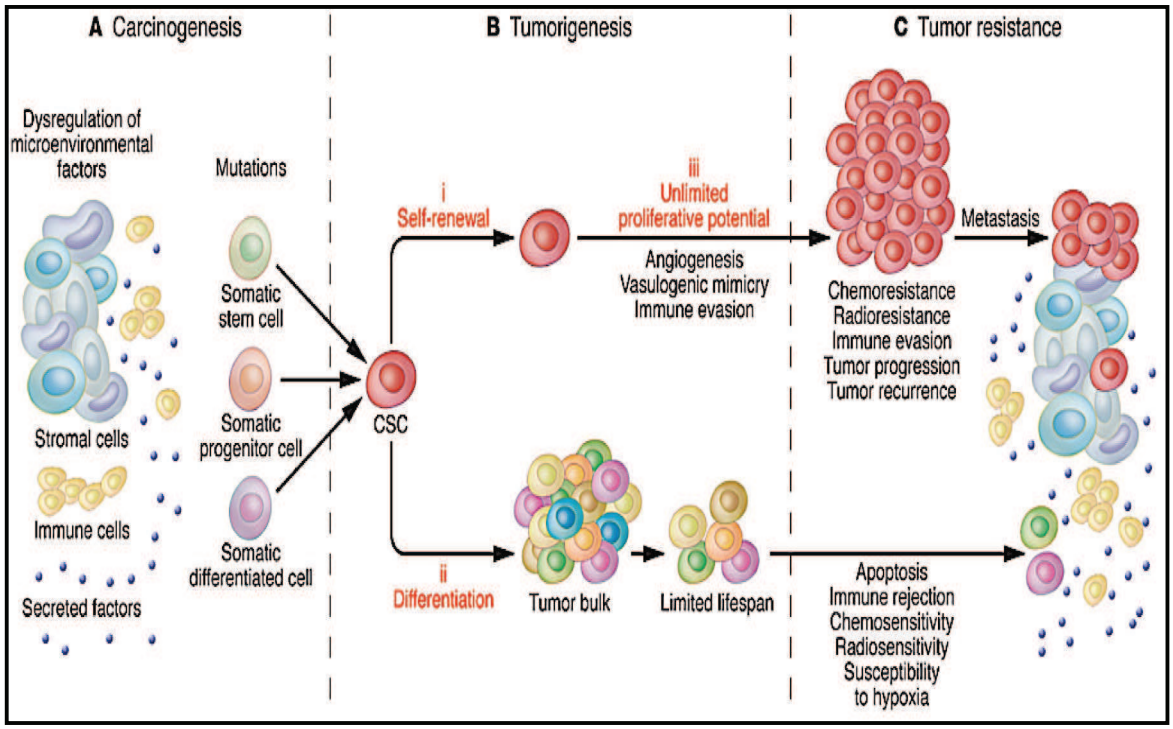

Fig. 4. Carcinogenesis, tumorigensis and resistance in CSCs . (Frank, Schatton et al. 2010).

Many evidences have proven the role of CSCs in the metastasis process. For instance, metastatic cancers such as prostate cancers (Collins, Berry et al. 2005), oligodendroglia tumors (Beier, Wischhusen et al. 2008), rectal cancers (Wang, Chen et al. 2009), high grade

of gastric adenocarcinoma (Zhao, Li et al. 2010), colon cancers (Zhang, Liu et al. 2013) , lung cancers (Wu, Qi et al. 2014) and pancreatic cancers (Nomura, Banerjee et al. 2015), correlate with the expression of CD133,a cell surface marker which is known to be expressed in normal and cancer SCs.

Epithelial to mesenchymal transition (EMT) is a key program that is activated during tumor invasion and metastasis. Immortalized human epithelial cells ectopically expressing specific transcription factors acquire a mesenchymal phenotype, with increasing migratory properties. Furthermore, isolated cells from highly invasive mouse or human mammary carcinoma show high expression of EMT markers (Mani, Guo et al. 2008) (Li and Li 2014). As it will be discussed later, Notch signaling pathway, known to regulate stem cell self-renewal, plays essential role in cell migration due to its participation in EMT (Yuan, Wu et al. 2014). Furthermore, Wnt/β-catenin signaling regulates the expression of the angiogenesis factor VEGF (Vascular Epithelial Growth Factor) in colon cancer and hepatocellular carcinoma and consequently increases vessel density (Easwaran, Lee et al. 2003) (Qu, Liu et al. 2014). On the other hand, knockdown of Oct4, un key regulator for the maintenance of the self-renewal process, inhibits cell migration and invasion of lung or colon cancer cells (Chiou, Wang et al. 2010) (Dai, Ge et al. 2013). All these examples finally demonstrate that cells with stemness properties have migratory and invasive potential.

The mechanisms involved in the resistance of CSCs to chemo- and radio-therapies have now been identified. The resistance to chemotherapy involves ATP-binding cassette transporters (ABC transporters), such as the multi drug resistance protein MDR1, which acts as efflux pump to reject drugs. Interestingly, hematopoietic stem cells show high levels of MDR1 (Chaudhary and Roninson 1991) , as well as hepatic or melanoma CSCs (Chow, Fan et al. 2012) (Keshet, Goldstein et al. 2008). On the other hand, CSCs have shown high potential to chemo- and radio-resistance by their strong ability to repair DNA damage and then to escape from apoptosis. For example, glioblastoma stem cells activate protein kinases ATM (Ataxia-Telangiectasia Mutated) and Chk1 (Checkpoint Kinase 1) more immediately after radiations than non-glioma stem cells, which then enable them to survive (Bao, Wu et al. 2006). Furthermore BRCA1 (Breast Cancer 1) and RAD51, known to be involved in the repair of damaged DNA, show increased expression levels in prostate CSCs. In other models of CSCs from colon and lung cancers, an efficient activity of Chk1 is observed, in contrast to their differentiated descendants, when treated with standard chemotherapeutic agents

(Maugeri-Saccà, Bartucci et al. 2012) . Resistance to chemo- or radio-therapies also involve other parameters, always linked with the possibility to induce a programmed cell death or inhibit the prosurvival pathway. For instance, breast cancer stem cells are considered resistant to apoptosis because they show, in comparison to non-CSCs, low levels of ROS (reactive oxygen species), which are known to induce DNA damage and to activate tumor suppressors (Diehn, Cho et al. 2009). Aldehyde dehydrogenase (ALDH), found to be highly expressed in many solid tumors (Abdullah and Chow 2013) and used as a marker to isolate CSCs, is implicated in chemo-resistance by protecting the cells against oxidative damage (Januchowski, Wojtowicz et al. 2013). Several studies demonstrate the role of the prosurvival protein BCL-2 (B-Cell Lymphoma-2) in drug resistance and these studies reveal that this protein is overexpressed in various CSCs lines (He, Zhou et al. 2014). EMT is also able to contribute to the resistance process of CSCs to the chemotherapy (Singh and Settleman 2010). Moreover CSC niche, which provides hypoxic conditions leads to increasing expression levels of hypoxia inducible factors (HIFs) which are correlated with CSCs resistance to the DNA-targeting agents through an activation of DNA repair enzymes (Vinogradov and Wei 2012). It has also been reported that inhibition of HIF-1α overcomes the resistance of lung cancer cells to topotecan and etoposide (Choi, Rho et al. 2009).

The chemoresistance has been also associated with the activity of the signaling pathways that play crucial role in the regulation of normal SCs or CSCs, especially those that promote self-renewal (see Table 1).

Table1. Summary of some chemoresistance-related signaling pathways (Abdullah and Chow 2013).

Transcription factor/ signaling pathway	Tumor type	Drug resistance	References
MYC	Hepatocellular Carcinoma, Leukemia,	Paclitaxel, Doxorubicin,	18, 56, 64
AKT/PKB	Hepatocellular Carcinomaxx	Doxorubicin, 5-Fluorouracil	68
WNT/β-Catenin	Hepatocellular Carcinoma, Neuroblastoma, Ovarian Cancer	Cisplatin, Doxorubicin, 5-Fluorouracil, Paclitaxel	74, 75, 76, 77
Notch	Colon Cancer, Ovarian Cancer, Glioma	Oxaliplatin, Cisplatin, Temozolomide	79, 80, 81, 82
Sonic hedgehog	Glioma, Gastric Cancer, Pancreatic Cancer, Ovarian Cancer, Prostate Cancer	Temozolomide, Oxaliplatin, Gemcitabine, Paclitaxel, Cisplatin	82, 84, 85, 86, 87
NF-κB	Ovarian Cancer, Breast Cancer	Paclitaxel, Carboplatin,	88, 89, 90

2.2.3. Cancer stem cell biomarkers

Since CSCs are implicated in the initiation and growth of tumors, in the metastasis and resistance to current therapies, it is crucial to identify them in order to develop appropriate and efficient anticancer treatments. Several cell surface antigens have been characterized as candidate markers for CSCs. For instance, CD133 has been recommended for a prospective isolation of CSCs. However different studies reveal that CD133 is also found in differentiated normal cells of various organs and cancer cells lacking CD133 can also initiate tumors. In glioblastoma tumors, subset of cancer cells with self-renewal capacity have been identified which negatively expressed CD133. In fact, a unique marker is not sufficiently specific for the isolation of one type of CSC (Chen, Nishimura et al. 2010). In general, markers of CSCs are also not steady in a single tumor or between patients, and therefore other tools, such as sphere forming, are required for the isolation of these cells (Wang, Chen et al. 2015) . Different biomarkers have been found in variety of tumors, as shown in Table 2 (Chen, Huang et al. 2013).

Table 2. Cell surface markers of CSCs.

Tumor type	Phenotype of CSCs markers	Reference
Leukemia	$CD34^{+}CD38^{-}$HLA-DR-$CD71^{-}CD90^{-}CD117^{-}CD123^{+}$	[18]
Breast cancer	$ESA^{+}CD44^{+}CD24^{-/low}Lineage^{-}$, $ALDH\text{-}1^{high}$	[4, 20]
Liver cancer	$CD133^{+}$, $CD49f^{+}$, $CD90^{+}$	[21, 22]
Brain cancer	$CD133^{+}$, $BCRP1^{+}$, $A2B5^{+}$, $SSEA\text{-}1^{+}$	[19, 23]
Lung cancer	$CD133^{+}$, $ABCG2^{high}$	[24, 25]
Colon cancer	$CD133^{+}$, $CD44^{+}$, $CD166^{+}$, $EpCAM^{+}$, $CD24^{+}$	[26–28]
Multiple myeloma	$CD138^{-}$	[29, 30]
Prostate cancer	$CD44^{+}$, $\alpha2\beta1^{high}$, $CD133^{+}$	[7]
Pancreatic	$CD133^{+}$, $CD44^{+}$, $EpCAM^{+}$, $CD24^{+}$	[31, 32]
Melanoma	$CD20^{+}$	[10]
Head and neck cancer	$CD44^{+}$	[33]

2.2.4. Control of self-renewal and differentiation

As described above, self-renewal is a process achieved by asymmetric or symmetric division to produce one or two daughter cells that are identical to the mother cell. Stem cells undergo a limited number of divisions under physiological conditions in adult tissues whereas

they undergo indefinite division in early embryonic development to generate embryo body. Self-renewal is controlled by extrinsic and/or intrinsic regulatory networks that suppress the expression of differentiation promoting genes and activate the expression of mitosis promoting genes (He, Nakada et al. 2009). Actually, self-renewal is expected to be extended to CSCs when it occurs through different deregulated processes.

2.2.4.1. The role of signaling pathways

The major extrinsic pathways that regulate stem cell self-renewal involve the Wnt/β-catenin, Notch and Hedgehog signaling networks.

2.2.4.1.1. Wnt/ β-catenin signaling pathway

Wnt signaling has been shown to regulate subset of processes in animal development and in several adult tissues. When Wnt ligands bind their receptors (namely frizzled) on the cytoplasmic membrane, they recruit a co-receptor, namely LRP (low-density lipoprotein receptor-related protein). Frizzled can then bind Dsh (disheveled) and induce the phosphorylation of LRP which in turn allows AXIN (alternative name of Axis inhibition protein) to be relocated to the cell membrane; as a consequence, an accumulation of β-catenin occurs which fosters its translocation to the nucleus, its binding to Tcf (T-cell-specific transcription factor) and Lef (lymphoid enhancer-binding factor) and finally the activation of certain transcription factor genes necessary for the maintenance of self renewal. At the opposite, the absence of Wnt ligands leads to the capture of β-catenin by APC (adenomatous polyposis coli) and AXIN; this fosters its phosphorylation by GSK3β (glycogen synthase kinase) or CK1α (casein-kinase 1α), thereby inducing its degradation by the proteasome (Fig. 5) (Clevers and Nusse 2012) (Blank, Karlsson et al. 2008).

The biological role of Wnt/β-catenin in the self-renewal process has been characterized only in some models of stem cells. For example, β-catenin is essential for maintenance of intestinal stem cells (Fevr, Robine et al. 2007), in normal hematopoietic stem cells (Reya, Duncan et al. 2003), neural stem cells (Gong and Huang 2012) and embryonic stem cells (ESCs) (Serio 2014). On the other hand, mutations of Wnt/β-catenin signaling components are known to cause an overexpression of β-catenin. This aberrant expression has been found in prostate cancer stem cell (Chen, Shukeir et al. 2004), in colorectal cancer stem

cells (Dalerba, Dylla et al. 2007), in breast cancer stem cells (Woodward, Chen et al. 2007), (Bisson and Prowse 2009) and glioblastoma stem cells (Kim, Seol et al. 2013).

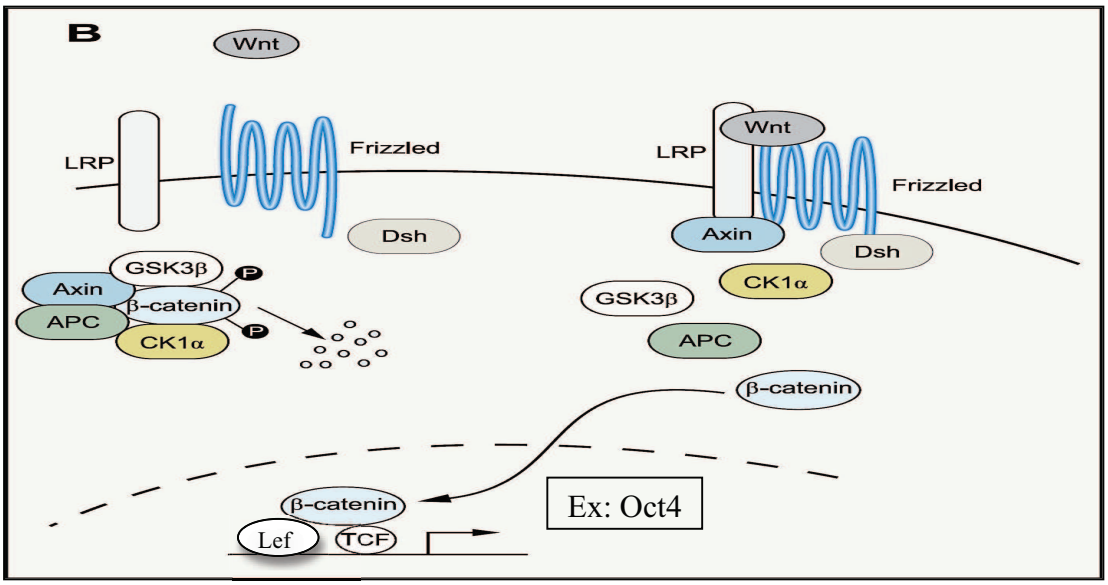

Fig. 5. Wnt/ β-catenin signaling pathway. Modified from (Blank, Karlsson et al. 2008).

2.2.4.1.2. Notch signaling pathway

Notch signaling is another known pathway that is involved in the control of self-renewal in several tissues (Krause 2002) . The activation of Notch by its ligands Delta and Jagged leads to the cleavage of NICD (Notch Intercellular Domain) by γ-secretase, and subsequently a complex between NICD, CSL transcription factor (CBF1, Suppressor of Hairless, Lag-1) and the MAML (co-factor Mastermind-like) is formed then activate the transcription of target genes (Fig. 6) (Blank, Karlsson et al. 2008).

During development of zebrafish brain, an asymmetric division allows the production of self-renewing and differentiated daughters, the later showing higher levels of notch ligands. This demonstrates the precise role of Notch pathway for the regulation of self-renewal and differentiation within an asymmetric division process (Dong, Yang et al. 2012). Notch signaling is also found to balance self-renewal and differentiation in hematopoietic stem cells (Mancini, Mantei et al. 2005) and mammary gland stem cells (Liu, Dontu et al. 2005) . As expected, different studies show that Notch deregulation is implicated in various types of cancers, such as pancreatic cancers (Wang, Ahmad et al. 2011), ovarian cancers (McAuliffe,

Morgan et al. 2012), glioma cancers (Wang, Wang et al. 2012) and liver cancers (Zhu, Wang et al. 2015).

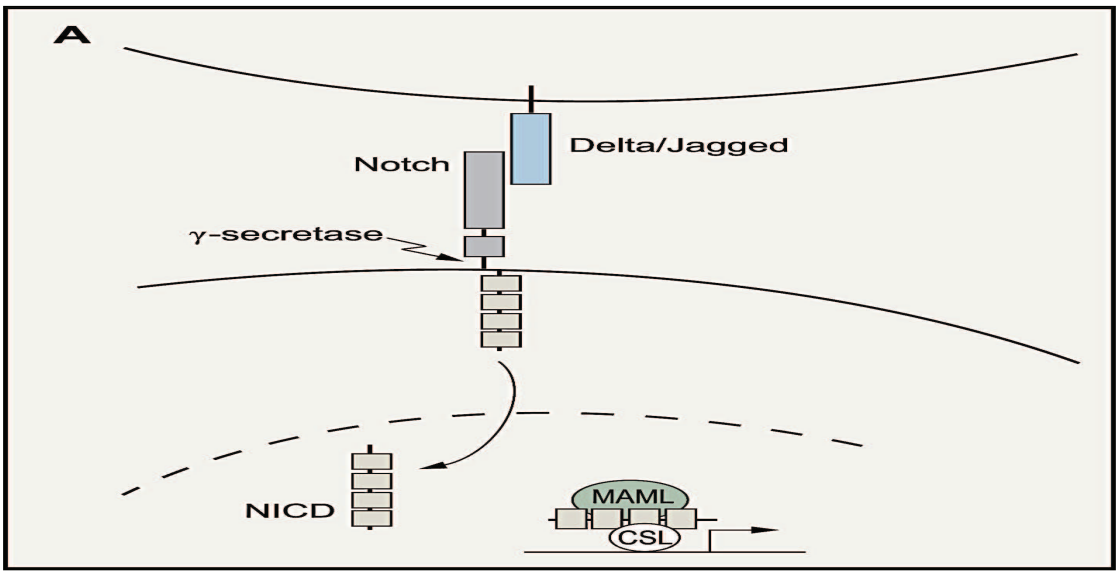

Fig. 6. Notch signaling pathway. (Blank, Karlsson et al. 2008).

2.2.4.1.3. Sonic Hedgehog (Shh) signaling pathway

Shh signaling pathway plays a crucial role in early embryonic development and is involved in the regulation of self-renewal and differentiation of ESCs. Three ligands are identified as yet: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh). When hh ligands bind to the transmembrane protein PTCH 1 (patched protein 1), the trans-membrane protein SMO (smoothened) is recruited and will activate the downstream transcription factor GLI (glioma-associated oncogene homolog), by dissociating it from the SUFU protein (Suppressor of Fused) or the KIF17 protein (Kinesin Family Member 17); finally this allows GLI transcription factor to activate genes necessary for the self renewal process such as Bmi-1 (Polycomb complex protein) as showed in (Fig. 7) (Kumar and Fuchs 2015) (Manoranjan, Venugopal et al. 2012).

In neuronal stem cells, hh signaling activity increases symmetric division and thus expands their number (Ferent, Cochard et al. 2014), as in hematopoietic stem cells (Merchant, Joseph et al. 2010) .In tongue mouse ,it promotes self-renewal of taste buds (Miura, Kusakabe et al. 2001). This signaling pathway also regulates the self-renewal of normal and malignant mammary epithelial stem cells (Liu, Dontu et al. 2006). Actually, Shh ligands are expressed in several pathological tissues and stages of carcinogenesis. For example, Shh is clearly

involved in the self-renewal process of breast, ovarian and mammary cancers (Tanaka, Nakamura et al. 2009) (Chen, Gao et al. 2013) (Memmi, Sanarico et al. 2015).

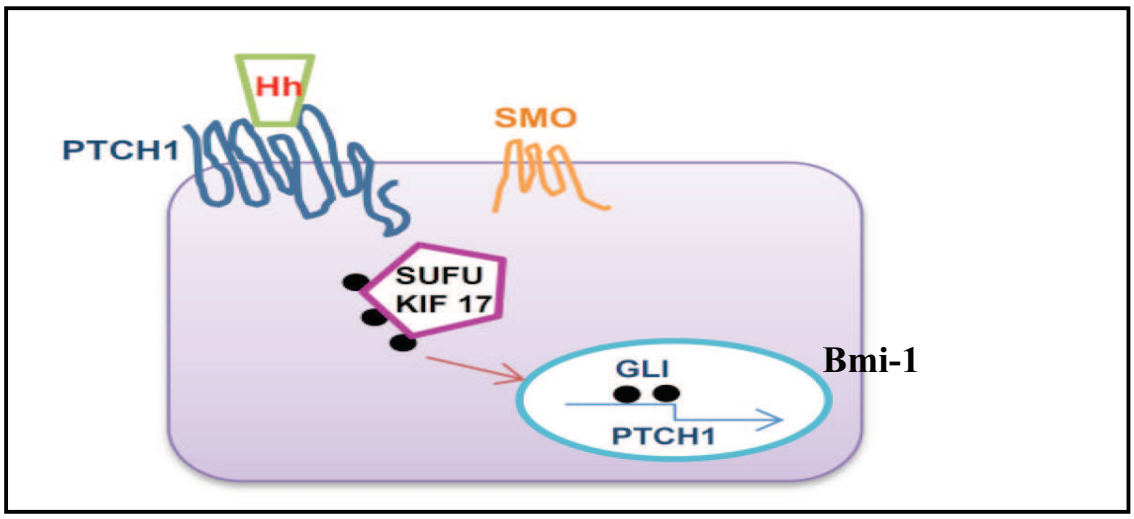

Fig.7. Sonic hedgehog signaling pathway. (Modified from (Kumar and Fuchs 2015).

The molecular interactions between Wnt/β-catenin, Hedgehog and Notch pathways for the regulation of both self-renewal and differentiation have been partly identified. For example, in the stem cell niche, the full activity of Notch to promote activation of genes that prevent differentiation induction, needs the participation of the Shh pathway (Weli, Fink et al. 2010).

As a conclusion, Wnt/β-catenin, Hedgehog and Notch pathways play a major role in normal stem cell self-renewal and their deregulation by mutations or epigenetic alterations increases the tumorigenic and metastatic potential (Czerwinska and Kaminska 2015) .

2.2.4.2. The role of transcription factors

There are three main transcription factors that are key modulators of the maintenance of self-renewal and the repression of the differentiation in normal stem cells. These factors have been also implicated in the development of many tumors.

2.2.4.2.1. Oct4

Oct4 (octamer binding transcription factor) is encoded by Pou5f1 gene. This protein belongs to the POU family (**P**ituitary-specific, **O**ctamer and neural **U**nc-86 transcription

factors). Oct4 binds to the octameric sequence motif AGTCAAAT and activates or suppresses the expression of numerous target genes, leading to a precise regulation of the self-renewal and to repression of the differentiation process (Pan, Chang et al. 2002).

Oct4 plays a major role in the maintenance of ESC potency and the establishment of the germ cell lineage. Several studies have revealed that Oct4 is expressed in early embryos, unfertilized oocytes (Scholer, Hatzopoulos et al. 1989) and in different germ cell tumors, namely the embryonal carcinoma cells (ECCs) (Lenardo, Staudt et al. 1989). Actually, the protein is downregulated during differentiation of ESCs and then its expression is restricted to germ cell lineage. It has been proven that Oct4-deficient mice were not able to develop beyond blastocyst stage because they have a lack of pluripotent cells from the inner cell mass (Nichols, Zevnik et al. 1998). On the other hand, the expression of Oct4 is repressed upon induction of differentiation by retinoic acid in ESCs and ECCs. In addition, recent observations have shown the role of this transcription factor in the reprogramming of "differentiated" somatic cells into induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). Therefore, all these facts confirm that Oct4 is indispensible for the regulation of self-renewal and stem cell identity. The recapitulation of in *vivo* and in *vitro* expression of Oct4 in human normal and cancer SCs are presented in Fig. 9 (Sharif Tanveer 2011) .

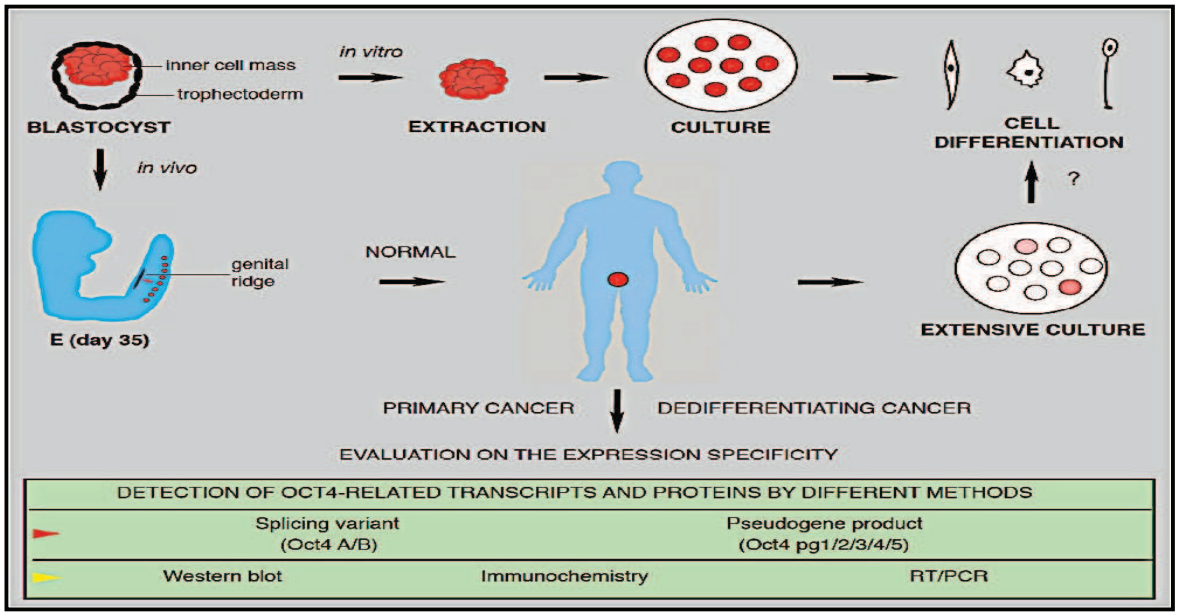

Fig. 8. In vivo and in vitro expression of Oct4 in human normal and cancer SCs. Oct4 expression is depicted in red; the brightness of the color represents the amount of expression (Sharif Tanveer 2011).

There are two master isoforms of this protein, Oct4A and Oct4B. Oct4A is responsible for pluripotency and self-renewal regulation, while the Oct4B isoform does not contribute to the regulation of stem cell identity and its role remain unclear (Bhartiya 2013). Recent studies have reported that Oct4 can be expressed in some somatic cells; actually this expression seems to be linked to the existence of Oct4-like proteins (Wang and Dai 2010) ; for instance, proteins from pseudogenes Oct-4pg1, Oct-4pg3 and Oct-4pg4 have been detected in human adult fibroblasts, without evidence of an expression of Oct4A. Therefore the expression of a functional and active Oct4 in somatic cells remains still controversial (Jez, Ambady et al. 2014). However the ability of isolating stem cells by using cell-sorting techniques enables the researchers to identify whether Oct4 is expressed in adult stem cells. Isolated stem cells from adult tissues, *i.e.* breast, kidney, pancreas or liver, show the presence of Oct4, as detected by specific antibody against this protein or by RT-PCR (Tai, Chang et al. 2005). Human normal tissue sections from skin show that few cells from the basal layer are expressing Oct4. Furthermore, Oct4 expression is markedly reduced in cells which are undergoing differentiation (White, Al-Turaifi et al. 2011).

The regulation of Oct4 expression is controlled by two enhancers located, near or far the promoter. The distal enhancer has been found to be active in ESCs and germ cells, while the proximal enhancer is active during gastrulation and in ECCs (Jerabek, Merino et al. 2014). It has been reported that SF-1 (Steroidogenic Factor 1) and LRH-1 (Liver Receptor Homolog-1) bind to Oct4 promoter which in turn activates the expression of the protein and maintains self-renewal, whereas the binding of GCNF (Germ cell nuclear factor) to the promoter leads to suppression of Oct4 expression and consequently induces cell differentiation.

Different signaling pathways are involved in the regulation of Oct4 expression. For example, activation of the pathway JAK/STAT3 (Janus Kinase/Signal Transducer and Activator of Transcription) leads to transcriptional activation of Oct4 (Do, Ueda et al. 2013). In addition, PI3/Akt (phosphatidylinositol-3-kinase/protein kinase B) is another pathway that can activate the transcription of Oct4 through its phosphorylation and thus enhance survival and self-renewal processes (Lin, Yang et al. 2012). Furthermore, it has been shown that Wnt/β-catenin activates LRH-1, and consequently controls the balance between self-renewal and differentiation of pluripotent SCs, by controlling Oct4 expression (Jerabek, Merino et al. 2014). The posttranscriptional regulation of Oct4 has been recently explored. For Instance, miRNA-145, by pairing Oct4 mRNA, prevents its translation. On the other hand,

posttranslational events also contribute to the regulation of Oct4 expression. The ubiquitination by E3 ubiquitin-protein ligase of the WW domain-containing Protein 2 (WWP2), mediates Oct4 proteasomic degradation. In the opposite, sumoylation by SUMO-1 (Small Ubiquitin-related Modifier, 1) stabilizes the protein and increases its transactivation potential (Sharif Tanveer 2011).

The regulation of stem cell pluripotency and differentiation by Oct4 has been well characterized. Chromatin immunoprecipitation with DNA microarray (ChIP- on chip) on ESCs demonstrate that 90% of genes that are occupied by Oct4 are genes correlated with pluripotency regulation. Interestingly, this study also shows that Nanog and Sox2 are bound to these genes, which indicate a relationship and cooperation between all these transcription factors (Jerabek, Merino et al. 2014).

Many data have demonstrated the role of Oct4 in the suppression of cell differentiation. For example, silencing of Oct4 has been shown to favour differentiation in human ESCs by activating the tumor suppressor p53, through the reduction of the expression of Sirt1, a deacetylase inhibitor of p53 activity (Zhang, Chung et al. 2014). Furthermore, the localization of Oct4/ Cdk1 (cycline-dependent kinase1) complex on the promoter of Cdx2 (Caudal type homeobox transcription factor 2) gene leads to the repression of its expression and prevent differentiation induction (Li, Wang et al. 2012).
Table 3 shows a set of Oct4 target genes that are involved in various developmental and functional processes (Sharif Tanveer 2011).

Table 3. Example of some genes involved in different cellular processes regulated by Oct4 .

Target gene	Protein function	Cell process	Reference
CDX2 (caudal type homeobox transcription factor 2)	Transcription factor	Differentiation induction	Babaie et al., 2007
FGF4 (fibroblast growth factor-4)	Signaling molecule	Differentiation repression	Chew et al., 2005
Nanog	Transcription factor	Stem cell identity	Rodda et al., 2005
NDUFA3 (NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 3)	Mitochondrial electron carrier	Cell metabolism	X. Chen et al., 2008
Oct4	Transcription factor	Stem cell identity	Chew et al., 2005
p21 (WAF1 ; CIP1)	Cyclin-dependent kinase inhibitor	Proliferation inhibition	Lee et al., 2010
SOX2	Transcription factor	Stem cell identity	Chew et al., 2005
TP53 (p53)	Tumor suppressor	Cell death	Campbell et al., 2007
SUZ12 (suppressor of zeste 12 homolog)	Polycomb repressive complex 2	Chromatin remodeling	Sharov et al., 2008

Recently, several studies revealed that Oct4 is expressed in various tumors, such as breast or cervical cancers (Tai, Chang et al. 2005). In oral cancers Oct4 expression is correlated with advanced stages and poorly differentiated status (Chiou, Yu et al. 2008). Also abnormal expression of this protein has been found in human rectal adenocarcinoma (Xing, Lu et al. 2010). Moreover, Oct4 is considered as a hallmark of the aggressivity of human hepatocellular carcinoma cells, since its knock-down led to a loss of their self-renewal, chemoresistance and tumorigenicity properties (Murakami, Ninomiya et al. 2015). The expression of Oct4 has also been correlated with the invasive properties of ovarian cancers (Liu, Zhang et al. 2015) and the grade of tumor malignancy in human glioma is linked to the levels of expression of Oct4 and its partners, Nanog and Sox2 (Guo, Liu et al. 2011). Interestingly, increased Oct4 gene expression is observed in human lung cancer cell lines when cultured as spheroids which favour the selection of undifferentiated cell populations (Zhang, Lou et al. 2015). It has been reported that Oct4 is involved in the regulation of 25 pro-apoptotic genes. For example, Oct4 regulates the expression of miRNA-125b, which is involved in the inhibition of the proapoptotic protein BAK1 (BCL2-Antagonist/Killer 1) (Wang, Cai et al. 2013).

The mechanisms by which Oct4 is reactivated in human cancers has been recently elucidated. The exposure of isolated non-cancerous stem cells from patient samples to ionizing radiation reprograms them into highly undifferentiated cells, with reexpression of Oct4, Nanog and Sox2. These reprogrammed cells show increasing rate in spheroid formation. This indicates the contribution of Oct4 in the resistance and tumor recurrence after radiotherapy (Lagadec, Vlashi et al. 2012). Tryptophan derivatives such as ITE (2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester) function as endogenous ligand for AhR receptor (aryl hydrocarbon). Bindings of ITE to this receptor lead to binding of AhR to Oct4 promoter and thereby repress its expression. Recently it was found that the increase of Oct4 expression in cancers was related to low levels of ITE due to tryptophan deprivation or hypoxia, which characterize tumor microenvironment. Accordingly, administration of synthetic ITE reduced Oct4 levels, induced differentiation of cancer stem-like cell and decreased tumorigenicity in xenograft tumor model (Cheng, Li et al. 2015). Furthermore CaMKIIγ (Ca2+/calmodulin-dependent protein kinase IIγ) is highly expressed in many cancers and enhances Oct4 expression via activation of Akt and β-catenin signaling pathways (Chai, Xu et al. 2015). Moreover, the translocation of the cleaved intercellular domain of CD44 to the nucleus led to activation of the expression of stemness factors Oct4, Nanog and

Sox2 and consequently promoted tumorigenicity of breast cancer (Cho, Lee et al. 2015).

As a conclusion, Oct4, through different ways, has been found to be reactivated in many cancers, inducing a reversion from a highly differentiated state to a highly undifferentiated state which contributes to cancer development and aggressivity.

2.2.4.2.2. Nanog

Nanog is a homeobox protein that binds to specific sequences of DNA and then regulates genes involved in the maintenance of the self-renewal and pluripotency state. As an example, reactivation of Nanog in adult human fibroblasts leads to the acquisition of a stem cell identity which enforces its role as stemness factor (Takahashi, Tanabe et al. 2007). Actually, Nanog regulates the self-renewal process by targeting it epigenetically. For instance, Nanog, in cooperation with Oct4, upregulates Dnmt1 (DNA methyltransferase 1) which, in turn, promotes DNA methylation of genes necessary for differentiation induction and consequently maintains an undifferentiated state (Tsai, Su et al. 2012). On the other hand, Nanog directly targets specific genes, like FOXD3 gene (forkhead box 3), which is needed for the maintenance of pluripotency during the pre-implantation embryo development (Loh, Wu et al. 2006) .

Nanog is regulated by different ways. Signaling cascades, such as Wnt/ β-catenin or Jak/STAT pathways, are positively regulating Nanog expression. Moreover Nanog can be regulated by epigenetic factors; for example, tumor suppressor p53 binds to Nanog promoter and recruits HDACs (histone deacetylases), reducing acetylation of H3 (Histone 3) and thereby suppressing Nanog expression (Lin, Chao et al. 2005).

The expression of Nanog is thought to be confined to ESCs, but now several studies show that the protein is also expressed in some adult stem cells, such as in mesenchymal stem cells (Riekstina, Cakstina et al. 2009).

Nanog has been found to be highly expressed in certain cancers, like breast cancers (Lu, Mazur et al. 2014) or hepatocellular carcinoma (Shan, Shen et al. 2012). In ovarian cancer, Nanog stimulates cell migration through the deregulation of E-cadherin which is crucial for tissue integrity and suppression of cell motility (Siu, Wong et al. 2013) .

2.2.4.2.3. Sox2.

SRY (SEX determining Region Y) -box2, also known as Sox2, is essential for the maintenance of self-renewal and undifferentiating state of stem cells. Accordingly, many studies indicate that Sox2 is required for homeostasis and regeneration of some adult tissues. Sox2 regulates genes involved in cell proliferation, stem cell identity and metabolism (Boumahdi, Driessens et al. 2014). Sox2 cooccupies, with its partners Oct4 and Nanog, several target genes that are implicated in the differentiation process (Fig. 8) (Sarkar and Hochedlinger 2013).

Expression of Sox2 has been detected in adult tissues such as glandular stomach (Sarkar and Hochedlinger 2013), retina (Taranova, Magness et al. 2006) ,as well as in tongue epithelium (Okubo, Pevny et al. 2006). Sox2-positive cells have been identified in several cancers, like glioma (Ikushima, Todo et al. 2009) or melanoma (Laga and Murphy 2010).

Sox2 is regulated by different mechanisms. Extracellular signals, such as Shh or Notch, play an important role in Sox2 gene regulation (Sarkar and Hochedlinger 2013).

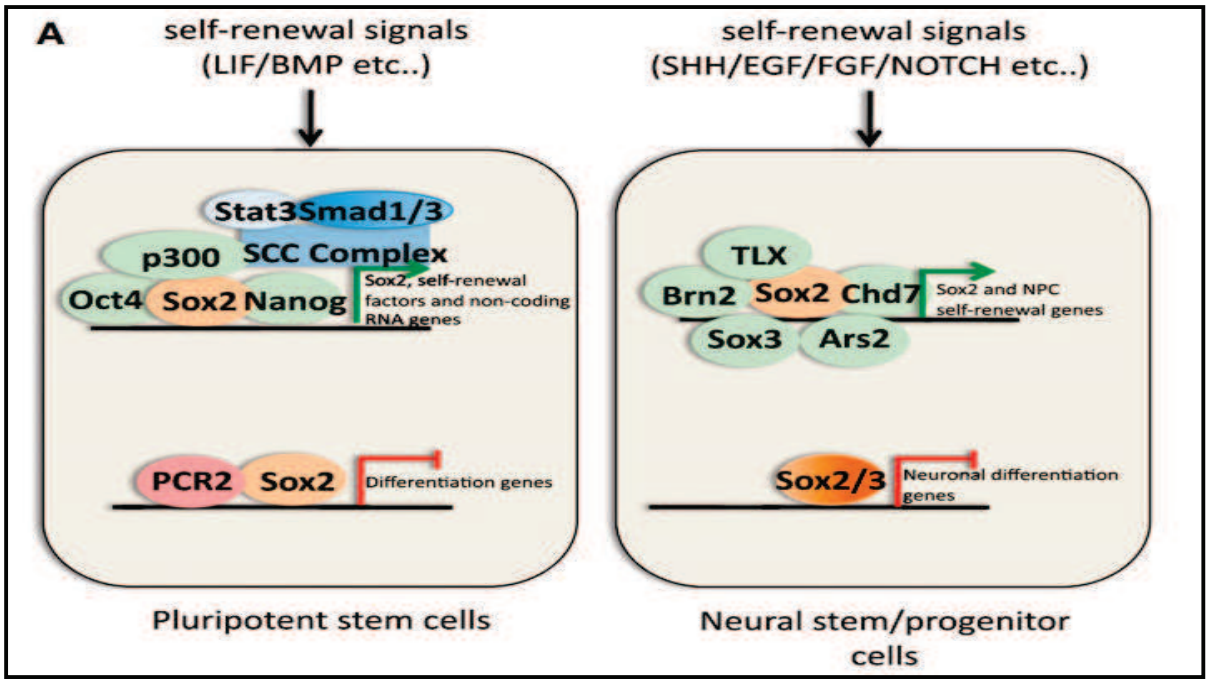

Fig. 9. Sox2 network in the regulation of self-renewal and differentiation. (Sarkar and Hochedlinger 2013).

3. Human embryonal carcinoma cells (hECCs)

This section will identify the model of CSCs used in our studies and by previous works.

3.1. Origin, derivation and culture system

Human embryonal carcinoma (known also Teratocarcinomas) is a subset of germ cell tumors that occur in testes of young people. They are composed of pluripotent stem cells capable to undergo differentiation into a large variety of cell types. Actually, EC cell is very close to the cell of the pluripotent inner cell mass.

EC cell is able to form secondary tumors after serial xenograft (Chambers and Smith 2004). It has been shown that after grafting a single EC cell to a host mouse, a new tumor will be produced (Kleinsmith and Pierce 1964).

Teratocarcinoma is a highly malignant tumor which loses it aggressiveness after differentiation. Since differentiated EC cells exert limited proliferation potential, it is postulated that they are subjected to severe selected mutations that slow-down their capacity to differentiate (Singec, Jandial et al. 2007).

Embryonal carcinoma cell lines are derived from heterogeneous surgically isolated germ tumors, via the combination of immunomagnetic isolation and single-cell selection. The first established germ cell line from teratocarcinoma was named TERA2. This cell line is derived from a lung metastasis of a 22-year-old patient with primary embryonal carcinoma. EC cells were isolated by using SSEA-3 (Stage-Specific Embryonic Antigen 3) immunoreactive magnetic particles and were then grown until the formation of a homogeneous confluent monolayer in tissue culture plates in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS (Fetal calf serum) and 2 mM L -glutamine. The NTERA2.cl.D1 (also abbreviated NT2/D1) cell line has been derived from a xenograft tumor of TERA2. When NT2/D1 cells are cultured in the presence of retinoic acid in vitro, they sustain their differentiation potential into diverse somatic tissues (Andrews, Damjanov et al. 1984) (Przyborski, Christie et al. 2004)

3.2. Embryonal carcinoma as a model to study the biology of CSC

CSC self-renews and differentiates; its aggressiveness and invasion capacity is mostly due to a reexpression of the stemness factors Oct4, Nanog and Sox2. EC cell shares all these properties. Therefore these cells represent a suitable model to study the biology of the CSCs and to develop efficient therapy that could target them (Emhemmed, Ali Azouaou et al. 2014) (Lin, Yang et al. 2012) (Silvan, Diez-Torre et al. 2009).

4. Therapeutic strategies against CSCs

Targeting CSCs means targeting their self-renewal capacity and reducing their aggressiveness by the induction of a proapoptotic and/or prodifferentiating process. For that reason, it is necessary to develop therapeutic strategies that affect their molecular signature and the mechanisms which permit its maintenance. We will therefore focus here on the critical regulatory pathways which could be targeted in CSCs, when exposed to a pharmacological agent.

4.1. Targeting cancer stem cells by apoptosis induction

The term apoptosis, known also as programmed cell death, is a controlled process that eliminates cells in an organ without aggravating the immune response or host inflammatory process. This phenomenon plays a central role for development, homoeostasis and tumor suppression. Apoptosis induction is triggered by two main ways: the intrinsic and extrinsic pathways (Iannolo, Conticello et al. 2008).

4.1.1. The intrinsic pathway

4.1.1.1. Generalities

The signaling pathway is activated in response to stress induced by oncogene-mediated DNA damage or lack of growth factors. These stresses cause disruption of the mitochondrial membrane and consequently activate the proapoptotic mitochondrial pathway. This activation involves the release of apoptotic factors, such as AIF (Apoptosis Inducing Factor), Smac /DIABLO (Second Mitochondria-derived Activation of Caspase /direct LAP

binding protein with low pI) and cytochrome C from the mitochondria to cytosol. For example, cytochrome C associates then with Apaf-1 (Apoptotic Protease-Activating Factor-1), leading to oligomeric apoptosome formation which then binds and cleaves procaspase 9. Finally activation of the intrinsic pathway will lead to DNA fragmentation and protein degradation by the effector caspases, such as caspase 3, 6 and 7 (corresponding to the caspase-dependent cell death or CDCD) (Fig.10).

The activity of the intrinsic pathway is controlled by a family of proteins, namely Bcl-2. These proteins include promoters (i.e. Bad, Bax, Bid) or suppressors (*i.e.* Bcl-2, Bcl-xL, p-Bad) of apoptosis (Iannolo, Conticello et al. 2008).

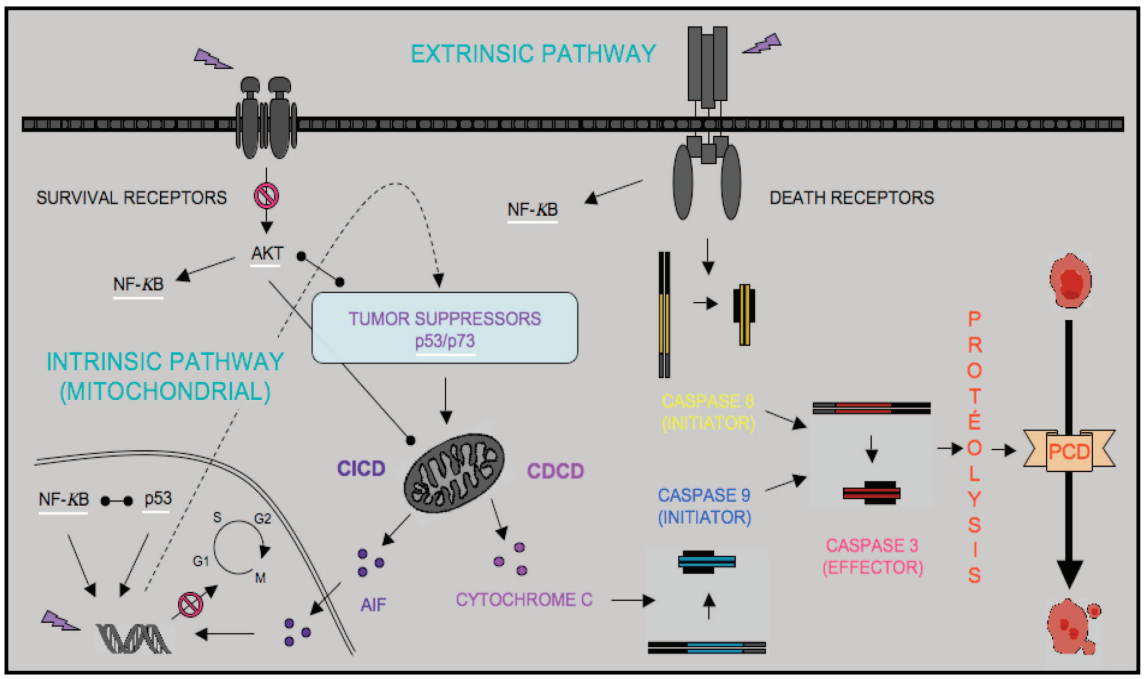

Fig. 10. Proapoptotic and prosurvival pathways that can be targeted by anticancer agents (Sharif Tanveer 2011).

4.1.1.2. Oxidative stress and tumor suppressors

Oxidative stress is reflecting the abundant generation of reactive oxygen species (ROS) into the cell. ROS consist of two groups, free radicals like superoxide and non-radicals such as hydrogen peroxide. Generation of ROS may cause mutations and favour carcinogenesis, and can increase proliferation. In contrast, they can also trigger a cascade of signals that induce cell death. ROS is mostly generated during the activation of the intrinsic

pathway. For example, treatment with anticancer drugs that act as pro-oxidants, induce DNA damage, leading to ROS formation, via the activation of PARP (ADP-ribose polymerase) or NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) (Mizutani 2007). When the DNA damage is severe and non-repairable, expression and activity of tumor suppressors, such as p53 and p73, will be increased, and consequently apoptosis will be induced, via the intrinsic pathway (Zhou, Ahn et al. 2001).

Unlike non-cancerous stem cells, CSCs have shown weak levels of intracellular ROS, due to low rate of oxygen in their niche; this partly explains the failure of apoptosis induction when they are exposed to radio- and some chemotherapy. On the other hand, CSCs exhibit high activity in DDR (DNA damage response) and, as a consequence, increased activity in DNA repair process. They can therefore escape from anticancer-induced ROS dependent cell death through their enhanced ability to repair damaged DNA (Liu and Wang 2015). It should be noted that the inactivation of tumor suppressor p53 in CSCs favours symmetric division and therefore contributes to tumor growth (Cicalese, Bonizzi et al. 2009). A link between mutation in p53 and drug resistance has also been observed (Shetzer, Solomon et al. 2014). Finally, targeting CSCs by pharmacological agents that are able to induce oxidative stress represents a promising strategy only in limited cases; for instance, these CSCs should express a functional p53 and high levels of ROS-induced oxidative stress are needed to overcome CSC resistance and reactivity.

4.1.1.3. p38 MAPK

Increasing evidences have highlighted the proapoptotic role of p38 MAPK. The protein promotes cell death by different ways. For example, p38 MAPK induces phosphorylation and translocation of the proapoptotic proteins of the Bcl-2 family to the mitochondria, resulting in a release of cytochrome C and finally induction of apoptosis via the intrinsic pathway (Lenassi and Plemenitaš 2006). p38 MAPK can also trigger apoptosis by overexpressing the GADD- inducible genes (Growth arrest and DNA damage) (Sarkar, Su et al. 2002).

p38 MAPK and its role in the biology of SCs and CSCs has been studied. Inhibition of this kinase promotes pluripotency by preventing differentiation. Accordingly, phosphorylation of p38 MAPK in SCs leads to a loss of their self-renewal capacity (Ito, Hirao et al. 2006) Actually, p38 MAPK has other functions; for instance, its activation suppresses the migration

of mesenchymal stem cells and prevents metastasis of breast cancer (Hong, Li et al. 2015). Regarding to these data, p38 MAPK could represent a promising therapeutic target which could affect the pathological behavior of CSCs.

4.1.2. The extrinsic pathway

The signaling pathway involves the death receptors that belong to the TNF (Tumor necrosis factor) receptor gene superfamily. These receptors have essential role in sustaining tissue homeostasis, particularly in the immune recognition. The intercellular domain of these receptors is known as death domain. There are several death receptors which, when activated by their corresponding ligands, can initiate extrinsic apoptosis including CD95 R/ CD95L, TRAIL-R1 and R2/ TRAIL-1and 2 ligands (TNF-Related Apoptosis-Inducing Ligand) and TNFα/ TNFRI (TNF receptor 1). For example, after CD95L binding to its receptor, the adaptor protein FADD (Fas-associated death domain) that resides in the cytosol, binds to the intercellular domain of CD95 receptor. The later then associates with and activates caspase 8, which in turn activates the downstream caspase cascade (Elmore 2007) (Fig. 10). Moreover, there are relationships between the intrinsic and extrinsic pathways. For instance, activation of the death receptor CD95 can stimulate the activity of NADPH oxidase, which will increase ROS production, and finally will induce apoptosis via caspase 8 and 9 (Kruyt and Schuringa 2010).

Most importantly, CSCs have shown their resistance to TRAIL-induced apoptosis because they express high levels of FLIP (FLICE-like inhibitory protein), an inhibitor of the death receptor-mediated apoptosis, CSCs are also known to express low levels of caspase 8 (Ning, Shu et al. 2013).

4.2. Targeting CSCs by differentiation induction

4.2.1. Targeting self-renewal signaling pathways

Many studies have shown that there is an inverse relationship between the differentiation level of CSCs and their resistance to therapy. Furthermore, the aggressiveness of CSCs is inversely correlated to their stage of differentiation (Al-Hajj, Becker et al. 2004). It has been observed that traditional therapies are efficient on differentiated cancer cells, while having no effect on their dedifferentiated counterparts. It is therefore expected that targeting

CSC by inducing their differentiation should alter their malignancy. Several pharmacological agents have been proposed to induce CSC specification. Retinoid derivatives, including ATRA (all-trans retinoic acid), are well-known examples. This compound successfully cure about 90% of patients with acute myelocytic leukemia. Alternatively, SAHA (Suberoylanilide hydroxamic acid), an inhibitor of HDAC, which targets tumor epigenetic changes is also identified as a strong differentiation inducer of many CSCs lines (Campos, Wan et al. 2010) .

Despite the efficiency of ATRA on leukemic patients, this prodifferentiating agent has limited effect on certain solid tumors. For example, several gliomas are resistant to ATRA, due to a high catabolism activity or to a lack of expression or affinity of its endogen receptor RARs (for instance, the retinoic acid receptor). Moreover, long term treatment with ATRA or SAHA have side effects, due to their cytotoxicity (Campos, Wan et al. 2010) (Xu, Zhang et al. 2014) (Ding, Zhang et al. 2015).

As yet, no ideal prodifferentiating agent has been identified, with efficient curative impact on CSCs, especially of solid tumors. It is expected that the therapeutic agents (alone or in combination) should target several signaling pathways in order to force CSCs to differentiate and to lose their self-renewal capacity (Fig. 11) (Han, Shi et al. 2013).

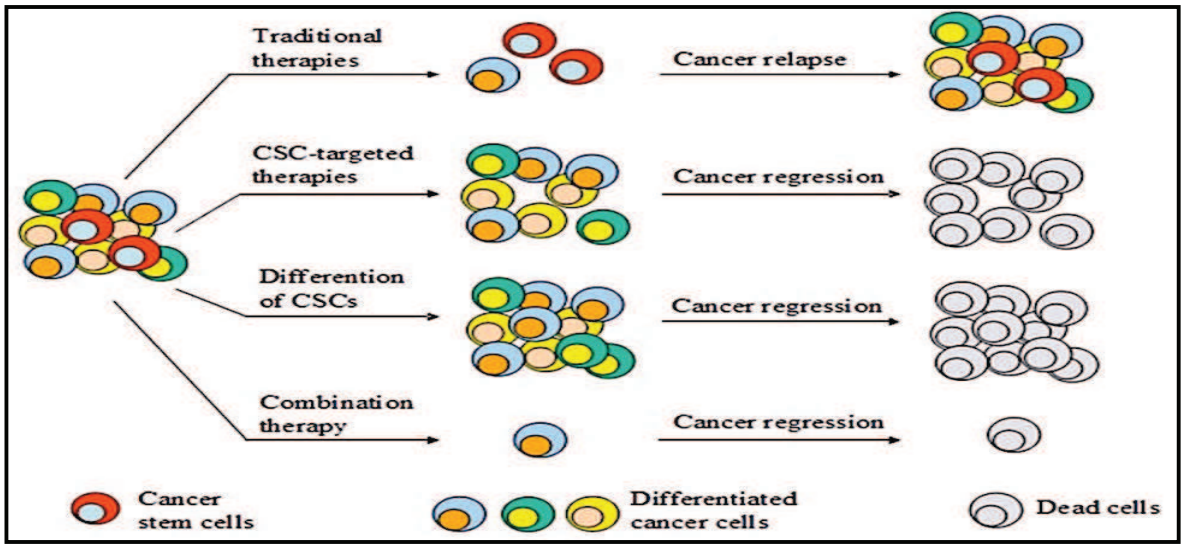

Figure 11. Therapeutic strategy to eliminate CSCs by anticancer agents. (Han, Shi et al. 2013).

4.2.2. Targeting Oct4 function in CSCs

Oct4,(as discussed in section 3.4.2.1), has been implicated in the growth, metastasis and drug resistance of many cancers. Therefore targeting its activity by pharmacological agents represents a promising anticancer approach. ATRA has been shown to exert its prodifferentiating effect by suppressing Oct4 gene expression. By extrapolation, pharmacophores of interest should be able to target selectively Oct4 network in CSCs, without having any effect on their normal counterparts (Fig. 12) (Sharif Tanveer 2011).

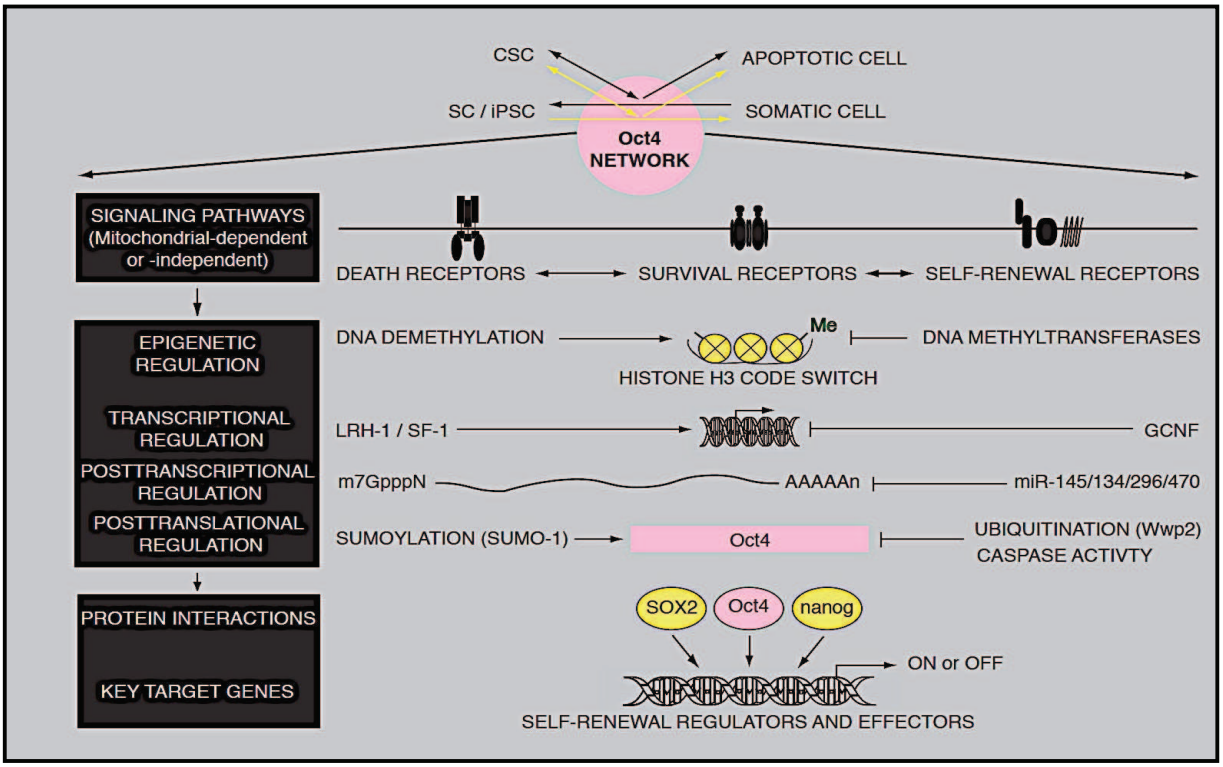

Fig. 12. Potential targets of the Oct4 network by anticancer drugs. (Sharif Tanveer 2011).

4.3. Targeting cell surface biomarkers of CSCs

Like normal SCs, CSCs have been identified and isolated by various cell surface markers, which could be interesting targets for anticancer drugs. For example, knocking down the expression of CD133 by siRNA of several types of cancer cells has led to a reduction of their growth and motility. On the other hand, the use of monoclonal antibodies against CD133 epitopes in melanoma cells has shown an anticancer effect. These data suggest therefore that cell surface biomarkers of CSCs might be potential therapeutic targets, to specifically eradicate them (Smith, Nesterova et al. 2008).

It has been reported that drug efflux pumps or ABC drug transporters are overexpressed in normal SCs, to protect them from toxic agents. This overexpression is also

characteristic of CSCs. Actually ABCG2, one of the ABC transporter family, is considered as the main marker to detect CSC side population. Interestingly, treatment of glioma cell lines with curcumin triggers a decrease of their CSC side population. Thus, inhibition of ABC transporters by small molecules could increase the efficiency of various anticancer drugs (Fong and Chan 2012).

4.4. Targeting miRNAs expression of CSCs

MicroRNAs are non-coding RNA molecules that consist of oligomers of 20-22 nucleotides and cause a post-transcriptional gene silencing. Recently, it has been demonstrated that the expression of specific miRNAs are dysregulated in several tumors. For instance, miRNA-34a, which targets mRNA of some oncogenes such as Notch-1 and -2 and CD44, is downregulated in various cancers (Guessous, Zhang et al. 2010) In contrast, other miRNAs, like miRNA-21 and -205, are overexpressed in specific tumors where they function as oncogenes and disrupt the mRNAs of tumor suppressors (Han, Shi et al. 2013). Furthermore, Delivery of miRNA-145 in Oct4-positive glioma cells suppresses Oct4 and Sox2 expressions and attenuates the number of CD133 positive cells, thereby inducing cell differentiation (Yang, Chien et al. 2012). All these data suggest that specific miRNAs are involved in tumor progression or regression and could served as anticancer agents that target CSCs.

4.5. Targeting CSC hypoxic niche

As discussed above, the microenvironment where stem cells reside, has an important role for their protection and their maintenance in a quiescent state. The location of CSCs in their niche provides low vascular support and hypoxic conditions. Actually hypoxic environment correlates with increased expression of HIF-1α and HIF-2α, due to the setting up of an anaerobic glycolysis. Both hypoxia-inducible factors are key inducers of the expression of stemness factors in various cancer cell lines derived either from brain, liver, lung, kidney, cervix, colon, prostate, or breast cancers (Mathieu, Zhang et al. 2011). Hypoxia can therefore be considered as a favoring factor that could allow a dedifferentiation process of cancer cells (Li, Zhou et al. 2013) (Santoyo-Ramos, Likhatcheva et al. 2014). While HIF-2α activity is restricted to the regulation of the expression of stemness factors, HIF-1α seems to have additional functions; it enhances the expression of target genes involved in drug resistance and angiogenesis, such as MDR1 or VEGF genes (Lu, Forbes et al. 2002) (Arsham, Plas et al.

2004) (Keith and Simon 2007). Therefore, targeting the hypoxic tumor microenvironment, and more specifically HIF-1α and HIF-2α, could influence or even block the factors that contributed to CSC development.

4.6. Targeting CSCs by inhibiting PI3K/Akt/mTOR/STAT3 survival pathway

One of the most important pathways of cell survival is the PI3K/Akt/mTOR pathway. Phosphorylated form of these proteins are involved in the inhibition of apoptosis, by inhibiting caspase activity and modulating the expression of the antiapoptotic proteins of Bcl-2 family and of specific pro-proliferative factors. Inactivation of the survival pathway triggers apoptosis by attenuating the expression of the antiapoptotic protein Mcl-1 (myeloid cell leukemia 1) and reducing the phosphorylation of proapoptotic protein Bim (Bender, Opel et al. 2011). Actually, PI3K kinase is linked to the intracellular portion of RTKs (receptor tyrosine kinases) and GPCRs (G protein-coupled receptors). Bindings of various growth factors on these receptors activate the PI3K/Akt cascade, which will trigger the activation of mTOR (mammalian target of rapamycin), and finally that of the transcription factor STAT3. This transcription factor has been considered as oncogene and its activation leads to a regulation of the expression of different survival factors, such as Bcl-xl, c-Myc and cyclin D1 (Martelli, Evangelisti et al. 2010) (Schroeder, Herrmann et al. 2014).

The PI3K/Akt/mTOR signaling pathway plays an important role in the survival of liver, glioblastoma and leukemia CSCs. Indeed, its inhibition abolishes several CSC properties. For instance, selective knock-down of STAT3 in glioblastoma cells is correlated with decreased tumorsphere growth and blocked of the self-renewal process (Yu, Lee et al. 2014). Interestingly, PI3K/Akt/mTOR pathway appears to mediate the radio- and chemo-resistance in many CSC types, by increasing the expression of drug resistance transporters, such as ABCG2. These effects are abolished in the presence of inhibitors of the different actors of this pathway (Martelli, Evangelisti et al. 2010). In addition, Akt has been identified as a direct regulator of Oct4 activity and correlates with the resistance to apoptosis and tumorigenic potential of CSCs (Lin, Yang et al. 2012) (Ghislin, Deshayes et al. 2012). Akt is therefore a promising target for anti-CSC treatment.

5. Flavaglines and cancer therapy

The use of medicinal plants, as traditional drug suppliers to cure various human diseases, has been reported since about 5000 years ago. From approximately 250,000 plant species, only several have been identified with specific bioactive substances (Hussain, Majeed et al. 2009). In the 19th century, the extraction, purification and identification of active compounds from plants attracted a large interest. Currently, despite the existing of new approaches that are used for therapy purposes, such as radiation administration, medicinal plants remain however a central source for new therapeutic agents against different human diseases (D. Brown 2006).

5.1. Generalities

Flavaglines are a family of natural substances that are extracted from traditional Asian plants of the genus *Aglaia.* In 1982, (King, Chiang et al. 1982) have identified and isolated the first flavagline with a cyclopenta [*b*] benzofuran skeleton, called rocaglamide 1. This compound has been described as a strong antileukemic agent. Subsequently, other cyclopenta [b] benzofurans have been isolated, such as rocaglaol and silvestrol (Fig. 13). However, the difficulty of extracting natural flavaglines has required the synthesis of chemical molecules with a similar structure and with the same benefit effect. Recent results have provided some important clues to improve their activity, through modifications of their chemical structures. For example flavagline 3 (FL3), the molecule that is used in all my studies, derived from rocagloal where one methoxy group is replaced by a bromide atom (Fig. 13). This formula enhances its cytotoxic activity against several cancer cell lines.

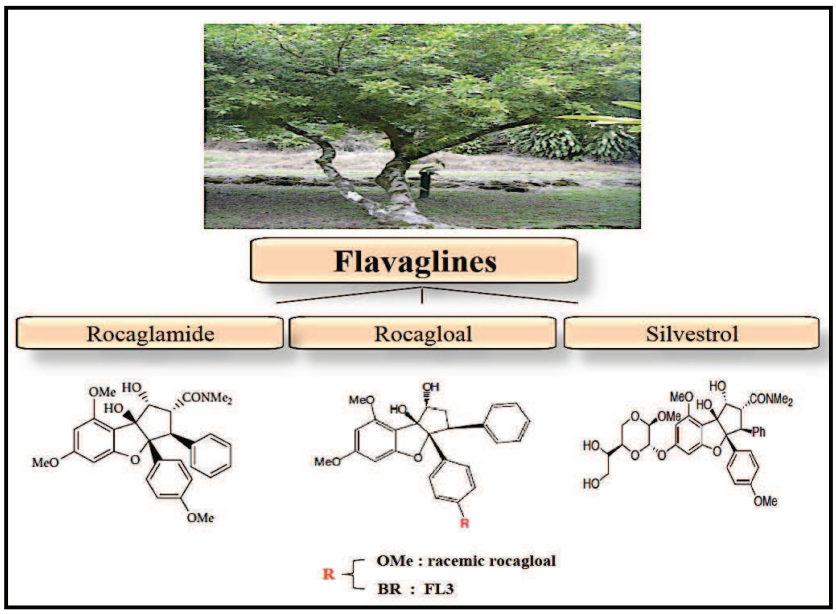

Fig.13.chemical structure of different flavagline compounds. Flavagline 3 is synthetized from rocaglaol after replacement of the methoxy group (OMe) by bromide (BR).

5.2. Biological activity

Many studies have examined the biological activity of different flavaglines. They exhibit anti fungal, insecticidal, anti-inflammatory and anticancer activities (Thuaud, Bernard et al. 2009). Here I will focus only on the anticancer activity of flavaglines.

5.2.1. Anticancer activity

Flavaglines have been shown to inhibit the proliferation of cancer cells in a nanomolar range, depending upon the compound or the studied cell line (Thuaud, Bernard et al. 2009). In vivo, flavaglines are able to reduce tumor growth of mouse lymphoma, after only 16 days of exposure. Interestingly, flavaglines show no toxicity towards many normal cells, either *in vitro* and *in vivo* as it will be discussed later. Actually, the anticancer activity of flavaglines involves several molecular mechanisms which do not seem to be linked.

5.2.1.1. Inhibiting translation

Translation inhibition has been described as a main mechanism by which flavaglines target cancer cells. Treatment of human lung carcinoma cells with 4′ -demethoxy-3′ , 4′ -methylenedioxy-methyl rocaglate leads to tumor inhibition, accompanied with an arrest of cell cycle in G1-phase and a strong inhibition of protein synthesis, suggesting that this compound is able to prevent the protein translation process (Lee, Cui et al. 1998). Accordingly, FL3 has been proved to bind to eIF4A (eukaryotic initiation translation factor-4A) and to inhibit its activity. This factor is required for the initiation of the translation, by favoring the binding of mRNAs to the ribosomal subunits 40S. More recently, it has been shown that FL3 and other flavagline derivatives can also inhibit the formation of the complex consisting of eIF4E, eIF4G and eIF4A. This complex, involved in the initiation of the protein translation, has been described as a main driver of resistance in BRAF-(V600)-mutant melanoma, thyroid and colon cancer cell lines to anti-BRAF drugs (Boussemart, Malka-Mahieu et al. 2014). Accordingly, FL3 -treated breast cancer cell line MCF7R, which is known to express P-glycoprotein (Also known MDR1), is able to overcome drug resistance (Thuaud, Bernard et al. 2009). This suggests that FL3, by interfering with the translation initiation process, can target various molecular mechanisms involved in the cell life.

5.2.1.2. Targeting prohibitins

One of the molecular targets of flavaglines is PHBs (prohibitins). PHB-1 and -2 have been involved in many signaling pathways that control cell proliferation, inflammation process and metabolism activity. PHBs are implicated in metastasis process of different cancers and in the resistance to apoptosis, by modulating the prosurvival Ras-C Raf-MEK-ERK signaling pathway. It has been shown in human T cell leukemia and Hela cells that flavaglines are direct targets of PHB1 and 2, by binding them and preventing their stimulatory effects on Ras-C Raf-MEK-ERK signaling pathway (Polier, Neumann et al. 2012).

5.2.1.3. Targeting apoptosis

Flavaglines have shown their potential to trigger programmed cell death via both the extrinsic and intrinsic pathways. Indeed, flavaglines reduce the expression of the major inhibitor of caspase 8, called c-FLIP, upregulate the expression of CD95L and sensitizes cancer cells to undergo apoptosis (Zhu, Giaisi et al. 2009). Flavaglines are also able to induce programmed cell death through the mitochondrial pathway. In a model of leukemia cell line, FL3 triggers apoptosis via the translocation of the apoptotic inducible factor AIF and caspase 12 to the nucleus (Thuaud, Bernard et al. 2009). Furthermore, flavaglines are able to induce a mitochondrial depolarization and a caspase-dependent cell death, through the activation of ERK (Extracellular-Signal-Regulated Kinases), JNK (c-Jun N-terminal Kinases) and p38 MAPK (Zhu, Lavrik et al. 2007). Moreover it has been observed in leukemia cells that rocaglamide reduces the levels of several antiapoptotic proteins, such as Bcl-2, Bcl-XL and Mcl-1. On the other hand, Flavaglines increase the activity of the tumor suppressor p53, via its phosphorylation, and decrease the expression of the oncogene c-Myc that is involved in the genesis of several human tumors (Callahan, Minhajuddin et al. 2014).

In vivo studies have shown that flavagline derivatives are able to reduce tumors after transplantation into mice. 4′ -demethoxy-3′ ,4′ -methylenedioxy-methyl rocaglate delays tumor growth of transplanted human breast cancer cell line when mice receive three times per week at10mg/kg body weight . 0,5mg/kg of silvestrol for 8 days is however enough to inhibit tumor growth (Ebada, Lajkiewicz et al. 2011). Transplantation of human primary acute myelogenous leukemia cells followed by silvestrol treatment at 0,75mg/kg every 5 days for a total period of 3 weeks also reduces tumor population in mice (Callahan, Minhajuddin et al.

2014). FL3 has also been found to reduce the tumor growth of engrafted mice with human melanoma cell lines when animals recevied 15mg/kg interaperitoneally injected each day for 13 days (Boussemart, Malka-Mahieu et al. 2014).

5.2.2. Cytoprotective activity

Dissimilar to other anticancer agents, flavaglines do not show any toxicity on various normal cells, such as normal hematopoietic and hematopoietic stem cells (HSCs), normal lymphocyte cells, human umbilical vein endothelial cells and intestinal epithelial cell line IEC18. Interestingly, flavaglines have also a cardioprotective and neuroprotective activity. For example, Administration of 100 nM or 0,1mg/kg of FL3 protects cardiomyoblasts against doxorubicin-induced toxicity in vitro and in vivo respectively. This protective effect is mediated by an activation of the HSP27 (heat shock protein- 27), a protein that plays an important role in cell protection against many injuries and stresses (Bernard, Ribeiro et al. 2011). Furthermore rocaglamide exhibits a neuroprotection activity both in vitro and in vivo. For instance, injection of 1mg/kg of rocaglamide in rats with traumatic brain injury (to induce an acute neurodegenerative condition) reduces the cerebral infarct volume. The neuroprotective effect of rocaglamide involves an inhibition of the activities of NF-kB (Nuclear factor –Kappa B) and AP-1 (Activator Protein-1), which both are known to be implicated in chronic neurodegenerative diseases (Fahrig, Gerlach et al. 2005).

Aims and objectives

Aims and objectives

There is no longer any doubt of the involvement of the cancer stem cell (CSC) in the emergence, metastasis and recurrence of cancer; this pathological cell shows deregulation of its self-renewal process which in turn allows unlimited division without the possibility to escape through a differentiation process.

Advances in cell-sorting technology enable the isolation, identification and characterization of CSCs in many cancers. In this point of view, we are focusing our study on a highly aggressive and poorly differentiated CSC, namely the embryonal carcinoma cell. This cell model is known to express the stemness marker Oct4 that is a major regulator of the maintenance of the self-renewal and cell pluripotency. Accordingly, recent studies have demonstrated that Oct4 expression is reactivated in many cancers and this expression is associated with tumor development, metastasis and resistance to radio- and chemotherapy.

Flavaglines are a family of natural cyclopenta [b] benzofurans compounds extracted from the plant genus *Aglaia.* Several members of this family, such as rocaglamide, rocaglaol and silvestrol have shown an anticancer activity in vivo and in vitro. We are focusing our study on a synthetic flavagline, named FL3, with exhibits enhanced cytotoxicity against several types of cancer cells.

The aim of this work is to examine the selective anticancer activity of FL3 on the pluripotent cancer stem-like cells and to identify the molecular mechanisms (in particular the signaling pathways) that underly this activity. It is expected that the pharmacological agent will be able to induce a proapoptotic process after a short-term treatment with high concentrations, while it should induce a prodifferentiation process after long-term treatment with low concentrations. Particular emphasis will be made on the study of the effects of FL3 on Oct4 expression, at both the transcriptional and translational levels. Indeed, it is assumed that a downregulation or suppression of Oct4 expression, after administration of the drug, should initiate either apoptosis or cell commitment. Such differentiation therapy would then represent a novel attractive tool for the reduction of CSC aggressiveness. On the other hand, the cellular and molecular effects of FL3 will also be studied on normal fibroblastic cells, which can be defined as normal stem-like cells with limited differentiation capacity. This final aspect of our study should highlight the selective activity of the synthetic flavagline which targets cancer SCs, while having no significant effect on normal SCs.

Results

Article1

Selective anticancer effects of a synthetic flavagline on human Oct4-expressing cancer stem-like cells via a p38 MAPK-dependent and caspase3-dependet pathway

The results obtained from this study have been published in Biochemical pharmacology (2014)

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Selective anticancer effects of a synthetic flavagline on human Oct4-expressing cancer stem-like cells via a p38 MAPK-dependent caspase-3-dependent pathway

Fathi Emhemmed [a,1], Sarah Ali Azouaou [a,1], Frédéric Thuaud [b], Valérie Schini-Kerth [a], Laurent Désaubry [b], Christian D. Muller [b], Guy Fuhrmann [a,*]

[a] UMR 7213 CNRS, Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France
[b] UMR 7200 CNRS, Laboratoire d'Innovation Thérapeutique, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

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ABSTRACT

Cancer stem cells (CSCs) are considered as the initiators of the carcinogenic process and are therefore emerging targets for innovative anticancer therapies. In order to evaluate the anticancer chemopreventive activity of flavagline derivatives, we used the pluripotent teratocarcinomal cell as a model of Oct4-expressing cancer stem-like cell and determined the underlying cellular and molecular mechanisms induced by a synthetic flavagline. We precisely investigated the effects of the flavagline derivative FL3 on the human embryonal carcinoma (EC) cell line NT2/D1 and compared the responses to those of a normal more restrictive pluripotent stem cell line (i.e. BJ fibroblast cell line). FL3 selectively inhibited the proliferation of NT2/D1 cells by inducing G_1 phase cell cycle arrest in a dose-dependent manner. Moreover, FL3 treatment specifically triggered apoptosis in association with an induction of the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and caspase-3 activation followed by a drastic downregulation of the master regulator of stemness Oct4. Forced inhibition of p38 MAPK activity by the specific pharmacological inhibitor SB203580 or by p38 MAPK gene knockdown using small-interfering RNA (siRNA) counteracted the effects of FL3, demonstrating that its chemopreventive action is related to growth inhibition and a p38-dependent caspase-3-dependent induction of apoptosis in Oct4-expressing CSCs. This study also shows that FL3 selectively kills poorly differentiated and highly aggressive carcinomal cells, but has little effect on normal stem-like cells. Thus FL3 offers great promise for cancer treatment since it is able to target the carcinogenic process without affecting normal cells.

1. Introduction

Cancer stem cells (CSCs) are considered to be responsible for tumor initiation and development, metastatic spreading, and resistance to radio- and chemo-therapies [1]. A large body of observations supports the 100 year old hypothesis which predicted a clonal genetic background of the heterogeneous cell population found in a tumor outgrowth [2]. Growing evidence suggests now that CSCs arise from embryonic, fetal or adult stem cells (SCs) exposed to repetitive mutation-inducing stress injuries [3]. CSCs could also arise, after additional oncogenic hits, from closely related dedifferentiated descendants which possess de facto higher lineage-specific competencies [4,5]. Such a hierarchical and dynamical relationship between these two cell type populations thus indicates that future anticancer treatments should not only target cancer cells, but also CSCs.

The chemotherapeutic properties of various pharmacological agents on different cancer cell types have been extensively studied over the past few decades [6,7]. By modulating specific signaling pathways, the anticancer compounds target different cell processes, including those leading to apoptotic or nonapoptotic cell death. However their effects on CSCs have only recently been accessible to experimentation with the development of new cell models. Accordingly, it is now accepted that serial xenotransplantation and tissue cultivation allows to recapture the malignant phenotype and to isolate CSCs from any tumor tissue [8]. In this point of view, we and others have suggested that the malignant counterparts of the embryonic stem cell lines, namely the embryonal

* Corresponding author.
E-mail address: guy.fuhrmann@unistra.fr (G. Fuhrmann).
[1] *Present address:* UMR 7200 CNRS, Laboratoire d'Innovation Thérapeutique, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France.

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carcinoma stem cell lines, could be suitable models of CSCs [8–10]. These cell lines are poorly differentiated pluripotent germinal stem cells with a highly aggressive malignant phenotype and could be used as surrogated investigational tools for the evaluation of potential anticancer chemopreventive agents. Alternatively, there is now growing evidence that CSCs can be isolated on the basis of their multidrug resistance (MDR) properties from numerous cell lines as side-populations [10–12] and could therefore be promising experimental models for carcinogenicity studies.

Flavaglines are a family of natural cyclopenta[*b*]benzofurans extracted from Asian plants of the genus *Aglaia*. The first described flavagline, rocaglamide, was discovered by King et al. [13] and found to exhibit strong antileukemic activity. Since then, more than 100 flavaglines, such as rocaglaol or silvestrol, have been characterized. Flavaglines selectively induce the death of cancer cells, without affecting non-cancerous cells, through a direct action on two apparently unrelated targets, the scaffold proteins prohibitins (PHB1 and PHB2) and the eukaryotic translation initiation factor 4A (eIF4A) [14]. In the course of our medicinal chemistry program, we have identified a synthetic flavagline, FL3, which displays enhanced cytotoxicity on cancer cells compared to natural flavaglines; in addition FL3 activity is unaffected by multidrug resistance [15]. Actually this flavagline has a similar structure than its parent compound rocaglaol, but one of the electron-donating methoxy has been replaced by a more lipophilic bromide [14,15].

The first aim of this work was to evaluate the in vitro anticancer properties of flavagline FL3 on human embryonal teratocarcinoma stem cells NT2/D1 (also known as NTERA-2 cl.D1). This cell line is described as a highly pluripotent undifferentiated cell line, a property associated to a strong expression of the stemness factor Oct4. The transcription factor is known to be essential for toti/pluripotency maintenance and self-renewal. Indeed, Oct4 interacts with the octamer motif ATGCAAAT (or certain variants) located at the promoters and/or regulatory regions of numerous target genes which are associated with proliferation and differentiation processes [16,17]. To achieve a higher specificity, Oct4 may form protein complexes with other transcriptional regulators, including the homeobox protein Nanog and the SRY-related HMG-box protein Sox2 [18]. It is now accepted that Oct4 is detectable in CSCs from diverse tumor origin [19] and its presence as a part of the pluripotency signature has been observed in poorly differentiated and highly aggressive cancers [10,20,21].

The second aim of the work is to evidence a selective effect of FL3 on CSCs with no activity on normal stem-like cells. Several studies have shown that pharmacological agents could interact with compounds of the cell culture media [9,22]. We therefore

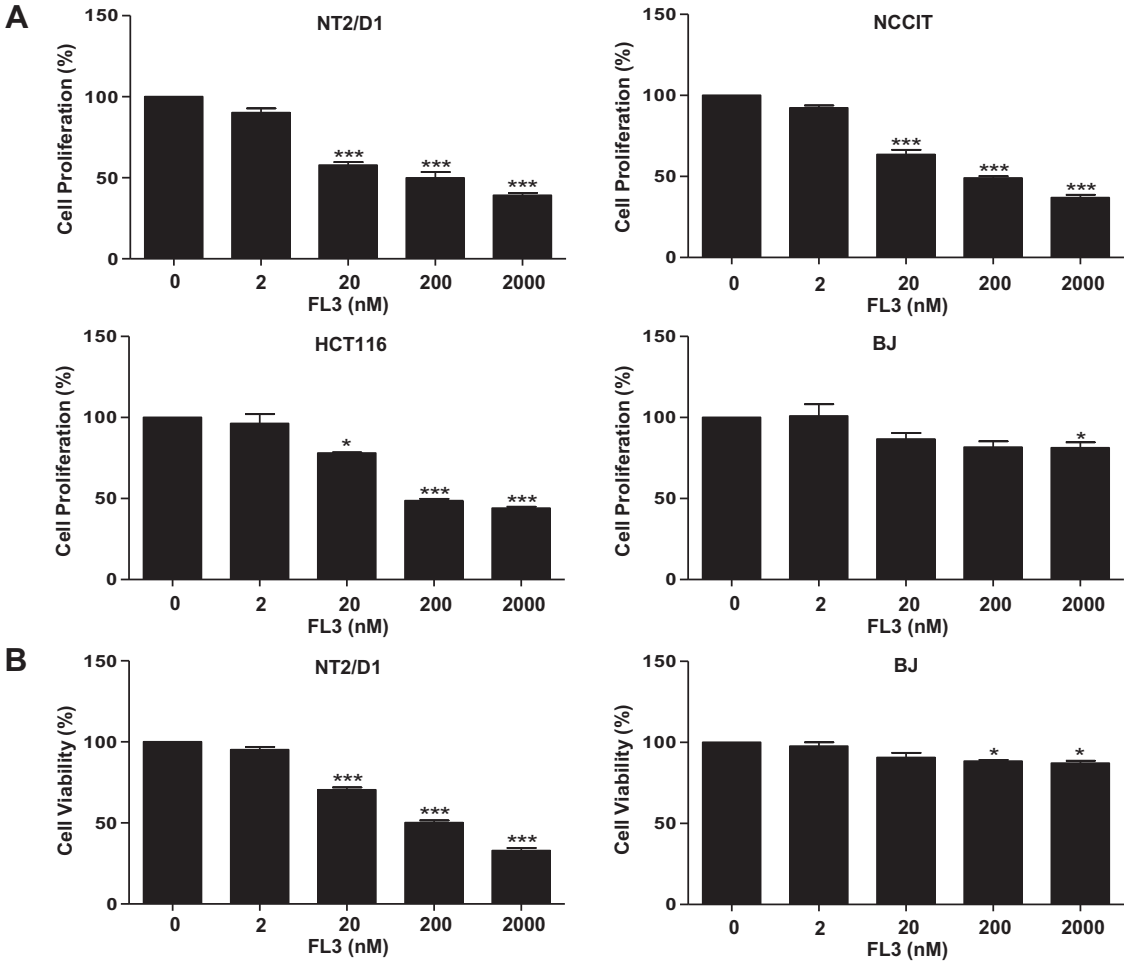

Fig. 1. Concentration-dependent cytotoxic effects of FL3 on different cell lines. NT2/D1, NCCIT, HCT116 or BJ cells were exposed to FL3 at the indicated concentrations and incubated for 24 h. (A) Cell proliferation rate was assessed by colorimetry using the MTS assay. (B) Cell viability rate was assessed by cell counting using trypan blue dye exclusion assay. The absolute value obtained for each FL3-treated sample is expressed in a second step as percent relative to the corresponding absolute value obtained for the untreated sample and set at 100%. Values are means ± S.E.M. of three independent experiments; statistically significant: * $p < 0.05$; *** $p < 0.001$ (versus the corresponding untreated group).

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focused on a precise hybrid media which can be used for both the EC cell lines and the most appropriate normal cell line counterpart, the fibroblastic BJ cell line. These cells have limited differentiation potential [9], but are now currently used for the generation of induced pluripotent stem cells [23].

Here we show that FL3 selectively kills pluripotent cancer stem-like cells but not normal stem-like cells. This chemopreventive effect involves a p38 MAPK-dependent caspase-3-dependent pro-apoptotic pathway, leading to a downregulation of the major gardian of a highly pluripotent cell state, namely Oct4. Our study therefore provides insights into the mechanisms controlling the selective cell reactivity after exposure to phamacological agents (e.g. flavaglines) with chemotherapeutic and chemopreventive properties.

2. Material and methods

2.1. Cell lines and culture conditions

NT2/D1 (CRL-1973), NCCIT (CRL-2073), HCT116 (CCL-247) and BJ (CRL-2522) cell lines, all purchased from ATCC (LGC Standards, Molsheim, France) were cultivated in the physiological nutrient-rich DMEM-based media (Sigma-Aldrich, Saint-Quentin Fallavier, France), supplemented with 10% (v/v) fetal bovine serum (Lonza, Verviers, Belgium), 2 mM glutamine, 50 μM non essential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich). Cells were grown in Petri dishes to 70–80% confluency prior to treatment. All plates were incubated in humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Cell treatment

FL3 was synthetized and diluted at 10 mM in DMSO [15]. For our purpose, cell lines were treated with the appropriate working concentrations of FL3 mixed with the cell culture medium. The highest concentration of DMSO (for treated and untreated cells) never exceeded 0.02% (v/v) to avoid side effects, like cell toxicity or induction of differentiation. Experiments using a specific inhibitor of caspase (Z-VAD-FMK from BD Biosciences Pharmingen, San Diego, CA, USA) or p38 MAPK (SB203580 from SelleckChem, Houston, TX, USA) were carried out according to the respective manufacturer's instructions. Transient transfections of p38 MAPK

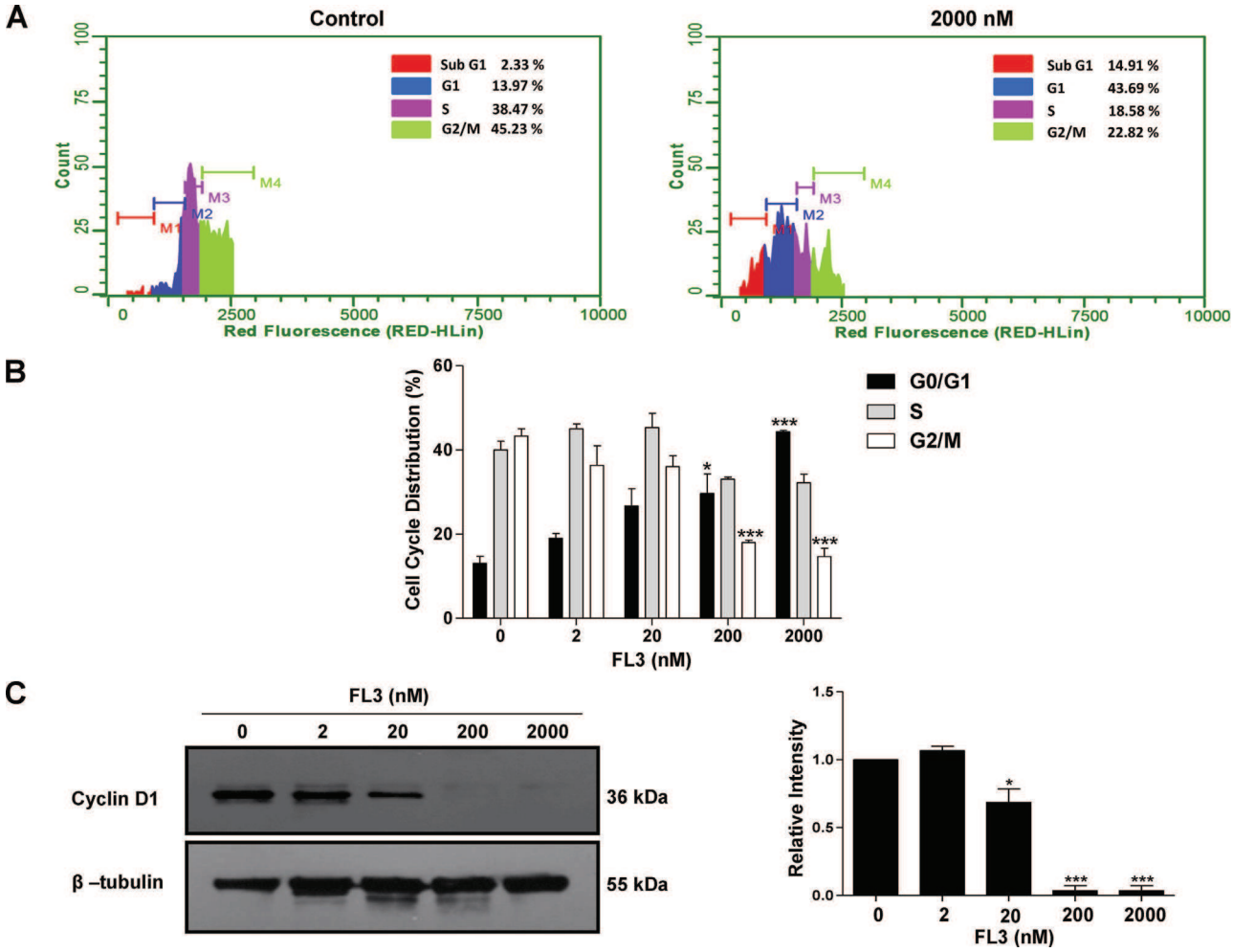

Fig. 2. Concentration-dependent effects of FL3 on the cell cycle progression of NT2/D1 cells. Cells were exposed to FL3 at the indicated concentrations and incubated for 24 h. Cell cycle distribution was assessed by a capillary cytometry detection assay. (A) shows representative DNA content histograms for treated (right panel) or untreated (left panel) cells. (B) recapitulates in a bar graph form the distribution of cells in G_0/G_1, S or G_2/M phase; the number of cells in each mitosis phase was determined and expressed as percent relative to the total cell number. (C) Immunoblotting analyses were performed as described in Section 2 with the corresponding antibodies. Specific bands were detected with their expected apparent molecular weight. The left panel shows representative immunoblotting results. The right panel shows densitometry results of protein expression normalized to β-tubulin expression and given as ratios relative to the value obtained for the untreated sample. Values are means ± S.E.M. of three independent experiments; statistically significant: * $p < 0.05$; *** $p < 0.001$ (versus the corresponding untreated group).

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siRNA (no. 6564; Cell Signaling Technology, Danvers, MA, USA) were performed with INTERFERin (Polyplus-transfection, Illkirch, France), following the manufacturer's recommendations.

2.3. MTS assay

Cells were seeded in triplicate on 96-multiwell plates at a density of 3×10^3 cells/well, grown for 24 h and exposed to FL3 at different concentrations for an additional 24 h. Cell proliferation rate was then assessed by colorimetric assay using the CellTiter 96 AQueous One® Solution Cell Proliferation Assay (MTS), following the manufacturer's recommendations (Promega, Charbonnières-les-Bains, France). Absorbance was measured at 490 nm on a multiwell ELISA plate reader. The percentage of living cell was calculated as a ratio of the OD value of each FL3-treated cell sample to the OD value of the corresponding control FL3 vehicle.

2.4. Trypan blue exclusion assay

Cells were seeded on 6-multiwell plates at a density of 5×10^3 cells/well, grown for 24 h, exposed to FL3 at different concentrations for an additional 24 h and then collected after trypsinization. Cell viability was determined by haemocytometric cell counting using the trypan blue exclusion method, according to the manufacturer's instructions (Sigma-Aldrich). The viability rate was obtained by dividing the number of trypan blue-negative cells by the total number of cells.

2.5. Cell cycle phase distribution analysis and quantitation of hypodiploid sub-G_0/G_1 cell population

Cells were cultured in 6-multiwell plates at a density of 2×10^5 cells/ml, grown for 24 h and exposed to FL3 at different concentrations for an additional 24 h. Cells were then washed with phosphate-buffered saline (PBS), resuspended in ethanol 70% and incubated for 1 h at 4 °C. After centrifugation at 200*g* for 10 min at 4 °C, cells were washed twice with PBS buffer. Cells were then resuspended in 500 μl PBS, 50 μg/ml of the DNA fluorochrome propidium iodide (Sigma-Aldrich) and RNase solution (Sigma-Aldrich) and kept in the dark at room temperature for 30 min. Cellular DNA content was then assessed by flow cytometry in a Guava EasyCyte Plus system (EMD Millipore, Billerica, MA, USA). A minimum of 20,000 cells were acquired per sample and the data were analyzed by using InCyte

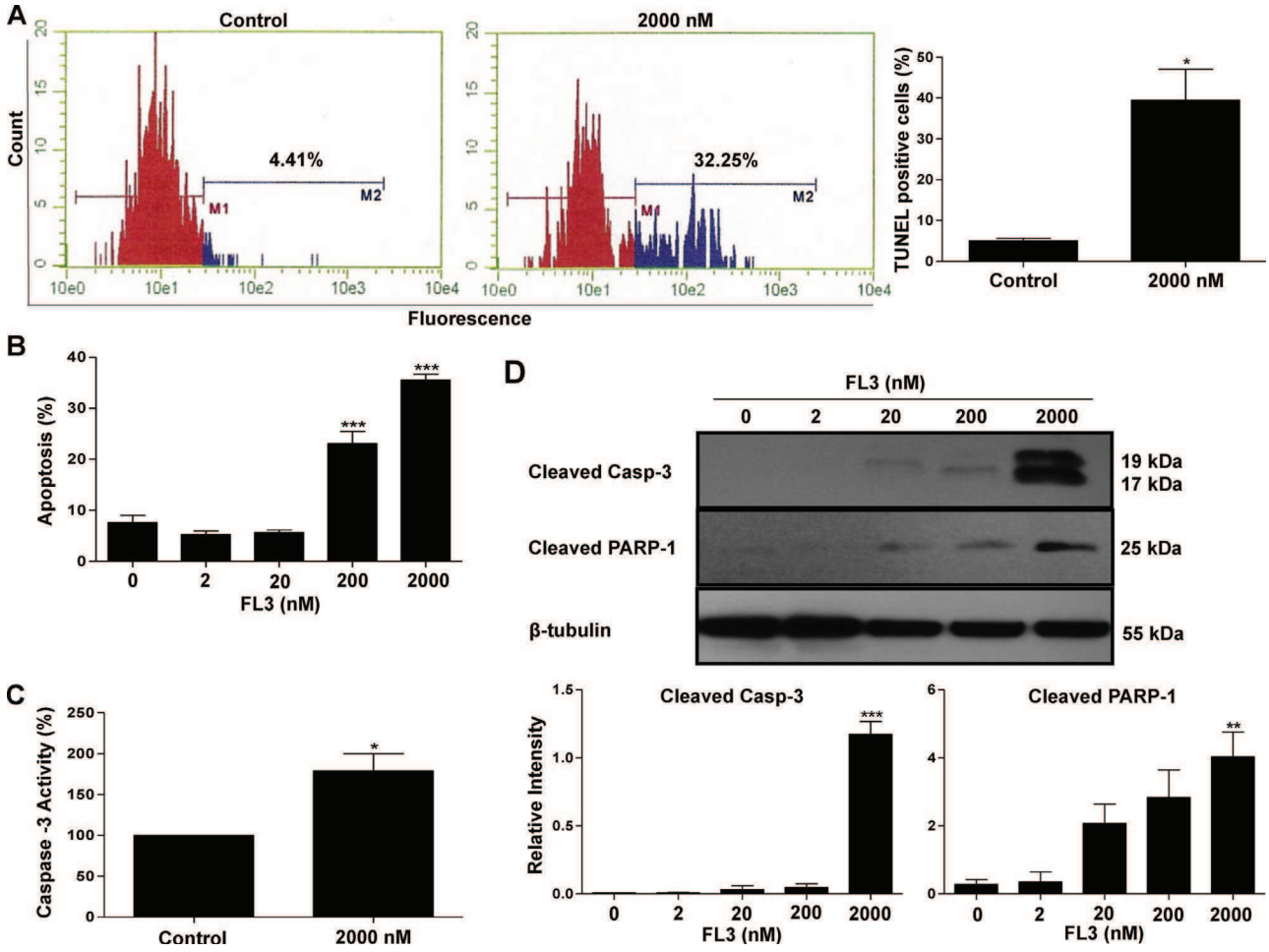

Fig. 3. Concentration-dependent effects of FL3 on the apoptosis rate of NT2/D1 cells. Cells were exposed to FL3 at the indicated concentrations and incubated for 24 h. (A) Cell apoptosis rate was assessed by capillary cytometry using the TUNEL staining assay. Representative histograms of TUNEL data are shown for treated (middle panel) and untreated (left panel) cells. The right panel recapitulates the rate of TUNEL-positive cells, expressed as percentage of total cell number. (B) Cell apoptosis rate was assessed by imaging cytometry using the Annexin V-FITC/PI staining assay. The number of apoptotic cells is expressed as percent relative to the total cell number. (C) Caspase-3 activation was detected by colorimetry using an in vitro caspase-3 activity assay. The panel shows in a bar graph form the absorbances at 405 nm, expressed as percent relative to the value obtained for the untreated sample. (D) Immunoblotting analyses were performed as explained in the legend of Fig. 2C. The upper panel shows representative immunoblotting results. The two sequential lower panels show densitometry results of cleaved caspase-3 and PARP-1 expression normalized to β-tubulin expression and given as ratios relative to the value obtained for the untreated sample. Values are means ± S.E.M. of three independent experiments; statistically significant: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (versus untreated group).

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software. The percentage of cells in G_0/G_1, S, G_2/M and sub-G_0/G_1 was determined from DNA content histograms.

2.6. Apoptosis rate analysis by TUNEL assay

Apoptosis was assessed by measuring the DNA fragmentation with a Terminal deoxy-transferase-mediated dUTP Nick-End Labeling (also termed TUNEL) kit. For that purpose, cells were cultured, treated with FL3 and collected as described above (see Section 2.4). Apoptosis rate was then evaluated by capillary cytometry (Guava EasyCyte Plus, EMD Millipore) using the "*In situ* cell death detection kit, Fluorescein" (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's recommendations. At least 10,000 events were recorded to assess the percentage of apoptotic cells.

2.7. Apoptosis rate analysis by annexin V assay

Apoptosis was checked by evaluating the externalization of phosphatidylserine and nucleus labeling of propidium iodide (PI). For that purpose, cells were cultured, treated with FL3 and collected as described above (see Section 2.4). Apoptosis rates were assessed either by imaging cytometry (Celigo, Cyntellect, San Diego, CA, USA) or flow cytometry (Guava EasyCyte Plus, EMD Millipore) using the Annexin V-FITC/PI Apoptosis Assay (BD Biosciences Pharmingen), according to the manufacturer's recommendations.

2.8. Western blot analysis

Exponentially growing cells were treated either with FL3 at different concentrations or with the vehicle and incubated for 24 h. Cells were then harvested and centrifuged at 200*g* for 10 min at room temperature; the pellets were resuspended in RIPA buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Sigma-Aldrich). Proteins from cell lysates were then extracted, separated on 10–15% SDS–polyacrylamide gels and transferred to membranes as previously described [24,25]. Immunoblotting was performed by using either a mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnology, Heidelberg, Germany), a mouse monoclonal anti-p73 antibody (BD Biosciences Pharmingen), a mouse monoclonal anti-cyclin D1 antibody (BD Biosciences Pharmingen), a rabbit monoclonal phospho-p38 MAPK antibody or a rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology), a rabbit monoclonal PARP-1 (Cleaved p25) antibody (Abcam, Paris, France), a rabbit polyclonal anti-Oct4 antibody (Active Motif, La Hulpe, Belgium), a rabbit polyclonal anti-Nanog or Sox2 antibody (GeneTex, Irvine, CA, USA), according to the manufacturer's instructions. Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Immunoreactive bands were detected using ECL chemiluminescence substrate solution (GE Healthcare Europe, Saclay, France). Membranes were stripped subsequently and reprobed with a mouse monoclonal anti-alpha tubulin antibody (GeneTex), a mouse polyclonal anti-beta tubulin antibody or a mouse monoclonal anti-beta actin antibody (Abcam). Autoradiographic signals were captured on a GeneGenius Bio Imaging System (Syngene, Cambridge, UK) using the GeneSnap software and analyzed by the NIH's Image J software.

2.9. Caspase-3 activity determination

Cells were cultured at a density of 5×10^5 cells/ml in 100 mm cell culture dishes, grown for 24 h and exposed to FL3 for an

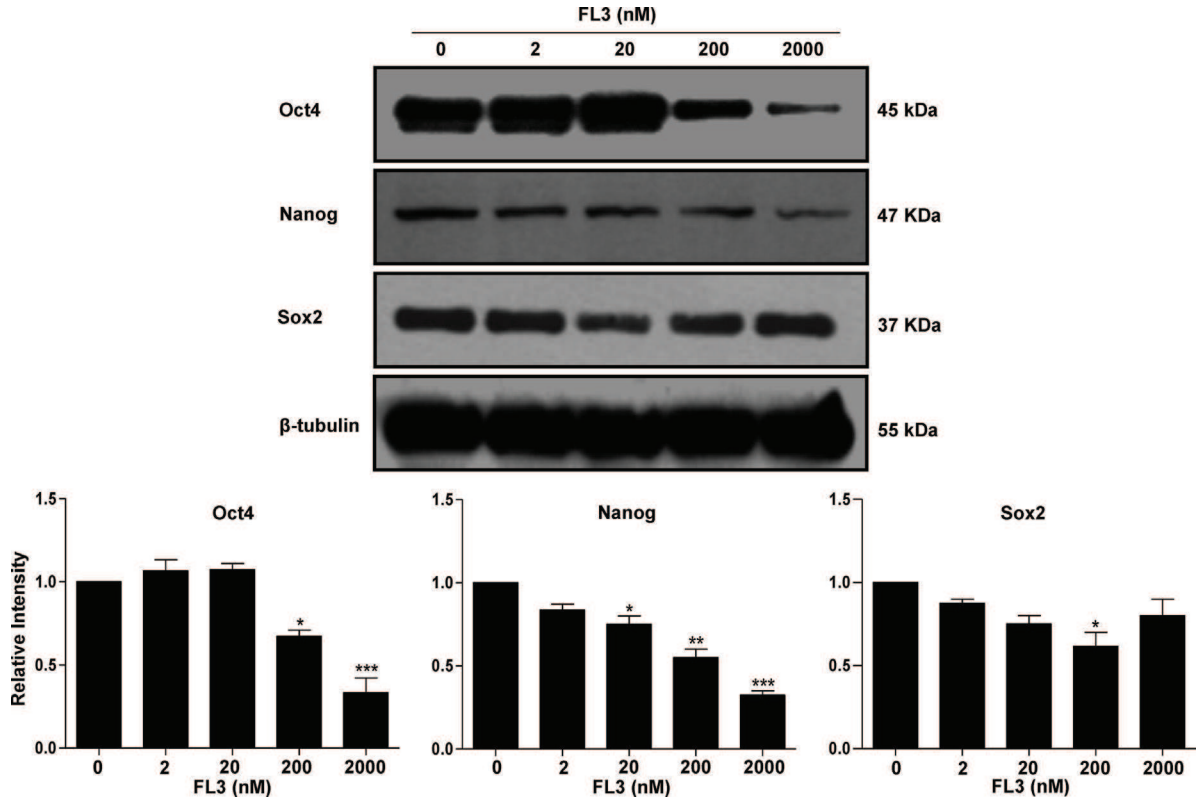

Fig. 4. Concentration-dependent effects of FL3 on the expression levels of the main stemness factors in NT2/D1 cells. Cells were exposed to FL3 at the indicated concentrations and incubated for 24 h. Immunoblotting analyses were performed as explained in the legend of Fig. 2C. The upper panel shows representative immunoblotting results. The three sequential lower panels show densitometry results of Oct4, Nanog and Sox2 expression normalized to β-tubulin expression and given as ratios relative to the value obtained for the untreated sample. Values are means ± S.E.M. of four independent experiments; statistically significant: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (versus untreated group).

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additional 24 h. After trypsinization, the cells were collected by centrifugation and resuspended in 500 μl of cell lysis buffer and incubated on ice for 10 min. After centrifugation for 5 min at 10,000 *g*, supernatant was transferred to a 96-multiwell plate and caspase-3 activity was determined by Chemicon's caspase-3 colorimetric activity assay kit (EMD Millipore), according to the manufacturer's recommendations.

2.10. Bioinformatic analysis

Potential caspase cleavage sites from primary substrate sequences were determined using the online prediction webserver Cascleave, freely accessible at http://sunflower.kuicr.kyoto-u.ac.jp/~sjn/Cascleave/[26].

2.11. Statistical analysis

Data are presented in a bar graph form and expressed as means ± S.E.M. of at least three independent experiments. Statistical evaluation was performed with either the one-way ANOVA test followed by Tukey's post hoc analysis or the Student's *t*-test using GraphPad Prism software (Prism version 5.04 for Windows, GraphPad Software, La Jolla, CA, USA); a p value less than 0.05 was considered as significant.

3. Results

3.1. FL3 selectively inhibits cell growth of cancer stem-like cells and cancer cells

Measuring the growth parameters of different cell lines after short-term administration first assessed the cytotoxic effects of FL3. Cell proliferation (Fig. 1A) following 24 h of FL3 treatment was decreased in a concentration-dependent manner in the teratocarcinomal stem-like cell lines NT2/D1 and NCCIT, and the colon carcinoma cell line HCT 116. In our experimental conditions, extrapolated calculated concentrations of FL3-induced half-maximal effects on cell proliferation were, respectively, of 152 ± 21 nM, 189 ± 10 nM and 294 ± 14 nM for NT2/D1, NCCIT and HCT116 cells. When treated with FL3 for 48 h, the IC_{50} value for NT2/D1 cells dropped to 30 ± 9 nM (data not shown). These results indicate that CSCs and cancer cells optimally respond to FL3 within 24 h and at a nanomolar concentration of one to two decimal places. In contrast, proliferation of the fibroblast cell line BJ was only slightly affected by FL3 and only when administered at high concentrations (Fig. 1A). Since both cancer stem-like cell lines showed a similar decrease in growth rate when exposed to FL3, we chose the teratocarcinomal NT2/D1 cell line as the referential model for the next steps of our work.

Cell viability (Fig. 1B) following 24 h of FL3 treatment was decreased in a concentration-dependent manner in the teratocarcinomal stem-like cell line NT2/D1. In our experimental conditions, a calculated concentration of FL3-induced half-maximal effects on cell viability was of 220 ± 30 nM. These results indicate that FL3 acts on the two growth parameters (i.e. cell proliferation and cell viability) of cancer stem-like cells in a similar fashion. Accordingly, cell viability of BJ fibroblasts was slightly altered by the drug but only when given at high concentrations (Fig. 1B).

3.2. FL3 induces cell cycle arrest of cancer stem-like cells

Since cell growth is a result of the progression of the cells through the different phases of the cell cycle, we next determined the effects of FL3 on the cell cycle distribution in the teratocarcinomal cancer stem-like cell line NT2/D1 (Fig. 2A). Slight modifications were already detectable at 20 nM of FL3, but a significant accumulation of the cells in G_0/G_1 phase was observed at 200 nM and 2000 nM (Fig. 2B), concomitantly with the appearance of a substantial sub-G_0/G_1 cell population (Fig. 2A). It appears therefore that FL3 is able to inhibit within 24 h the growth of teratocarcinomal cells by promoting cell cycle arrest at G_1 phase.

Inhibition of cell cycle progression from G_0/G_1 to S phase is known to be associated with decreased expression of cyclin D1 [27]. Accordingly we observed a sharp down-regulation of the expression of this protein (Fig. 2C) at concentrations of FL3 which inhibit cell growth.

3.3. FL3 induces apoptosis of cancer stem-like cells

As mentioned above, increased number of hypodiploid sub-G_0/G_1 cells could be observed when teratocarcinomal cancer stem-like

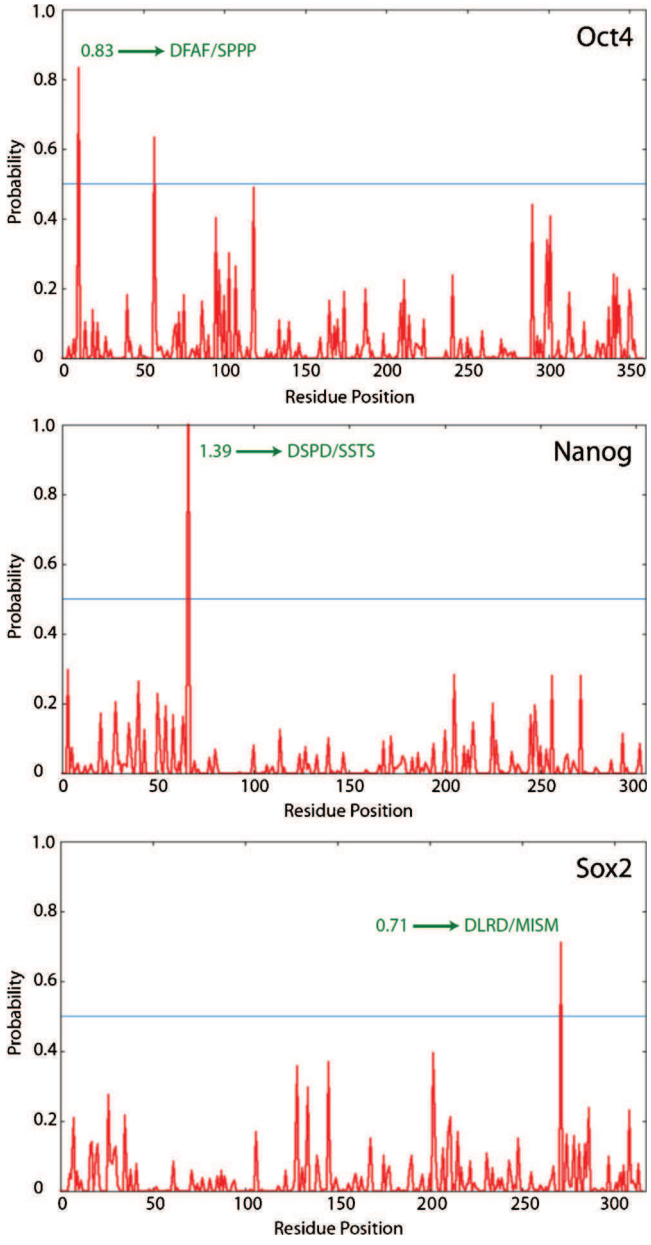

Fig. 5. Potential caspase cleavage sites in the primary sequences of the main stemness factors. The different histograms depict the propensity score assigned to each amino acid residue of Oct4, Nanog and Sox2 sequences. A threshold value of 0.5 to emphasize a positive cleavage site prediction is denoted by a blue solid line. Each substrate has one potential cleavage site (denoted in green); Sox2 has the lowest score. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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cells NT2/D1 were exposed to FL3 for 24 h. We therefore investigated whether FL3 induces apoptosis. Accordingly, TUNEL-positive cells were detected at high concentrations of the drug (Fig. 3A), suggesting that a pro-apoptotic event, namely DNA laddering, occurs. Actually, 24% and 35% of Annexin V-FITC positive cells were observed after FL3 treatment at 200 and 2000 nM, respectively (Fig. 3B). These data, consistent with those obtained by the TUNEL assays, suggest an induction of DNA fragmentation in teratocarcinomal cancer stem-like cells, when exposed to the flavagline. In agreement with the appearance of this hallmark of apoptotic cell death, we noticed that FL3-treated cells at 2000 nM showed an 75% increase of caspase-3 activity when compared to untreated cells (Fig. 3C). Moreover caspase-3 cleaved subunits, as well as cleaved PARP-1 (Poly ADP-Ribose Polymerase type 1) could be detected when cells were exposed to the flavagline (Fig. 3D). It is noteworthy to mention that the appearance of active caspase-3 could also be observed in NCCIT cells following FL3 treatment (data not shown). All these results suggest therefore the occurrence of a caspase-3-dependent proapoptotic process in the teratocarcinomal cell after exposition to the drug.

3.4. FL3 down-regulates the expression of stemness factors in cancer stem-like cells

Oct4 plays a critical role for the maintenance of cell toti/pluripotency and self-renewal [16,28]. Increased activity is acquired if Oct4 can interacts with two additional transcription factors, Nanog and Sox2 [18]. There are several lines of evidences that induced apoptosis in toti/pluripotent normal or potential cancer stem-like cells is accompanied by a down-regulation of Oct4 [9,10,21]. We therefore analyzed the expression levels of Oct4, Nanog and Sox2 during FL3-induced apoptosis of teratocarcinomal stem-like cells. As shown in Fig. 4, NT2/D1 cells treated with 200 nM of FL3 exhibited decreased Oct4 and Nanog levels, whereas at higher concentrations, only faint bands could be visualized on the different Oct4 and Nanog immunoblots. In contrast, a down-up expression pattern was recorded for Sox2 when cells were exposed to increasing doses of FL3. It should be noted that we could also observed a strong decrease of Oct4 expression in flavagline-treated NCCIT cells (data not shown). Taken together, these results indicate that FL3 kills teratocarcinomal cells by a pro-apoptotic process which is accompanied by a selective downregulation of core regulators (i.e. Oct4 and Nanog) involved in maintaining a stemness state.

3.5. FL3 induces apoptosis in cancer stem-like cells via a caspase-dependent mechanism

We suspected that FL3, at a concentration corresponding at least to its half-maximal effects, could trigger apoptosis in teratocarcinomal cells by a caspase-3-dependent pathway which selectively leads to the proteolytic degradation of Oct4 and Nanog, but not that of Sox2. Accordingly, in silico analysis of caspase cleavage sites from primary sequences showed with more than

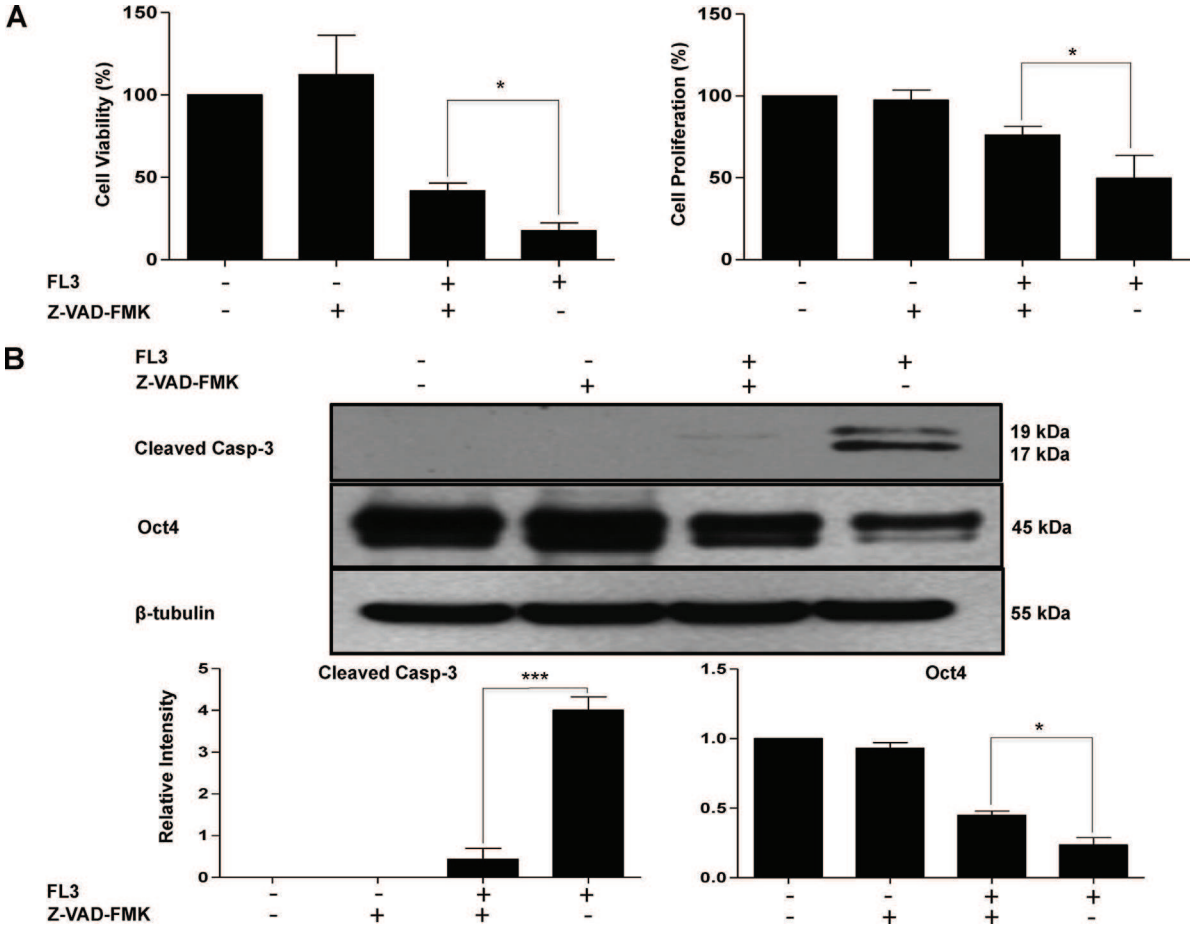

Fig. 6. Rescue effects of a treatment with a pan-caspase inhibitor on FL3-induced inhibition of NT2/D1 cell growth. Cells were treated or not with 20 μM of the caspase inhibitor Z-VAD-FMK for 2 h and then grown for further 24 h in the presence or absence of 2000 nM of FL3. (A) shows the cell viability (left panel) and cell proliferation (right panel) rates, as explained in the legend of Fig. 1, after the different treatments. (B) shows representative immunoblotting results for cleaved caspase-3, Oct4 and β-tubulin expressions (upper panel), and normalized densitometry results for cleaved caspase-3 and Oct4 expressions (lower panels), as explained in the legend of Fig. 2C. Values are means ± S.E.M. of three independent experiments; statistically significant: * $p < 0.05$; *** $p < 0.001$.

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80% of accuracy that the stemness factors Oct4 and Nanog could be potentially cleaved by the cysteine-aspartic acid proteases (Fig. 5).

We further examined the effects of a caspase inhibitor on FL3 activity in teratocarcinomal cells. As shown in Fig. 6A, the pan-caspase inhibitor Z-VAD-FMK was able to partially countervail the inhibition of cell viability and proliferation triggered by FL3 at 2000 nM. Moreover, as expected, the decrease of caspase-3 expression after exposure of FL3-treated cells to the caspase inhibitor was accompanied by a further sustained expression of Oct4 (Fig. 6B). The results suggest therefore that FL3 activation of the caspase-dependent pathway in cancer stem-like cells induces Oct4 down-regulation and the observed apoptosis.

3.6. FL3 induces apoptosis in cancer stem-like cells via a p38 MAPK-dependent signaling pathway

In order to define the nature of the cell cycle checkpoint signaling pathway which could be activated in response to FL3-induced apoptosis, we first examined the expression status of p53 and p73. A concentration-dependent decrease of the expression levels of the two proteins could be observed in the teratocarcinomal cells, when exposed to the drug (Fig. 7A). This suggests that the tumor suppressors p53 and p73 are not the upstream candidates of the caspase-dependent proapoptotic pathway activated by FL3.p38 MAPK acts as a regulator of caspase activities in many cell death models [29,30]. We therefore analyzed the expression levels of the MAPK during FL3-induced apoptosis of teratocarcinomal stem-like cells. As shown in Fig. 7B, phosphorylated p38 MAPK remained undetectable in NT2/D1 cells at low drug concentrations, whereas at higher concentrations (i.e. above the half-maximal effects), increased levels could be observed. These results suggest therefore the occurrence of a p38-dependent proapoptotic process in the cancer stem-like cells, when exposed to FL3.

To determine whether p38 MAPK could act in NT2/D1 cells as an upstream regulator of the caspase-3-dependent pro-apoptotic signaling pathway activated by FL3, we attempted a reversible inhibition of its activity by using the p38 MAPK inhibitor SB203580. As shown in Fig. 8A, this compound partially counterbalanced the inhibitory effects of FL3 on both cell viability and proliferation. Furthermore concomitantly to a decrease of FL3-induced phospho-p38 MAPK expression, the p38 MAPK inhibitor triggered a downregulation of the expression of the active cleaved caspase-3. As expected, these modifications were accompanied by an accumulation of Oct4 (Fig. 8B). Accordingly, siRNA targeted against p38 MAPK transcripts could rescue FL3-treated teratocarcinomal cells from apoptosis (Fig. 9A and B); moreover, p38 MAPK transient gene knockdown alleviated the overexpression of cleaved caspase-3 and the downregulation of Oct4 induced by FL3, concomitantly with a disappearance of p38 MAPK (Fig. 9C and D). All these findings demonstrate that the cytotoxic activity of FL3 on

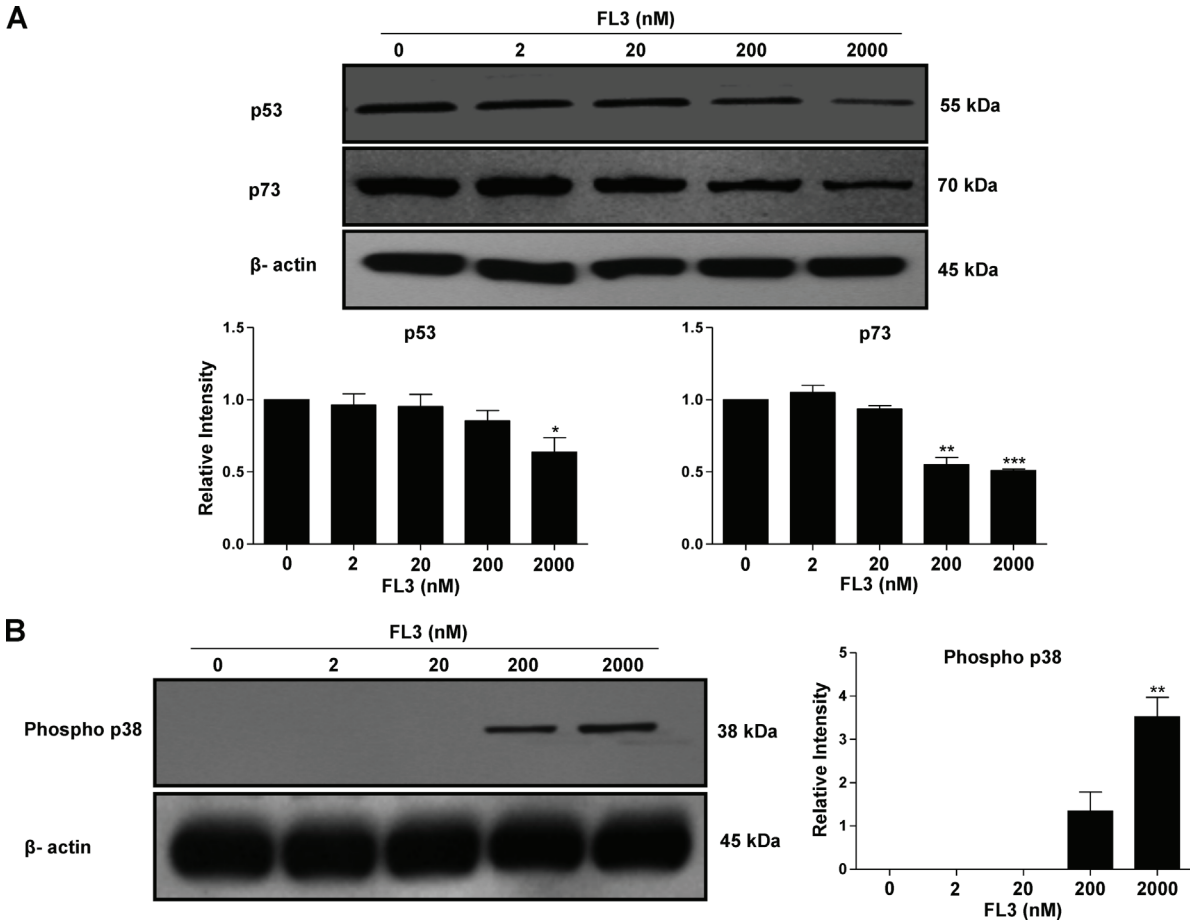

Fig. 7. Concentration-dependent effects of FL3 on the expression levels of tumor suppressors in NT2/D1 cells. Cells were exposed to FL3 at the indicated concentrations and incubated for 24 h. Immunoblotting analyses were performed as explained in the legend of Fig. 2C. (A) shows representative immunoblotting (upper panel) and normalized densitometry (lower panels) results for p53 and p73. (B) shows representative immunoblotting (left panel) and normalized densitometry (right panel) results for phosphorylated p38 MAPK. β-actin was used for the normalization of the expression levels of the different proteins. Values are means ± S.E.M. of three independent experiments; statistically significant: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (versus untreated group).

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cancer stem-like cells involves a p38 MAPK-dependent caspase-3-dependent pathway which likely leads to the proteolytic downregulation of the stemness factor Oct4 and the promotion of apoptosis.

4. Discussion

It has been reported that the cytotoxic effect of flavaglines on cancer cells involves the release of apoptosis-related mitochondrial-associated proteins into the cytoplasm [31]. Accordingly, the pro-apoptotic activity of FL3 implicates a mitochondrio-nuclear translocation of the caspase-independent death effector AIF which leads to DNA fragmentation and death of cancer cells [15]. In CSCs however, the molecular mechanisms involved in flavagline-induced reactivity are not yet documented.

The present study demonstrates that the flavagline FL3 triggers apoptosis in teratocarcinomal cancer stem-like cells through the activation of p38 MAPK and consequently of caspase-3. Actually, DNA damage-induced activation of a precise proapoptotic pathway is known to be cell type- and genotoxin-specific, depending amongst others on the p53/p73 status, the death receptor reactivity or MAPK responsiveness [30]. It is therefore not surprising that cancer cells and CSCs respond in a divergent manner (e.g. caspase involvement) to a specific pharmacophore (like flavagline) since the molecular determinants of their reactivity are different. Indeed, stemness factors-expressing cancer cells have a complex pathogenesis with numerous crosstalks between the different signaling pathways, including Wnt/β-catenin, Notch, Hedgehog and the "classical" proapoptotic pathways [10]. Such redundancy and crosstalk between the signal transduction pathways are known to trigger a drug-related regulation of various effectors and this could even negate the effect of a drug on a single downstream target [32]. Moreover, it is expected that drugs, like flavagline derivatives, could act as multi-target agents able to modulate, depending on their concentration and their contact time, the activity of specific components of the prosurvival or self-renewal machineries. Accordingly, it has been recently demonstrated that a drug, alone or in combination, can induce a dynamic rewiring of the signaling pathways depending on the order and duration of drug exposure [33]. All this explains why a specific pharmacological agent can have different molecular effects, although leading to a same cellular response, e.g. apoptosis.

The cytotoxicity of a pharmacological agent on CSCs and cancer cells, but not on normal SCs and normal cells, provides a promising opportunity for a chemotherapy with limited side effects. As FL3 has a strong cytotoxic effect on CSCs, the selectivity of its activity needed to be clarified. To address this question, we studied the effects of the flavagline derivative on a fibroblast cell line, a model of normal SC line with restricted pluripotency and enhanced reprogramming capacity; as expected, the results showed that high doses of FL3 which were able to strongly decrease the viability of cancer cells (HCT116) and cancer stem-like cells (NT2/D1 and NCCIT cells), had no obvious toxic effect on normal SCs. Indeed our data revealed that FL3 treatment, in a reasonable range of concentrations, had no significant effect on the proliferation of BJ cells. Annexin V assay confirmed that the drug, even at high

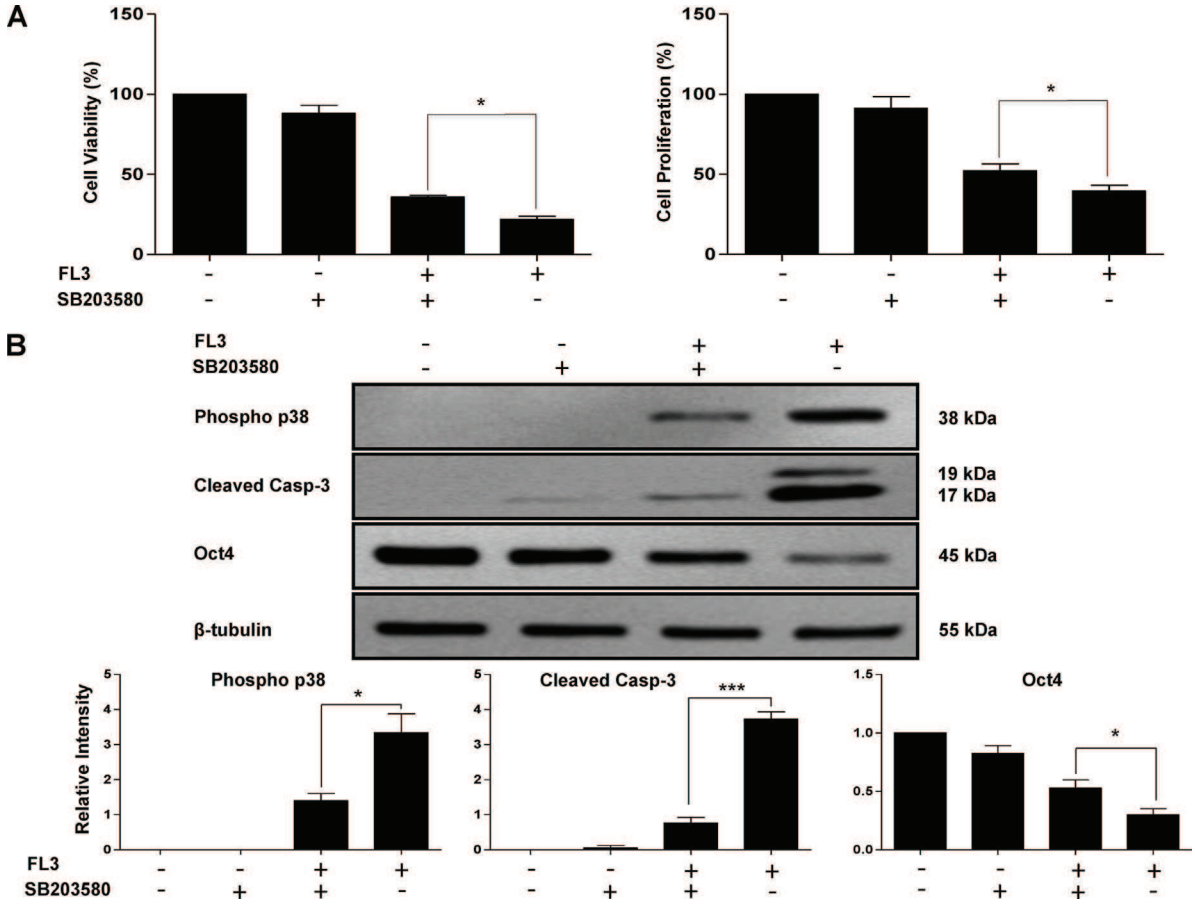

Fig. 8. Rescue effects of a treatment with an inhibitor of p38 MAPK activity on FL3-induced inhibition of NT2/D1 cell growth. Cells were treated or not with 20 μM of the p38 MAPK inhibitor SB203580 for 3 h and then grown for further 24 h in the presence or absence of 2000 nM of FL3. (A) shows the cell viability (left panel) and cell proliferation (right panel) rates, as explained in the legend of Fig. 1, after the different treatments. (B) shows representative immunoblotting results (upper panel), and normalized densitometry results (lower panels) for phosphorylated p38 MAPK, cleaved caspase-3 and Oct4 expressions, as explained in the legend of Fig. 2C. β-tubulin was used for the normalization of the expression levels of the different proteins. Values are means ± S.E.M. of three independent experiments; statistically significant: $^{*}p < 0.05$; $^{***}p < 0.001$.

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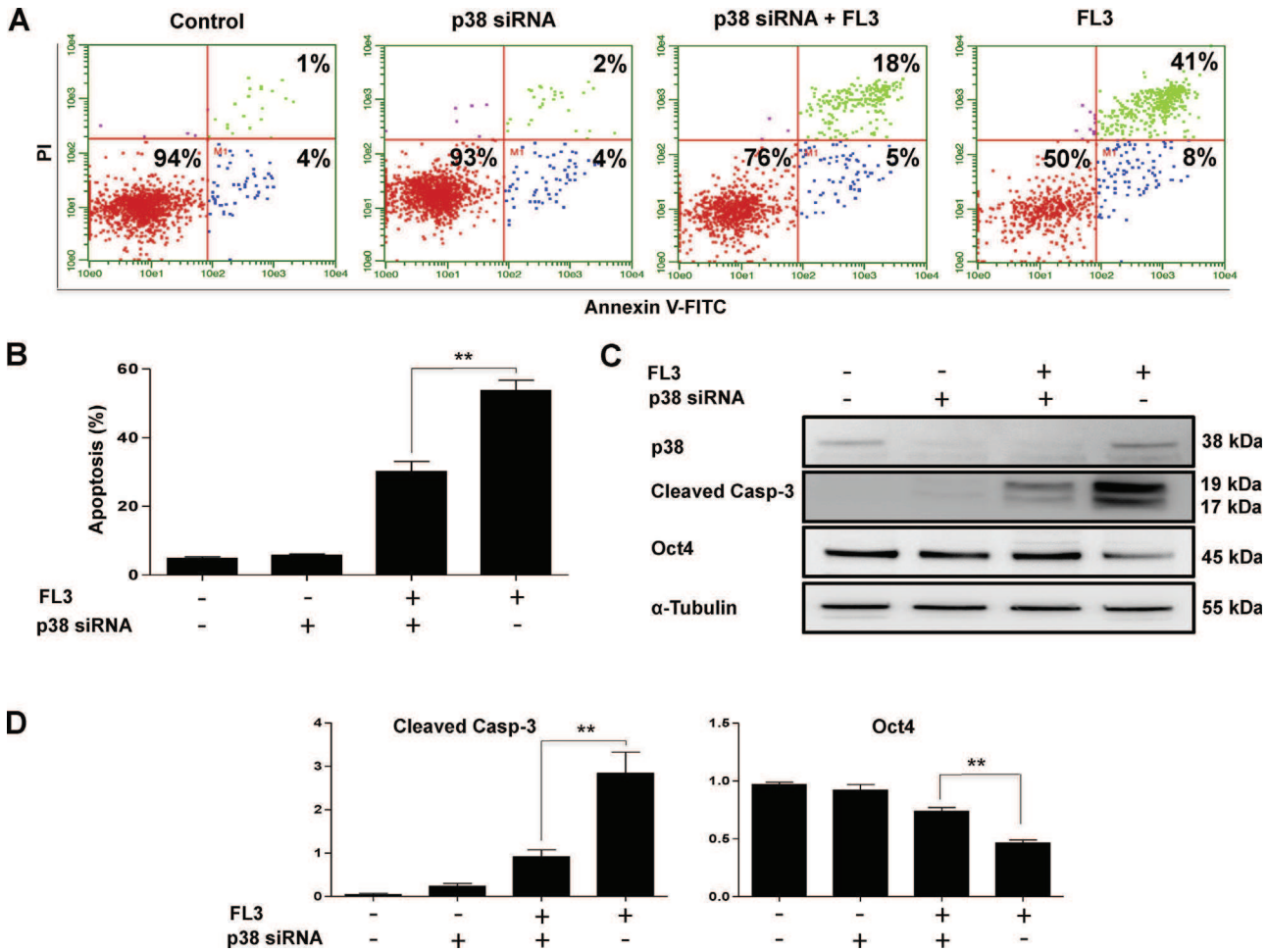

Fig. 9. Rescue effects of p38 MAPK post-transcriptional gene silencing on FL3-induced inhibition of NT2/D1 cell growth. Cells were transfected or not with 100 nM of p38 MAPK siRNA for 48 h and grown for further 24 h in the presence or absence of 2000 nM of FL3. Cell apoptosis rate was then assessed by flow cytometry using the Annexin V-FITC/PI staining assay. Expression levels of proteins were determined as described in Section 2. (A) shows the results of representative scatter plots obtained after the different treatments; cells of the lower left quadrant are viable; cells of the upper and lower right quadrants are in late and early apoptosis, respectively. The number of cells in the different states, expressed as percent relative to the total cell number, is indicated. (B) recapitulates in a bar graph form the percentage of cells undergoing apoptosis after the different treatments. (C) shows representative immunoblotting for p38 MAPK, cleaved caspase-3 and Oct4 expressions. (D) shows normalized densitometry results for cleaved caspase-3 and Oct4 expressions, as explained in the legend of Fig. 2C. α-Tubulin was used for the normalization of the expression levels of the different proteins. Values are means ± S.E.M. of three independent experiments; statistically significant: ** $p < 0.01$.

concentration, had no effect on fibroblasts (data not shown). This selective pro-apoptotic effect of FL3 on CSCs and cancer cells, but not on normal SCs and normal cells, is still largely unexplained. It is known that cells with an oncogenic phenotype have increased anabolic demands which need high translational flux to support enhanced ribosome biogenesis. Accordingly, rocaglamide has recently been identified as a strong inhibitor of the activity of the heat-shock transcription factor HSF1 which is known to be a key effector of protein translation activation in cancer cells [34]. This suggests that FL3 could act in a similar manner on cells with cancer-associated genetic aberrations, like CSCs, by modifying the protein translation activity and targeting, along with prohibitins, the eukaryotic initiation factor-4A, as it was suggested previously [15]. As a consequence, it could be hypothesized that FL3, like rocaglamide, triggers a repression of the activity of HSF1 which is a prime transducer of the translational flux specifically accelerated in the malignant state.

The present findings show that FL3 modulates the activity of p38 MAPK, a critical intermediate of the stress-induced pro-apoptotic pathway. It has been previously reported that rocaglamide kills leukemia cells and its action has been shown to be associated with a consistent activation of p38 MAPK [35]. We demonstrated in the present work that this protein kinase is the effector of the flavagline FL3 in cancer stem-like cells, since the forced repression of its signal transduction or expression by specific inhibitor SB203580 or siRNA, respectively, partially abolishes its antiproliferative effects. It should be noted that FL3 activates p38 MAPK through a mechanism which does not implicate reactive oxygen species generation (data not shown). Interestingly, p38 MAPK is involved in the translational regulation [36,37]; this suggests that flavagline action implicates a p38-dependent pathway which triggers the modulation of the protein synthesis rates and consequently could inactivate HSF1. Moreover we observed that forced repression of p38 MAPK activity only partially antagonizes FL3-induced growth inhibition, suggesting that other signaling pathways may contribute to the effects of the drug. For instance, an activation of a caspase-independent death process could be considered, as described previously [14,15]. However further investigations are necessary in order to validate the attempt to link the different partners which could be recruited during FL3-associated reactivity of CSCs.

Our findings show that the flavagline FL3 exerts cytotoxic activity on Oct4-expressing cancer stem-like cells. We observed that this drug targets the stemness factor via a caspase-3-dependent process that is abolished by a pan-caspase inhibitor. Indeed, FL3 treatment of teratocarcinomal cells leads to a dramatic

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increase of the expression levels of cleaved caspase-3, the main executor of stress-induced apoptosis. Interestingly the drug activates a p53/p73-independent proapoptotic pathway and therefore could be a promising tool to target cancers which harbor mutations for these tumor suppressors and are known to be highly resistant to current chemotherapy [38]. Actually the overexpression of cleaved caspase-3 is a consequence of FL3-induced p38 MAPK activation since p38 chemical or post-transcriptional gene inactivation counteracts its expression in FL3-treated cells. It should be noted that a treatment with a pan-caspase inhibitor had no effects on FL3-upregulated phosphorylated p38 MAPK (data not shown), which clearly demonstrates that the increase of the expression levels of the post-translationally modified p38 MAPK are upstream of caspase-3 activation.

Pluripotency and self-renewal are the main properties of normal SCs, as well as of CSCs. These characteristics are controlled by various proteins, also involved in cell proliferation and survival [9,10]. Oct4, as a marker of the stemness and unrestricted pluripotency, plays a central role in the survival of poorly differentiated and highly aggressive CSCs [10,20,21]. Our results show that FL3 treatment of EC cells markedly decreased the expression levels of Oct4. Interestingly, in contrast to Nanog which expression is strongly decreased, Sox2 expression remains unaffected by FL3 treatment; this suggests that the three stemness transcription factors display differences in the pathways regulating their expression. At the top of the pluripotency regulatory network, Oct4, Nanog, and Sox2 work cooperatively to activate or repress numerous target genes. Modifications in the expression levels of the three transcription factors induce a loss of cell pluripotency and promote cell death. However it could be possible that the remaining living cells undergo differentiation. This point is now currently under investigation.

It was proposed that Oct4 acts as a multi-functional protein during cancer development [39]. The ability of Oct4 to potentiate tumor growth and to work as an oncogene is associated with its competency to regulate transcriptionally pro- and anti-apoptotic genes [40]. Actually, it could indirectly be implicated in the control of the different cell cycle checkpoints [10] and the expression of various cell cycle regulators, like cyclin D1 [41]. Finally our findings clearly show that the synthetic flavagline FL3 is able to target Oct4 and therefore could be a strong anticarcinogenic drug killing CSCs, without having significant effects on their normal counterparts.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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Article 2

Bad phosphorylation is required for the protection of normal stem-like cells against the cytotoxic effects of the synthetic flavagline FL3

This work under preparation

Bad phosphorylation is required for the protection of normal stem-like cells against the cytotoxic effects of the synthetic flavagline FL3

Fathi Emhemmed [1], Sarah Ali Azouaou [1], , Valérie Schini-Kerth [2], Laurent Désaubry [1], Christian D. Muller [1] and Guy Fuhrmann [2 *]

[1] UMR 7200 CNRS, Laboratoire d'Innovation Thérapeutique, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

[2] UMR 7213 CNRS, Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

* Corresponding author: Guy Fuhrmann, PhD
UMR 7213 CNRS
Laboratoire de Biophotonique et Pharmacologie
Faculté de Pharmacie
74 route du Rhin, B.P. 60024, 67401 Illkirch
France
Tel: (33) 3 68 85 42 17
Fax: (33) 3 68 85 43 13
E-mail: guy.fuhrmann@unistra.fr

ABSTRACT

The molecular pathways by which flavagline derivatives exert their anticancer activity against various cancer cell lines are well documented, while the mechanisms that prevent their cytotoxic effects on normal cells remain to be clarified. We previously showed that the synthetic flavagline FL3 selectively induces apoptosis in Oct4-expressing cancer stem-like cell (*e.g.* NT2/D1 cell) through a p38MAPK/Caspase3- dependent pathway, without having any effect on the normal more restrictive pluripotent stem-like cell (*e.g.* BJ fibroblast). Here we provide the underlying mechanism by which normal cells remain unaffected after exposure to FL3. We first show that FL3 fails to trigger apoptosis in fibroblats. Accordingly, FL3 is unable to induce depolarization of their mitochondrial membrane and the release of cytochrome C in the cytoplasm. Most importantly, FL3 selectively phosphorylates Akt at Ser473 and Bcl-2-associated death promoter protein (Bad) at Ser112 and Ser136 in normal stem-like cells but not in cancer stem-like cells. The fact that the active form of Akt promotes cell survival and that the phosphorylated form of Bad serves as antiapoptotic protein, could explain the resistance of fibroblasts to FL3. Indeed, forced inhibition of the phosphorylation of the upstream regulator Akt by the specific inhibitor LY-294002 or gene knockdown of Bad by siRNA sensitize normal stem-like cells to FL3, by undergoing a caspase-3 dependent proapoptotic process, as measured by annexin V-FITC and western blot assays. These findings, therefore, provide new insights into the signaling pathway that could underly the resistance of normal stem-like cells against the potent anticancer agent FL3.

Key words: normal stem-like cell; cytotoxicity; resistance; flavagline; Bad protein

1-Introduction

Potent anticancer agents without apparent toxicity on normal cells attract most of the attention in the drug discovery field. Annually, thousands of new chemicals are subjected to the evaluation of their activity on various human cancer and cancer stem cells (CSCs) *in vitro*, but as a result of their toxicity towards healthy cells, these chemicals fail to get the status of efficient drugs [1].

Flavaglines, like the leader rocaglamide, are natural compounds of plants from the genus *Aglaia* [2]. They show at nanomolar concentrations high efficacy against many human cancer cell lines *in vitro* [3] and are able to reduce growth of different mouse tumors *in vivo* ([4]. Interestingly and dissimilar to other known anticancer agents, flavaglines exert no obvious toxicity on various human normal cells , such as hematopoietic and hematopoietic stem cells (HSCs) ([5]), lymphocyte cells [6], umbilical vein endothelial cells [7], intestinal epithelial cells [8] and cardiomyoblast cells [10]. Moreover we recently observed that flavaglines have no effects on BJ fibroblast cells [9]. These cells were originally established from primary human newborn foreskin and were spontaneously immortalized with stable growth rate, without oncogenic transformation; actually, the BJ cell can be defined as normal stem-like cell since it is currently used for the generation of induced pluripotent stem cell and has limited differentiation capacity by itself [9].

Flavagline derivatives are also described for their capacity to act as cardio- or neuro-protective agents [10]. For instance, pretreatment of cardiomyocytes with the synthetic flavagline FL3 attenuates doxorubicin-induced cardiotoxicity via a phosphorylation of the heat shock protein HSP27 [11]. The natural product rocaglaol (compound A) reduces neuronal cell death by inhibiting the activity of transcription factors, like nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) which are implicated in chronic neurodegenerative diseases [12]. We and others have previously been reported that flavaglines trigger the intrinsic proapoptotic pathway linked to cellular stress and induce the activation of p38-MAPK in teratocarcinomal stem-like cells and leukemia cells, without cytotoxic effect on fibroblasts and lymphocytes.

There is a general agreement that the intrinsic proapoptotic process is characterized by a disruption of the mitochondrial membrane, leading to release of cytochrome C to the cytosol which, in turn, activates the main executioner of apoptosis, namely caspase3 [13] [14]. The mitochondrial membrane depolarization occurs by homoligomerization of proapoptotic effector proteins such as Bcl2-associated X protein (BAX) or Bcl2-antagonist/killer protein

(BAK); this induces the generation of pores in mitochondrial membrane, which leads to an escape of cytochrome C into the cytoplasm and then cause activation of caspase cascade and induction of apoptosis. However, oligomerization requires association with BH3-only proapoptotic proteins such as PUMA (p53-upregulated modulator of apoptosis), Bim and Bid [15, 16]. Another BH3-only proapoptotic protein, namely Bcl2-associated death promoter protein (Bad), acts indirectly as proapoptotic factor, by displacing and freeing BAX from antiapoptotic proteins Bcl-2/Bcl-xl [17] [18]. However Bad phosphorylation at Ser112 or ser136 leads to a formation complex with 14-3-3 protein in the cytosol, thereby remaining BAX associated with the antiapoptotic proteins Bcl2/Bcl-xl and inhibiting the apoptosis process [19] [20].

We speculate that inactivation of caspase3 and absence of apoptosis in normal cells exposed to FL3 could be due in part to molecular events that sustain the integrity of their mitochondries. For this purpose, we sought to explore the underlying mitochondrial mechanisms by which fibroblast cells do not respond to flavaglines and remain alive. In the current study, we provide evidences that FL3 induces Bad phosphorylation at Ser112 and Ser 136. Moreover, knockdown of Bad by small interfering RNA (siRNA) markedly activates caspase 3 and causes cell death, when cells are treated with FL3. Our work therefore provides the molecular mechanism involved in the protection of normal stem cells (NSCs) against flavagline toxicity.

2- Material and methods

Cell culture and treatment

Human BJ fibroblast cell line (CRL-2522) and NT2/D1 teratocarcinoma cell line (CRL-1973) were purchased from ATCC (LGC Standards, Molsheim, France). Both cell lines were cultured in high-glucose DMEM (4,5 g glucose) (Sigma-Aldrich, Saint-Quentin Fallavier, France), supplemented with 10% (v/v) fetal bovine serum (Lonza, Verviers, Belgium), 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma- Aldrich). Cell culture was maintained in humidified incubator at 37°C in 5% CO2 atmospher. FL3 was synthetized and diluted in DMSO as described in (9). To avoid cytotoxicity or cell differentiation, DMSO concentration never rise 0,02% (v/v) in culture medium.

Apoptosis assay

Cells at density of 4 x 10^3 cells/ml were seeded in 96-well-plates, incubated overnight

and then treated for 24 h. After treatment, cells were trypsinized, resuspended in medium and stained with annexin V-FITC and propidium iodide -PI- (Immunotools, Friesoythe, Germany) for 10 min at 37C. Cells then analyzed by using flow cytometry (Guava EasyCyte Plus, EMD Millipore, Billerica, MA, USA).

Fluorescence detection of mitochondrial membrane potential

Untreated or treated cells at density of 2 x10^5 cells/ml were harvested, resuspended in culture medium without serum and incubated with 40 nM of 3,30-dihexyloxacarbocyanine iodide staining dye (DiOC6; Sigma Aldrich) for 20 min at 37C. At least, 5000 cells were then analyzed by using flow cytometry (Guava EasyCyte Plus, EMD Millipore).

Western Blot analysis.

Western blot was performed as described previously (9). Target proteins were visualized with following antibodies: a rabbit polyclonal phospho-p38 MAPK antibody, a rabbit polyclonal anti-cleaved caspase-3 antibody, a mouse monoclonal anti-phospho-Bad Ser112 antibody, a rabbit polyclonal anti-phospho-Bad Ser 136 antibody, a rabbit polyclonal anti-phospho-Akt Ser473 antibody (Cell Signaling Technology, Danvers, MA, USA), a rabbit polyclonal anti-Bad antibody (Santa Cruz), a mouse anti-cytochrome C antibody (BD Pharmingen), a rabbit monoclonal anti-CoxIV antibody (Thermo scientific), a mouse polyclonal anti-beta tubulin antibody or a mouse monoclonal anti-beta actin antibody (Abcam). Blotted membrane were imaged by ImageQuant LAS4000 and band density was quantified using Image J software.

Mitochondria and cytosol Fractionation

Isolation of Mitochondria and cytosol fractions was performed as previously described [21]. Briefly, cells were washed, trypsinized and pelleted by using centrifugation at 4°C. Pellets were then resuspended in lysis buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA and 30 mM Tris-HCL pH 7.4) and sonicated for 10s. Cytosolic lysate was collected after two fractioning by centrifuging at 600g/5min and 7000g/10min respectively, and stored at -80C. Remaining pellet was again resuspended in the same lysis buffer without EGTA and centrifuged two times at 7000g and then 10000g for 10min. Pellet containing mitochondria fraction was resuspended in a buffer containing 250 mM mannitol, 5 mM HEPES (pH 7.4) and 0.5-mM EGTA and stored at -20C.

siRNA transient transfection

Cells at density of 2 x 10^5 cells/well were seeded in 6-well plates, cultured with free antibiotic Opti-MEM reduced serum (Gibco-Life Technology) and then transfected with 120 nM of Bad siRNA (Cell Signaling Technology) in presence of INTERFERin (Polyplus-transfection, Illkirch, France), as described by the manufacturers. Efficient knockdown was achieved after 48h incubation.

Statistical analysis

All data were obtained from at least three independent experiments and were expressed as means ± SEM. Statistical analysis was achieved with student t-test by using GraphPad Prism software (Prism version 5.04 for Windows, GraphPad Software, La Jolla, CA, USA). *P* value < 0,05 was taken as statistically significant for all analyzed data.

3- Results

Fibroblast cells are unresponsive to FL3

Previously we showed that FL3 selectively inhibited the cell growth of cancer stem-like cells (*e.g.* teratocarcinomal NT2/D1 cells), while slight affect was observed on normal stem-like cells (*e.g.* fibroblastic BJ cells) only when exposed for 24h to high concentration (2000 nM), as measured by MTS or trypan blue assays. To further investigate the selective action of FL3, we exposed both cell lines to FL3 at the same conditions mentioned above and a potential apoptotic effect was examined. Consistent with our previous study, 50% of NT2/D1-treated cells were annexin V-FITC positive, whereas there was no detectable fluorescence for fibroblast cells (Fig.1a, b). Furthermore, as expected, immunoblot analysis on teratocarcinomal cells showed increased levels of cleaved caspase 3 and phosphorylated form of p38 MAPK known to act as its upstream activator. In contrast, cleaved caspase 3 could not be detected in treated normal cells. Nevertheless, p38MAPK was significantly activated in fibroblats (Fig.1c, d). These findings therefore reinforce the postulate of an absence of cytotoxic effect of FL3 on normal stem-like cells.

Mitochondrial membrane potential and cytochrome-C location are unchanged in fibroblasts

p38 MAPK activity is known to trigger an intrinsic caspase 3 dependent pro-apoptotic pathway, through a disruption of the mitochondrial membrane [22] [6]. Therefore, we sought

to determine whether FL3 could selectively affect the mitochondrial membrane permeability. For this purpose, we stained both teratocarcinomal and fibroblastic cells with DiOC6, a cell-permeant fluorescence dye that is used to detect the mitochondrial membrane potential ΔΨm. Flow cytometric analysis showed that the number of NT2/D1 cells that exhibit low levels of DiOC6 uptake was significantly increased when exposed to FL3, reflecting a loss of ΔΨm. In contrast, no change was observed for normal FL3-treated cells compared to untreated cells (Fig.2a and b), demonstrating that the mitochondrial membrane of fibroblasts remain intact, while exposed to the drug.

The subcellular localization of cytochrome C is an additional parameter which gives information about the integrity of the mitochondria. For that purpose, mitochondrial and cytosolic fractions were collected from both cell lines and analyzed by immunoblotting assays. In a first step, the purity of the fractions were monitored by determining the expression levels of COX IV and beta-tubulin as integral protein of the mitochondria or cytosol respectively. Our results showed that cytochrome C was significantly decreased in the mitochondria of NT2D1-treated cells, whereas, elevated levels were observed in the cytosolic fraction, when compared to untreated cells (Fig.2c and d). Interestingly, cytochrome C was mainly restricted to the mitochondria of fibroblastic cells, even after exposure to FL3. Taken together, these observations well supported that the failure of FL3-induced apoptosis in normal stem-like cells is mainly due to its inability to breakdown their mitochondrial membrane.

FL3 selectively phosphorylates the proapoptotic protein Bad in fibroblasts

It has been shown that Bad can initiate the activation of a mitochondrial proapoptotic pathway. At the opposite, when phosphorylated, Bad acts as an antiapoptotic factor [17] [18]. We suspected therefore that FL3 might selectively target Bad in normal BJ cells and consequently could prevent the occurrence of an proapoptotic process. For that purpose, total cell lysates of both cell lines were subjected to western blot analysis by using specific-antibodies against phospho-Bad at Ser112 or Ser136. Accordingly, normal FL3-treated cells showed significant amount of phosphorylated Bad at both sites,when compared to untreated cells. In contrast, Bad was clearly unphosphorylated in NT2/D1 treated cells (Fig.3a and b). These results suggest therfeore that phosphorylation of Bad could be a major event of the cytoprotection process of fibroblast cells against FL3-induced apoptosis.

Knockdown of Bad is sufficient to sensitize fibroblast cells to FL3

To verify the implication of Bad phosphorylation in the protection of normal cells against the cytotoxic affect of FL3, we next targeted Bad by silencing its expression with siRNA. Prior the treatment, significant transient gene knockdown in transfected cells was achieved, when compared to non-transfected cells. Upon treatment with FL3 for 48h, transfected cells significantly responded to the toxic effect of FL3 and underwent apoptosis, as measured by annexin V-FITC assay (Fig.4a and b). Accordingly, western blot analysis confirmed this response since increasing levels of cleaved caspase 3 could only be detected in transfected cells, concomitantly with an absence of Bad protein and its phosphorylated forms (Fig.4c and d). Collectively, these results strongly suggest that phosphorylation of Bad is the major molecular event implicated in the protection of fibroblasts against FL3 cytotoxicity.

FL3 markedly phosphorylates Akt in fibroblast cells

It has been reported that phosphorylation of protein kinase B (Akt) at Ser473 plays a central role in inhibiting the activation of the mitochondrial proapoptotic pathway [23]. We therefore examined by western blot analysis whether FL3 could be able to phosphorylate Akt at Ser473. The results showed that the protein was phosphorylated in fibroblast cells, but not in teratocarcinomal cells, although the levels of total Akt remain unchanged in both cell lines (Fig.5a and b). To explore the involvement of the Ser473 phosphorylated form of Akt in the survival process, we used LY294002 known to be a prominent inhibitor of Akt activity. As shown in Fig.5c and d, the presence of the inhibitor sensitizes the normal cells to FL3 which undergo a pronounced apoptosis after 48h ofFL3 treatment, as measured by annexin V-FITC assays. These data showed therfore the involvement of Akt phosphorylation in the cytoprotection mecanism of normal stem-like cells against FL3.

4- Discussion

The molecular mechanisms by which flavagline derivatives exert their anticancer activity against variety of cancers are well documented. However, the mechanism that protect normal cells against their cytotoxicity remains to be clarified. In this study, we investigated the underlying process implicated in the absence of effects of the synthetic flavagline FL3 towards fibroblast cells. Indeed, we previously showed that this drug selectively induces apoptosis in teratocarcinomal cancer stem-like cell, whereas it has no effect on fibroblast which can be considered as a model of normal stem-like cell with restricted differenciation

capacity [9] [24].

The present work provides, at first, further evidences for the selective effects of FL3. As expected, the drug is unable to induce apoptosis in fibroblasts, as shown by annexin V-FITC assays. Accordingly, cleaved caspase 3 could not be detected in treated cells. moreover we previously demonstrated that FL3 triggers apoptosis in Oct4-expressing cancer stem-like cells through the activation of p38MAPK and caspase 3 [9]. An earlier study also revealed that the activation of p38MAPK and caspase 3 by rocaglamide is the main cause of apoptosis induction in leukemia cells, while normal mature lymphocytes were unaffected by the drug, due to the inactivation of p38MAPK [6]. The two reports stated therefore that any flavagline derivative could selectively target cancer and cancer stem-like cells, but not normal and normal stem-like cells, by modulating p38MAPK activity. Surprisingly however, the current study shows that FL3 also induces phosphorylation of p38MAPK in fibroblast cells. This suggests that the effect of FL3 involves p38MAPK in both NSCs and CSCs, but the downstream targets are different, since the activation of the main executioner of apoptosis, namely caspase 3, could not be observed in NSCs. Actually, the mitochondrial integrity and sequestration of cytochrome C in mitochondria of fibroblast cells support the fact that caspase 3 remains inactivated in these cells, despite the activation of its upstream regulator p38MAPK. It has been reported that this protein may switch from a proapoptotic to a survival function by upregulating the antiapoptotic members of Bcl-2 family [25]. As an example, activation of p38MAPK is known to protect endothelial cells against acidic preconditioning-induced apoptosis, through an overexpression of the antiapoptotic protein Bcl-xL [26].

The present study provides novel insights into the mechanisms that prevent the proapoptotic activity of FL3 on normal stem-like cells. Indeed, our results reveal a phosphorylation of Bad at Ser 112 and Ser136 in FL3-treated fibroblasts. Such phosphorylation is known to allow Bad to be sequestred in the cytosol by binding to the cytosolic protein 14.3.3 and therefore prevents the displacement of Bax from Bcl-2 which in turn impedes apoptosis [17] [27]. The protective role of Bad phosphorylation is further highlighted by knock down experiments. Indeed, loss of Bad function with small interfering RNA sensitizes the normal stem-like cells to the proapoptotic activity of FL3. This strongly suggests that Bad phosphorylation is the central molecular event for the protection of NSCs against the cytotoxic effects of the synthetic flavagline.

The present results show that FL3 targets the activity of Akt, a major intermediate of the survival pathway. More specifically, the drug induces Akt phosphorylation in normal stem-like cells but not in cancer stem-like cells, whereas total amount of Akt remains

unchanged in both cell types. In addition, by inhibiting Akt activity with the specific inhibitor LY294002, a sensibilization of fibroblasts to the cytotoxic effect FL3 sets in, suggesting an essential role of Akt activation in the resistance of NSCs to the drug. It should be noted that LY294002 inhibits FL3-induced AKT phosphorylation 24h after the treatment (data not shown), whereas evidence of a proapoptotic cell reactivity is only observed after 48 h of treatment. These results are similar to previous observations, which showed that the complete inhibition of Akt phosphorylation was rapidly achieved in presence of LY294002, but remains transitory, although delayed apoptosis can be detected. This events unfold is likely linked to the short half-life of LY294002 which does not allow sustained inhibition of Akt activity [28].

It is known that Akt activity prevents cell-intrinsic death by blocking the pro-apoptotic function of Bad through its phosphorylation [17]; we therefore hypothesize that Akt activation may serve as an upstream inductor of Bad phosphorylation. Accordingly, inhibition of FL3-induced phosphorylation of Akt by LY294002 triggers in NSCs a transient downregulation of Bad phosphorylation at Ser 112 (data not shown). This suggests that Bad activation initiated in FL3-treated fibroblasts involves an Akt-dependent mechanism. However it should be noted that total Akt expression is stable and unchanged in both NSCs and CSCs. Therefore how FL3 selectively phosphorylates Akt in non cancerous cells, remains to be clarified.

The interplay between Akt and p38 MAPK in the survival process is still controversial. It has been found that the inhibition of Akt and p38MAPK activities enhances doxorubicin-induced apoptosis in NIHT3 fibroblasts [29]. Furthermore, it has been shown that p38MAPK phosphorylates Akt (Ser473) / Bad (Ser112) and prevents the induction of apoptosis of skeletal muscle cells [30]. In contrary, activation of Akt inhibits p38MAPK activity and mediates endothelial and lung cell cytoprotection [31, 32]. We observed that both Akt and p38MAPK are activated in FL3-treated fibroblasts; however inhibition of FL3-induced activation of p38MAPK with the specific inhibitor SB203580 does not trigger apoptosis (data not shown); this suggests an absence of a direct effect of p38MAPK on Akt activation, thereby on cell survival. It can be hypothetized that FL3 targets different interconnected signalling pathways and that the resulting effect depends on the pathological background. In the present case, the precise role and interrelationship of Akt and p38MAPK phosphorylation in the selective protection of NSCs against FL3 need therfeore further investigations.

Taken as a whole, our findings provide novel evidence of how human normal stem-

like cells are protected towards the toxicity of the anticancer compound FL3. This protection mechanism may explain the absence of the cytotoxic effect of other flavaglines derivatives on the different normal cells that were used in other studies. The synthetic flavagline FL3, as an antiapoptotic agent, may be a useful tool to treat certain apoptosis-associated diseases that involve an activation of p38 MAPK, like heart diseases [33, 34] or Alzheimer's disease [35, 36]. Indeed, FL3, by its ability to interrupt the intrinsic apoptotic pathway in normal cells despite p38 MAPK activation, could be a suitable drug lead to alleviate cardiovascular and neurodegenerative diseases related to p38 MAPK activity. Finally flavagline derivatives are potent anticancer compounds with cytoprotective properties on noncancerous cells and therefore should attract more much attention in the future for therapeutic purposes.

Conflict of interest

The authors declare no conflict of interest.

Authorship Contributions

Participated in research design: FE, LD, GF

Conducted experiments: FE, SAA, GF

Contributed new reagents or analytic tools: VSK, CDM, LD

Performed data analysis: FE, SAA, GF

Wrote or contributed to the writing of the manuscript: FE, SAA, LD, GF

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Figures and legends

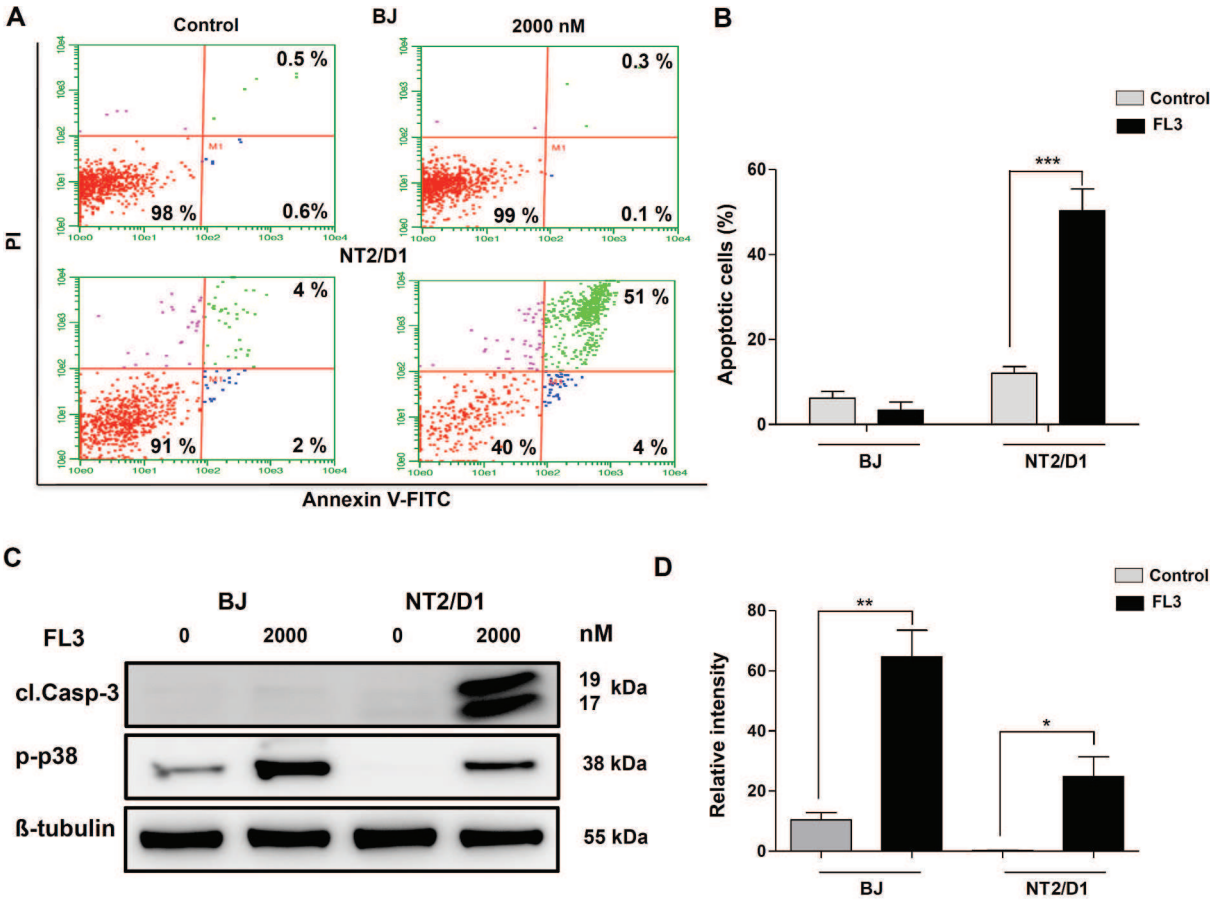

Fig.1. Effect of FL3 on the apoptosis rate, p38 MAPK and cleaved caspase 3 activities of fibroblasts and teratocarcinomal cells. Cells were treated with FL3 at the indicated concentration for 24h. (**A**) Histograms show percentage of live (lower left panel), early apoptotic (lower right panel), late apoptotic (upper right panel) and necrotic (upper left panel) cells, measured by flow cytometry using annexin V-FITC/PI staining assay. (**B**) Bar charts represent the percentage of annexin V-positive cells. The number of apoptotic bodies is expressed as percent relative to the total cell number. (**C**) Total cell lysates were subjected to immunoblotting analysis with the corresponding antibodies, as described in "material and methods". Specific bands were detected with their expected apparent molecular weight. The panel shows representative immunoblotting results. (**D**) show densitometry results of p38 MAPK and cleaved caspase 3 expression levels normalized to β-tubulin expression levels. Values are mean ± S.E.M of at least three independent experiments; *, $p< 0,05$; **, $p<0,01$; ***, $p<0,001$.

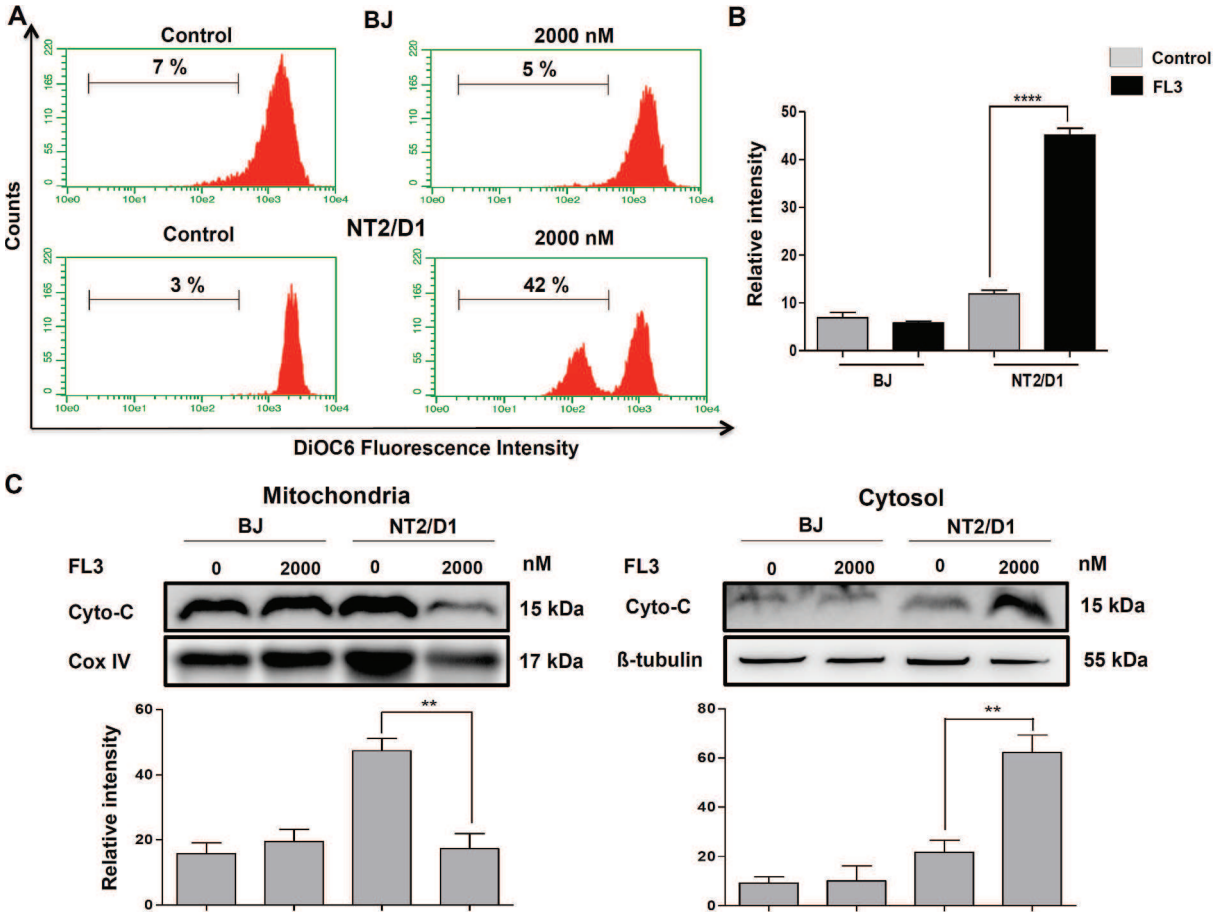

Fig.2. Effect of FL3 on mitochondrial integrity of fibroblasts and teratocarcinomal cells. Cells were treated with FL3 at the indicated concentration for 24h. (**A**) Histograms show the percent of BJ or NT2/D1 cells exhibiting ΔΨm changes, as measured by flow cytometry using DiOC6 staining assay. Fluorescence intensity shifts from right to left indicate a loss of ΔΨm. (**B**) Bar charts represent the percentage of BJ or NT2/D1 cells with mitochondrial permeability alteration, expressed as percent relative to the total number of cells. (**C**) Expression levels of Cytochrome C, COX IV and β-tubulin were assessed in BJ or NT2/D1 cells by western blot analysis. Specific bands were detected with their expected apparent molecular weight. The panel shows representative immunoblotting results. COX IV and β-tubulin were used as mitochondrial and cytosolic internal controls respectively. (**D**) Show densitometry results of mitochondrial and cytosolic cytochrome C expression levels, normalized to either COXIV or β-tubulin expression levels. Values are mean ± S.E.M of three independent experiments; **, p<0,01; ****, p<0,0001.

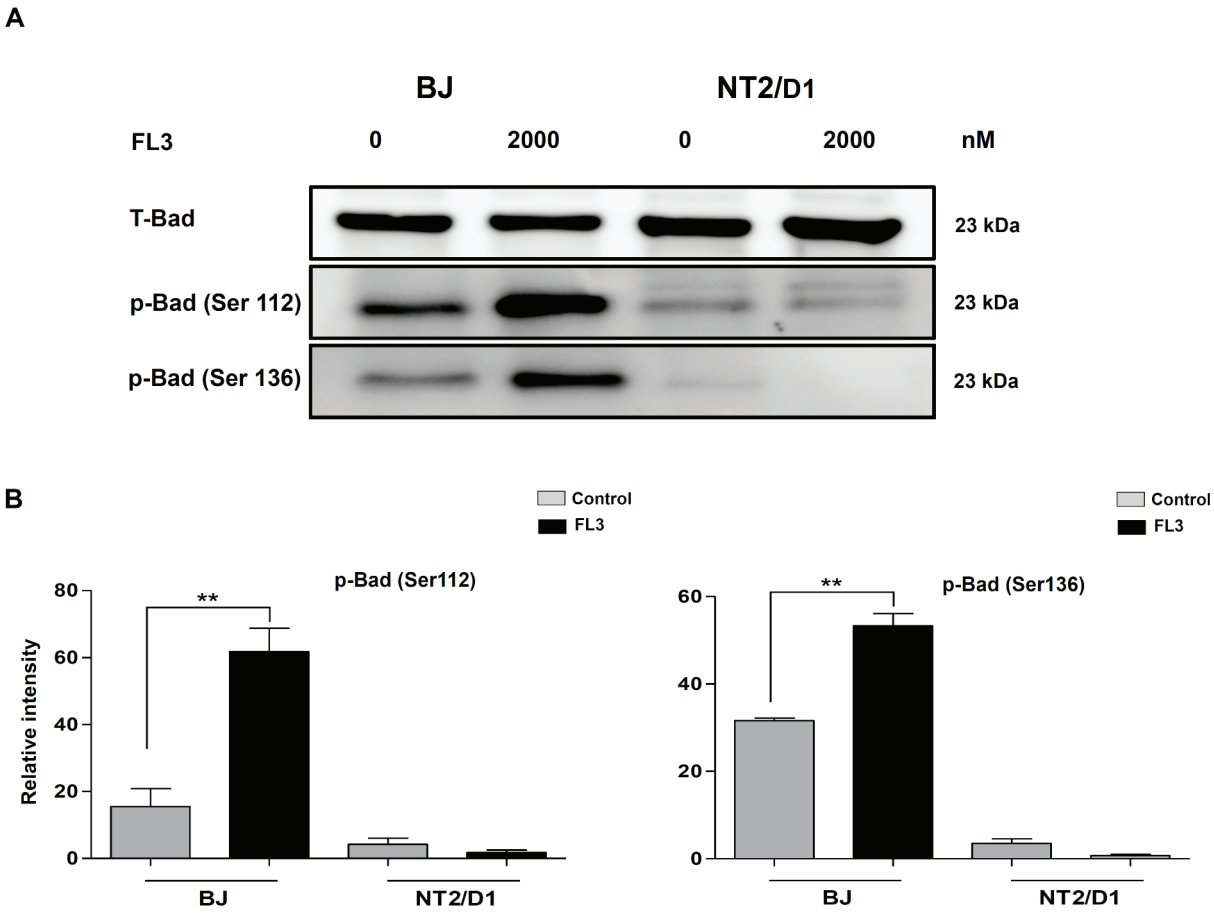

Fig.3. Effects of FL3 on Bad phosphorylation in fibroblasts and teratocarcinomal cells. Cells were exposed to the indicated concentration of FL3 for 24h. (**A**) Total extracts of either BJ or NT2/D1 cells were subjected to immunoblotting and analyzed by using antibodies against total Bad, p-Bad Ser112 or p-Bad Ser136. Specific bands were detected with their expected apparent molecular weight. The panel shows representative immunoblotting results. (**B**) shows densitometry results of the indicated proteins, normalized to total Bad expression levels and given as ratios relative to the value obtained for the untreated sample. Values are means ± S.E.M. of at least three independent experiments; statistically significant: ** p, < 0.01 (versus untreated group).

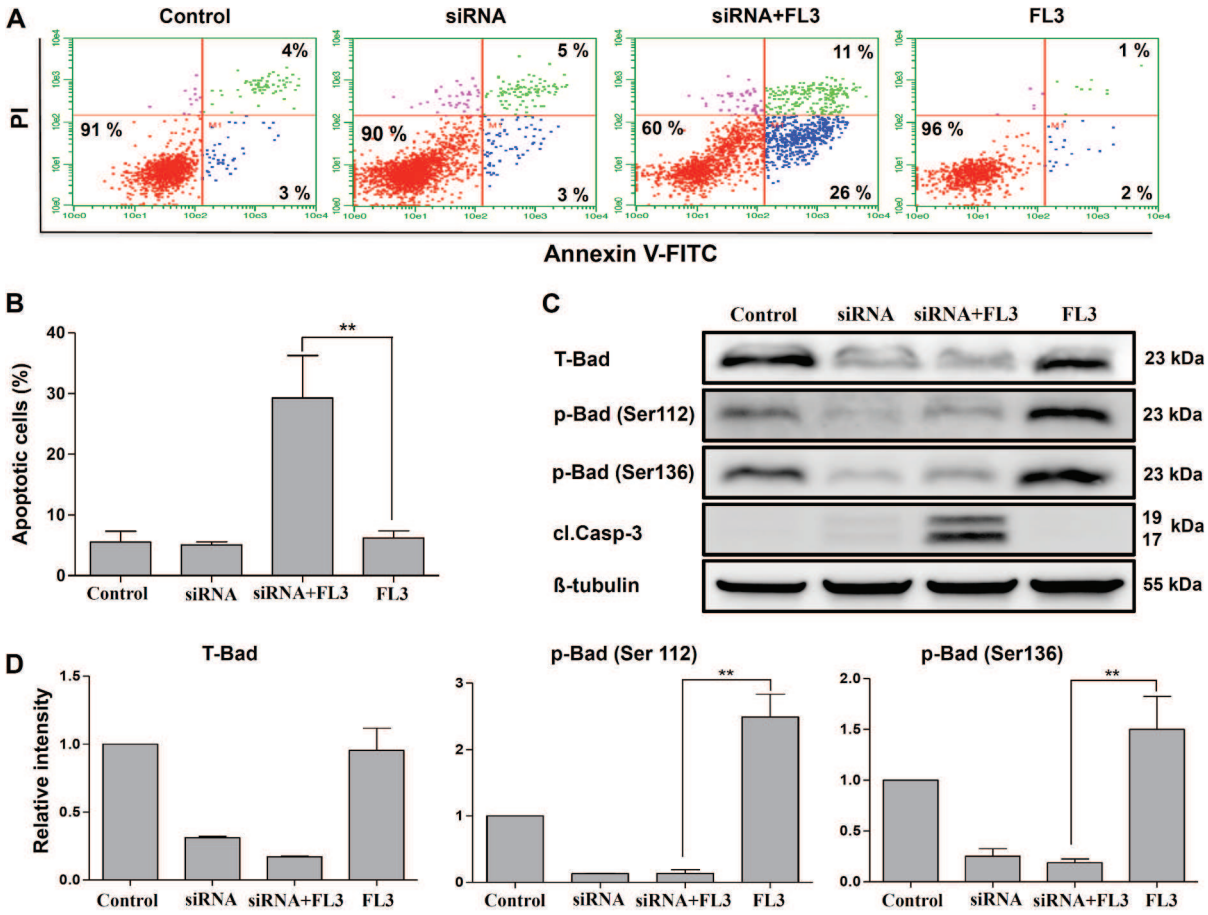

Fig.4. Abrogation of the cytoprotection against FL3 of fibroblasts by posttranscriptional silencing of Bad. Cells were transfected with 120 nM of bad siRNA for 48h, followed by an additional 48h incubation in the presence or absence of 2000 nM FL3. (**A**), Histograms show the percentage of cells in different stages, as mentioned in Fig.1A. (**B**), Bar graph represents the number of total apoptotic fibroblasts, calculated as sum of the percentage of both early and late apoptotic bodies. (**C**) show representative immunoblotting results for total Bad, p-Bad Ser112 or p-Bad Ser136. Specific bands were detected with their expected apparent molecular weight. (**D**) show densitometry results for the indicated proteins, after normalization against β-tubulin expression levels. Values are means ± S.E.M. of three independent experiments; statistically significant: **, $p < 0.01$ (versus untreated group).

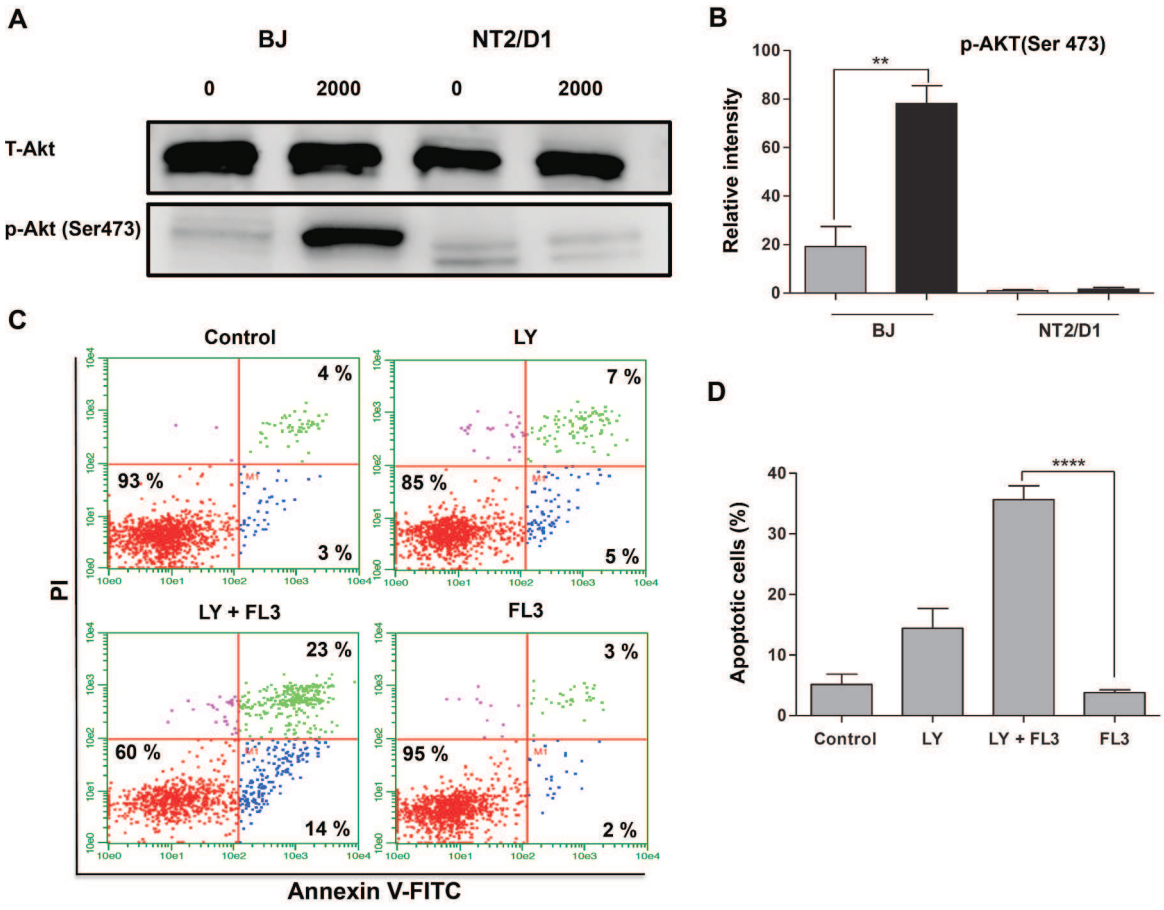

Fig.5. Implication of phospho-Akt in the cytoprotection of fibroblasts against the cytotoxic effect of FL3. (**A**), Total cell lysates of untreated or 24h FL3-treated BJ or NT2/D1 cells were collected and analyzed by western blotting. The panel shows representative results of the expression levels of p-Akt Ser473. (**B**), Bar graph shows quantitative densitometric analysis of p-Akt expression levels, normalized to the corresponding total Akt expression levels. (**C**), Fibroblasts were pretreated for 1h with 40 µM of LY249002, followed by an additional 48h incubation, in the presence or absence of 2000 nM FL3. Histograms show the percentage of cells in different stages, as mentioned in Fig.1A. (**D**), Bar graph represents the number of total apoptotic fibroblasts incubated at the indicated conditions. The apoptosis rate is calculated as sum of the percentage of both early and late apoptotic bodies. Values are means ± S.E.M. of three independent experiments; statistically significant: **, $p < 0.01$; ****, $p < 0.0001$ (versus untreated group).

Article 3

Pro-differentiating effects of a synthetic flavagline on human Oct4-expressing cancer stem-like cells

This work under preparation

Pro-differentiating effects of a synthetic flavagline on human Oct4-expressing cancer stem-like cells

Fathi Emhemmed [2], Sarah Ali Azouaou [2], Valérie Schini-Kerth [1], Christian D. Muller [2], Laurent Désaubry [2] and Guy Fuhrmann [1 *]

[1] UMR 7213 CNRS, Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

[2] UMR 7200 CNRS, Laboratoire d'Innovation Thérapeutique, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

* Corresponding author: Guy Fuhrmann, PhD

UMR 7213 CNRS

Laboratoire de Biophotonique et Pharmacologie

Faculté de Pharmacie

74 route du Rhin, B.P. 60024, 67401 Illkirch

France

Tel: (33) 3 68 85 42 17

Fax: (33) 3 68 85 43 13

E-mail: guy.fuhrmann@unistra.fr

Abstract

As initiators of the carcinogenic process, cancer stem cells (CSCs) are considered as emerging targets for innovative anticancer therapies. However these cells are hidden in the cancer bulk and remain insensitive to chemotherapy which precisely targets only their proliferative capacities. Alternatively, growing evidences have pointed out that a differentiation therapy could adversely affect these cells which consequently should lose their self-renewal properties and become less aggressive.

In order to evaluate the differentiation potential of flavaglines, we used the poorly differentiated pluripotent teratocarcinomal cell as a model of Oct4-expressing cancer stem-like cell and determined the molecular mechanisms induced by the synthetic compound FL3. The drug, administrated at sub-lethal doses and for long period, was able to significantly downregulate the expression levels of the stemness factor Oct4 at both the transcriptional and translational level, concomittantly with a decrease of clonogenicity. The appearance of specific neural markers further demonstrates the differentiation properties of FL3. Interestingly, the drug treatment first induced the appearance of caspase-3 cleaved fragments followed by significant increase of the expresion levels of the germ cell nuclear factor; this suggests that the extinction of Oct4 expression necessary for the induction of differentiation involves overlapping mechanisms of protein degradation and gene repression. Finally this study shows that FL3, like all-trans retinoic acid (ATRA), is acting as a novel differentiation inducer of CSCs. Thus FL3 could offer an alternative possibility for cancer treatment since it is able to target the carcinogenic process by inducing differentiation in ATRA-resistant cancers.

Keywords: cancer stem cells; teratocarcinoma; flavagline; differentiation; Oct4

1. Introduction

Cancer stem cells (CSCs) are responsible for tumor initiation, invasion and metastasis, and are considered to be the main contributors of therapy resistance and cancer recurrence. CSCs might arise from normal stem cells (NSCs) exposed to repetitive mutation-inducing stress injuries. Recent studies suggest that CSCs could also arise from closely related dedifferentiated descendants, which possess more restricted lineage-specific competencies (Sharif et al., 2011a).

A growing number of reports show that chemotherapeutic agents are usually able to act on both cancer cells and CSCs, by targeting similar cell processes, including those leading to apoptotic cell death (Emhemmed et al., 2014; Ali Azouaou et al., 2015). This emerging concept of pluralist therapeutic tools has been developed on the basis of accumulating data obtained for several CSC models. Indeed, identification of specific markers has allowed to isolate and characterize CSCs of various cancer types and, as a consequence, has initiated prospective analyses of putative chemotherapeutic drugs which could be able to efficiently target them (Sharif et al., 2011a). In this point of view, we and others have hypothetised that the malignant counterparts of the embryonic stem cell lines, namely the embryonal carcinoma stem cell lines, could be suitable models of CSCs (Sell, 2004; Sharif et al., 2011b). These cell lines are poorly differentiated pluripotent germinal stem cells with a highly aggressive pathological phenotype and can used as surrogated investigational tools for the evaluation of potential anticancer agents (Sharif et al., 2011b).

It has been reported that the aggressiveness of a CSC is proportional to its lineage-specific competencies (Ben-Porath et al., 2008; Sharif et al., 2013). Actually several studies have reported that the differentiation level of a CSC type is inversely correlated with its resistance capacity to radio- and chemo-therapy (Al-Hajj et al., 2004; Sharif et al., 2011a). Disrupting the molecular pathways which control CSC self-renewal is therefore an attractive alternative to weaken the aggressiveness and resistance phenotype of the tumor bulk. By targeting these pathways, it is assumed that the CSC switches from a highly proliferative and undifferentiated state to a harmless low-growing and mature state (Sharif et al., 2013). The most exhaustively studied differentiation-inducing compounds are retinoids, including vitamin A and its derivatives. As an adjunct to clinical therapy, all-*trans* retinoic acid (ATRA) treatment allows a long-lasting remission of more than 90% of patients with acute

promyelocytic leukemia (Freemantle et al., 2003). This example shows the strongly positive impact of the differentiation strategy which is able to block the tumor development and to prevent its recurrence. However several cancers remain refractory to ATRA and therefore new therapeutic strategies have to be developed to cure them (Grimwade et al., 2010).

Flavaglines are a family of natural products with a cyclopenta[*b*]benzofuran skeleton which are extracted from the widespread plant genus *Aglaia*. Their leader compound, rocaglamide, has been found to exhibit strong antileukemic activity (King et al., 1982). Other flavaglines, like rocaglaol or silvestrol, also induce the death of cancer cells, without affecting non-cancerous cells, by acting on two distinct targets, the scaffold proteins prohibitins and the eukaryotic translation initiation factor 4A (Basmadjian et al., 2013). Interestingly, we recently identified a synthetic flavagline, FL3, which displays enhanced cytotoxicity on cancer cells compared to natural flavaglines (Thuaud et al., 2009). Moreover we showed that the pharmacological compound kills cancer stem-like cells by a p53/p73-independent p38 MAPK-dependent caspase-3 dependent pro-apoptotic pathway at micromolar concentrations (Emhemmed et al., 2014).

A growing number of *in vitro* and *in vivo* studies have shown that various plant-derived compounds are potentially anticancer agents since they are able to target specifically the self-renewal properties of CSCs (Li et al., 2011). Moreover, prosurvival and self-renewal signaling pathways share several common molecular components, pointing out the fine-tuned balance which controls cell proliferation and differentiation (Konopleva and Jordan, 2011). As a consequence, it is not surprising that some phytochemicals could target specific nodal points of the self-renewal and differentiation machineries (Sarkar et al., 2009). In this point of view, it has been observed that rocaglamide induces the differentiation of HL-60 promyelocytic cells, in correlation with an arrest of proliferation (Mata-Greenwood et al., 2001), suggesting that other members of the flavagline family could have similar bidirectional activity.

The aim of our work was to evaluate the *in vitro* pro-differentiating properties of the flavagline FL3 on human embryonal teratocarcinoma stem cells NT2/D1 (also known as NTERA-2 cl.D1). This cell line is described as a highly pluripotent undifferentiated cell line, a property associated to a strong expression of the stemness regulator Oct4. This transcription factor is known to be essential for pluripotency maintenance and self-renewal. Indeed, Oct4 binds at the promoters and/or regulatory regions of numerous target genes which are

associated with proliferation and differentiation processes (Pesce and Schöler, 2001; Jung et al., 2010). To achieve a higher specificity, Oct4 may form protein complexes with other transcriptional regulators, including the homeobox protein Nanog and the SRY-related HMG-box protein Sox2 (Boyer et al., 2005). It is now accepted that Oct4 is detectable in CSCs from diverse tumor origin (Kang et al., 2009) and its presence as a part of the pluripotency signature has been observed in poorly differentiated and highly aggressive cancers (Sharif et al., 2011a; Sharif et al., 2011b; Ben-Porath et al., 2008).

Here we show that FL3 selectively induces differentiation of pluripotent cancer stem-like cells at sub-lethal nanomolar concentrations. This chemopreventive effect involves a downregulation of the major gardian of a highly pluripotent cell state, namely Oct4 by targeting its degradation and its transcriptional level. As a consequence, cells undergo diffrentiation in the neural pathway, with the appearance of specific markers like bIII-Tubulin (a microtubule element expressed in neurons) and glial fibrillary acidic protein -GFAP- (an intermediate filament expressed in astrocytes). Our study therefore highlights the pro-differentiation properties of a synthetic flavagline which could be a promising substitution chemotherapeutic compound in ATRA-resistant cancers.

Material and methods

Cell lines and culture conditions

NT2/D1 (CRL-1973) cell line, purchased from ATCC (LGC Standards, Molsheim, France) was cultivated in DMEM-based media (Sigma-Aldrich, Saint-Quentin-Fallavier, France), supplemented with 10% (v/v) fetal bovine serum (BioWhittaker, Verviers, Belgium), 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich). Cells were grown in Petri dishes to 30% confluency prior to treatment. All plates were incubated in humidified atmosphere with 5% CO_2 at 37 °C.

Cell Treatment

FL3, synthetized as previously described (Thuaud et al., 2009) and ATRA (Sigma-Aldrich) were diluted at 10 mM in DMSO - dimethyl sulfoxide - . For our purpose, cells were treated with either 10 nM of FL3 or 5 µM ATRA mixed with the cell culture medium.

Treatment was carried out till 12 days and FL3- or ATRA-supplemented media was changed every two days.

Clonogenic assay

Spheroids were generated by seeding 200µl/well of a cell suspension at a density of 1.5 x 10^4 cells/ml in ultra-low attachment 96-well plates (Sigma-Aldrich). After four days incubation, spheroids were exposed to either vehicle (DMSO) or FL3 for different times. Images analysis of tumor spheroids and determination of their diameter was carried out by using a Celigo cytometer (Cyntellect Inc, San Diego, CA, USA), as previously described (Vinci et al., 2012).

Immunocytochemistry analysis

Cells at a density of 4 x 10^4 were seeded in imaging m-Dishes (Ibidi, Martinsried, Germany) and incubated for 24 h. Cells were then fixed with 4% paraformaldehyde for 15 min, permeabilized with 0,3% Triton X-100 in PBS (Phosphate-Buffered Saline) for 15 min and blocked in 2% normal goat serum for 30 min at room temperature. After rinsing, cells were incubated with either a rabbit polyclonal anti-Oct4 or anti-Nanog antibody (GeneTex, Irvine, CA, USA), a rabbit poyclonal anti-GFAP antibody (Abgent, San Diego, CA, USA) or a mouse monoclonal anti-bIII-Tubulin antibody (Abnova, Taipei City, Taiwan). All the primary antibodies were diluted at 1:200-1:400 in 2% normal goat serum. After 2 h of incubation at room temperature, cells were washed with PBS and exposed for an additional half hour to either a mouse or a rabbit Alexa fluor 488-conjugated secondary antibody (Life Technologies, Saint Aubin, France), diluted at 1:2000 in 2% normal goat serum. After rising, cells were stained with Hoechst 33342 (Life Technologies) diluted at 1:10000 for 5 min. Images were captured using a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Nanterre, France).

Western blot analysis

Exponentially growing cells were treated with the vehicle or FL3 and incubated at different times. Cells were harvested, centrifuged at 200 *g* for 10 min at room temperature and

the pellets were resuspended in RIPA buffer (25 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% sodium deoxycholate) containing protease inhibitors (Sigma-Aldrich). Proteins of cell lysates were then extracted, separated on 8-15% SDS-polyacrylamide gels and transferred to membranes. Immunoblotting was performed as previously described (Sharif et al., 2011b), by using either a rabbit polyclonal anti-Oct4, anti-Nanog or anti-Sox2 antibody (GeneTex), a rabbit polyclonal anti-GFAP antibody (Abgent), a mouse monoclonal anti-bIII-Tubulin antibody (Abnova), a rabbit monoclonal anti-STAT3 (phospho Y705) antibody (Abcam, Paris, France), a rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA, USA), or a rabbit polyclonal GCNF antibody (Abcam). Membranes were subsequently reprobed with a mouse polyclonal anti-beta tubulin antibody (Abcam), a rabbit polyclonal anti-beta actin antibody (Abcam) or a rabbit polyclonal anti-glyceraldehyde-3-phosphate deshydrogenase (GAPDH) antibody (Abcam).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT/PCR) analysis

Total RNA from untreated and treated cells was extracted using the EZ-10 DNAaway RNA Mini-prep kit (BioBasic Inc., Toronto, Canada) following the manufacturer's instructions. PCR reaction was then carried out with 10 ng RNA and 200 nM of specific primers (QuantiTect Primer Assays, Qiagen, Courtaboeuf, France), using the KAPA SYBR Fast 1-step qRT-PCR kit (Kapa Biosystems Ltd., London, UK), according to the manufacturer’s specifications. Amplification was performed on an iCycler MyiQ system (Bio-rad, Marnes-la-Coquette, France).

Statistical analysis

Data are presented in a bar graph form and expressed as means ± S.E.M. of at least three independent experiments. Statistical evaluation was performed with the one-way ANOVA test followed by Tukey's post hoc analysis or the Student’s t-test using GraphPad Prism software (GraphPad Software Inc, CA, USA); a p value less than 0.05 was considered as significant.

3. Results

FL3 treatment is able to induce the disappearance of nuclear stemness factors and the appearance of cytoplasmic neural markers in highly pluripotent cancer stem-like cells

Differentiation involves a steady reduction and finally the disappearance of the stemness factors in poorly differentiated CSCs. Accordingly, teratocarcinomal cells treated for 12 days with 10 nM of FL3 showed morphological changes, concomittantly with an absence of Oct4 and Nanog, in contrast to untreated cells which exhibit strong nuclear labelling of both proteins (Fig. 1). Moreover FL3-treated teratocarcinomal cells exhibited a strong cytoplasmic labelling of GFAP and bIII-Tubulin which are two main markers of neural differentiation (Fig. 2). These results therefore suggest that FL3 is able to repress the stemness maintenance and to induce a neuro-ectodermic differentiation of embryonal carcinoma cells.

Long lasting treatment with FL3 at low concentration induces a gradual downregulation of the stemness factors in highly pluripotent cancer stem-like cells

The expression levels of the stemness factor Oct4 and its interactive partners Nanog and Sox2 were monitored during the 12 days of treatment with 10 nM of FL3. As shown in Fig. 3, a progressive, but significant decrease of the expression rates of these transcription factors is observed. At day 8 of treatment, only faint bands were visualized by immunoblotting, suggesting that the embryonal cells have definitively lost their highly pluripotent state. Interestingly, a more precise analysis of Fig. 3 showed a transitory and reproductible upregulation of Oct4 at day 2 of treatment; the reasoning behind this event remains at that stage of the study unknown.

The expression levels of Oct4 during long lasting treatment of cancer stem-like cells with FL3 or ATRA treatment show overlapping, but delayed patterns

ATRA is a known differentiation factor of teratocarcinomal cells which acts as a gene repressor of the stemness factor Oct4. We therefore investigated the behavior of this transcription factor, by monitoring its expression pattern during a long lasting treatment with either FL3 (10 nM) or ATRA (1 mM). As shown in Fig. 4, a globally similar decrease of Oct4 expression levels along the time was observed with the two treatments in the teratocarcinomal cells. It should be noted however that ATRA seemed to act earlier than FL3; indeed, a

reduction of Oct4 expression levels of over 50 % was detected by day 4 in ATRA-treated cells, whereas at that time Oct4 content remained unchanged in FL3-treated cells.

Long lasting treatment with FL3 at low concentration induces a gradual upregulation of neural markers in highly pluripotent cancer stem-like cells

The expression levels of several neural factors, namely bIII-Tubulin (neuronal marker) and GFAP (astrocyte marker), were monitored during the 12 days of treatment with 10 nM of FL3. As shown in Fig. 5, a progressive and significant increase of the expression rates of these neural factors was observed. From day 8 of treatment, strong bands were visualized by immunoblotting, suggesting that the embryonal cells have definitively switched from an highly undifferentiated state to a neural phenotype. Accordingly, we also observed enhanced levels of phospho-STAT3 Y-705 which are absolutely crucial for astrocyte commitment (Nagao et al., 2007; Cheng et al., 2011). These results clearly demonstrated that FL3-treated teratocarcinomal cells have acquired a neural phenotype, with a production of both neurons and astrocytes.

Long lasting treatment with FL3 at low concentration induces a gradual downregulation of the transcript levels of the stemness factors in highly pluripotent cancer stem-like cells

The expression levels of Oct4 and Nanog mRNAs were monitored during the 12 days of treatment with 10 nM of FL3. As shown in Fig. 6, a progressive, but significant decrease of the expression rates of both transcripts was observed. These results were in agreement with those obtained at the protein level, suggesting that the drug could target the regulatory mechanisms involved in both the neosynthesis and the degradation of the two stemness factors.

Long lasting treatment with FL3 at low concentration inhibits the formation of embryoid bodies from highly pluripotent cancer stem-like cells

The growth of embryonal stem cell-derived spheres was monitored during the 12 days of treatment with 10 nM of FL3. As shown in Fig. 7, the drug significantly repressed the

formation of embryoid bodies. At day 12 of the treatment, their diameter was roughly a half of that observed for either the vehicle or the control. Thus these results suggest that the synthetic flavagline is able to interfere with the self-renewal capacities of the cancer stem-like cells and, as a consequence, to induce differentiation.

Long lasting treatment with FL3 at low concentration induces a transient expression of cleaved caspase-3 and a gradual upregulation of GCNF in highly pluripotent cancer stem-like cells

The expression levels of active caspase-3 were monitored during the 12 days of treatment with 10 nM of FL3. As shown in Fig. 8, a transient appearance of the main effector of apoptosis could be vizualized by immunoblotting, with a peak of expression at day 2 and 4 of the treatment. Otherwise, a gradual, but significant increase of the expression rates of a repressor of Oct4 gene expression, namely GCNF (for Germ Cell Nuclear Factor) was observed. These results suggest therefore that the drug targets two different regulatory mechanisms of the Oct4-ome; the first one involves a caspase-3-dependent degradation of the stemness factor, as we described previously (Emhemmed et al., 20141). The second one implicates a transcription factor which acts as the initial repressor of Oct4 promoter activity and which expression is known to be upregulated when teratocarcinomal cells are differentiating, like when they are treated with ATRA (Fuhrmann et al., 2001).

4. Discussion

By means of different experimental approaches, we demonstrate that the synthetic flavagline FL3 has a strong potential to erase the stemness signature and to induce a neural differentiation of embryonal stem-like cells. In a previous work, we reported that FL3 triggers apoptosis in teratocarcinomal cancer stem-like cells through the activation of p38 MAPK and consequently of caspase-3 (Emhemmed et al., 2014). Actually it is expected that drugs, like flavagline derivatives, could act as multi-target agents able to modulate, depending on their concentration and their contact time, the activity of specific components of the prosurvival or self-renewal machineries. Accordingly, it has been recently demonstrated that a drug, alone or in combination, can induce a dynamic rewiring of the signaling pathways depending on the order and duration of drug exposure (Lee et al., 2012). All this explains why a specific

pharmacological agent can have different molecular effects, leading to different cellular response, *i.e.* apoptosis or differentiation.

The present study demonstrates that the flavagline FL3, administrated at sub-lethal doses and for long period, is able to repress in embryonal cancer stem-like cells the expression of stemness factors at both the transcriptional and translational levels. As a consequence, cells lose their capability to selfrenew and to form embryoid bodies. In that point of view, FL3 acts like ATRA which has been described as a strong repressor of embryonic sphere formation *in vitro* (Huang et al., 2013). The blockage of the selfrenewal process has a direct impact on stem cells, either normal or pathological, which undergo then differentiation. Accordingly, we observed that long lasting treated teratocarcinomal cells with FL3 directs their specification towards neural cells, as demonstrated by the appearance of specific markers of either the neuronal or glial commitment. Actually similar observations have been reported for ATRA which preferentially induces a neuroectodermic differentiation of both embryonic and embryonal stem cells (Rohwedel et al., 1999; Xia et al., 2007).

Pluripotency and self-renewal are the main properties of normal SCs, as well as of CSCs. These characteristics are controlled by various proteins, also involved in cell proliferation and survival (Sharif et al., 2011a; Sharif et al., 2011b). Oct4, as a marker of the stemness and unrestricted pluripotency, plays a central role in the survival of poorly differentiated and highly aggressive CSCs (Ben-Porath et al., 2008; Sharif et al., 2011b; Sharif et al., 2013). Actually, at the top of the pluripotency regulatory network, Oct4, Nanog, and Sox2 work cooperatively to activate or repress numerous target genes. Modifications in the expression levels of the three transcription factors therefore induce a loss of cell pluripotency and promote either cell death or differentiation.

The molecular determinants of the cell reactivity to a long lasting treatment of low concentration of FL3 seem to be similar as those described for ATRA. Accordingly, it has been previously observed that caspase activity mediates the differentiation of embryonic cells, suggesting that the major component of the programmed cell death pathway could also be involved in the regulation of stem cell development (Abdul-Ghani et al., 2008; Fujita et al., 2008). More recently, it has been reported that drug-induced differentiation of embryonal cancer stem cells involves the degradation of stem-cell-specific proteins by caspases (Munsch et al., 2010). Oct4 and Nanog, as targets of caspase-3 activity (Emhemmed et al., 2014), are therefore concerned by this degradation-inducing differentiation process. On the other hand, we observed a progressive increase of the expression levels of GCNF in FL3-treated cancer stem-like cells. Actually, this orphan nuclear receptor represses Oct4 gene activity by

specifically binding within the proximal promoter and its upregulation in teratocarcinomal cells treated with ATRA initiates Oct4 transcript and protein downregulation during cell differentiation (Fuhrmann et al., 2001). Taken as a whole, long lasting treatment with low-dose of the synthetic flavagline activates two different regulatory mechanisms which lead to the loss of pluripotency. The first one involves a caspase-3-dependent degradation of the stemness factors, second one likely involves a GCNF-dependent gene repression of these factors. Further investigations are now necessary in order to validate an attempt to link the two different processes recruited during FL3-associated cell reactivity and Oct4 repression.

Finally, our results show that the synthetic flavagline FL3, known as a strong anticarcinogenic drug killing CSCs, could also be a novel differentiation factor, since it acts on similar molecular mechanisms as ATRA. Therefore FL3 could be a promising substitution chemotherapeutic compound in ATRA-resistant cancers.

Conflict of interest

The authors declare no conflict of interest.

Authorship Contributions

Participated in research design: FE, LD, GF

Conducted experiments: FE, SAA, GF

Contributed new reagents or analytic tools: VSK, CDM, LD

Performed data analysis: FE, SAA, GF

Wrote or contributed to the writing of the manuscript: FE, SAA, LD, GF

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Figures and legends

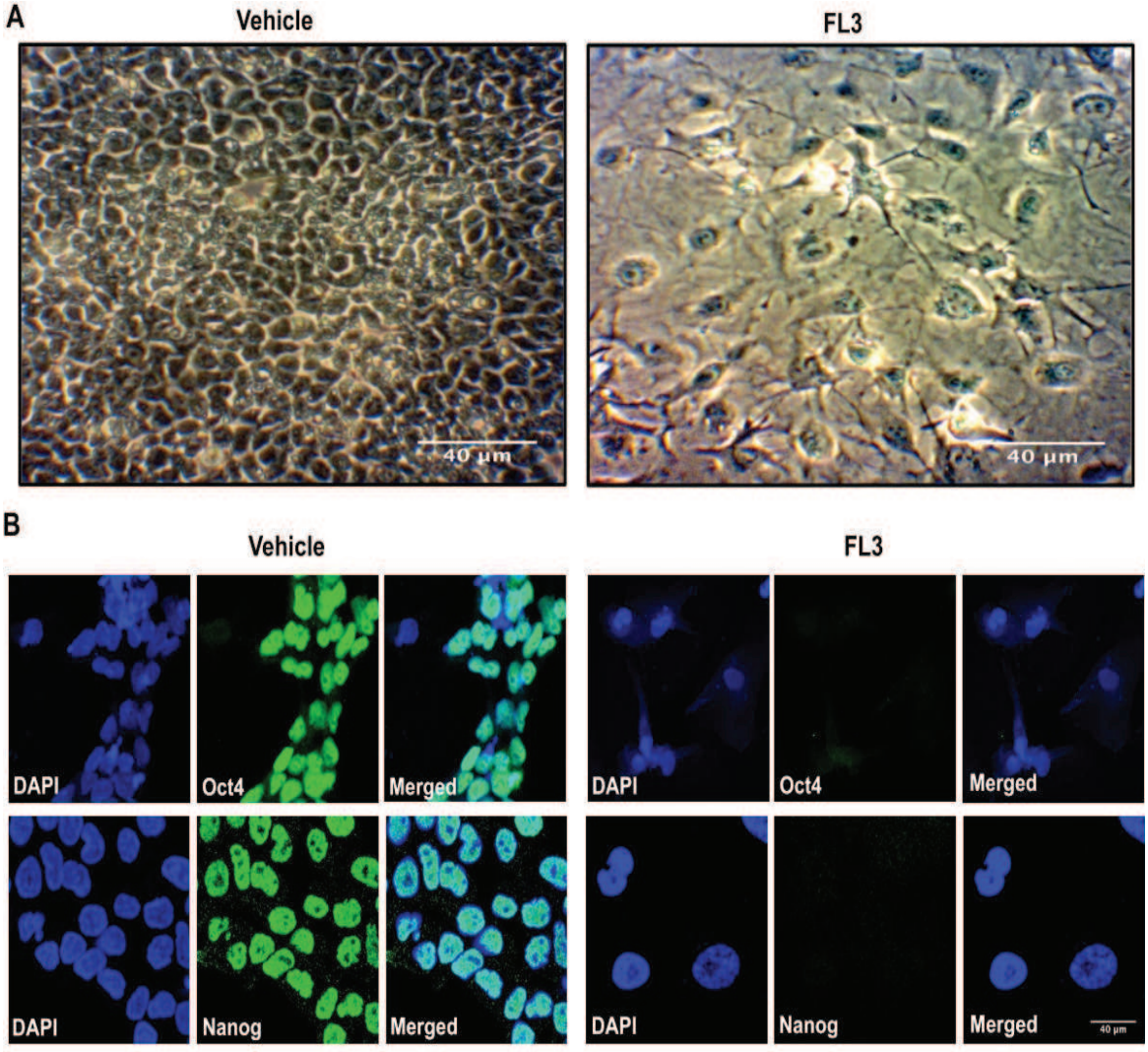

Fig. 1. FL3 treatment induces the disappearance of stemness factors in highly pluripotent cancer stem-like cells. NT2/D1 cells were exposed to either 10 nM of FL3 or to the vehicle. Panel A shows phase contrast image of cells after12 days of FL3 treatment, revealing morphological changes consistent with differentiation. Panel B shows an absence of a nuclear labelling, in relation with an expression of either Oct4 or Nanog, of cells which nuclei were stained with DAPI. Scale bar 40 mm.

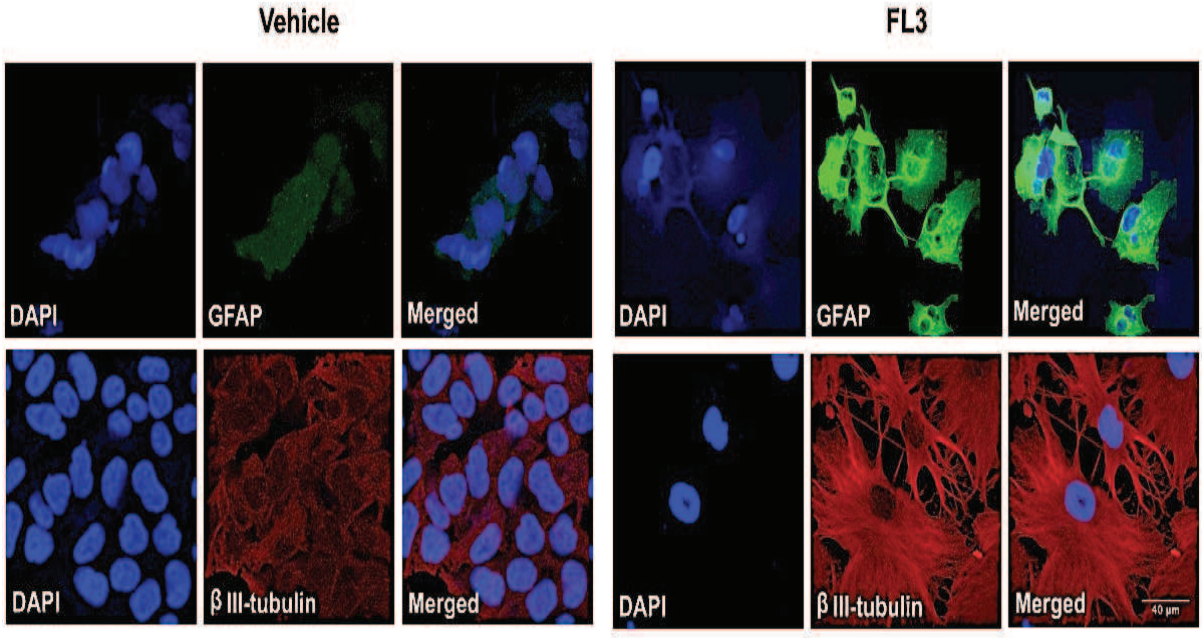

Fig. 2. FL3 treatment induces the appearance of neural markers in highly pluripotent cancer stem-like cells. NT2/D1 cells were exposed to either 10 nM of FL3 (right panel) or to the vehicle (left panel). The right panel shows the presence of a cytoplasmic labelling, in relation with an expression of either GFAP or bIII-Tubulin, of cells which nuclei were stained with DAPI. Scale bar 40 mm.

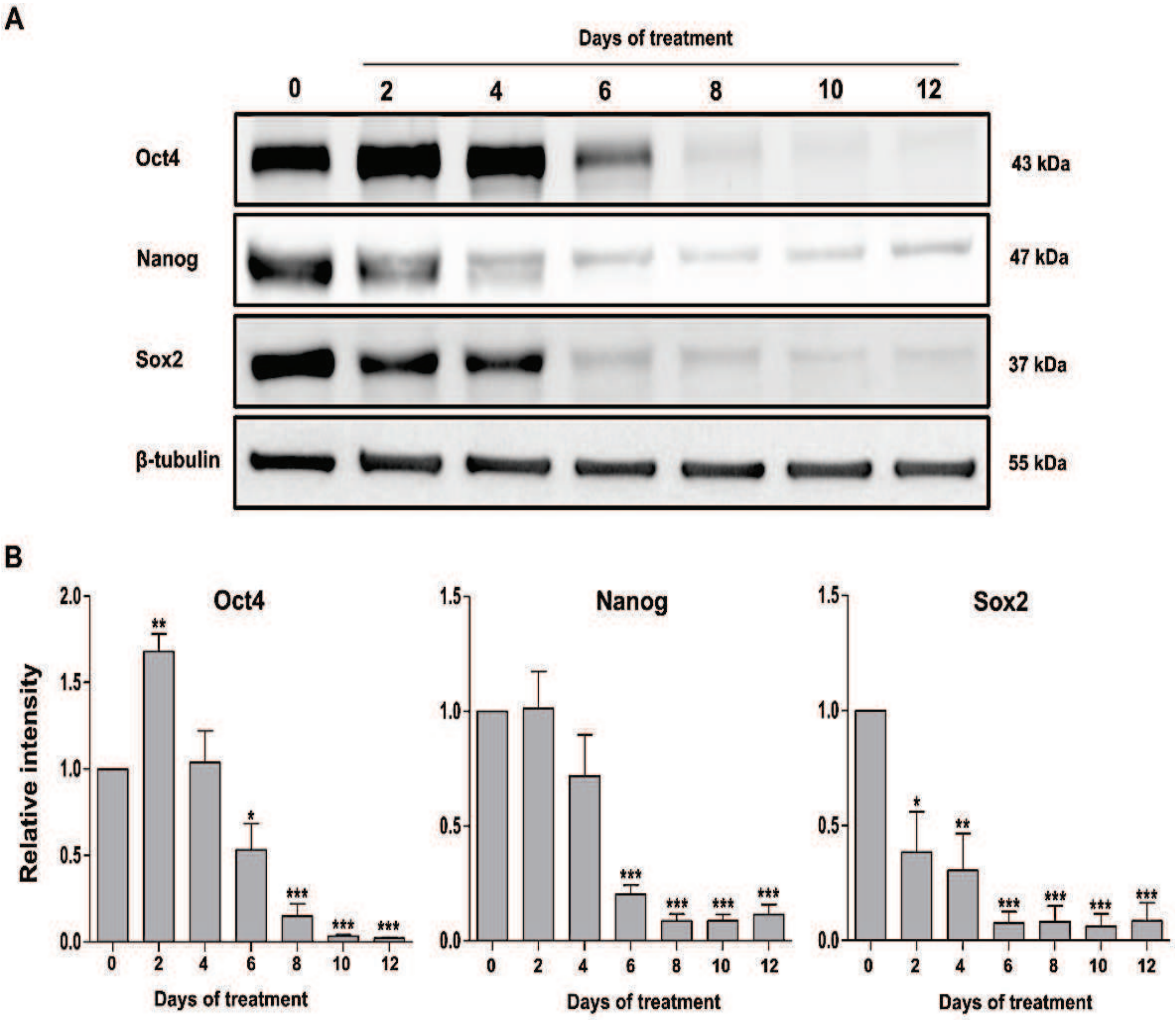

Fig. 3. Time-dependent effects of FL3 on the expression levels of the main stemness factors in NT2/D1 cells. Cells were exposed to 10 nM of FL3 and incubated at the indicated times. Immunoblotting analyses were performed as described in "Material and methods" with the corresponding antibodies. Specific bands were detected with their expected apparent molecular weight. The upper panel shows representative immunoblotting results. The three sequential lower panels show densitometry results of Oct4, Nanog and Sox2 expression normalized to b-tubulin expression and given as ratios relative to the value obtained for the untreated sample. Values are means ± S.E.M. of four independent experiments; statistically significant: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus untreated group).

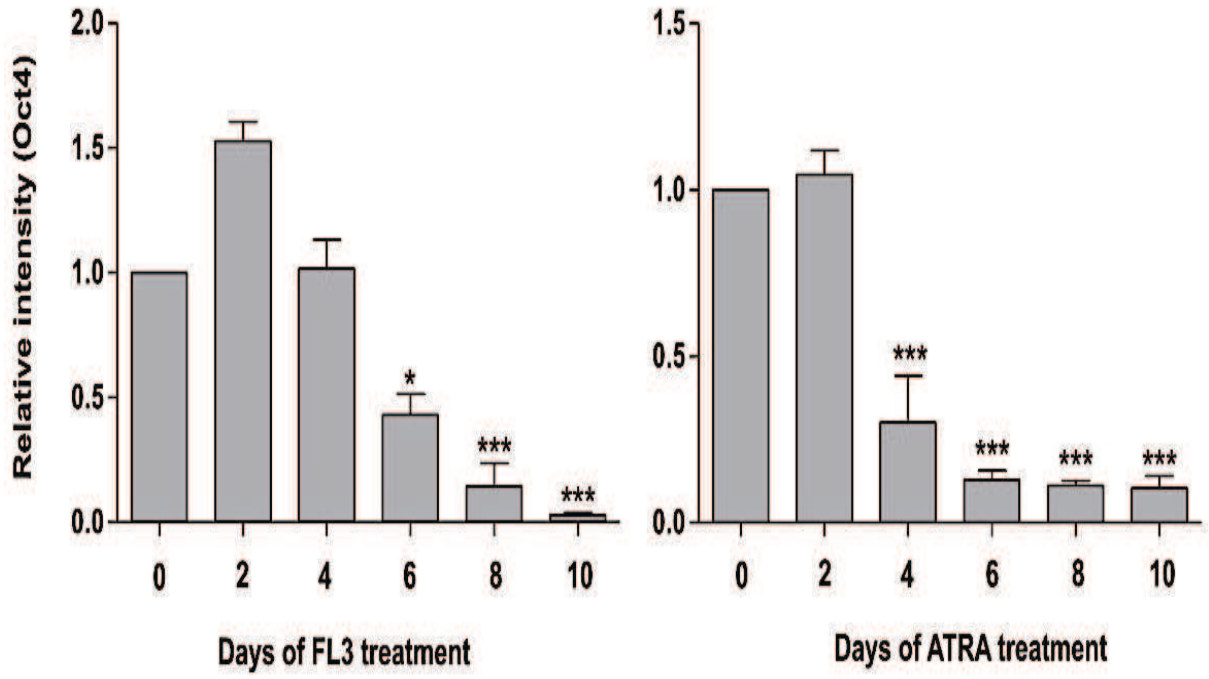

Fig. 4. Comparative time-dependent effects of FL3 and ATRA on the expression levels of Oct4 in NT2/D1 cells. Cells were exposed to either 10 nM of FL3 or 1 mM ATRA, and incubated at the indicated times. Immunoblotting analyses were performed as described in "Material and methods" with Oct4 antibody. Specific band was detected with its expected apparent molecular weight. The two panels show, for FL3 or ATRA treatment respectively, the densitometry results of Oct4 expression normalized to b-tubulin expression and given as ratios relative to the value obtained for the untreated sample. Values are means $\pm$ S.E.M. of three independent experiments; statistically significant: *, $p < 0.05$; ***, $p < 0.001$ (versus untreated group).

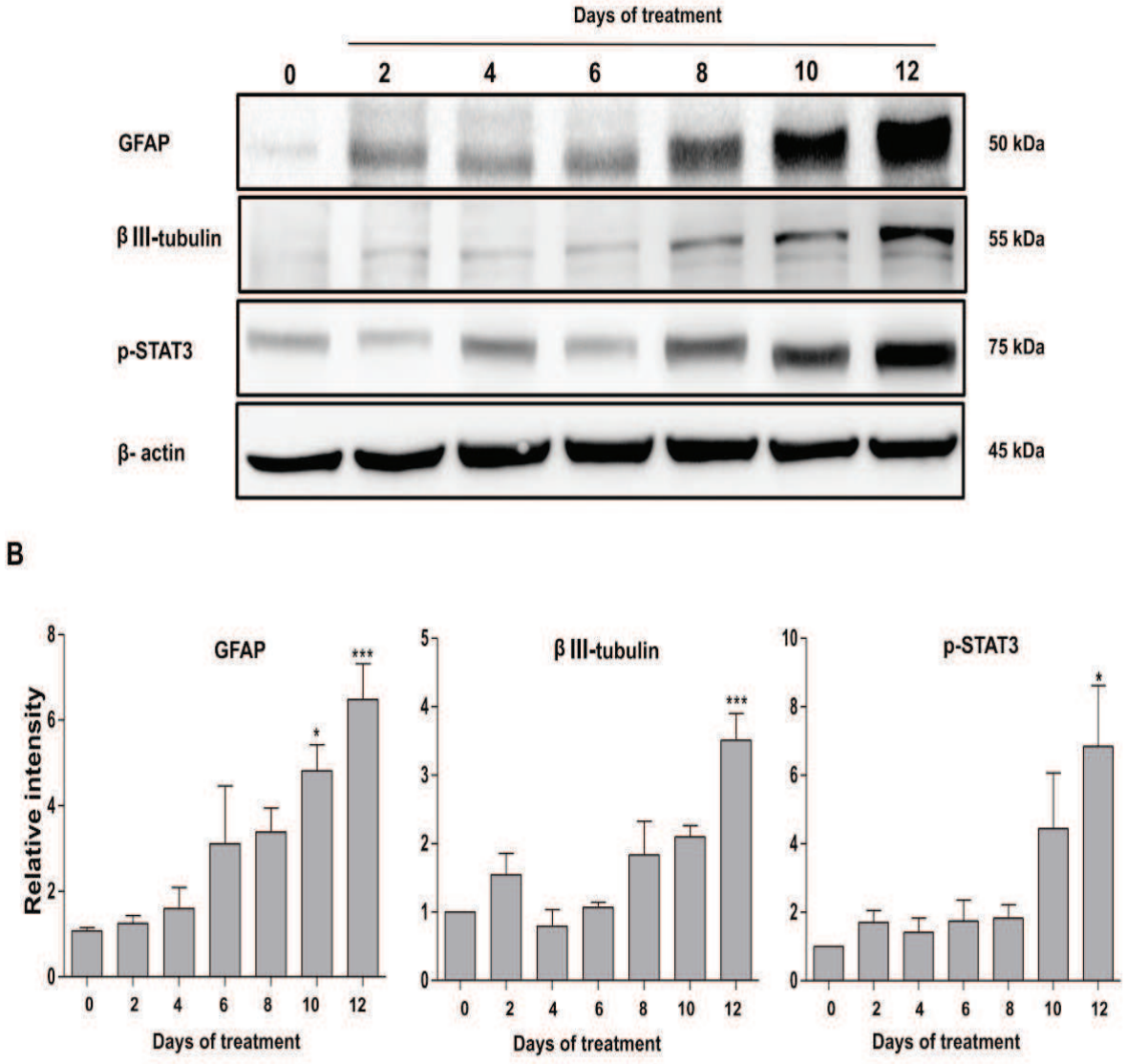

Fig. 5. Time-dependent effects of FL3 on the expression levels of neural markers in NT2/D1 cells. Cells were exposed to 10 nM of FL3 and incubated at the indicated times. Immunoblotting analyses were performed as described in "Material and methods" with the corresponding antibodies. Specific bands were detected with their expected apparent molecular weight. The upper panel shows representative immunoblotting results. The three sequential lower panels show densitometry results of GFAP, bIII-Tubulin and phospho-STAT3 expression normalized to b-actin expression and given as ratios relative to the value obtained for the untreated sample. Values are means ± S.E.M. of four independent experiments; statistically significant: *, $p < 0.05$; ***, $p < 0.001$ (versus untreated group).

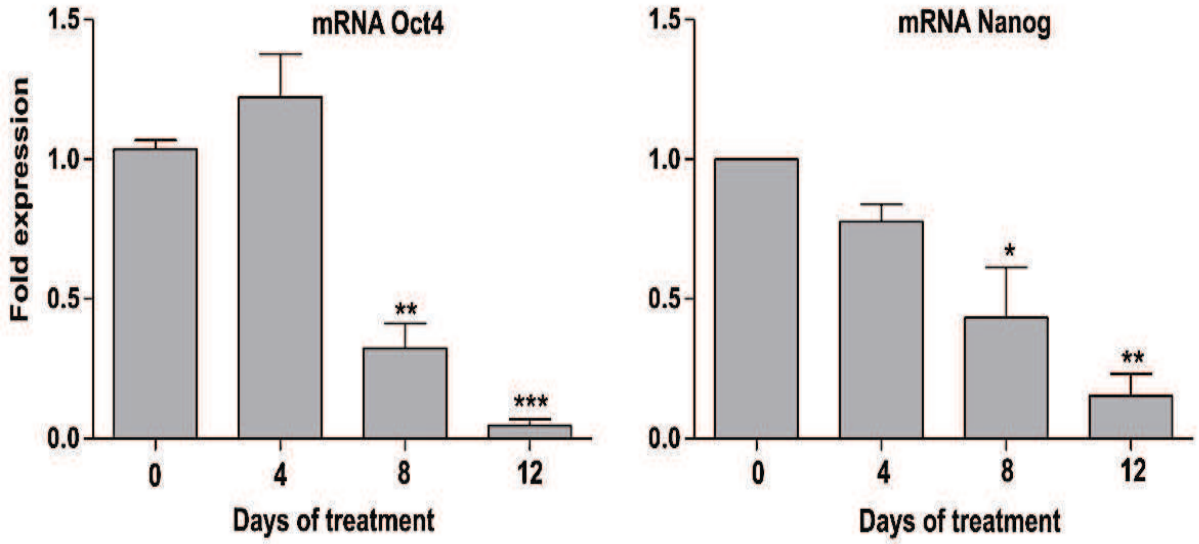

Fig. 6. Time-dependent effects of FL3 on the mRNA expression levels of stemness factors in NT2/D1 cells. Cells were exposed to 10 nM of FL3 and incubated at the indicated times. RT/PCR were performed as described in "Material and methods". The two panels show the results obtained for the expression levels of Oct4 and Nanog transcripts, normalized to the expression levels of GAPDH mRNA and given as ratios relative to the value obtained for the untreated sample. Values are means $\pm$ S.E.M. of four independent experiments; statistically significant: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (versus untreated group).

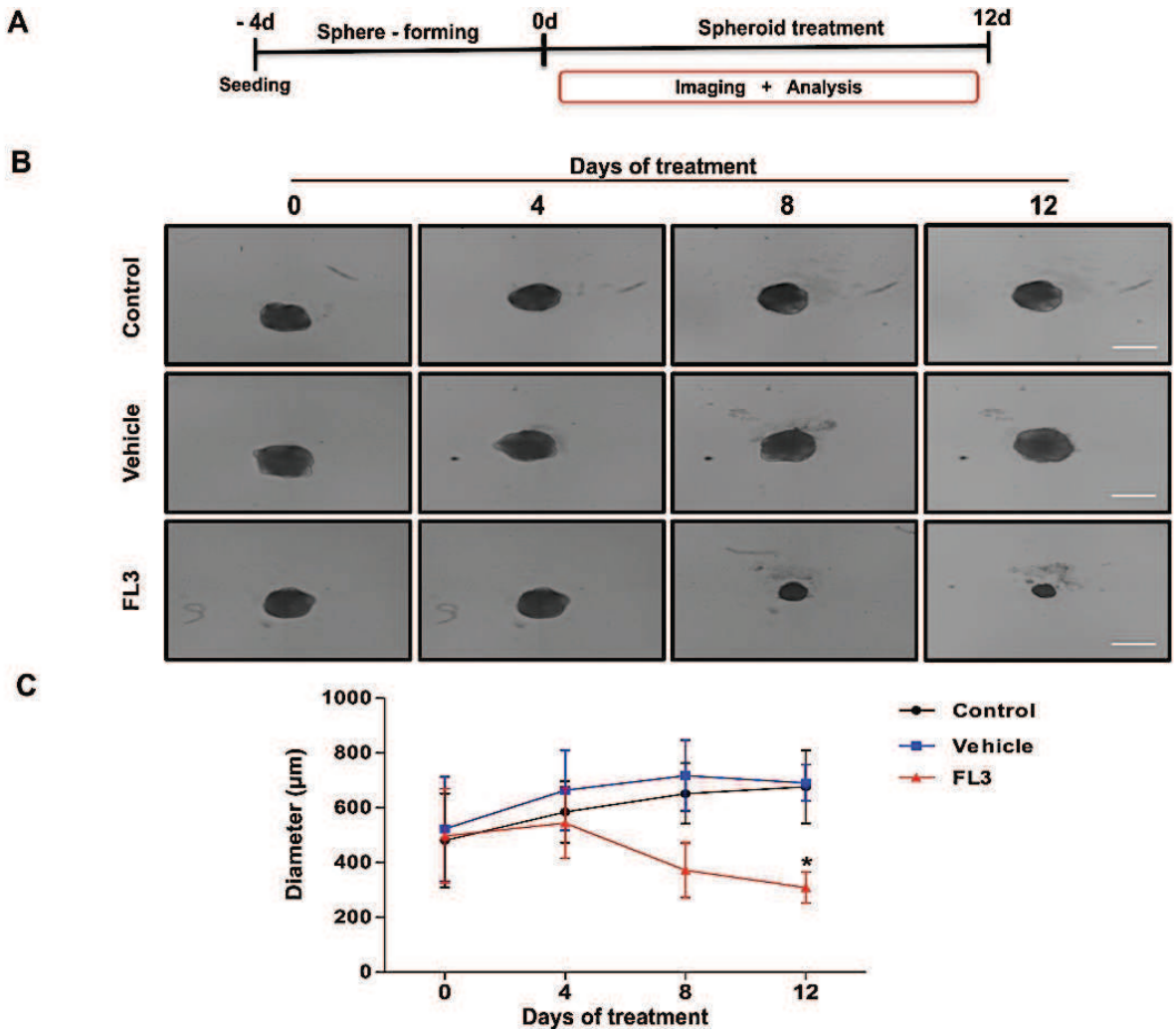

Fig. 7. FL3-dependent inhibition of tumor spheroid growth. Panel **A** shows a schematic illustration of NT2/D1 spheroid growth kinetics and FL3 treatment procedure. Cells, seeded at day -4, formed spheroids which were treated from day 0 until day 12 with 10 nM of FL3. Imaging and analysis were performed at the indicated times with a Celigo cytometer. Panel **B** shows representatives images of spheroids treated or not with either FL3 or the vehicle, and for the indicated times. Panel **C** shows on a curve graph the diameter variations of spheroids after treatment with either 10 nm FL3 or the vehicle, and for the indicated times. Values are means $\pm$ S.E.M. of four independent experiments; statistically significant: *, $p < 0.05$ (versus untreated group). Scale bar 300 mm.

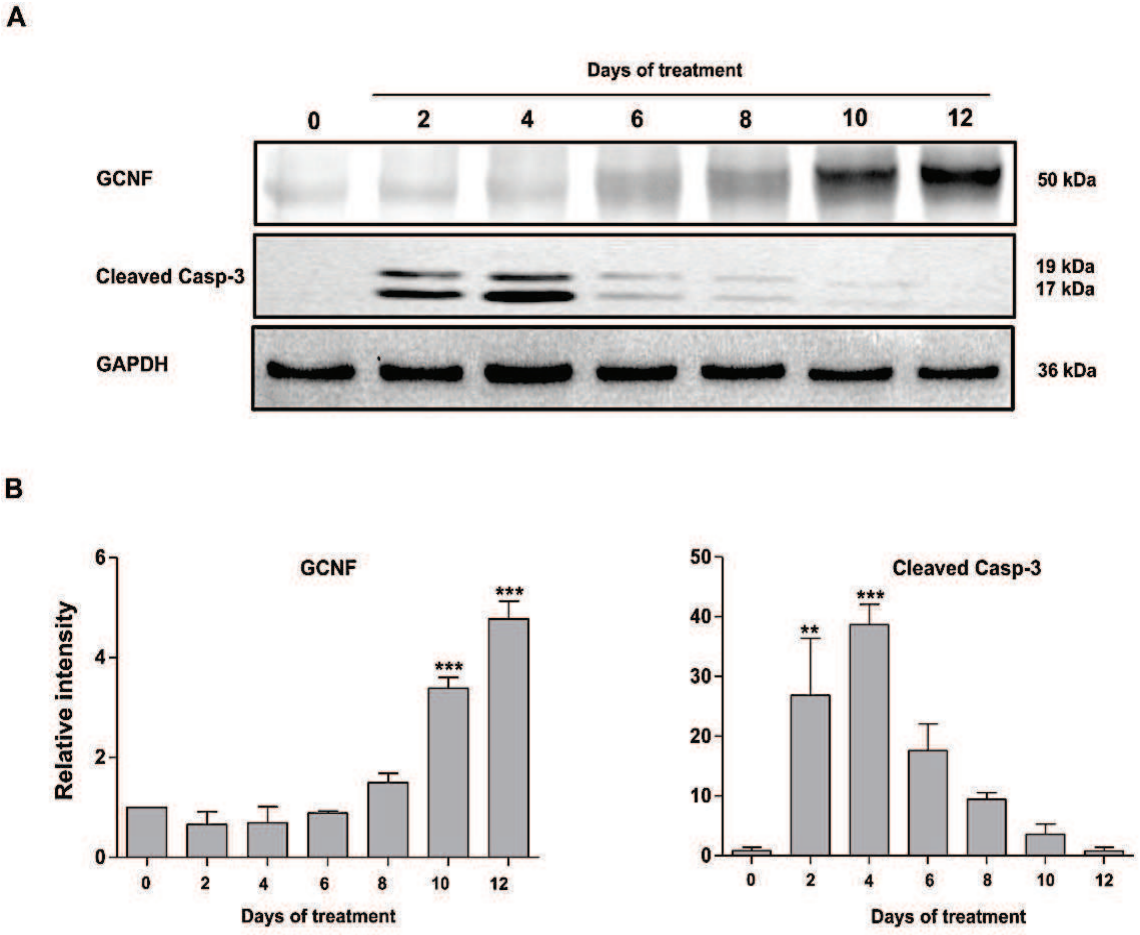

Fig. 8. Time-dependent effects of FL3 on the expression levels of GCNF and cleaved caspase-3 in NT2/D1 cells. Cells were exposed to 10 nM of FL3 and incubated at the indicated times. Immunoblotting analyses were performed as described in "Material and methods" with the corresponding antibodies. Specific bands were detected with their expected apparent molecular weight. The upper panel shows representative immunoblotting results. The two sequential lower panels show densitometry results of GCNF and active caspase-3 expression normalized to GAPDH expression and given as ratios relative to the value obtained for the untreated sample. Values are means $\pm$ S.E.M. of four independent experiments; statistically significant: **, $p < 0.01$; ***, $p < 0.001$ (versus untreated group).

Discussion and perspective

Discussion and perspective

The concept of cancer stem cell (CSC) has attracted much attention in the last two decades since the work of Dick's team which demonstrates for the first time the existence, in leukemia, of malignant stem cells (SCs), able to self-renew and to differentiate. Since then, numerous studies have also reported the presence of small populations of CSCs in solid tumors, suggesting that they can arise from embryonic, fetal or adult SCs or closely related dedifferentiated descendants. It is now admitted that CSCs are responsible for tumor initiation, development and relapse, metastasis and resistance to radiotherapy and *a priori* to numerous natural or synthetic chemical compounds (Tan, Park et al. 2006). As a direct consequence, future anticancer treatment must take into account this cell population. However the advances in drug discovery that should yield lead compounds able to target CSCs are slow. Different molecules have been proposed, such as salinomycin, the retinoid derivative (ATRA) or the HDAC inhibitor (SAHA); however these compounds, by inducing apoptosis and/or differentiation of CSCs, exhibit some cytotoxic effects on normal SCs, more specifically when they are administrated for long period (Campos, Wan et al. 2010) (Bhat-Nakshatri, Goswami et al. 2013) (Xu, Zhang et al. 2014) (Ding, Zhang et al. 2015).

Flavaglines are a family of natural cyclopenta [b] benzofurans compounds extracted from plant genus *Aglaia.* The synthetic flavagline FL3, like other members of the family (namely rocaglamide or silvestrol), is known to exert an anticancer activity on various types of cancer cells, without any cytotoxic effect on normal cells. The overall aim of our study was then to evaluate the selective anticancer activity of the drug on a model of highly malignant and pluripotent cancer stem-like cells, namely the embryonal carcinoma cells. The study includes therefore three different aspects corresponding to the FL3' properties that we have identified during the PhD training: 1) FL3 proapoptotic activity; 2) FL3 selective cytotoxicity; 3) FL3 prodifferentiating activity.

1. Pro-apoptotic activity of FL3 against CSCs and underlying molecular mechanisms involved

Our study demonstrates that FL3 triggers apoptosis in teratocarcinomal cancer stem-like cells through the activation of p38 MAPK and consequently of caspase-3 (Emhemmed,

Ali Azouaou et al. 2014). It seems therefore that the molecular mechanisms involved in flavagline-induced reactivity are different in cancer cells and CSCs. Indeed the pro-apoptotic activity of FL3 in cancer cells implicates a mitochondrio-nuclear translocation of the caspase-independent death effector AIF which leads to DNA fragmentation and cell death (Thuaud, Bernard et al. 2009). Actually, DNA damage-induced activation of a precise proapoptotic pathway is known to be cell type- and genotoxin-specific, depending amongst others on the p53/p73 status, the death receptor reactivity or MAPK responsiveness. Surely, studies of FL3 activity on other models of CSCs (including non expressing Oct4-CSCs), are needed to support our data. However it is not surprising that cancer cells and CSCs respond in a divergent manner (e.g. caspase involvement) to a specific pharmacophore (like flavagline), since the molecular determinants of their reactivity are different, although they lead to the same cellular response, e.g. apoptosis. For instance, stemness factors-expressing cancer cells have a complex pathogenesis with numerous crosstalks between the different signaling pathways, including Wnt/β-catenin, Notch, Hedgehog and the "classical" proapoptotic pathways. Such redundancy and crosstalk between the signal transduction pathways are known to trigger a drug-related regulation of various effectors and this could even negate the effect of a drug on a single downstream target (Sharif Tanveer 2011). It should also be noted that forced repression of p38 MAPK activity only partially antagonizes FL3-induced growth inhibition of embryonal carcinoma cells, suggesting that other signaling pathways may contribute to the effects of the drug. Further investigations are therefore necessary in order to identify the different partners which could be recruited during FL3-associated reactivity of CSCs.

Interestingly the drug activates a p53/p73-independent proapoptotic pathway and therefore could be a promising tool to target cancers which harbor mutations for these tumor suppressors (Muller and Vousden 2013) and which are known to be highly resistant to current chemotherapy.

We found that FL3, like many flavagline derivatives, does not generate ROS. Previous studies in our laboratory have shown that many phytochemicals, such as polyphenols or rooperol, act as prooxidant agents and exhibit an anticancer activity through a ROS-dependent activation of p53 and /or p73 (Sharif, Stambouli et al. 2013) (Azouaou, Emhemmed et al. 2015). It would therefore be interesting to study the effects of a combination of a prooxidant and non prooxidant compounds (like rooperol and FL3), which may achieve a more efficient therapeutic impact against cancer cells and CSCs.

Our findings show that the synthetic flavagline FL3 selectively exerts its cytotoxic activity on cancer stem-like cells, by inducing in particular a downregulation of the stemness factor Oct4 (Emhemmed, Ali Azouaou et al. 2014). Such modifications are known to induce a loss of cell pluripotency and promote cell death, which is in agreement with our observations. However it cannot be excluded that the remaining living cells undergo differentiation (see below). This point, as well as the "selectivity" aspect, will be discussed below.

2. Molecular mechanisms of the protection of normal cells against the cytotoxic effect of FL3

The cytotoxicity of a pharmacological agent on CSCs and cancer cells, but not on normal SCs and normal cells, provides a promising opportunity for chemotherapy with limited side effects. As FL3 has a strong cytotoxic effect on CSCs, the selectivity of its activity needed to be clarified. To address this question, we have studied the effects of the flavagline derivative on a fibroblast cell line, a model of normal SC line with restricted pluripotency and enhanced reprogramming capacity; as expected, the results have shown that high doses of FL3 which were able to strongly decrease the viability of cancer cells and cancer stem-like cells, had no obvious toxic effect on normal SCs, in a reasonable range of concentrations.

Previous studies have reported the cardioprotective and neuroprotective activities of flavagline derivatives against doxorubicin-induced cardiotoxicity and NF-kB-dependent acute neurodegenerative condition (Bernard, Ribeiro et al. 2011) (Fahrig, Gerlach et al. 2005). However, the molecular mechanism of resistance of normal cells and normal SCs to the cytotoxic effects of flavaglines remained to be elucidated.

We observe that, in contrast to cancer stem-like cells, FL3 is unable to alter the mitochondrial integrity of normal stem-like cells. Actually, the selective phosphorylation of the proapoptotic protein Bad, via an activation of Akt, is the key event for the protection of fibroblasts against the cytotoxic effects of the synthetic flavagline. However the precise mechanism by which FL3 activates Akt only in non-cancerous cells, needs further investigation.

We and others have stated that any flavagline derivative could selectively target cancer and cancer stem-like cells, but not normal and normal stem-like cells, by modulating p38MAPK activity (Zhu, Lavrik et al. 2007),(Callahan, Minhajuddin et al. 2014)

(Emhemmed, Ali Azouaou et al. 2014). Surprisingly however, our present study shows that FL3 also induces phosphorylation of p38MAPK in fibroblast cells. This suggests that FL3 activates p38MAPK in both normal and cancer SCs, but the downstream targets could be different. Actually, an interplay between Akt and p38MAPK in the survival process remains controversial. Our study suggests rather an absence of a direct effect of p38MAPK on Akt activation, thereby on cell survival. It can therefore be hypothesized that FL3 targets different interconnected signaling pathways and that the resulting effect depends on the pathological background. In the present case, the precise role and interrelationship of Akt and p38MAPK phosphorylations in the selective protection of normal SCs against FL3 need further investigations. Targeting both kinases with inhibitor tools might give more insights about their respective role in the inefficiency of FL3 to kill different types of normal stem cells, including fibroblast cells.

Taken as a whole, our findings provide novel evidence of how human normal stem-like cells are protected towards the toxicity of the anticancer compound FL3. This protection mechanism may explain the absence of the cytotoxic effect of other flavaglines derivatives on other normal cells used in previous studies. Finally flavagline derivatives are potent anticancer compounds with cytoprotective properties on noncancerous cells and therefore should attract more much attention in the future for therapeutic purposes.

3. Pro-differentiation activity of FL3 on CSCs

It has been reported that the aggressiveness of a CSC is proportional to its lineage-specific competencies. Actually several studies have reported that the differentiation level of a CSC type is inversely correlated with its resistance capacity to radio- and chemotherapy. Disrupting the molecular pathways that control CSC self-renewal is therefore an attractive alternative to weaken the aggressiveness and resistance phenotype of the tumor bulk. By targeting these pathways, it is assumed that the CSC switches from a highly proliferative and undifferentiated state to a harmless low-growing and mature state (Sharif, Stambouli et al. 2013). For instance, ATRA treatment allows a long-lasting remission of more than 90% of patients with acute promyelocytic leukemia (Ohno, Asou et al. 2003). This example shows the strongly positive impact of the differentiation strategy which is able to block the tumor development and to prevent its recurrence. However several cancers remain refractory to

ATRA and therefore new therapeutic strategies have to be developed to cure them. We therefore evaluate the in vitro pro-differentiating properties of flavagline derivative on embryonal teratocarcinoma stem cells.

Our study shows that FL3 selectively induces differentiation of pluripotent cancer stem-like cells at sub-lethal concentrations. This chemopreventive effect involves a downregulation of the major guardian of a highly pluripotent cell state, namely Oct4. As a consequence, cells undergo differentiation towards neural specification.

The molecular determinants of the cell reactivity to a long lasting treatment of low concentration of FL3 seem to be similar as those described for ATRA. Accordingly, we observe that the drug-induced differentiation of embryonal cancer stem cells involves, like for ATRA, the degradation of stem cell-specific proteins by caspases, suggesting that the major components of the programmed cell death pathway could also be involved in the regulation of stem cell differentiation.

Oct4, as a target of caspase-3 activity (Emhemmed, Ali Azouaou et al. 2014), is concerned by the protein degradation process accompanying FL3-induced cell differentiation. Interestingly, we also observe, in FL3-treated cancer stem-like cells, a progressive increase of the expression levels of GCNF, concomitantly with a decrease of Oct4 mRNA content. Actually, this orphan nuclear receptor is known to repress Oct4 gene activity and its upregulation in teratocarcinomal cells treated with ATRA initiates a downregulation of Oct4 transcript and protein during cell differentiation (Fuhrmann, Chung et al. 2001). Taken as a whole, our study shows therefore that long lasting treatment with sub lethal doses of the synthetic flavagline activates two different regulatory mechanisms which lead to the loss of pluripotency in cancer stem-like cells. The first one involves a caspase-3-dependent degradation of the stemness factor Oct4, the second one involves a GCNF-dependent gene repression of this factor. Further investigation, by means of knock-down experiments, is now necessary in order to specify the potential link between the two processes recruited during FL3-induced CSC differentiation.

Our whole study reports that the synthetic flavagline FL3 can act as a selective anticarcinogenic drug which is able to kill or to differentiate embryonal carcinoma stem cells, depending on the concentrations and duration of the treatment. This study needs now to be extended to other models of CSCs, including those showing increased resistance to existing chemotherapies. The challenge is to decipher all in vitro and in vivo aspects of the biological activity of FL3, in terms of cellular and molecular targets, prior to clinical trials.

Discussion et perspective en français

Le concept de cellules souches cancéreuse (SCC) a attiré beaucoup d'attention dans les deux dernières décennies, depuis les travaux de l'équipe de Dick qui démontre pour la première fois l'existence, dans la leucémie, de cellules malignes souches (CS), capables d'auto-renouvellement et de se différencier. Depuis, de nombreuses études ont également été signalé la présence de petites populations de cellules souches cancéreuses dans les tumeurs solides, ce qui suggère qu'elles peuvent provenir de cellules embryonnaires, fœtales ou adultes SC ou de descendants dédifférenciés étroitement liées. Il est maintenant admis que les CSC sont responsables de l'initiation de la tumeur, le développement et la rechute, la métastase et la résistance à la radiothérapie à de nombreux composés chimiques naturels ou synthétiques (Tan, Park et al. 2006). Comme conséquence directe, les futurs traitements anticancéreux devront tenir compte de cette population de cellules. Toutefois, les progrès dans la découverte de médicaments qui devraient donner des composés capables de cibler les CSC sont lents. Différentes molécules ont été proposées, telles que la salinomycine, dérivé de l'acide rétinoïque (ATRA) ou l'inhibiteur de HDAC (SAHA); cependant ces composés, par induction de l'apoptose et / ou de la différenciation de cellules souches cancéreuses, présentent des effets cytotoxiques sur les cellules normales, plus particulièrement lorsqu'ils sont administrés pendant une longue période (Campos, Wan et al. 2010) (Bhat-Nakshatri, Goswami et al. 2013) (Xu, Zhang et al. 2014) (Ding, Zhang et al. 2015).

Les flavaglines sont une famille de cyclopenta [b] benzofuranes, composés naturels extraits des plante du genre Aglaia. La flavagline synthétique FL3, comme d'autres membres de la famille (à savoir rocaglamide ou silvestrol), est connu pour exercer une activité anticancéreuse sur divers types de cellules cancéreuses, sans aucun effet cytotoxique sur les cellules normales. L'objectif global de notre étude était d'évaluer l'activité anticancéreuse sélective du médicament sur un modèle de cellules souches un cancer hautement malin et pluripotent, à savoir les cellules de carcinome embryonnaire. L'étude comprend donc trois différents aspects correspondant aux propriétés de FL3 que nous avons identifiés au cours de notre doctorat: 1) FL3 et activité pro-apoptotique; 2) FL3 et cytotoxicité sélective; 3) FL3 et activité de différenciation.

7.1. Activité pro-apoptotique de FL3 contre les CSC : mécanismes moléculaires sous-jacents.

Notre étude démontre que FL3 déclenche l'apoptose dans les cellules souches comme le cancer du tératocarcinome par l'activation de p38 MAPK et par conséquent de la caspase-3 (Emhemmed, Ali Azouaou et al. 2014). Il semble donc que les mécanismes moléculaires impliqués dans la réactivité à la flavagline sont différents dans les cellules cancéreuses et les cellules souches cancéreuses. En effet, l'activité pro-apoptotique de FL3 dans les cellules cancéreuses implique une translocation nucléaire et mitochondriale de l'effecteur de mort AIF caspase-indépendant qui conduit à la fragmentation de l'ADN et la mort cellulaire (Thuaud, Bernard et al. 2009). L'activation faite, les dommages à l'ADN induit d'une voie pro-apoptotique précise connue pour être type cellulaire et génotoxine, en fonction, entre autres, du statut p53 / p73 et de la réactivité des récepteurs de mort ou MAPK réactivité. Certes, les études de l'activité FL3 sur d'autres modèles de CSC (y compris n'exprimant pas Oct4-CSC), seront nécessaires pour infirmer nos données. Toutefois, il n'est pas surprenant que les cellules cancéreuses et les cellules souches cancéreuses réagissent d'une manière divergente (par exemple par la participation de la caspase) à un pharmacophore spécifique (comme la flavagline), puisque les déterminants moléculaires de leur réactivité sont différentes, même si elles conduisent à la même réponse cellulaire, par exemple, l'apoptose. Par exemple, les facteurs de souchitude exprimé par les cellules cancéreuses sont associés à la pathogenèse de nombreuses redondances entre les différentes voies de signalisation, y compris Wnt / β-caténine, Notch, Hedgehog et des voies pro-apoptotiques "classiques". Ces redondances entre les voies de transduction du signal sont connus pour déclencher un dérèglement de différents effecteurs lié à la molécule et cela pourrait même annuler l'effet d'un médicament sur une seule cible en aval (Sharif Tanveer 2011). Il convient également de noter que l'inhibition forcée de l'activité de MAPK p38 antagonise partiellement l'inhibition de la croissance induite par FL3 chez les cellules du carcinome embryonnaire, ce qui suggère que d'autres voies de signalisation peuvent contribuer aux effets du médicament. D'autres investigations sont donc nécessaires afin d'identifier les différents partenaires qui pourraient être recrutés lors de la réactivité FL3-associé des CSC.

Il est intéressant de noter que la molécule active une voie pro-apoptotique p53/p73 indépendante et donc pourrait être un outil prometteur pour cibler les cancers qui hébergent

des mutations de ces suppresseurs de tumeurs (Muller and Vousden 2013)connus pour être très résistante à la chimiothérapie actuelle.

Nous avons constaté que FL3, comme de nombreux dérivés de flavagline, ne génère pas de ROS. Des études antérieures dans notre laboratoire ont montré que de nombreux composés phytochimiques, tels que les polyphénols ou rooperol, agissent comme des agents pro-oxydants et présentent une activité anticancéreuse par une activation ROS dépendant de p53 et / ou p73 (Sharif, Stambouli et al. 2013) (Azouaou, Emhemmed et al. 2015). Il serait donc intéressant d'étudier les effets d'une combinaison d'un composé pro-oxydant et d'un composé non pro-oxydants (comme rooperol et FL3), qui peuvent avoir un impact thérapeutique plus efficace contre les cellules cancéreuses et les cellules souches cancéreuses.

Nos résultats montrent que la flavagline synthétique FL3 exerce son activité cytotoxique sélectivement sur les cellules souches comme le cancer, en induisant notamment une régulation à la baisse du facteur de souchitude Oct4 (Emhemmed, Ali Azouaou et al. 2014). De telles modifications sont connues pour induire une perte de pluripotence des cellules et de promouvoir la mort des cellules, en accord avec nos observations. Toutefois, il ne peut être exclu que les cellules vivantes restantes subissent une différenciation. Ce point, ainsi que l'aspect "sélectivité", seront discutés ci-après.

7.2. Mécanismes moléculaires de la protection des cellules normales contre l'effet cytotoxique de FL3

La cytotoxicité d'un agent pharmacologique sur les CSCs et les cellules cancéreuses, mais pas sur les CSN et les cellules normales, offre une opportunité prometteuse pour la chimiothérapie avec des effets secondaires limités. Comme FL3 a un effet cytotoxique fort sur les cellules souches cancéreuses, la sélectivité de son activité se doit d'être clarifié. Pour répondre à cette question, nous avons étudié les effets du dérivé FL3 de la flavagline sur une lignée de cellules de fibroblastes, un modèle de lignée de CSN présentant une pluripotence restreinte et un renforcement de la capacité de reprogrammation; comme prévu, les résultats ont montré que des doses élevées de FL3 qui étaient en mesure de diminuer fortement la viabilité des cellules cancéreuses et les cellules souches cancéreuses, n'a eu aucun effet toxique évident sur CS normales, dans une gamme de concentration raisonnable.

Des études antérieures ont rapporté les activités cardioprotectrices et neuroprotectrices des dérivés de la flavagline dans la cardiotoxicité induite par la doxorubicine et la neuro-dégénération NF-kB-dépendante aiguë (Bernard, Ribeiro et al. 2011) (Fahrig, Gerlach et al. 2005). Cependant, le mécanisme moléculaire de la résistance des cellules normales et des cellules souches normales aux effets cytotoxiques de la flavagline reste à élucider. Nous observons que, contrairement aux cellules souches cancéreuses, FL3 est incapable d'altérer l'intégrité mitochondriale des cellules souches normales. En fait, la phosphorylation sélective de la protéine pro-apoptotique Bad, via une activation de Akt, est l'événement clé pour la protection des fibroblastes contre les effets cytotoxiques de la flavagline synthétique. Toutefois, l'étude du mécanisme précis par lequel FL3 active Akt uniquement dans les cellules non cancéreuses se doit d'être approfondie. Nous, comme d'autres avant nous, avions indiqués que tout dérivé de flavagline pourrait cibler sélectivement les cellules souches comme le cancer et le cancer, mais pas les cellules souches normales, en modulant l'activité p38MAPK (Zhu, Lavrik et al. 2007),(Callahan, Minhajuddin et al. 2014) (Emhemmed, Ali Azouaou et al. 2014). Étonnamment cependant, notre étude actuelle montre que FL3 induit également la phosphorylation de p38MAPK dans les cellules de type fibroblaste. Ceci suggère que FL3 active la p38MAPK dans les cellules souches normales et cancéreuses, mais que les cibles en aval pourraient bien être différentes. En fait, une interaction entre Akt et p38MAPK dans le processus de survie reste controversée. Notre étude suggère plutôt une absence d'un effet direct de p38MAPK sur l'activation d'Akt et de ce fait sur la survie de la cellule. On peut donc émettre l'hypothèse que FL3 cible différentes voies de signalisation interconnectées et que l'effet qui en résulte dépend du phénotype pathologique. Dans le cas présent, le rôle exact et l'interrelation entre Akt et la phosphorylation de P38MAPK dans la protection sélective des cellules souches normales contre FL3 a besoin d'investigations complémentaires pour être explicité plus avant. Cibler les deux kinases avec des inhibiteurs pourrait donner de nouvelles idées au sujet de leur rôle respectif dans l'inefficacité de FL3 de tuer différents phénotypes de cellules souches normales, y compris les cellules de fibroblastes.

Pris dans son ensemble, nos résultats fournissent des indices sur comment les cellules souches humaines normales sont protégées de la toxicité du composé anticancéreux FL3. Ce mécanisme de protection peut expliquer l'absence de l'effet cytotoxique d'autres dérivés de flavagline sur d'autres cellules normales utilisées dans les études précédentes. Enfin les dérivés de flavagline sont des composés anticancéreux puissants ayant des propriétés

cytoprotectrices sur les cellules non cancéreuses et devraient donc attirer à l'avenir beaucoup plus d'attention à des fins thérapeutiques.

7.3. FL3 stimule la différenciation des CSCs

Il a été rapporté que l'agressivité d'un CSC est proportionnelle à ses compétences spécifiques de lignée cellulaire. En fait, plusieurs études ont indiqué que le niveau de différenciation d'un type CSC est inversement corrélé à la capacité de résistance à la radio- et chimiothérapie. Perturber les voies moléculaires qui contrôlent l'auto-renouvellement des CSC est une alternative intéressante afin d'affaiblir l'agressivité et changer le phénotype de résistance de la masse tumorale. En ciblant ces voies, il est supposé que la CSC passe d'un état hautement prolifératif et indifférencié à un état de faible croissance et maturation rendant les cellules inoffensives (Sharif, Stambouli et al. 2013). Par exemple, le traitement par l'ATRA permet une rémission de longue durée de plus de 90% des patients atteints de leucémie promyélocytaire aiguë (Ohno, Asou et al. 2003). Cet exemple montre l'impact fortement positif de la stratégie de différenciation capable de bloquer le développement de la tumeur et de prévenir sa récurrence. Cependant plusieurs cancers restent réfractaires à l'ATRA et donc de nouvelles stratégies thérapeutiques se doivent d'être développées pour guérir ces patients. Nous avons ainsi évalué les propriétés in vitro de pro-différenciation des dérivés de la flavagline sur les cellules souches embryonnaires de tératocarcinome.

Notre étude montre que FL3 induit sélectivement la différenciation des cellules souches cancéreuses pluripotentes à des concentrations sub-létales. Cet effet de chimio-prévention implique une régulation négative du gardien principal de l'état hautement pluripotent de la cellule, à savoir Oct4. En conséquence, les cellules engagent une différenciation vers une spécification neuronale. Les déterminants moléculaires de la réactivité des cellules à un traitement de longue durée à faible concentration de FL3 semblent être similaires à celles décrites pour l'ATRA. Par conséquent, nous observons que la différenciation induite par le médicament des cellules souches cancéreuses embryonnaires implique, comme pour l'ATRA, la dégradation des protéines spécifiques des cellules souches par les caspases, ce qui suggère que les principaux composants de la voie de la mort cellulaire programmée pourraient également être impliqués dans la régulation de différenciation des cellules souches.

Oct4, une cible d'activité de la caspase-3 (Emhemmed, Ali Azouaou et al. 2014), est concernée par le processus de dégradation des protéines accompagnant la différenciation cellulaire induite par FL3. Fait intéressant, on observe également, dans les cellules souches cancéreuses traitées par FL3, une augmentation progressive des niveaux d'expression de GCNF, de manière concomitante à une diminution de la teneur en ARNm d'Oct4. En fait GCNF, ce récepteur nucléaire orphelin, est connu pour réprimer l'activité du gène Oct4 et sa régulation à la hausse dans les cellules teratocarcinomales traitées avec l'ATRA déclenche une régulation à la baisse de transcription d'Oct4 et des protéines au cours de la différenciation des cellules (Fuhrmann, Chung et al. 2001) Pris dans son ensemble, notre étude montre donc que le traitement de longue durée à des doses sous létales par la flavagline synthétique FL3 active deux mécanismes différents de régulation qui conduisent à la perte de la pluripotence des cellules souches cancéreuses. La première implique une dégradation de la caspase-3 dépendante du facteur de souchitude Oct4, le second comporte un gène de répression GCNF dépendante de ce facteur. Une prospection plus poussée, au moyen d'expériences "knock-down", est maintenant nécessaire afin de préciser le lien potentiel entre les deux processus recrutés lors de la différenciation des CSC induite par FL3.

L'ensemble de notre étude indique que flavagline synthétique FL3 peut agir comme un médicament anticancéreux sélectif capable de tuer ou de différencier les cellules souches embryonnaires de carcinome, en fonction des concentrations et la durée du traitement. Cette étude doit maintenant être étendue à d'autres modèles de CSC, y compris ceux présentant une résistance accrue aux chimiothérapies existantes. Le défi est de déchiffrer *in vitro* et *in vivo* tous les aspects de l'activité biologique de FL3, en termes de cibles cellulaires et moléculaires, avant de passer aux essais cliniques.

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Annexes

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The polyphenolic-rich *Aronia melanocarpa* juice kills teratocarcinomal cancer stem-like cells, but not their differentiated counterparts

*Tanveer Sharif, Mouni Stambouli, Benjamin Burrus, Fathi Emhemmed, Israa Dandache, Cyril Auger, Nelly Etienne-Selloum, Valérie B. Schini-Kerth, Guy Fuhrmann**

UMR 7213 CNRS, Laboratoire de Biophotonique et Pharmacologie, Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, 67401 Illkirch, France

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ABSTRACT

A diet rich in plant-derived products is expected to have anticancer chemopreventive effects by acting on the appearance and growth of cancer stem cells (CSCs). Thus the effects of *Aronia melanocarpa* juice (AMJ) on the mouse embryonal carcinoma (EC) stem cell line P19 were investigated. AMJ inhibited cell proliferation, induced cell cycle arrest in S phase and triggered apoptosis. A pronounced upregulation of tumour suppressors p53 and p73 was observed in association with caspase-3 activation and a downregulation of the anti-apoptotic protein UHRF1 and the stemness factor Oct-4. Overall the results strongly suggest that AMJ is functionally able to counteract the carcinogenesis process by targeting CSCs. Interestingly AMJ selectively kills undifferentiated EC cells, without significant effects on normal restricted pluripotent cells (*i.e.* NIH/3T3 fibroblasts) or even differentiated EC cells. This argues that a differentiation therapy might normalize the pathological phenotype of a CSC which becomes insensitive to further plant-derived pharmacological treatment.

1. Introduction

On the basis of epidemiological data, it has recurrently been reported that a diet rich in herbs, spices, fruits and vegetables has cancer-protective properties (Surh, 2003). Indeed, increased consumption of fruits and vegetables has been shown to be associated with a reduction of risk of cancer death by 6% (Pauwels, 2011; Sofi, Cesari, Abbate, Gensini, & Casini, 2008). Similarly, prevention of cancer has been linked with intake of tea or grape-derived products (Chung, Schwartz, Herzog, & Yang, 2003; Walter et al., 2010). The presence in these plant-derived products of both micronutrients (for instance vitamins and minerals) and non-nutritive components (known as phytochemicals), especially polyphenols, could explain such anticancer effects. High intake of such functional food is therefore essential in the prevention, management and treatment of cancer (Manchali, Kotamballi, Murthy, & Patil, 2012; Tsuda et al., 2004). Accordingly numerous bioactive compounds isolated from plants of terrestrial or marine origins have shown strong chemotherapeutic potential *in vitro* (Isa et al., 2012; Kang et al., 2012; Lee & Pan, 2012). Although their effects on cancer cells are well documented (Shu, Cheung, Khor, Chen, & Kong, 2010), their impact on cancer stem cells (CSCs) remains poorly understood, to a large extent because of the absence of well characterized experimental models.

* *Corresponding author.* Tel.: +33 3 68 85 42 17; fax: +33 3 68 85 43 13.
E-mail address: guy.fuhrmann@unistra.fr (G. Fuhrmann).
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PRECLINICAL STUDIES

Selective ROS-dependent p53-associated anticancer effects of the hypoxoside derivative rooperol on human teratocarcinomal cancer stem-like cells

Sarah Ali Azouaou · Fathi Emhemmed · Noureddine Idris-Khodja · Annelise Lobstein · Valérie Schini-Kerth · Christian D. Muller · Guy Fuhrmann

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Abstract Cancer stem cells (CSCs) are potential targets for innovative anticancer therapies that involve natural products with potential chemopreventive effects. We therefore analyzed the antineoplastic activity of rooperol, the aglycone of the plant-derived compound hypoxoside, on a model of Oct4-expressing cancer stem-like cell, i.e. the human embryonal carcinoma (EC) cell NT2/D1.

Rooperol selectively inhibited the proliferation of NT2/D1 cells in a concentration-dependent manner and had no effect on either normal embryonic fibroblasts which are more restrictive pluripotent stem cells or on NCCIT p53-mutant EC cells. Accordingly, rooperol only eliminates colon carcinoma cells expressing p53.

Rooperol treatment triggered cell death on NT2/D1 cells through the alteration of mitochondrial membrane potential and production of reactive oxygen species (ROS). Rooperol-induced apoptosis was associated with activation of p53 and concentration-dependent changes of the expression levels of both caspase 3 and poly ADP ribose polymerase type 1 cleaved subunits. These modifications were accompanied by a downregulation of Oct4 and its two partners involved in the maintenance of cell pluripotency and self-renewal, Nanog and Sox2.

Treatment with intracellular membrane permeant O_2^- scavengers prevented rooperol-induced apoptosis and upregulation of the expression of p53 and active caspase-3. Our findings indicate that rooperol mediates its growth inhibitory effects on CSCs via a mitochondrial redox-sensitive mechanism. We propose that abrogating the expression of the stemness regulators is a prerequisite for rooperol to fully exert its pro-apoptotic properties on wild-type p53-bearing CSCs.

Keywords Cancer stem-like cells · Rooperol · Apoptosis · Reactive oxygen species · p53 · Oct4

S. Ali Azouaou · F. Emhemmed · N. Idris-Khodja · V. Schini-Kerth · G. Fuhrmann (✉)
UMR 7213 CNRS, Laboratoire de Biophotonique et Pharmacologie, Faculté de Pharmacie, Université de Strasbourg, 74 route du Rhin, 67401 Illkirch, France
e-mail: guy.fuhrmann@unistra.fr

A. Lobstein · C. D. Muller
UMR 7200 CNRS, Laboratoire d'Innovation Thérapeutique, Faculté de Pharmacie, Université de Strasbourg, 74 route du Rhin, 67401 Illkirch, France

Present Address:
S. Ali Azouaou · F. Emhemmed
UMR 7200 CNRS, Laboratoire d'Innovation Thérapeutique, Faculté de Pharmacie, Université de Strasbourg, 74 route du Rhin, 67401 Illkirch, France

Present Address:
N. Idris-Khodja
Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, 3755 Côte-Ste-Catherine Rd., Montreal, QC H3T 1E2, Canada

Introduction

The cancer stem cell (CSC) is believed to be the initiator of tumor development and metastatic outgrow, as well as the causal factor of cancer resistance to radiotherapy and numerous natural and synthetic anticancer chemical compounds [1, 2]. It is now accepted that CSCs arise from normal stem cells (NSCs) exposed to repetitive mutation-inducing stress injuries [2, 3]; actually CSCs share many characteristics with NSCs and can be defined by their ability to undergo self-renewal and to differentiate into more restricted cell types (from pluripotency to monopotency). The capacity to self-renew allows the expansion of NSC or CSC pool in response to

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Towards New Anticancer Strategies by Targeting Cancer Stem Cells with Phytochemical Compounds

Sharif Tanveer, Emhemmed Fathi and Fuhrmann Guy
Laboratoire de Biophotonique et Pharmacologie –UMR 7213 CNRS-
Faculté de Pharmacie –UDS-
France

1. Introduction

Cancer stem cells (CSCs) are believed to be responsible for tumor initiation and development, metastasis and resistance to radio-therapy and *a priori* to numerous natural or synthetic chemical compounds. A large body of observations support now the 100 year old hypothesis which predicted a clonal genetic background of the heterogeneous cell population found in a tumor outgrowth [Paget, 1889]. Accordingly, since Dick's laboratory pioneering work in 1994 [Lapidot et al., 1994], growing realizations suggest that CSCs arise from embryonic, fetal or adult stem cells (SCs) or closely related dedifferentiated descendants. Interestingly, the concept that CSCs give rise to the bulk cancer cells is in accordance with the germ theory of disease developed by Koch in the 19th century [Garcion et al., 2009]. This theory points out that any disease has a unique causative agent. Although Koch's dogma suggests that tumors should arise from CSCs, it must also be borne in mind that their descendant cancer cells can generated dedifferentiated cells with a parental phenotype and therefore can be involved in the outburst of a secondary cancer.

On the basis of epidemiological data, it has been recurrently reported that diet rich in fruits and vegetables has cancer-protective properties; this suggests that plant-derived compounds are able to restrict the expansion of CSCs and even to kill them. The chemotherapeutic benefits of different natural or synthetic phytochemical agents on cancer cells are well documented. However their effects on CSCs are poorly understood, to a large extent because on the absence of well characterized experimental models. The objective of this chapter is therefore to recapitulate some aspects of the biology of CSCs and to propose different cellular tools and molecular preys for thorough pharmacological studies on CSCs, on the basis of the most recent data concerning the stemness factor Oct4. After reviewing known effects of specific phytochemicals on CSCs, we will focus on related promising strategies which could target the Achilles' heel of CSCs, in particular those harboring a selective sensitivity to oxidative stress and/or present in weakly differentiated Oct-4 expressing cancers.

Publications and communications

Publications

❖ Articles

1- Tanveer Sharif, Mouni Stambouli, Benjamin Burrus, **Fathi Emhemmed**, Israa Dandache, Cyril Auger, Nelly Etienne-Selloum, Valérie B. Schini-Kerth, Guy Fuhrmann. The polyphenolic-richAronia melanocarpa juice kills teratocarcinomal cancer stem-like cells, but not their differentiated counterparts. Journal of Functional Foods, 2013, 5:1244-52.

2- Fathi Emhemmed, Sarah Ali Azouaou, Frédéric Thuaud, Valérie Schini-Kerth, Laurent Désaubry, Christian D. Muller, Guy Fuhrmann. Selective anticancer effects of a synthetic flavagline on human Oct4-expressing cancer stem-like cells via a p38 MAPK-dependent caspase-3-dependent pathway. Biochemical Pharmacology, 2014, 89:185-196.

3- Sarah Ali Azouaou, **Fathi Emhemmed**, Noureddine Idris-Khodja, Annelise Lobstein, Valérie Schini-Kerth, Christian D. Muller, Guy Fuhrmann. Selective ROS-dependent p53-associated anticancer effects of the hypoxoside derivative rooperol on human teratocarcinomal cancer stem-like cells. Investigational New Drugs, 2015, 33:64-74.

4- Fathi Emhemmed, Sarah Ali Azouaou, Valérie Schini-Kerth, , Laurent Désaubry, Christian D. Muller, Guy Fuhrmann. Bad phosphorylation is required for the protection of human normal fibroblast cells against the cytotoxic effects of the anticancer synthetic flavagline FL3 (Under preparation).

5- Fathi Emhemmed, Sarah Ali Azouaou, Valérie Schini-Kerth, Christian D. Muller, Laurent Désaubry, Guy Fuhrmann. Pro-differentiating properties of the synthetic flavagline FL3 on teratocarcinomal stem-like cells (Under preparation).

❖ Review (Book chapter)

1- Sharif Tanveer, **Fathi Emhemmed**, Fuhrmann Guy (2011). Towards New Anticancer Strategies by Targeting Cancer Stem Cells with Phytochemical Compounds, Cancer Stem Cells - The Cutting Edge-, Prof. Stanley Shostak (Ed.), InTech. pp 432-56.

Communications

❖ Oral presentation

Fathi Emhemmed, Christian Muller, Guy Fuhrmann. Anticarcinogenic effects of FL3. 13ème journée scientifique de la Ligue Contre le Cancer, Ecole Supérieure de Biotechnologie, Illkirch, novembre 2012.

❖ Posters

Fathi Emhemmed, Guy Fuhrmann, Jean Peluso, Serge Dumont, Annelise Lobstein and Christian Muller. Role of Oct4 transcription factor in the selectivity of the pro-apoptotic process in cancer stem cells after exposition to bioactive substances of marine origin**.** Medalis public scientific meeting, Ecole Supérieure de Biotechnologie, Illkirch, mars 2012.

UNIVERSITÉ DE STRASBOURG

EMHEMMED Fathi

École Doctorale des Sciences de la Vie et de la Santé STRASBOURG

Evaluation des effets anticarcinogéniques de la flavagline synthétique FL3 sur les cellules souches cancéreuses; caractérisation des mécanismes moléculaires mis en jeu

Résumé : Il est connu aujourd'hui qu'une petite sous-population de cellules au sein des tumeurs possède une puissante capacité d'auto-renouvellement et est impliquée dans la progression tumorale, l'agressivité et la résistance à la fois à la chimiothérapie et la radiothérapie. Ces cellules, nommées cellules souches cancéreuses (CSC), sont connues pour exprimer les facteurs de souchitude Oct4 et Nanog, quand elles sont pluripotentes. Le but de ma thèse était d'analyser les effets de petites molécules pharmacologiques en mesure de cibler chez les SCC ces régulateurs d'auto-renouvellement, afin d'apporter de nouvelles thérapies efficaces contre le cancer. Plus précisément, ma thèse avait pour but d'étudier l'activité anticancéreuse sélective d'une flavagline synthétique, à savoir FL3, sur un modèle de SCC peu différencié et très malin (tératocarcinome) qui exprime les facteurs de souchitude. Nous avons également utilisé un modèle de cellules souches normales restreintes (NSC) (de type fibroblastique), pour évaluer l'effet sélectif de ce médicament. Nous avons constaté que, contrairement aux NSCs, FL3 était capable de déclencher un processus pro-apoptotique mitochondriale dans les CSCs, via l'activation de p38 MAPK et de la caspase 3, suivie par une régulation négative de Oct4 et Nanog. Nous avons ensuite étudié le mécanisme moléculaire impliqué dans la protection des NSCs contre les effets cytotoxiques de la drogue. Nous avons constaté que FL3 active sélectivement les protéines pro-survie Akt et Bad dans les NSCs. En effet, l'inhibition de la sur-expression de ces protéines a déclenché un processus pro-apoptotique lié à la caspase-3 dans les NSCs traitées par FL3. Dans une deuxième étape, nous avons montré que FL3 à faible concentration, était capable d'induire la différenciation des CSCs par la régulation négative de l'expression d'Oct4 et de Nanog, tant au niveau de la traduction que de la transcription. Cet effet a coïncidé avec une régulation à la hausse de l'expression de plusieurs marqueurs neuronaux. Pris dans leur ensemble, les résultats présentés dans ma thèse démontrent clairement que la flavagline synthétique FL3 est un composé anticancéreux puissant, agissant comme un agent sélectif pro-apoptotique et pro-différenciation sur les cellules souches cancéreuses, sans effets sur les cellules souche normales.
Mots-clés: cellules souches cancéreuse, apoptose, différenciation, flavaglines.

Abstract: It is believed that small subpopulation of cells within the tumor, with powerful self-renewal capacity, are involved in tumor progression, aggressiveness and resistance to both chemo- and radio-therapy. These cells, named cancer stem cells (CSCs), are known to express the stemness factors Oct4 and Nanog, when they are highly pluripotent. The aim of my thesis was therefore to analyze the effects of small pharmacological molecules which are able to target in CSCs these self-renewal regulators, in order to bring new effective therapies for cancer. More specifically, this thesis was aimed to study the selective anticancer activity of a synthetic flavagline, namely FL3, on a poorly differentiated and highly malignant CSC model (*i.e.* the teratocarcinomal stem-like cell) that expresses the stemness factors. Herein we also used a model of very restricted normal stem cell (NSC) (*i.e.* the fibroblastic stem-like cell), to evaluate the selective effect of this drug. We found that, unlike in NSCS, FL3 was able to trigger a mitochondrial pro-apoptotic process in CSCS, via the activation of p38 MAPK and caspase3, followed by a downregulation of Oct4 and Nanog. We newt investigated the molecular mechanism involved in the protection of NSCS against the cytotoxic effects of the drug. We found that FL3 selectively activated the prosurvival proteins Akt and Bad in NSCS. Indeed, forced inhibition of the expression of these proteins triggered a caspase-3 proapoptotic process in FL3-treated NSCS. In a next step, we showed that the drug, at low concentration, was able to induce the differentiation of CSCS, by downregulating the expression of Oct4 and Nanog at both transcription and translation levels. This effect coincided with an upregulation of the expression of several neural markers. Taken as a whole, the results reported in my thesis clearly demonstrate that the synthetic flavagline FL3 is a powerful anticancer compound, since it acts as a selective proapoptotic and pro-differentiating agent on cancer stem-like cells, without having any effect on normal stem-like cells.
Key words: *cancer stem cells, apoptosis, differentiation, flavaglines.*