THESIS

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Genetics of male infertility

Genes implicated in non-obstructive azoospermia and severe oligozoospermia

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The important thing is not to stop questioning.

Curiosity has its own reason for existing.

Albert Einstein

1879-1955

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Finally, I would like to thank all my family. Thank you for your support and being there whenever I needed. Especially, an enormous thanks to my daughter Yoanna who was always there cheering me up.

Preface

This thesis has been written on the basis of studies conducted in the research team of Professor Stéphane Viville at the IGBMC, Strasbourg, France, that has taken place over a period of three years from 2012 until 2015. The goal of our team is to improve our knowledge on human gametogenesis by identifying genes that, when mutated, cause a male infertility phenotype. At the fundamental level this project open a way for basic science research, may help to identify factors involved in important processes during human spermatogenesis, allowing the fine dissection of these mechanisms. Deciphering such mechanisms will be highly relevant from the standpoint of the long-term impact on reproductive health and development.

So far, few genes involved in infertility have been identified and our team reveals two of them through the analysis of globozoospermia cases. By this study, we are now focusing our efforts on azoospermia and severe oligozoospermia. The first four chapters describe the background, methods and literature, while following three chapters present an analysis of datas and results. The first chapter gives an overview of male reproductive system with a detail explanation of testicular histology and organization, the second chapter provides definition of different steps of spermatogenesis. In chapter three, I give the overview of male infertility with definition of known causes and strategies to work on genetics of male infertility. Chapter four presents historical overview of assisted reproduction techniques and the process. The following three chapters concern the results of my thesis, in which three consanguineous families as well as 108 isolated cases and 107 fertile controls have been analysed. Chapter five concerns the identification of *TEX15*; an autosomal gene implicated in azoospermia and oligozoospermia which was identified by whole exome screening. Chapter

six defines the X-linked *MAGEB4* gene in the quest of mutation causing an infertility phenotype in a consanguineous Turkish family including male triplets. Chapter seven gives result of a study with a large family comprising three azoospermic brothers and four infertile sisters. In this study, linkage analysis in combination with whole exome sequencing is used to identify underlying cause of infertility phenotype in the study. Finally I present conclusions and future perspectives.

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Abreviations

Ad	A-dark spermatogonium
AEs	Axial elements
Ap	A-pale spermatogonium
ART	Assisted reproductive technologies
AX	Axoneme
AFA	Acetic acid/formaldehyde/alcohol
AZF	Azoospermia factor
AZFa	Azoospermia factor a
AZFb	Azoospermia factor b
AZFc	Azoospermia factor c
B	B spermatogonium
beta-hCG	Beta human chorionic gonadotropin
Bmp8b	Bone morphogenetic protein 8B, protein coding mouse gene
bp	Base pairs
BRWD1	Bromo domain and WD repeat domain containing 1
BTB	Blood-testis-barrier
CBAVD	Congenital bilateral absence of vas deferens
cDNA	Complementary DNA
CE	Central element
CESC1	Central element synaptonemal complex protein 1
CFTR	Cystic fibrosis transmembrane conductance regulator
CNVs	Copy number variations

COH	Controlled ovarian hyper-stimulation
CPEC	Circular polymerase extension cloning
CPNE1	Copine 1, protein coding human gene
CPP	Comité de Protection de la Personne
CSNK2A2	Casein Kinase 2, Alpha Prime Polypeptide, protein coding human gene
CSNK2B	Casein Kinase 2, Beta Polypeptide, protein coding human gene
DAPI	4',6'-diamino-2-phenylindole
DC	Distal centriole
ddNTPs	Dideocynucleotide triphosphates
DMEM	Dulbecco's modified Eagle medium
DMPK	Dystrophia myotonica-protein kinase
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNAH5	Dynein, Axonemal, Heavy Chain 5, protein coding human gene
DNAl1	Dynein, Axonemal, Intermediate Chain 1, protein coding human gene
DSBs	Double-stranded breaks
EAA	European Academy of Andrology
EIM	European IVF Monitoring
EMQN	European Molecular Genetics Quality Network
endo-siRNAs	Endogenous small interference RNAs
EP	Early pachytene spermatocytes
EPPIN	Epididymal Peptidase Inhibitor, protein coding human gene
ESHRE	European Society of Human Reproduction and Embryology

EVS	Exome Variant Server
ExAC	The Exome Aggregation Consortium
FAM181A	Family with Sequence Similarity 181, Member A, protein coding human gene
FKBP6	FK506 Binding Protein 6, 36kDa, protein coding human gene
FSH	Follicule stimulating hormone
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
GB	*Gigabyte*
GCC2	GRIP and coiled-coil domain containing 2, protein coding human gene
GTSF1L	Gametocyte Specific Factor 1-Like, protein coding human gene
GWAS	Genome-wide association studies
GnRH	Gonadotrophic-releasing hormone
GTYPE	GeneChip Genotyping Analysis Software
HH	Hypogonadotropic hypogonadism
HCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
IAM	Inner acrosomal membrane
ICE	Interchromosomal effect
ICMART	International Committee on the Monitoring of ART
ICSI	Intra-Cytoplasmic Sperm Injection
IGBMC	Institute of Genetics and Molecular and Cellular Biology
INSL6	Insulin-Like 6, protein coding human gene
INSL3	Insulin-like factor 3, protein coding human gene
IVF	In vitro fertilization

IVM	In-vitro maturation
KCl	Ptassium chloride
KO	Knock-out
KS	Kallmann syndrome
L	Leptotene spermatocytes
LEs	Lateral elements
LC	Leydig cells
LH	Lutenizing hormone
lncRNAs	Long noncoding RNAs
LP	Late pachytene spermatocytes
M	Mitochondria
MAF	Minor allele frequencies
MAGEA3	Melanoma-associated antigen 3, protein coding human gene
MAGEB4	Melanoma antigen family B4, protein coding human gene
MII	Metaphase II
MHD	MAGE homology domain
µg	Microgram
µl	Microliter
miRNAs	Micro RNAs
micro-TESE	Microdissection testicular sperm extraction
MP	Mid pachytene spermatocytes
mRNAs	Messenger ribonucleic acids
MSX1	Msh homeobox 1, protein coding human gene
ncRNAs	Non coding RNAs

NaCl	Sodium chloride
ng	Nanogram
NFM	Non-fat dry milk
NOA	Non obstructive azoospermia
NR5A1	Nuclear Receptor Subfamily 5, Group A, Member 1, protein coding human gene
OA	Obstructive azoospermia
OAM	Outer acrosomal membrane
OAT	Oligoasthenoteratospermia
OMIM	*Online Mendelian Inheritance in Man*
OR2T35	Olfactory receptor, family 2, subfamily T, member 35, human protein coding gene
PBS	Phosphate-buffered saline
PC	Proximal centriole
PCI	Protein C inhibitor, human protein coding gene
PCR	Polymerase chain reaction
PCOS	Polycystic ovarian syndrome
PGCs	Primordial germ cells
pg	Picogram
piRNAs	Piwi-interacting RNAs
Pl	Preleptotene spermatocytes
PRMs	Protamines
PTM	Peritubular myoid cell
qPCR	Quantitative PCR

RB	Residual body
r-hFSH	Recombinant human FSH
RGPD4	RANBP2-like and GRIP domain containing 4, protein coding human gene
RT-PCR	Reverse transcription PCR
Sa1 – Sd2	Developmental stages of spermatid maturation
SDS	Sodium dodecyl sulfate
SRY	Sex-determining region Y
SC	Synaptonemal complex
SCO	Sertoli cell only
SERPINA5	Serpin Peptidase Inhibitor, Clade A (Alpha-1 Antiproteinase, Antitrypsin), Member 5, protein coding human gene
SNPs	Single nucleotide polymorphisms
SO	Severe oligozoospermia
SOHLH1	Spermatogenesis and oogenesis specific basic helix-loop-helix 1, protein coding human gene
SPATA7	Spermatogenesis Associated 7, protein coding human gene
SPINT3	Serine Peptidase Inhibitor, Kunitz Type, 3, protein coding human gene
SYCE1	Synaptonemal complex central element protein 1
SYCP1	Synaptonemal complex protein 1
SYCP3	Synaptonemal complex protein 3
TAF4B	TAF4b RNA Polymerase II, TATA Box Binding Protein (TBP)-associated factor, protein coding human gene
TBS	Tris Buffer Saline buffer

TCEAL6	Transcription elongation factor A (SII)-like 6, protein coding human gene
TDRD9	Tudor Domain Containing 9, protein coding human gene
TESE	Testicular sperm extraction
TEX11	Testis expressed 11, protein coding human gene
TEX15	Testis expressed 15, protein coding human gene
TP53TG5	TP53 Target 5, protein coding human gene
TNPs	Transition proteins
USF	Uncontrolled slow freezing
USP26	Ubiquitin Specific Peptidase 26, protein coding human gene
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World Health Organisation
Wt1	Wilms Tumor 1, protein coding human gene
Yq	Long arm of the Y chromosome
ZMYND15	Zinc Finger, MYND-Type Containing 15, protein coding human

Introduction

Chapter I

MALE REPRODUCTIVE SYSTEM

1-Overview

Sexual reproduction is the process by which organisms create offsprings in which both the female and male reproductive systems are essential.

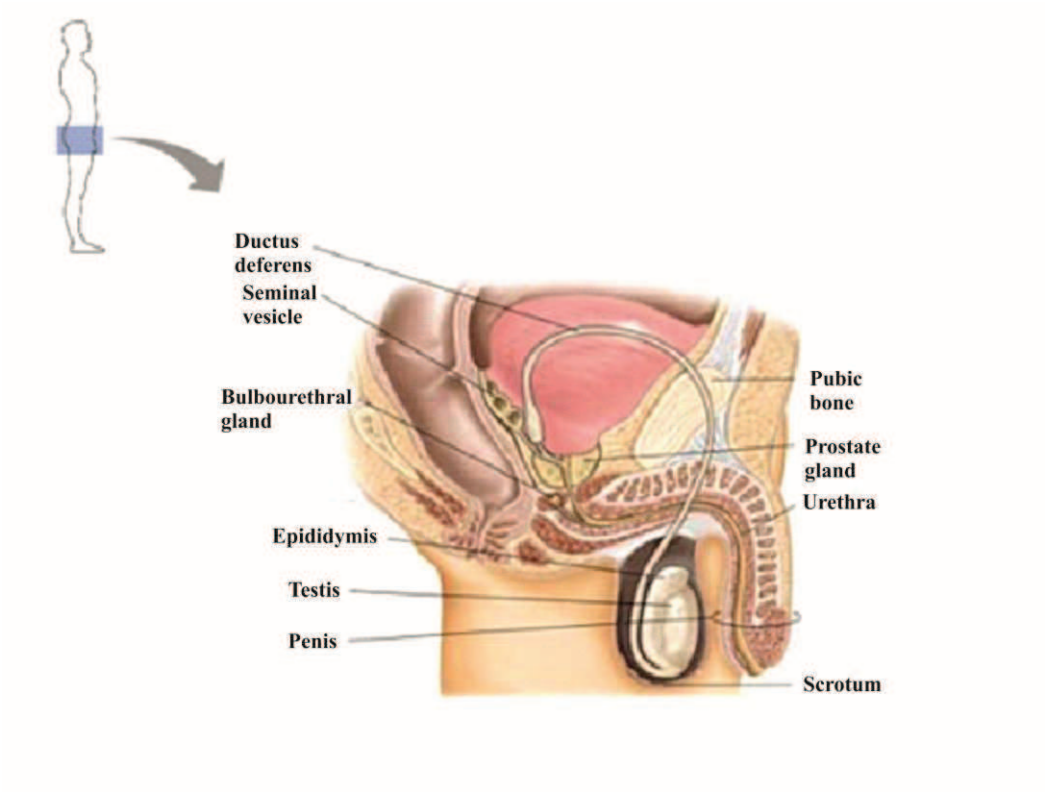
Reproductive organs are considered to be either *primary* or *secondary* organs. The primary reproductive organs are the gonads which are responsible for gamete and hormone production, whereas secondary organs are ducts and accessory glands which aid in the growth, maturation and transport of gametes (figure 1.1a). Testes are the primary male reproductive organs that differentiate from a sexually indifferent genital ridge (Sharpe 2010). In the scrotum, the mammalian testis is enclosed by a fibrous capsule, the Tunica albuginea, which is connected with the retracting muscles and is highly vascularized (Schlatt and Ehmcke 2014) (Figure 1.1b).

2-Compartments and cellular component of testes

There are two compartments of testes which morphologically and functionally distinguishable from each other; tubular compartment consisting of seminiferous tubules and interstitial compartment between seminiferous tubules.

The interstitium is responsible for blood supply and immunological responses (Witsuba *et al.*, 2007). Leyding cells are the most important cells of interstitial compartment, they are the source of testicular testosterone and of insulin-like factor 3 (INSL3). Aside from Leydig cells, the interstitial compartment contains immune cells, blood and lymph vessels, nerves, fibroblasts and loose connective tissue.

(A)



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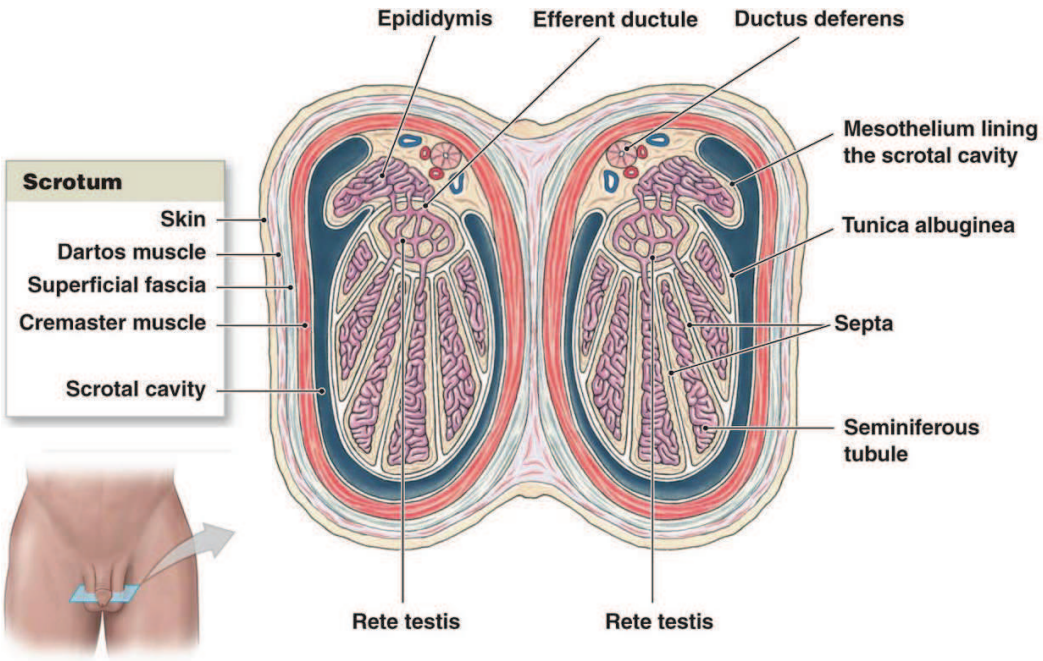


Figure1.1: Male Reproductive System (A) Testes are primary reproductive organs in male. Secondary organs can be internal or external; epididymis, dactus (vas) deferens, seminal vesicles, prostate gland, bulbourethal glands and urethra are internal organs while scrotum and penis are defined as external organs (adopted from Clark 2005). (B) A horizontal section through the scrotum showing the internal organization of the testes (adopted from www.highlands.edu, reproductive systems).

The seminiferous tubules are the functional units of the testis where spermatogenesis takes place; they represent 60-80% of total testicular volume. Tubules are covered by a lamina propria; which consists of a basal membrane**,** a layer of collagen and the peritubular cells. The peritubular cells are myoid and drive the peristalsis necessary to move the non-motile elongated testicular spermatozoa released from the nourishing sertoli cells in the direction of the efferent ducts (Witstuba *et al*., 2007). The inside of the tubules are lined with seminiferous epithelium, which consists of two general types of cells: spermatogenic cells and Sertoli cells (Figure 1.2).

The main function of Sertoli cells is to nurture the developing sperm cells through the stages of spermatogenesis and provide mechanical support for the spermatogenic cells. Spermatogenesis is explained in detail during the following chapter. Sertoli cells secrete two hormones - inhibin and activin - which provide positive and negative feedback on FSH secretion from the pituitary. Additionally, Sertoli cells control release of mature spermatids into the tubular lumen, phagocytosis of the degenerating germ cells and the excess cytoplasm (residual body) that remains from released sperm (Hess and de Franca 2008).

Sertoli cells proliferate in foetal life, in the immediate postnatal/neonatal period and just prior to puberty. In the prepubertal period Sertoli cells are relatively quiescent and seminiferous tubules grows slowly (Schlatt and Ehmcke 2014). Testosterone appears to be most important in the foetal and early neonatal period, whereas follicle-stimulating hormone (FSH) is probably more important subsequent to this and especially during puberty (Sharpe *et al*., 2003). Each individual Sertoli cell is in morphological and functional contact with a defined number of sperm; the number varied between species (Sharpe 1994). In men it was observed about 10 germ cells or 1.5 spermatozoa per each Sertoli cell (Zhengwei *et al*., 1998).

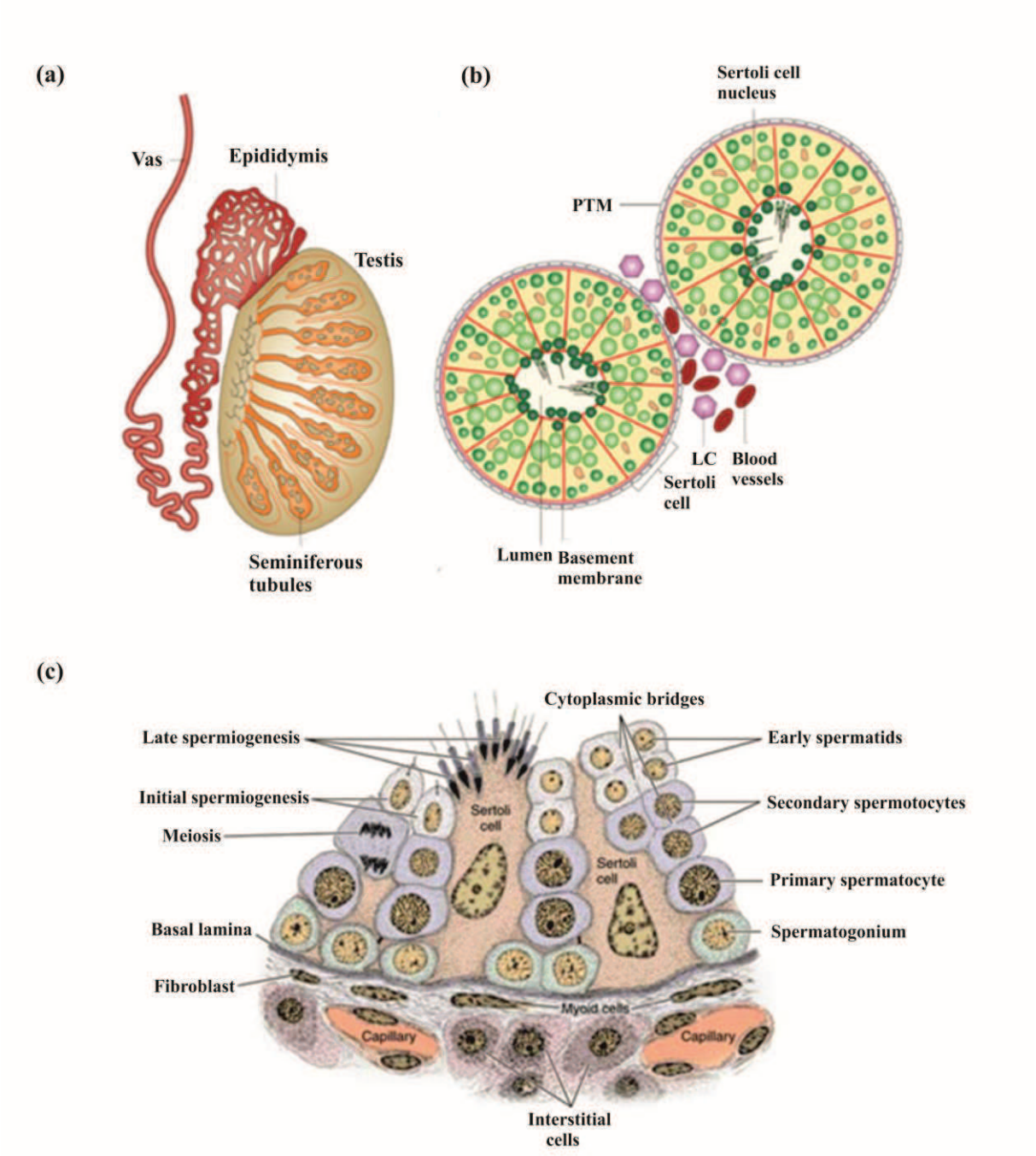


Figure 1.2 Organization of the testis (a) A cross-section through a testis, showing the location of the seminiferous tubules, the vas deferens and the epididymis (b) A diagrammatic cross-section through a testicular tubule, showing the germ cells (green) at different stages of maturation developing embedded in somatic Sertoli cells (each Sertoli cell is outlined in red). Leydig cells (LC) are present in the interstitium (pink). Maturing sperm are shown in the lumen of the tubules. PTM= peritubular myoid cell. (c) Part of a seminiferous epithelium with germ lines and surrounding tissues. The pre-meiotic cells (spermatogonia) are located in the basal region whereas the meiotic (spermatocytes) and the post-meiotic (round and elongating spermatids) cells are found organized in strict order of maturation towards the lumen (adopted and changed from Cooke and Saunders 2002, www.intranet.tdmu.edu.ua).

In adulthood, Sertoli cells are mitotically inactive. The division of Sertoli cells ends when the first germ cells undergo meiotic division and then Sertoli cells have built tight junctions between each other, the so-called blood-testis-barrier (BTB) (Figure 1.3). Through the BTB, the seminiferous epithelium is divided into two regions which are anatomically and functionally completely different from each other. It maintains a selective substance flow between luminal fluid, blood plasma and interstitial fluid, thereby creating an immune-privileged environment for haploid germ cells in the adluminal compartment of the seminiferous tubules (Philips *et al*., 2010). Two important functions are postulated for the BTB; the first one is the physical isolation of haploid and thereby antigenic germ cells to prevent recognition by the immune system, the second one is the preparation of a special milieu for the meiotic process and sperm development (Philips *et al*., 2010).

The tubules also contain the entire germ line which will be explained in detail in the following chapter. In a newborn mammal, only Sertoli cells, spermatogonia and preleptotene spermatocytes can be found, whereas advanced spermatocytes and spermatids appear in the peri-pubertal and adult testis only (Sutovsky and Manandhar 2006). Early germ cells are located in the basal region and the later stages of maturing germ cells in the adluminal region**.** During their development germ cells are displaced from the basal to the adluminal compartment (Figure 1.2c).

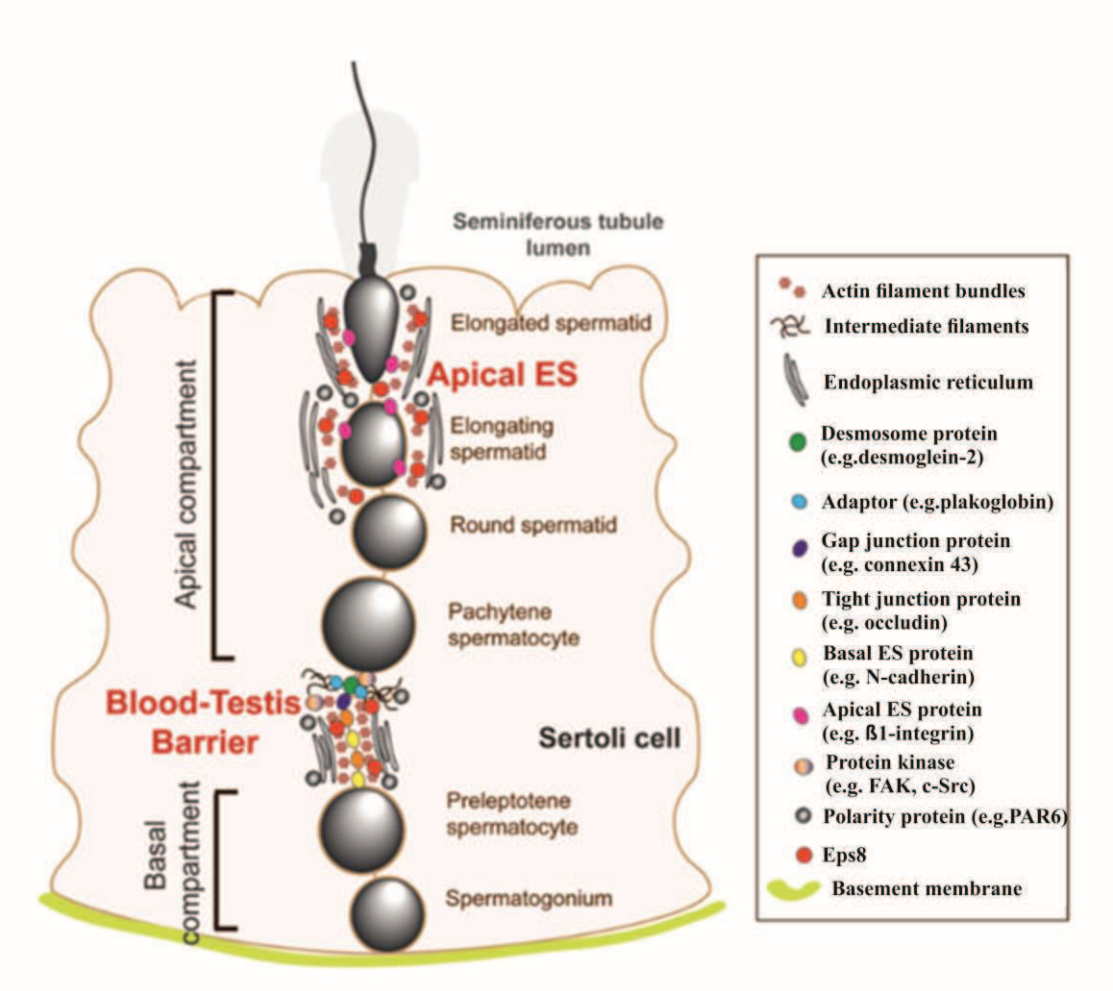


Figure 1.3: Schematic representation of BTB (adopted from Cheng *et al*., 2011).

Germ cells proliferate and differentiate normally during foetal life and in the postnatal period to ensure that adequate numbers are in place to facilitate normal spermatogenesis in adulthood (Sharpe 2010). Germ cell differentiation in perinatal life and the regulatory processes involved are poorly understood. Based on rodent studies, there are three important events (Orth *et al*., 2000 Culty 2009); (i) the foetal germ cells have to lose their pluripotency, (ii) the germ cells enter a period of quiescence, i.e. cease proliferation, (iii) the differentiated germ cells must migrate to the basal lamina and position themselves underneath the Sertoli cells so as to provide the spermatogonial stock from which

spermatogenesis will be delivered thereafter. In humans, a period of quiescence has not yet been defined (Mitchell *et al*., 2008) and it is uncertain when migration occurs (Sharpe 2010).

The germ cell cohorts in mammals are arranged in horizontal layers of an epithelium and their development is highly synchronized. This synchrony allowed us to recognize germ cell cohorts with specific morphological features in cross-sections of the seminiferous epithelium. Germ cells differentiate in distinctive associations and each association is classified as a stage. The number of stages within a spermatogenic cycle and the number of cycles required for the completion of spermatogenesis varies between species. 14 stages for the rat, 12 stages for the macaque and mouse are described (Leblond and Clermont 1952, Clermont and Leblond 1959, Clermont 1969, Oakberg 1956).

Six stages have been defined in human (figure 1.4), they followed each other in a temporal sequence; following stage VI, stage I reappear and a new cycle begins (*Clermont* 1963). The initial appearance of round spermatids following the second meiotic division is always defined as stage I of the seminiferous epithelial cycle. The last stage is characterized by the presence of secondary spermatocytes (Schlatt and Ehmcke 2014).

In contrast to most mammals investigated so far, in humans a given seminiferous tubule cross-section may contain two to five stages of spermatogenesis present as “islands” next to each other (Sharpe 2010). The reason for this appearance could be explained by two different events. First, the analysis of 3-dimensional reconstructions from small segments of seminiferous tubules shows the helical arrangement of the spermatogenic stage (Schulze and Rehder 1984) (figure 1.4). Spermatogenesis generations are wound around each other in spirals along the seminiferous tubule. Second, it was postulated that the arrangement of

the human spermatogenic stages along the seminiferous tubule is random and so that more than one consecutive stage could be found on serial sections (Johnson *et al.,* 1996).

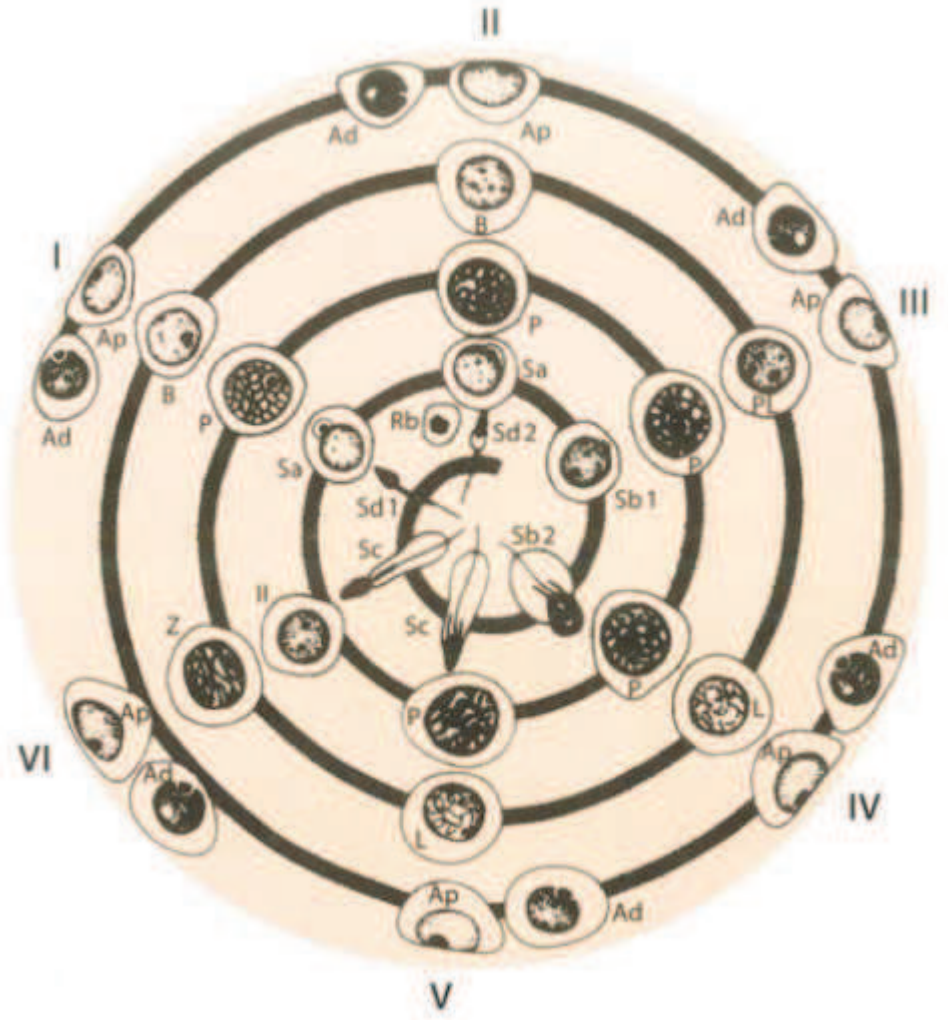


Figure 1.4: Six stages of human spermatogenic cycle. Stages are arranged in a helical manner in cross-sectioned seminiferous tubules. Ad= A-dark spermatogonium, Ap= A-pale spermatogonium, B= B spermatogonium, L= leptotene spermatocytes, P= pachytene spermatocytes, Z= zygotene spermatocytes II= 2nd meiotic division, Sa – Sd= developmental stages of spermatid maturation(adopted from Weinbauer *et al.,* 2001).

The spermatogenic stages are precisely ordered not only in time but also in space. This is known as the spermatogenic wave (fig 1.5). Spermatogenesis waves move in spriral towards the inner part of the lumen.

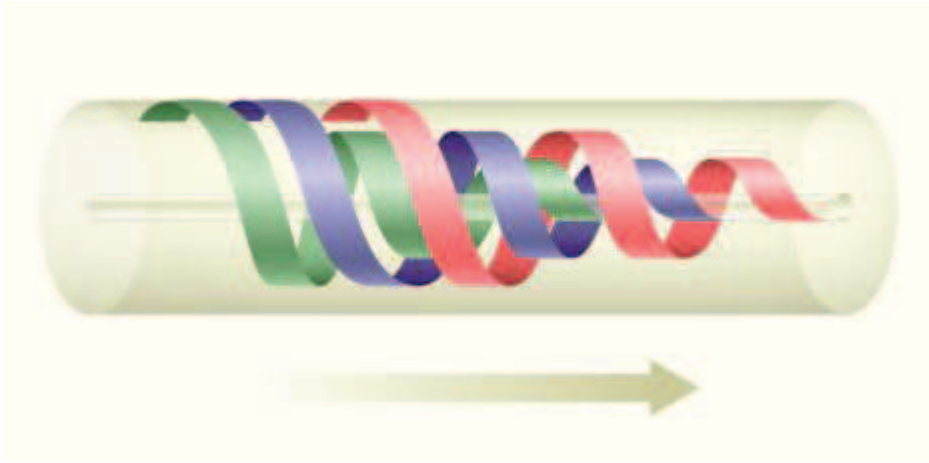
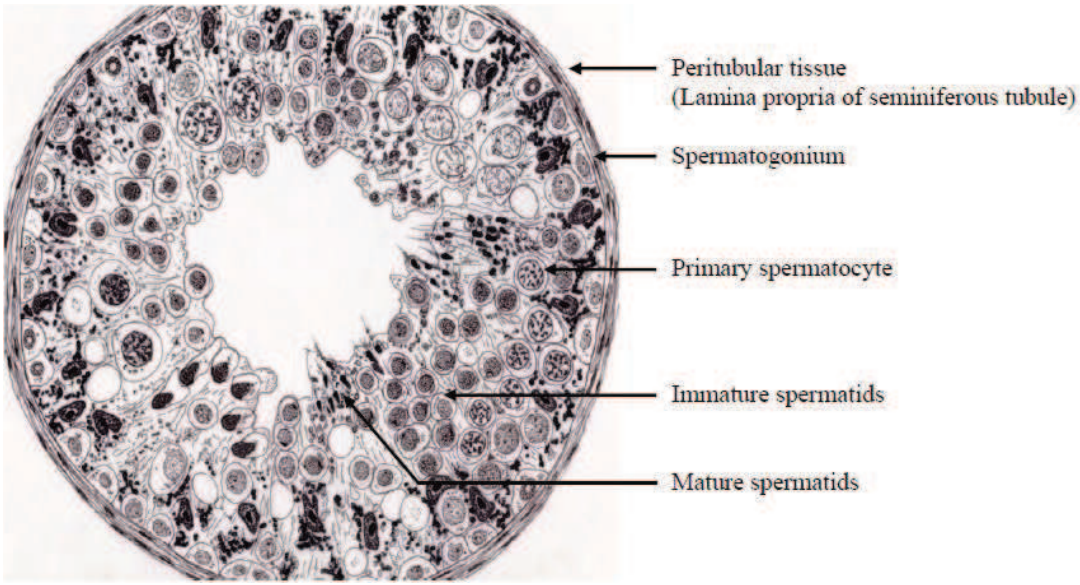


Figure 1.5: Spermatogenesis wave. This diagram illustrates three waves of human spermatogenesis (adopted from http://www.embryology.ch, module gametogenesis).

The duration of sequential occurrence of the all stages in a given area of the tubule over time is defined as a cycle of the seminiferous epithelium. The kinetics of spermatogenesis differs between species but is highly stable for a given species. It was accepted that one human spermatogenic cycle requires 16 days, and approximately 4.6 cycles are necessary for the development and differentiation of spermatogonium into a mature sperm; so the duration of spermatogenesis is calculated approximately 74 days in man (Heller and Clermont 1963, 1964) (Figure 1.6).

At the completion of spermatogenesis, mature spermatids are released from the seminiferous epithelium into the seminiferous tubule lumen and proceed through the excurrent duct system, known as the rete testis, until they enter the epididymis via the efferent ducts (Schlatt and Ehmcke 2014). The seminiferous tubules join together to become the epididymis. Within the epididymis the sperm complete their maturation and their flagella become functional. This is also a site to store sperm, nourishing them until the next ejaculation (figure 1.1).

(A)



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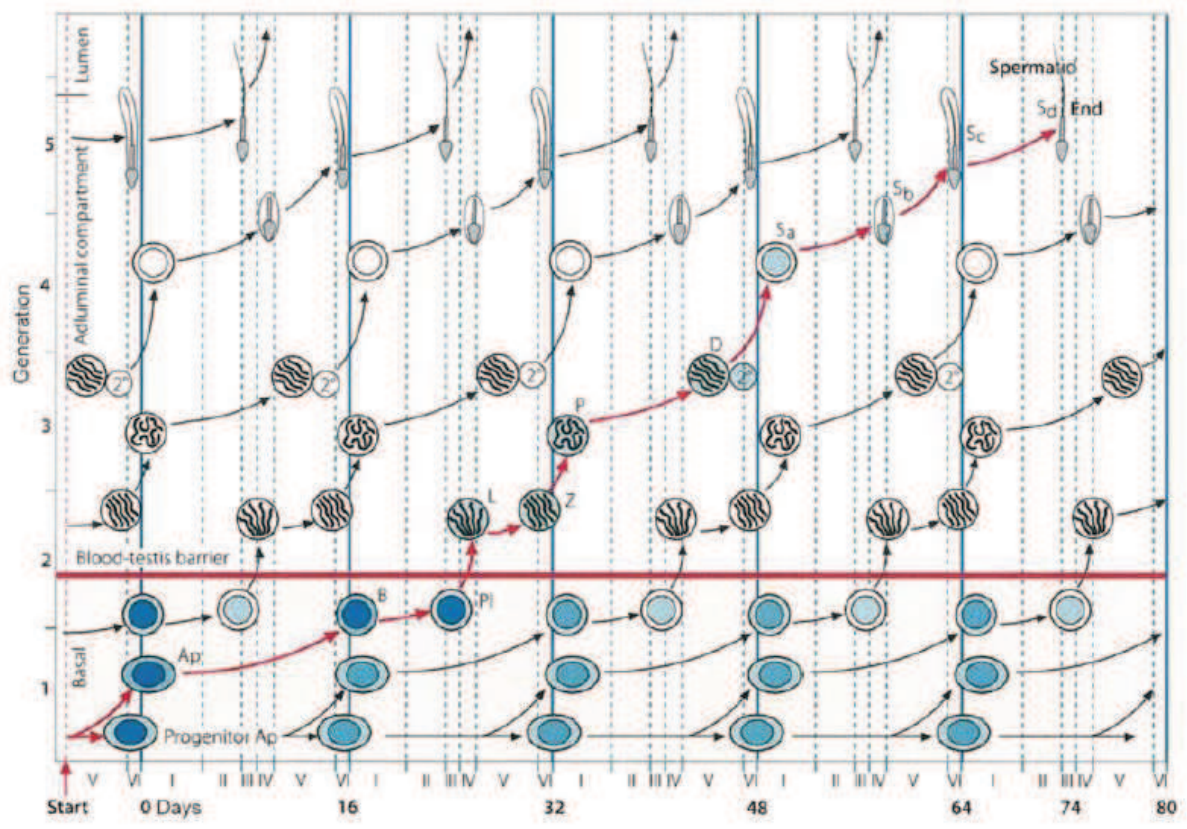

Figure 1.6: Human Seminiferous Epithelium (A) Cross section of a seminiferous tubule of a fertile man (adopted from Holstein *et al*., 2003) (B) Kinetics of the human seminiferous epithelium. Ap= A-pale type spermatogonia, progenitor stem cells Ad= A-darktype spermatogonia, B= B-type spermatogonia, Pl= prelepototene spermatocyte, L= leptotene spermatocyte, Z= zygotene spermatocyte, D= diplotere spermatocyte. 2°= 2nd meitotic division, Sa – Sd= developmental stages of spermatid maturation (adopted from Weinbauer *et al*., 2010).

Chapter II

SPERMATOGENESIS

Male germ cells, the spermatozoa, are produced in a unique process named spermatogenesis. The term “spermatogenesis” describes the development of the male gamete within the seminiferous epithelium from diploid spermatogonia up to the release of differentiated haploid germ cells into the lumen of the seminiferous tubule.

Regulation of spermatogenesis occurs at two major levels; (i) hormonal and endocrine and (ii) paracrine/autocrine. Several studies have indicated the need for both testosterone and FSH for the successful completion of spermatogenesis (De Kretzer et al 1998).

The entire spermatogenic process can be divided into four phases (Amann 2008, Weinbauer *et al.*, 2010):

1. Mitotic proliferation and differentiation of spermatogonia fallowed by their division to form preleptotene spermatocytes (***spermatogoniogenesis***)

2. Meiotic division of spermatocytes resulting in haploid germ cells called spermatids (***meiosis***)

3. Transformation of round spermatids into a spermlike mature spermatid (***spermiogenesis***)

4. Release of elongated spermatid into the tubular lumen (***spermiation***).

Schematic representation of all human germ cells according to stages of spermatogenesis is demonstrated in figure 2.1.

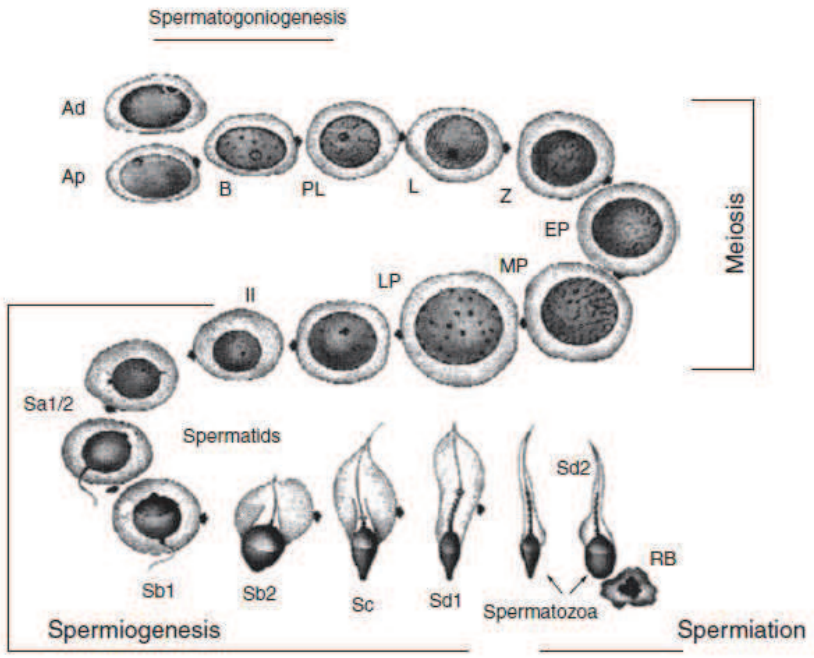

Figure 2.1: Schematic representation of all germ cell types that occur in the human seminiferous epithelium. Ad= A-dark spermatogonium, Ap= A-pale spermatogonium, B= B spermatogonium, Pl= preleptotene spermatocytes, L= leptotene spermatocytes, EP= early pachytene spermatocytes, MP= mid pachytene spermatocytes, LP= late pachytene spermatocytes, II= 2nd meiotic division, RB= residual body, Sa1 – Sd2= developmental stages of spermatid maturation (adopted from Weinbauer *et al*., 2010).

STAGES OF SPERMATOGENESIS

1) SPERMATOGONIOGENESIS

Germ cell lines are originated from primordial germ cells (PGCs). The precursors of PGCs are specified shortly after implantation in the epiblast (De Felici 2013, Yadav and Kotaja 2014). In human, PGCs appear at the end of 3rd week of development among endoderm cells in the wall of yolk sac close to the allantois (figure 2.2). The nascent PGCs migrate by ameboid movement from the yolk sac toward the developing gonads called genital ridges (Sadler 1995) and proliferate rapidly (Matsui 2010). In mouse embryos, this journey is aided by hindgut expansion and growth movements (Hara *et al*., 2009). During the migration, PGCs

begin extensive nuclear programming and reset of the genomic imprinting which is completed after arriving in to the genital ridges (De Felici 2013).

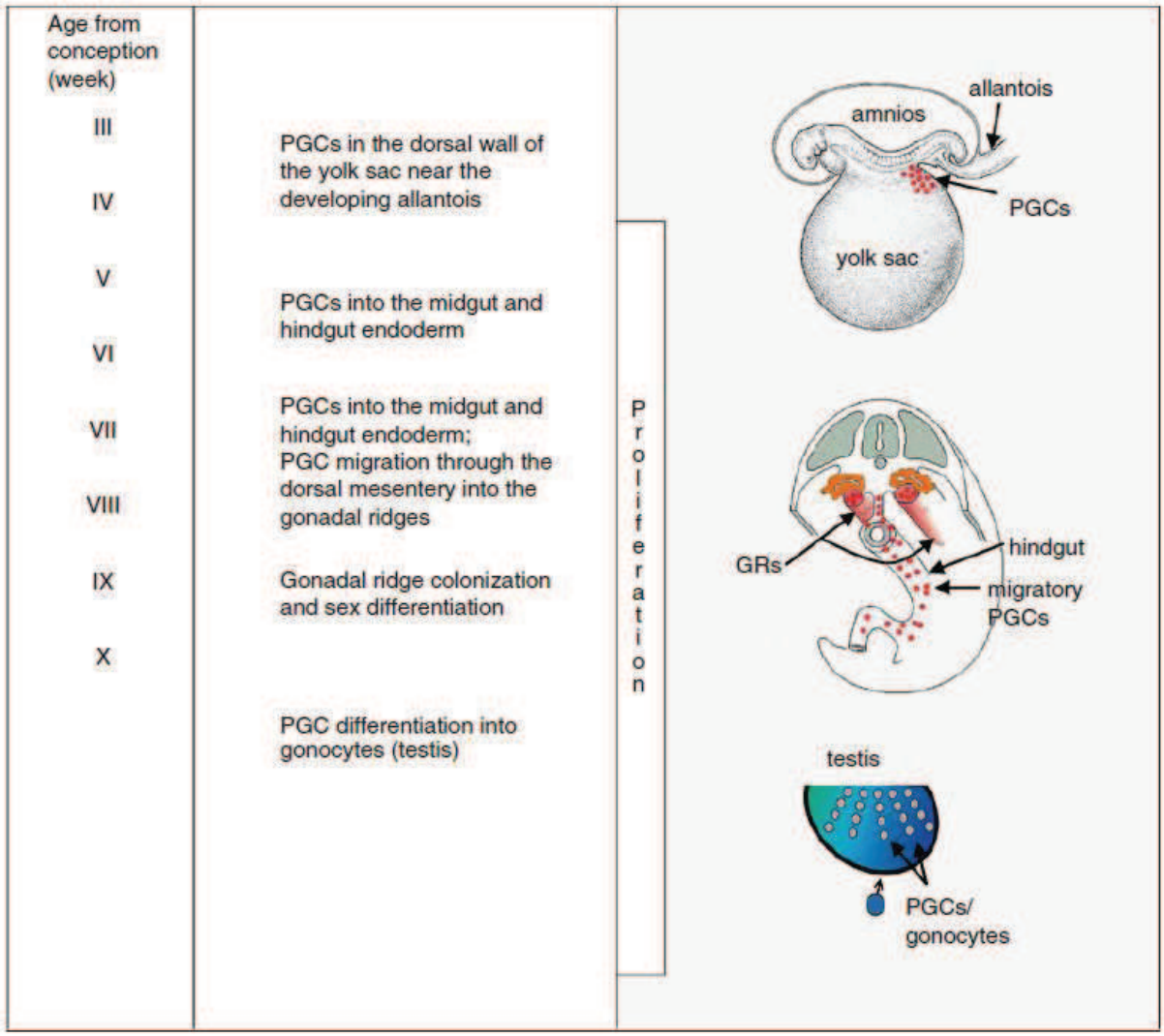

Figure 2.2. Chronology of human male primordial germ cell development (adopted from De Felici 2013).

PGCs arrive the ridges at the end of the 4th or the beginning of the 5th week. If they fail to reach the ridges, the gonads do not develop (Sadler 1995).

Shortly before and during arrival of PGCs, the coelomic epithelium of the genital ridge proliferates and epithelial cells penetrate the underlying mesenchyme. They form a number of irregularly shaped cords, the primitive sex cords. In males, under the influence of the Y

chromosome, which encodes testis -determining factor, the primitive sex cords continue to proliferate and penetrate deep into the medulla to form testis (Sadler 1995).

PGCs are usually termed gonocytes after reaching the developing testis (figure 2.2). During the first trimester, gonocytes are mitotically active then they arrest at the G0 phase of cell cycle, remaining mitotically quiescent until after birth; when they give rise to spermatogonia (de Rooij and Russell 2000, De Fellici 2013). Spermatogonia remain dormant until puberty.

Spermatogenesis starts just after birth by mitotic proliferation of spermatogonia. The initiation of spermatogenesis during puberty is probably regulated by the synthesis of bone morphogenetic protein 8B (BMP8B). Indeed in mice, *Bmp8b* appears to be required both for the initiation and maintenance of spermatogenesis (Zhao *et al*., 1996). When BMP8B reaches a critical concentration, the germ cells begin to differentiate. The differentiating cells produce high levels of *Bmp8b*, which can then further stimulate their differentiation. Mice lacking *Bmp8b* do not initiate spermatogenesis at puberty (Zhao *et al*., 1996).

The types and number of spermatogonial cells vary widely in different species of mammals. Three A type spermatogonium and four generations of B spermatogonia have been identified in non-human primates (Ehmcke *et al*., 2005) while in the mouse seven types of A spermatogonia, one intermediate and one B spermatogonia were defined (Chiarini-Garcia and Russell 2002). In rhesus monkey, the situation is different; two types of A spermatogonia are accompanied by four types of differentiating B type spermatogonia (Marshall *et al*., 2005).

In the human, A- dark (Ad), A-pale (Ap) and B spermatogonial forms can be distinguished based on their nuclear morphology (Heller and Clermont 1964). In the fully developed mammalian testis, the majority of undifferentiated cells of the germ line are type A

spermatogonia (Wistuba 2007). There are two distinct fates for a germ cell; either it duplicates itself for self-renewal or differentiate into spermatozoon. Meanwhile, in the adult human seminiferous epithelium, apoptosis occurs at the level of spermatogonia, spermatocytes and spermatids, as a rare event (Brinkworth *et al*., 1997). However the rate is increased in patients with impaired spermatogenesis (Lin *et al*., 1997), especially in primary spermatocytes and round spermatids associated with incomplete spermatogenic failure (Tesarik et al., 1998).

So far, the pattern, the timing of spermatogonial renewal and the proliferation to yield primary spermatocytes as well as the regulation of spermatogonial differentiation remain unknown (Amann 2008). In human, two models have been suggested for the renewal and proliferation of spermatogonia (figure 2.3). According to the model favored by Clermont, Ad-spermatogonia are in pairs, each member of a pair undergoes mitotic division in which one provides 2 Ad-spermatogonia while the other gives 2 Ap-spermatogonia. Then each Ap-spermatogonia undergoes one division to provide B spermatogonia (Clermont 1966) (fig-2.3A). An alternative model was proposed and favored by Ehmcke and Schlatt; in which spermatogenesis starts with a division of a pair or a quadruplet of Ap-spermatogonia, these clone of cells split and undergo second division. This second division not only leads to pairs of B-spermatogonia but also generates a pair of Ap-spermatogonia (Ehmcke and Schlatt 2006) (fig 2.3B).

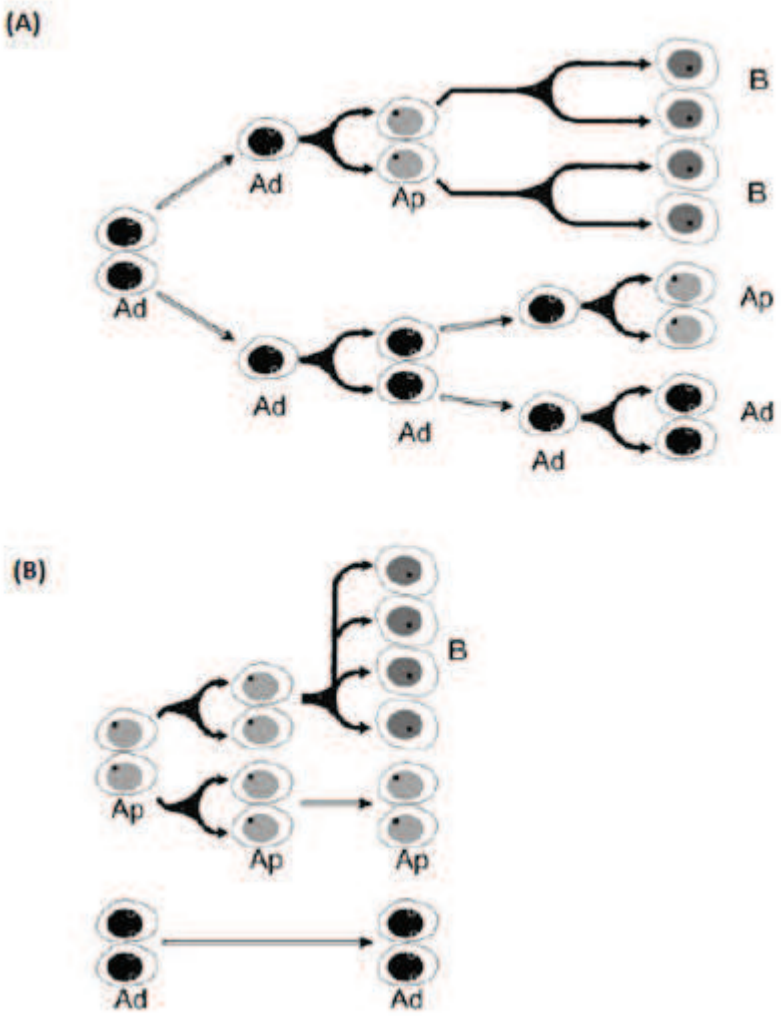

Figure 2.3: Schematic presentation of proliferation models for A-spermatogonia. (A) Clermont model (B) Ehmcke model (adopted from Ehmcke and Schlatt 2006).

In contrast to earlier model, Ehmcke model suggests that Ad-spermatogonia are not the source of Ap-spermatogonia during regular spermatogenic cycle. Ad-spermatogonia are considered to represent testicular stem cells (Ehmcke *et al*., 2006), they do not show proliferating activity under normal circumstances and are believed to divide only rarely (Ehmcke and Schlatt 2006). They, however, undergo mitosis when the overall spermatogonial population is drastically reduced, for example due to irradiation or toxic

exposure (van Alphen et al 1988a, b; de Rooij 1998). Ad-spermatogonia also show high-proliferative activity during prepubertal testicular development when the pool of both types of A spermatogonia is expanding (Simorangkir *et al*., 2005). In contrast, the Ap-spermatogonia shows typical characteristics of a progenitor; they divide, renew themselves and differentiate into two B spermatogonia. (Ehmcke and Schlatt 2006). Both model results in 8 preleptotene spermatocytes per original pair of Ap-spermatogonia.

In comparison to most animals, human spermatogenesis is less efficient. In rodents, for instance, the clonal expansion of germ cell is intense and in theory one stem cell could lead to production of 4096 spermatids while in man a maximum number of spermatids is 32 from one stem cell (Ehmcke *et al*., 2006).

2) MEIOTIC DIVISION

Throughout mammalian meiosis, one round of DNA replication is followed by two nuclear division; meiosis I and meiosis II respectively. During meiosis I, which is referred to as a *reductional division,* microtubules attach to the fused kinetochores of the sister chromatids, homolog chromosomes separate to opposite pole and it results in the reduction of the chromosome number from diploid to haploid. Meiosis II is an *equational division;* microtubes attach the individual kinetochores of single centromere and the sister chromatids are segregated, creating four haploid daughter cells.

Meiosis is initiated by the production of two preleptotene spermatocytes from type B spermatogonia via mitotic division. A primary spermatocyte is transformed into two secondary spermatocytes during meiosis I; these cells then are converted into spermatids during meiosis II.

Meiosis produces genetically different four haploid daughter cells; the genetic differences ensure siblings of the same parents are never entirely genetically identical. There are two ways that genetic diversity occurs during meiosis (Cox et al., 2012, Alford and Hill 2003):

(1) The physical exchange of homologous chromosomal regions during prophase I in a process called crossing over, also known as recombination

(2) The independent assortment of homologous chromosome pairs along the metaphase plate during metaphase I and random segregation of chromosomes to each daughter cell such that there is an equal probability of the daughter cell having either the maternal or paternal chromosome.

Both meiosis I and meiosis II are divided into four phases: prophase, metaphase, anaphase and telophase (figure 2.4). Meiosis is a slow process, largely because of the time that the cell spends in prophase I (O'Connor 2008). The prophase of the first meiosis lasts 1–3 weeks, whereas the other phases of the first meiosis and the entire second meiosis are concluded within 1–2 days, therefore very few secondary spermatocytes can be identified in histological sections (Weinbauer *et al*., 2010).

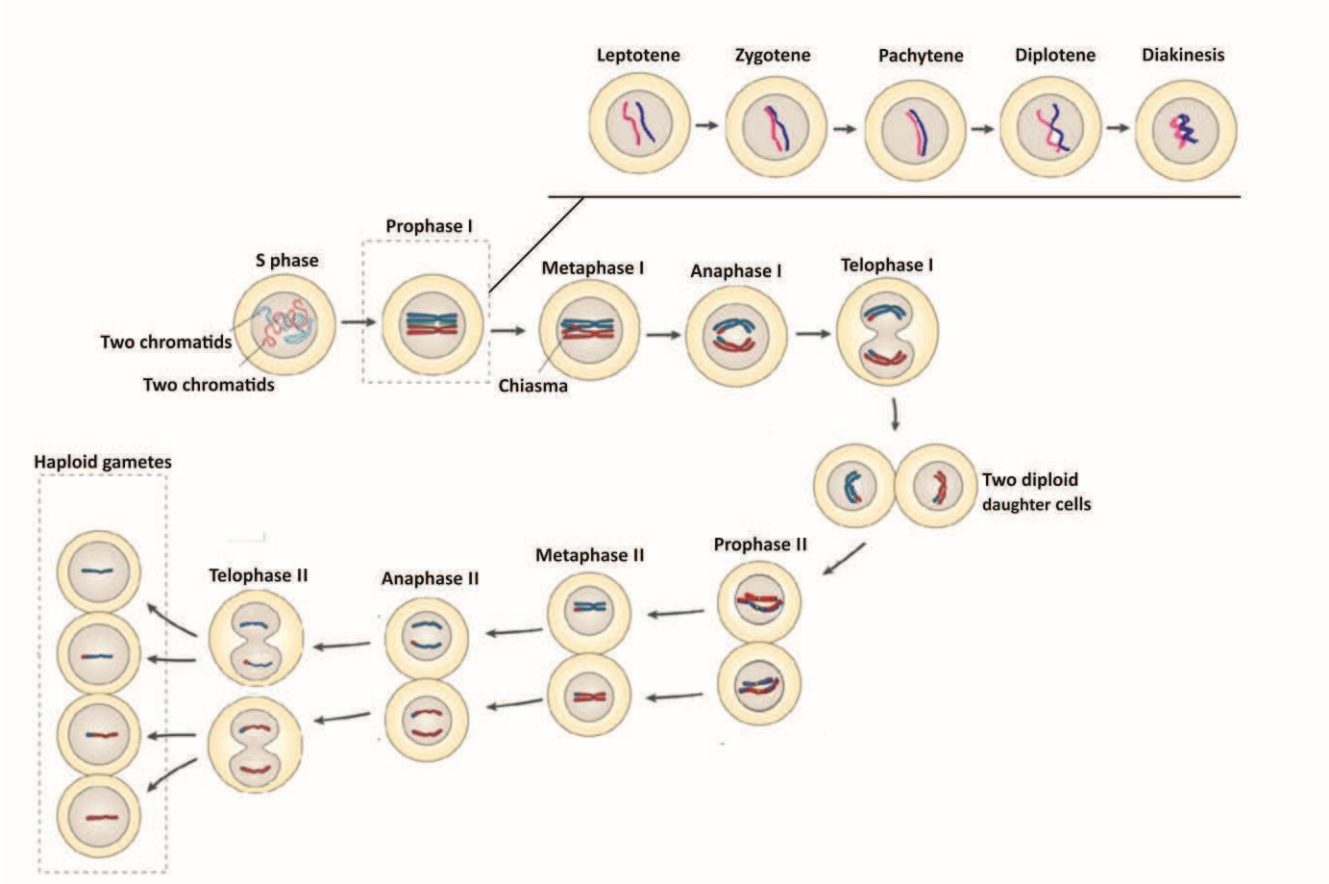

Figure 2.4: Overwiev of meiosis (modified from Handel and Schimenti 2010).

• Meiosis I

a-Prophase I: Prophase I is the first stage of meiosis, it is a crucial and highly regulated phase which can be subdivided into the stages on the basis of nuclear size, morphology and the association of homologous chromosomes during synapsis (Tsai and McKee 2011). Five stages can be identified; leptotene, zygotene, pachytene, diplotene and diakinesis (figure 2.4).

Several events occur during prophase I; including DNA double-strand break (DSB) formation and repair, homologous chromosome pairing and synapsis, crossing over-chiasmata formation. Before the leptotene phase, cells stay on the basement membrane, DNA begins to replicate, then nuclei form fine chromatin thread in leptotene and cells become transit.

Both leptotene and zygotene spermatocytes can move to adluminal compartment of the seminiferous tubule through the blood-testis barrier (or sertoli–sertoli barrier) (Hess and de Franca 2008). During zygotene stage synapsis is initiated and fully completed in pachytene spermatocytes (Hamer 2008). In virtually all mammalian species, the pachytene phase of meiosis occupies over a week and typically lasts 1.5-2 weeks, but has a fixed duration for each particular species (Cavalcanti 2008).

In humans, chromosomes do not pair with each other until double-stranded breaks (DSBs) appear in the DNA (Gerton and Hawley, 2005). In all organisms studied so far, the formation of DSBs is catalyzed by topoisomerase-like protein Spo11 (Zickler and Kleckner 2015). Genetic studies have shown that Spo11 activity is essential for meiosis in yeast, because *spo11* mutants fail to sporulate (O'Connor 2008). Distruption of *Spo11* in mouse leads to severe gonadal abnormalities from defective meiosis; spermatocytes arrest prior to pachytene with little or no synapsis and undergo apoptosis (Romanienko and Camerini-Otero 2000, Baudat *et al*., 2000). In mice, DSBs appear soon after the meiotic S phase, at leptotene, the beginning of prophase I. DSB repair takes place during the following stages and is completed before diplotene (Chicheportiche *et al*., 2007)

A large zipper-like protein complex called synaptonemal complex (SC) forms between homolog chromosomes and mediate chromosome pairing -synapsis (Page and Hawley 2004; Petronczki *et al*., 2003). SCs composed of two lateral elements (LEs) that are joined together by transverse filaments (reviewed in Page and Hawley 2004) (figure 2.5).

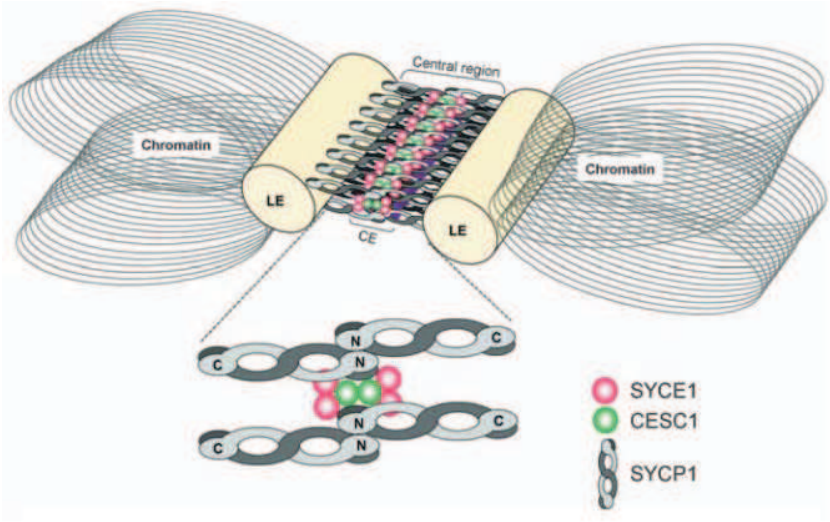

Figure 2.5: Model for synaptonemal complex. C= carboxyl-terminus, CE= central element, LE= lateral element, N= amino-terminus (adopted from Costa *et al*., 2005).

In early prophase I, the cohesin core, which is formed by meiosis specific cohesion subunits, starts to link sister chromatids together (Eijpe *et al*., 2003). During the zygotene stage, the paired homologous chromosomes become physically linked through transverse filaments which are composed of synaptonemal complex protein-1 (SYCP1) molecules (reviewed in Page and Hawley 2004). The C-terminal of SYCP1 is known to embed with the lateral element of SC while the N-terminal region is known to associate with the central element (Cohen and Holloway 2014). Two proteins, synaptonemal complex *central element* protein 1 (SYCE1) and *central element* synaptonemal complex protein 1 (CESC1) are defined to be localized to the central element of the mammalian synaptonemal complex (Costa *et al*., 2005).

Incorrect assembly of the synaptonemal complex leads to impaired recombination and cell death, which, in humans, causes infertility in males (Judis *et al*., 2004; Miyamoto *et al*., 2003) and a high aneuploidy rate in females (Hassold and Hunt 2001; Hunt and Hassold 2002). Recombination can occur between any two chromatids within this structure during

pachytene stage. The exchange of genetic material in between two non-sister chromatids is known as crossing-over. Crossover recombination in conjunction with sister-chromatid cohesion results in structures called chiasmata that tether homolog pairs and thereby facilitate their stable bi-orientation on the spindle (Jones 2007, Sakuno *et al*., 2011, Hirose *et al*., 2011). (figure2.5). Chiasmata become visible in diplotene after the synaptonemal complex has disappeared (Zickler and Kleckner 1999). Cells that fail to form chiasmata may not be able to segregate their chromosomes properly during anaphase, thereby producing aneuploid gametes with abnormal numbers of chromosomes (Hassold and Hunt 2001).

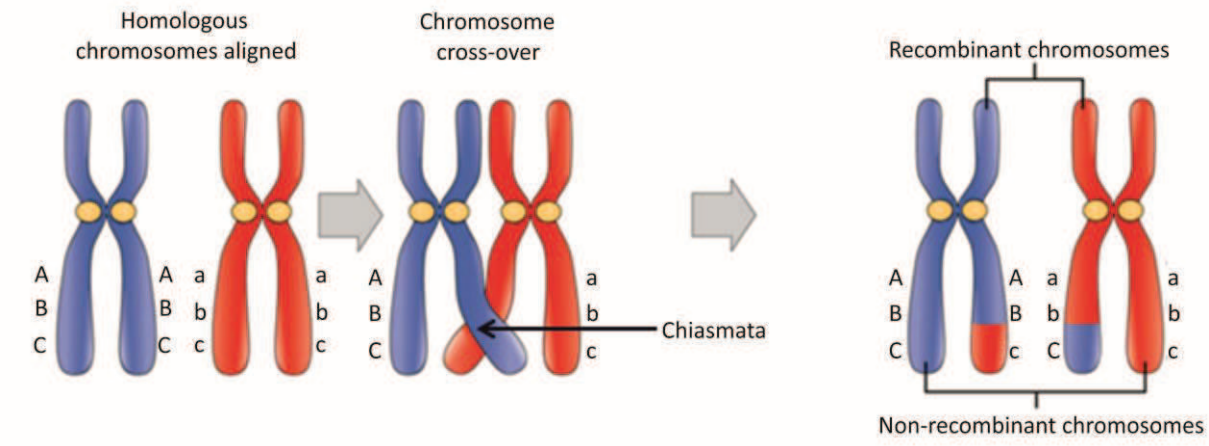

Figure 2.6: Illustration of crossing over. Crossover occurs between non-sister chromatids of homologous chromosomes. The result is an exchange of genetic material between homologous chromosomes (adopted from www.cnx.org).

In human males, the Y chromosome pairs and crosses over with the X chromosome through small regions of similarity near their tips called pseudoautosomal regions. There are three identified pseudoautosomal regions; PAR1, PAR2 (Mangs *et al*., 2007) and PAR3 (Veerappa *et al*., 2013). Crossover between these homologous regions ensures that the sex chromosomes will segregate properly when the cell divides (O'Connor 2008).

High levels of RNA synthesis have been confirmed in pachytene spermatocytes from mouse, rat, hamster and human testis (Eddy and O'Brien 1998). This phase takes some weeks, pachytene spermatocytes are seen frequently within the seminiferous epithelium (O'Donnel *et al*., 2005). A brief diplotene phase in which synaptonemal complex dissociate follows by diakinesis where chiasmata disappear and homologs begin to repel.

b-From metaphase I to telophase I: Nuclear envelope breakdown marks the start of metaphase I (Tsai and McKee 2011). Pairs of homologous chromosomes line up opposite each other on the metaphase plate, with the kinetochores on sister chromatids facing the same pole (O'Connor 2008). The pairs of homologous chromosomes separate to different daughter cells during anaphase I. Before the pairs can separate, however, the crossovers between chromosomes must be resolved. Failure to separate the pairs of chromosomes to different daughter cells is referred to as nondisjunction, and it is a major source of aneuploidy. During telomere I, chromosomes migrate to each pole and division yields secondary spermatocytes, which quickly progress through a second meiotic division. Frequency of disomy in secondary spermatocytes is higher than spermatozoa which suggest the existence of a post-meiotic checkpoint monitoring numerical abnormalities. (Uroz and Templado 2012).

• Meiosis II

Secondary spermatocytes have a very short lifespan of approximately 6h in the human (De Kretzer 1998). Following meiosis I, they enter meiosis II without passing through interphase and usually before the chromosomes have fully decondensed. Centrioles duplicate and spindle fibers rearrange throughout prophase II, then chromosomal pairs align along spindle

equator during metaphase II. This time sister chromatids seperate and move to the opposite poles in anaphase II and telophase II. When division ends, the products of meiosis II are four haploid cells that contain a single copy of each chromosome (figure 2.4). Since the second meiotic division is rapid, very few secondary spermatocytes can be identified in histological sections.

1) SPERMIOGENESIS

Spermiogenesis is the process by which the end product of meiosis II, round spermatid, transforms without further division into the specialized elongated spermatid via a series of complex cytodifferentiative steps. This process requires extensive cytoplasmic and nuclear remodeling. It can be mentioned of four phases; golgi, cap, acrosomal and maturation phase (Weinbauer *et al*., 2010) (figure 2.7).

Golgi phase: During the golgi phase, acrosomal bubbles and craniocaudal symmetry appear. Small pro-acrosomic granules fuse with each other to form a single large acrosomic granule that associates with the nuclear envelope (Abau-Haila and Tulsiani 2000). The centrioles start to migrate to a position beneath the nucleus that is opposite the acrosomic vesicle. The proximal centriole (PC) will give rise to the attachment point of the tail, whereas distal centriole (DC) will give rise to the developing axoneme (figure 2.7a).

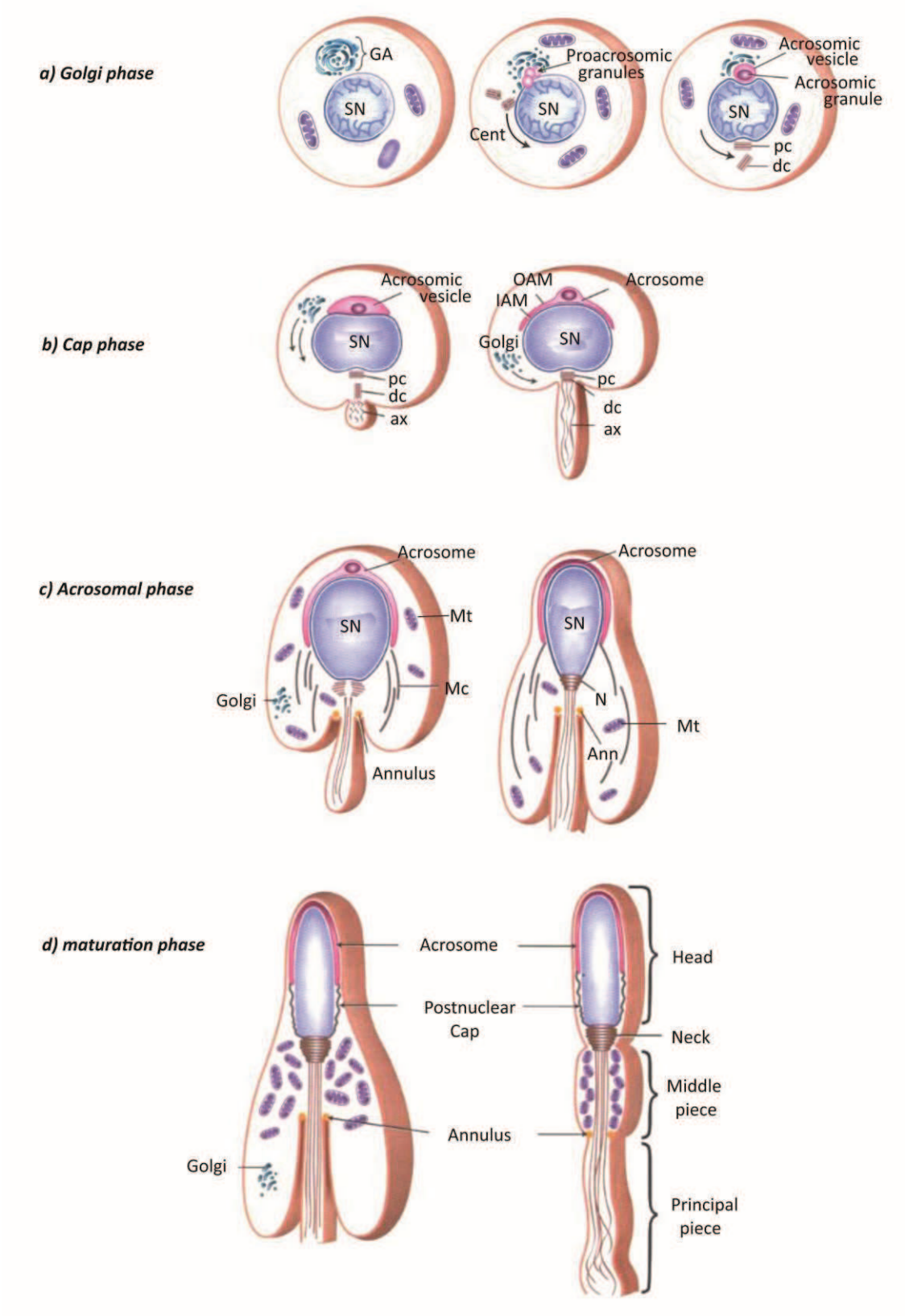

Figure 2.7: Phases of spermiogenesis. (a) golgi phase (b) cap phase (c) acrosomal phase (d) maturation phase. SN=spermatid nucleus, GA=golgi apparatus, cent=centrioles, pc=proximal centriole, dc=distal centriole, ax=axoneme, OAM=outer acrosomal membrane, IAM=inner acrosomal membrane, Mc=Manchette, Mt=mitochondria, N=neck, Ann=Annulus (adopted from www.webpages.uidaho.edu).

Cap phase: Throughout the subsequent cap phase (figure 7b), the acrosome increases its size and begins to spread over the anterior nuclear pole (Yao 2002). Acrosome is considered to be indispensable at fertilization; consequently, its biogenesis is a crucial event for sperm fertilizing potential. As the acrosomal cap is formed, the nucleus rotates so that the cap will be facing the basal membrane of the seminiferous tubule. This rotation is necessary because the flagellum is beginning to form and will extend into the lumen (Cavalcanti 2008).

Acrosomal phase: The nucleus changes its shape during the following acrosomal phase (figure 7c). At the chromosomal level, chromatin condensation begins with the replacement of histones, the predominant chromatin proteins of somatic cells, by the basic low molecular weight transition proteins (TNPs). These proteins are then replaced with protamines (PRMs) in the nuclear matrix producing a tightly compacted nucleus with extensive disulfide bridge crosslinking (Bukowska 2013) (fig 2.8).

Not all histones are evicted though, in human 10-15% of DNA keeps its histones (Brykczynska *et al*., 2010, Gatewood *et al*., 1987). It has now become clear that retained histones are not random; they are enriched at specific loci important for embryonic development providing a possibility for epigenetic inheritance, at the promoters of developmentally important messenger ribonucleic acids (mRNAs) in the zygote and embryonic developmental genes (Daxinger and Whitelaw 2010, Hammoud *et al*., 2009, Miller *et al*., 2010).

Spermatid nucleus becomes incapable of transcription as it condenses, and thus round spermatids transcribe high levels of mRNAs that are subject to translational delay until translation of protein is required during elongation (Braun 1998, Gilbert 2000).

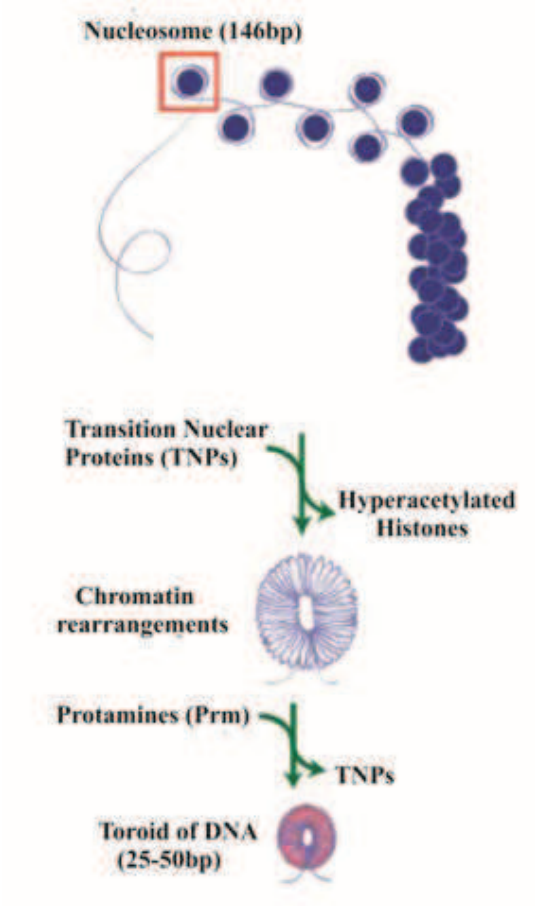

Figure 2.8: Schematic representation of the change in sperm chromatin organization. Replacement of most histones by transition proteins (TNPs) which in turn are replaced by protamines (PRMs) allow for the compaction of the majority of the sperm chromatin in toroidal structures. Each of torodial tructure is embedding 50 to 100 kb of DNA, thus permitting the great decrease in nuclear volume (adopted from Noblanc *et al*., 2014).

Maturation phase: Condensation is followed by migration of mitochondria during the maturation phase (figure 7b). Mitochondria forms a ring around the base of flagellum, becomes concentrated into the sheath of the middle piece (Gilbert 2000, Cavalcanti 2008). Excess of cytoplasm is discarded in the form of residual body which is then phagocytosed by Sertoli cells.

As a result of spermiogenesis, the shape of the germ cells is transformed from relatively roundish into small spindle-shaped cells. The spermatozoa are equipped with all the structures required for its prospective functions (Yadav and Kotaja 2014).

3) SPERMIATION

Spermiation is the final step of spermatogenesis, by which mature spermatids are released from the supporting somatic Sertoli cells into the lumen of the seminiferous tubule (figure 2.9). In that stage, cells are referred as spermatozoa and continue the journey to the epididymis. Seminiferous spermatozoa lack motility and fertilizing capacity. Transit of spermatozoa through the long convoluted epididymal duct is important for their final maturation and acquirement of the capacity for full motility (Yadav and Kotaja 2014).

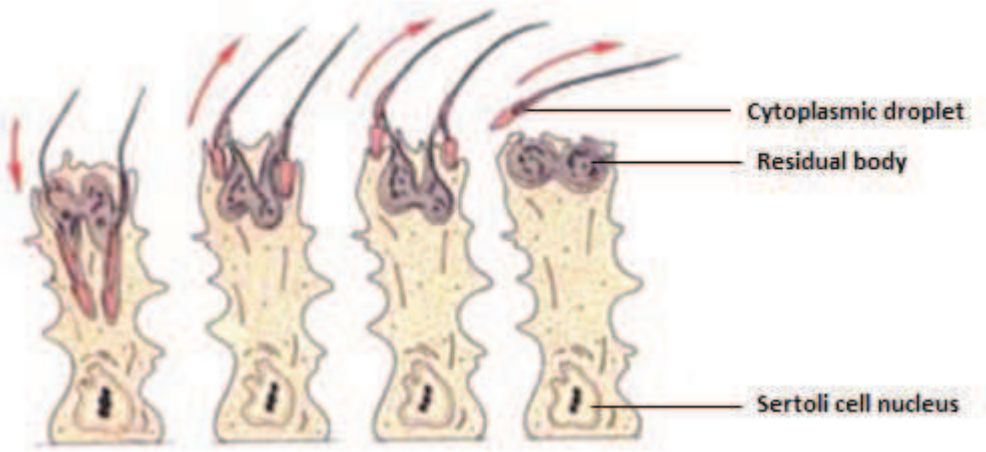

Figure 2.9: Spermiation process (adopted and changed from Jégou *et al*., 1995).

A small amount of cytoplasmic material, the cytoplasmic droplet, remains attached within the neck region or around the middle piece as the spermatozoon makes its way into the epididymis. During transit through the epididymis, which takes approximately two weeks, the cytoplasmic droplet migrates distally along the tail of the spermatozoon and falls off; this event is correlated with an increase in cellular motility (Cooper 2011). Nevertheless, in mammals the full fertilizing potential of spermatozoa is not gained until cells are affected by secretions of the female reproductive tract; these spermatozoa are said to be "capacitated" (Bedford 1970, Chang 1984).

Chapter III

MALE INFERTILITY

Infertility is described as the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild *et al*., 2009). The cause of infertility can be of female, male or mixed origin; a male factor is solely responsible for infertility in approximately about one third of all couples attempting to conceive.

Diagnosis of male infertility is generally based on standard semen analysis; problems commonly associated with abnormal semen parameters including sperm concentration, morphology, motility and vitality. A nomenclature related to semen quality was defined in World Health Organisation (WHO) manual (2010) as:

- Azoospermia (no spermatozoa in the ejaculate)
- Oligozoospermia (sperm concentration $<15x10^6$/ml)
- Asthenozoospermia (<32% progressively motile spermatozoa)
- Teratozoospermia (<4% morphologically normal spermatozoa)
- Oligoasthenoteratozoospermia (Disturbance of concentration, motility and morphological parameters all together)
- Cryptozoospermia (spermatozoa absent from fresh preparation but observed in a centrifuged pellet)
- Aspermia (No ejaculate)

1- KNOWN CAUSES OF MALE INFERTILITY

Male infertility has many causes ranging from hormonal imbalance to physical problems as well as lifestyle and psychological problems. So far, approximately 40 % of men seeking help at an infertility clinic, the etiology remains unknown (Chianese *et al*., 2014), and the infertility is thus classified as idiopathic. Known causes of male infertility can be grouped into five main categories.

A) Hormonal

The reproductive hormonal axis in men, known as hypothalamic-pituitary gonadal axis, consists of three main components; the hypothalamus, the pituitary gland and the testis. It functions in a tightly regulated manner to produce concentrations of circulating hormones required for normal male sexual development, sexual function and fertility.

Any disturbance within this system can lead to infertility. If the brain fails to release gonadotrophic-releasing hormone (GnRH) properly, this cause a lack of testosterone synthesis and a cessation in sperm production. GnRH deficiency leads to group of conditions known collectively as hypogonadotropic hypogonadism (HH). One of them is named as Kallmann syndrome (KS) which is characterized by altered sense of smell (anosmia) and pubertal failure (Kotan *et al*., 2014). Treatment options include sex steroids, gonadotropins, and pulsatile GnRH administration. Testosterone therapy in the form of injectable or transdermal routes of testosterone administration as well as hCG injections are typically used in order to promote testicular growth, normalize serum concentration of testosterone and induce development of secondary sexual characteristics (Buck *et al.,* 2013).

Similarly the pituitary failure to produce enough lutenizing hormone (LH) and follicule stimulating hormone (FSH) leads disruption in stimulation of testes and testosterone/sperm production (Babu *et al*., 2004). Pituitary failure is known as hypopituitarism, it is usually chronic and patients require lifelong hormone therapy. It can be associated with diabetes mellitus, dyslipidemia, cardiovascular diseases and osteoporosis (Schneider *et al*., 2007).

Conversely, elevated concentration of LH and FSH accompanied by low concentration of testosterone results in deficient spermatogenesis (Weinbauer and Nieschlag 1995, Subhan *et al*., 1995). For treatment high-dose testosterone or estrogen therapy could be an option since it suppresses FSH and LH (Shimon *et al.,* 2006) Elevated prolactin can decrease sperm production, reduces libido and may cause impotence. Hyperprolactinemia causes infertility in around 11% of oligospermic males (Masud *et al*., 2007). Dopamine agonist is the specific treatment of choice in most cases (Glezer and Bronstein 2015).

B) Physical

Physical problems either interfere with the sperm production or disrupt the pathway of ejaculation. Enlargement of the internal spermatic veins, namely variococele, is the most common example of physical problems affecting 40% of infertile men (Nagler *et al*., 1997). Twisting testis inside the scrotum is known as torsion, it can lead to testicular damage because of compression of sperm vessels and disturbed testicular blood flow. Acute and chronic genital tract infections are also well known causes of male infertility. Mumps viral infections could result in testicular atrophy and sterility (Senanayake 2008). Sexually transmitted diseases like gonorrhea and chlamydia can cause male infertility by blocking the epididymis (Ochsendorf 2008). In some cases, semen is ejaculated into the bladder rather than out through the urethra, and called retrograde ejaculation which affects less than 2% of

infertile men (Yavetz *et al*., 1994, Kamischke and Nieschlag 2002). It is caused by an anotomycal problem of bladder sphincter; treatment is generally only needed to restore fertility.

C) Coital

Several sexual problems are mostly both psychological and physical in nature. Erectile dysfunction, also known as impotence, premature ejaculation and ejaculatory incompetence are examples of coital problems (Hamada *et al*., 2012).

D) Environment and lifestyle

Men who are exposed to various substances at work, including lead, solvents, pesticides and radiation could have infertility problems. Toxins such as glues, solvents, silicones and physical agents may cause male infertility by affecting sperm concentration, motility and morphology (Mendiola *et al*., 2008).

Exposure to radiation can reduce sperm production, though it will often eventually return to normal except with high doses of radiation in which sperm production can be permanently reduced (Clifton and Bremner 1983, Meistrich and van Beek 1990).

Frequent use of saunas or hot tubs may temporarily lower sperm count. Repeated exposure to heat over an extended period of time, such as in occupations which involve long hours of sitting like drivers or workers exposed to high temperatures like bakers, may result in permanently impaired fertility (Sharpe 2010, Thonneau *et al*., 1998).

Estrogen-like hormone disrupting chemicals such as phthalates is ubiquitously used in plastics, personal care products and food packaging materials leading widespread general population exposure. This chemical was associated with increased DNA damage in human

sperm (Hauser *et al*., 2007). There is no conclusive agreement about the effects of cigarette smoking and alcohol use on sperm parameters and reproductive outcome, however progressive deterioration in semen quality may be related to increasing quantity of alcohol intake and cigarettes smoked (Gaur 2010). Poor nutrition plays also a role in male infertility, recently a lower sperm concentration and total sperm count in men with a high intake of saturated fat was found (Jensen 2013). Repeated drug consumption such as cannabinoids and cocaine was associated with significant lower concentration of urinary testosterone in males (Pichini *et al*., 2012).

E) Genetic factors

Genetic factors can be identified in about 15% of cases (Krausz 2011, Liebaers *et al.*, 2014), and can be classified as two groups; chromosomal abnormalities and monogenic mutations.

1-Chromosomal abnormalities

Any loss, gain or abnormal arrangement of genetic material at the chromosomal level is defined as chromosomal abnormalities and they have emerged as one of the major genetic factors contributing to male infertility. About 14% of azoospermic men and 5% of oligozoospermic men carry chromosomal abnormalities (Van Assche *et al*., 1996), these rates are much more frequent than the frequency in the general population which is approximately 0.6% (Berger 1975). Some chromosomal aberrations are inherited, while others arise de novo. Chromosomal abnormalities are subdivided further into sex chromosome aneuploidies, structural chromosome aberrations and copy number variations.

a) Sex chromosome aneuploidies: 47,XXY karyotype (Klinefelter syndrome) is the most common genetic cause of azoospermia, accounting for 14% of cases (Rives *et al*., 2000, Walsh *et al*., 2009). Y chromosome disomy, the 47,XYY karyotype could be linked to

spermatogenesis failure due to elevated FSH and aneuploidy in sperm (Wong 2008, Gonzalez-Merino 2007). Noonan's syndrome is the male counterpart of the Turner syndrome (45,X0), on chromosomal analysis males demonstrate X0/XY mosaicism. Mostly they have cryptorchidism and diminished spermatogenesis with elevated FSH (Griffin *et al*., 2005).

b) Structural chromosome aberrations: Structural chromosomal abnormalities are common especially in azoospermic males. Translocations, inversions and Y chromosome micro deletions can be grouped in this section.

- Translocations

Rearrangements between non-homologous chromosomes, known as translocations, are classified as "robertsonian" if the centromere of two acrocentric chromosomes fuse and giving rise to a dicentric chromosome or as "reciprocal" if the exchanges between terminal segments from the arms of two chromosomes are involved. Translocations are found in about 3% of patients with severe oligozoospermia *(*Foresta *et al*., 2005).

The translocation is considered to be balanced if there is no gain or loss of genetic information. In the vast majority of cases, carriers of balanced translocations are themselves phenotypically normal, unless one of the translocation breakpoints interrupts an important gene or *via* position effects (Harton and Tempest 2012). Nevertheless, carriers of balanced chromosomal translocations may experience reduced fertility, spontaneous abortions or birth defects since they are prone to produce genetically unbalanced gametes (Tempest 2010). The relative frequency of normal or unbalanced gametes appears to depend largely on the chromosomes involved, the size of the region involved, the presence of heterochromatin, location of the breakpoints (e.g., G-positive or G-negative bands) and

likelihood of recombinatorial events to take place within the translocated segments (Tempest 2010). So far, no rules are available to determine the level of unbalance gametes, sperm chromosome studies in translocation carriers show that the percentages of unbalanced sperm are in the range of 20–77% (Guttenbach *et al*., 1997).

For translocation carriers, it has been indicated an increased frequency of chromosomal abnormalities that involve chromosomes other than those involved in the rearrangement. This refers to an interchromosomal effect (ICE) and sperm aneuploidy studies have identified an ICE in 58% of Robertsonian translocation carriers and 64% of reciprocal translocation carriers (Martin 2008). The observed interchromosomal effect seems to be translocation, patient, and chromosome dependent. In case of patients with same translocation, the level of the disomy rate is variable and affected different chromosomes (Machev *et al*., 2005).

- Inversions

Inversion is a chromosomal rearrangement in which a segment of genetic material breaks and re-joins the same site of chromosome after 180°C rotation. There are two types of inversions according to whether inversion includes the centromere or not. Paracentric inversions do not include the centromere, and both breaks occur in one arm of the chromosome. Pericentric inversions, on the other hand, include the centromere and there is a break point in each arm.

Autosomal inversions are eight-fold more likely to occur in infertile than in fertile men (Sadeghi-Nejad and Farrokhi 2006). These types of chromosomal derangements tend to be balanced and result in phenotypically normal males, but with severe oligoasthenoteratospermia (OAT) or azoospermia (Griffin *et al*., 2005). Pericentric inversions

in chromosome 1, 3, 5, 6, 10 seem to interfere with meiosis leading to a reduced rate of postmeiotic sperm numbers or even azoospermia (Vogt 2004).

- Y chromosome microdeletions

A significant role for the Y chromosome in spermatogenesis was established a quarter of a century ago when Tiepolo and Zuffardi detected, on karyotype analysis, large terminal deletions of the long arm of the Y chromosome (Yq) in six men with azoospermia (Tiepolo and Zuffardi 1976). They named the defined region as azoospermia factor (AZF). Originally, three AZF regions were defined; AZFa, AZFb, and AZFc (azoospermia factors a, b, and c), which map on the long arm (Yq) in order from the centromere to the telomere and were thought to be non-overlapping (Vogt *et al*., 1996). However, subsequent studies showed that AZFb and AZFc overlap by 1.5Mb (Repping *et al*., 2002) (figure 3.1).

Microdeletions derive from the homologous recombination of identical segments within palindromic sequences (Patrizio *et al*., 2007). Prevalence of Yq microdeletions is 9.7 % in an azoospermic population, while in oligozoospermic men; the prevalence is 6.0% (Massart 2012). The most frequent deletion subtypes comprises the AZFc region (~80%) followed by AZFa (0.5-4%), AZFb (1-5%) and AZFb+c (1-3%) regions (Krausz *et al*., 2014).

Deletions encompassing the complete AZFa usually produce the severe phenotype of Sertoli-cell-only syndrome (SCOs) but this is based on a limited number of cases to date (Hopps *et al*., 2003; Kamp *et al*., 2001). Interstitial or terminal deletions that include AZFb and/or AZF b+c are mediated by recombination between palindromic repeats; P5/proxP1, P5/distP1, or P4/distP1. These deletions are uncommon and usually result in azoospermia (Repping *et al*., 2002, Silber 2011). Partial deletion of AZFb that removes the entire P4 palindrome decreases spermatocyte maturation but can be transmitted (Kichine *et al*., 2012). Common interstitial

or terminal deletions that include AZFc only are mediated by recombination between the b2/b4 palindromic repeats and result in a variable infertility phenotype, ranging from azoospermia and SCO to severe or mild oligozoospermia (Oates *et al*., 2002, Silber 2011).

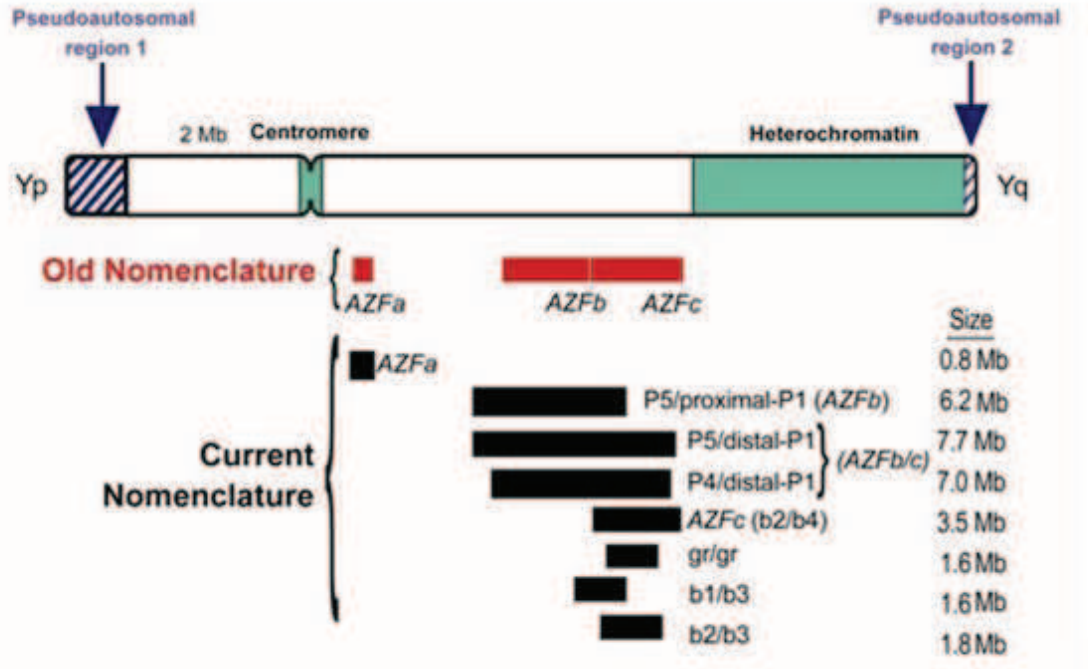

Figure 3.1: Schematic representation of AZF regions on Y chromosome. AZFa, AZFb, AZFc regions and the position of flanking palindromic repeats were indicated on Y chromosome (adopted from Silber 2011).

A partial deletion in AZFc region, termed "gr/gr", has been described in infertile men with varying degrees of spermatogenic failure (de Llanos *et al*., 2005). This deletion removes half of the AZFc gene content including two copies of DAZ gene, one copy of CDY1, and a few transcription units (Shamsi et al., 2011). "gr/gr" deletions may have an impact on fertility depending on ethnicity and geographic region (Stouffs et al., 2011). They are extremely common (25%) in Japanese men, for example, and represent simply a "risk factor" for male infertility (Silber 2011).

The literatures indicate that clinically relevant deletions are found in patients with azoospermia or severe oligozoospermia with sperm concentrations <2x 10^6/mL. Very rarely, deletions can be found in infertile patients with sperm concentration between 2 and 5x 10^6/mL (Maurer and Simoni 2000, Lo Giacco *et al*., 2014). The current guidelines by the European Academy of Andrology (EAA) and European Molecular Genetics Quality Network (EMQN) for the detection of Yq microdeletions recommend the use of two markers in each AZF region in two multiplex polymerase chain reactions (PCR). Each PCR also has to include a marker for sex-determining region Y (*SRY*), located on the short arm of the Y chromosome, and a marker for *ZFX/ZFY*, a gene located on the X and Y chromosomes (Krausz *et al*., 2014). Suggested primer sets for each mix are indicated in table 3.1. A DNA sample from a fertile male, a woman and a blank (water) control should be run in parallel with the set of primers.

MIX1	MIX2
ZFX/ZFY	ZFX/ZFY
SRY	SRY
sY84	sY254
sY134	sY86
sY255	sY127

Table 3.1: Recommended primer mix for Yq microdeletion (Krausz et al *et al*., 2014). sY84 and sY86 are located in AZFa region, sY127 and sY134 are located in AZFb region, sY254 and sY255 are located in AZFc region.

According to the current knowledge, once a deletion of both primers within a region is detected, the probability of a complete deletion is very high (Esteves 2015). The use of this primer set enables the detection of almost all clinically relevant deletions and of over 95% of the deletions reported in the literature (Krausz et al., 2014). If the analysis reveals only one marker deletion, PCR reaction should be repeated in single-plex; by using only the deleted

marker with appropriate controls. In case of two or more marker deletions, extension analysis is recommended acoording to the following flow chart (figure 3.2).

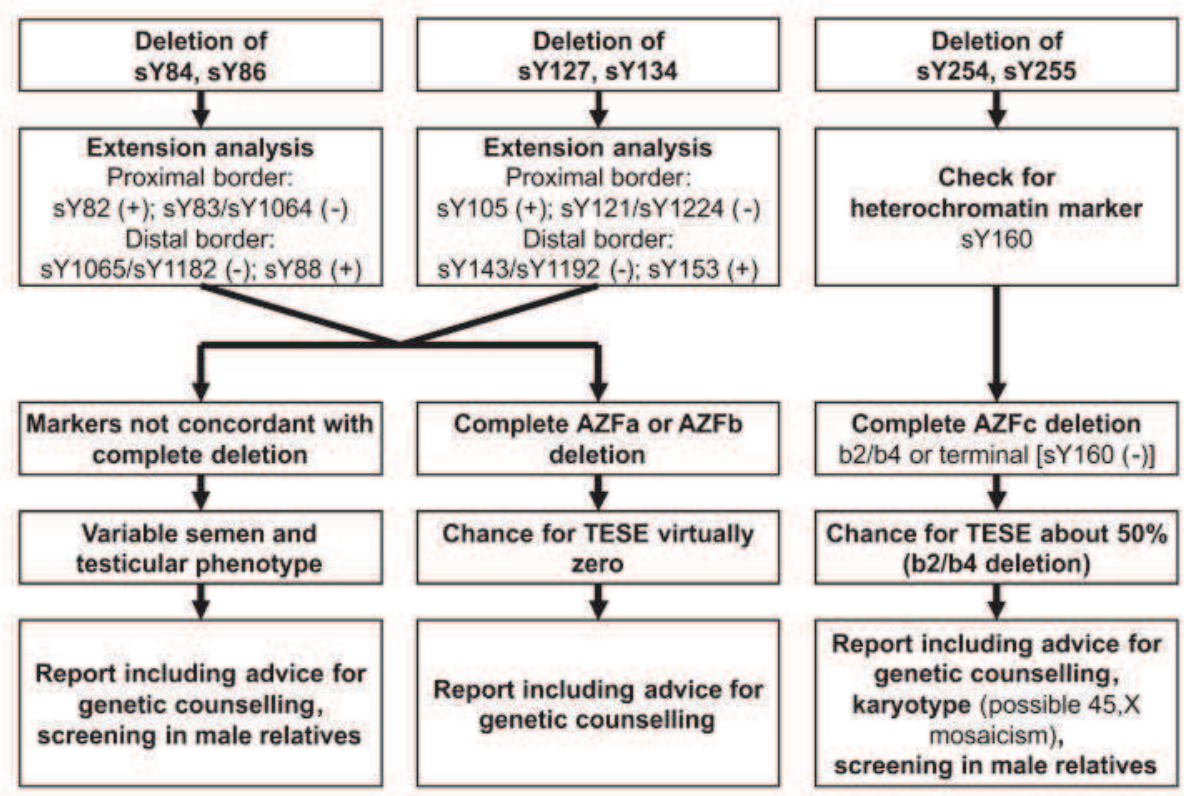

Figure 3.2: Flow chart for extension analysis. Common steps and consequences for cases with two or more deleted markers (adopted from Krausz *et al.*, 2014).

c) Copy Number Variations

Copy number variations (CNVs) are submicroscopic chromosomal duplications or deletions larger than 1000 bases and up to several Mb when compared with a reference genome (Eggers 2015).

Diseases and even susceptibility to diseases can be caused by CNVs (Henrichsen *et al.*, 2009). Although most CNVs are benign, alterations to the genomic architecture can have severe phenotypic implications through disruption of gene function and alteration to gene expression levels (Conrad *et al.*, 2010, Henrichsen *et al.*, 2009, Stranger 2007).

It is now established that CNVs cause genetic syndromes, isolated congenital defects, disease susceptibility, miscarriages or reproductive failure (McGuire et *al*., 2011, Aboura *et al*., 2009, Rajcan-Separovic *et al*., 2012). Recent examples of association of CNVs with diseases include Alzheimer disease (Rovelet-Lecrux *et al*., 2006), Crohn's disease (Fellermann *et al*., 2006), autism (Sebat *et al*., 2007, Weiss *et al*., 2008), psoriasis (Hollox *et al*., 2008), Parkinson's disease (Simon-Sanchez *et al*., 2008) and schizophrenia (Walsh *et al*., 2008).

The role of CNVs in male infertility is poorly defined, recently an excess of X-linked CNV number and DNA loss in patients with reduced sperm count was identified (Krausz *et al*., 2012). In the same study, a significant association between CNV number and sperm count in the infertile group was indicated. Similarly a significant inverse correlation has been found between sperm count and CNV number at the whole genome level in a German based study (Tüttelmann *et al*., 2011).

2-Monogenic mutations

Some gene mutations associated with pathological syndromes can also be related to infertility. For instance, congenital bilateral absence of vas deferens (CBAVD) leads obstructive azoospermia and in 80-90% of cases, it is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Bobadilla *et al*., 2002, Yu *et al*., 2002). Primary ciliary dyskinesia, also known as immotile cilia syndrome, is a genetically heterogeneous autosomal recessive disorder resulting from loss of normal ciliary function. Half of the male patients are infertile due to asthenozoospermia (Liabers *et al*., 2014). The most common genetic mutations include the Dynein, Axonemal, Heavy Chain 5 (*DNAH5* - in 15-28% of cases) and the Dynein, Axonemal, Intermediate Chain 1 *(DNAI1* - in 2-10% of cases) gene mutations (Boon et al., 2013). Another example is autosomal dominant

myotonic Dystrophy type 1 which is caused by CTG trinucleotide repeat expansion in the dystrophia myotonica-protein kinase (DMPK) gene. *Infertility* may occur in otherwise asymptomatic patients (Garcia de Andoin *et al*., 2005, Matsumura *et al*., 2009).

As a laboratory we are focusing on gene mutations which cause absent or abnormal spermatogenesis without any other problem, namely non syndromic infertility. So far few genes are defined as a cause of non syndromic infertility (table 3.2); we identified two of them, SPATA16 and DPY19L2 by studying globozoospermic patients. Since by definition male infertility causing mutation would have a very limited penetration into the general population, it is not surprising that a monogenic cause of male infertility is uncommon. Large-scale sequencing studies are essential to understanding a large percentage of aetiologies.

Phenotype		Genes	References
Teratozoospermia	Globozoospermia	*SPATA16*	Dam *et al.*, 2007
		DPY19L2	Koscinski *et al.*, 2011
		PICK1	Liu *et al.*, 2010
	Macrocephaly	*AURKC*	Dieterich *et al.*, 2007
	Sperm flagella	*DNAH1*	Ben Khelifa *et al.*, 2014
Asthenozoospermia		*CATSPER1*	Avenarius *et al.*, 2009
Spermatogenic failure	Azoospermia	*TEX11*	Yatsenko *et al.*, 2015
	Azoospermia/oligozoospermia	*TAF4B*	Ayhan *et al.*, 2014
		ZMYND15	Ayhan *et al.*, 2014
		NR5A1	Bashamboo *et al.*, 2010
		*TEX15**	Okutman *et al.*, 2015
		*MAGEB4**	*Article in preparation*
	Non obstructive azoospermia (NOA)	*SOHLH1*	Choi *et al.*, 2010
		Wt1	Wang *et al.*, 2013
		NAPS2	Ramasamy *et al.*, 2015

Table 3.2: Genes identified as a cause of non syndromic infertility in man. (*) Genes identified during this PhD.

2- STRATEGIES THROUGH THE QUEST OF MALE INFERTILITY GENE

There are two main approaches in male infertility studies; candidate gene approach and whole genome approaches.

A) Candidate gene approach

Candidate gene approach is depending on selecting genes from infertile animals, mostly mice, assuming that the gene function is conserved through evolution, and re-sequencing of candidate genes to detect allele frequency differences between cases and controls. Prior knowledge of the function and expression of the candidate genes are necessary to foreseen the biological plausibility of the role of the gene in studied phenotype.

Above all, ubiquitin-specific protease 26 (USP26) gene was the most studied one; multiple variations were detected in the human USP26 gene. A link between spertatogenesis defects and a cluster of variations were suggested (Stouffs *et al*., 2005, 2006), however the results from different groups are controversial (Christensen et al., 2008, Ribarski et al., 2009). According to a recent study, evidence from both enzymatic and meta-analyses did not support a direct association between USP26 variants and male infertility (Zhang *et al*., 2015). Due to the great genetic heterogeneity and the limited number of patients tested, the results brought by candidate gene approach have largely been unsuccessful or questionable. For example, mutation screening in some genes that might play a role in male reproductive function such as PCI (protein C inhibitor), FKBP6 (FK506 Binding Protein 6, 36kDa), CSNK2A2 (Casein Kinase 2, Alpha Prime Polypeptide) and CSNK2B (Casein Kinase 2, Beta Polypeptide), were not able to detect causal mutations (Gianotten *et al*., 2004, Westerveld *et al*., 2005, Pirrello *et al*., 2005). On the other hand, a heterozygous mutation identified in SYCP3

(Synaptonemal Complex Protein 3) gene (Miyamoto *et al.*, 2003) could not be confirmed by other groups (Stouffs *et al*., 2005, Martinez *et al*., 2007).

Considering more than 2000 genes with a possible function in mouse spermatogenesis, candidate gene approach is long and slow way to work about male infertility.

B) Whole genome approach

Recent technological improvements enable researchers to implement whole genome approaches such as SNP microarrays, high throughput sequencing technologies namely whole genome or exome sequencing for genetic testing studies.

- SNP microarrays

Single nucleotide polymorphisms, or SNPs, are minor variations in the genetic sequence that differ between members of a species or even between paired chromosomes in an individual. SNPs are broadly classified into common and rare based on minor allele frequencies (MAF) **(**Choudhury ***et al.,*** 2014)**.** A widely used cut-off for defining rare SNPs is with a MAF value less than 0.05 (Abecasis *et al*., 2010). SNPs are distributed uniformly throughout the genome. Commercial probe-based SNP array platforms can nowadays genotype, with >99% accuracy, about one million SNPs in an individual in one assay (LaFramboise 2009).

As a team, we are working with large families of well documented male infertility with some degree of consanguinity. From a genetic standpoint, intra-familial marriages greatly increase the emergence of pathologies following an autosomic recessive transmission. When husband and wife are related, they are susceptible to harbour the same rare dormant recessive mutation they have inherited from a common ancestor. Since the mutation comes from a recent common ancestor the whole region surrounding the mutation is identical,

therefore all the polymorphic markers, SNPs, of the area are homozygous and can be readily detected.

In the IGBMC sequencing platform, the Affymetrix 500K array set was utilized for our projects, the general protocol is summarised on figure 3.3. Regions of homozygosity are defined by a threshold of minimum 35 consecutive homozygous SNPs for a utilized array and visualized by the HOMOSNP program developed by IGBMC bioinformatic department. The homozygous regions are represented in different color according to consecutive homozygous SNPs; purple (less than 40 SNPs), light blue (40-44 SNPs) and dark blue (more than 44 SNPs).

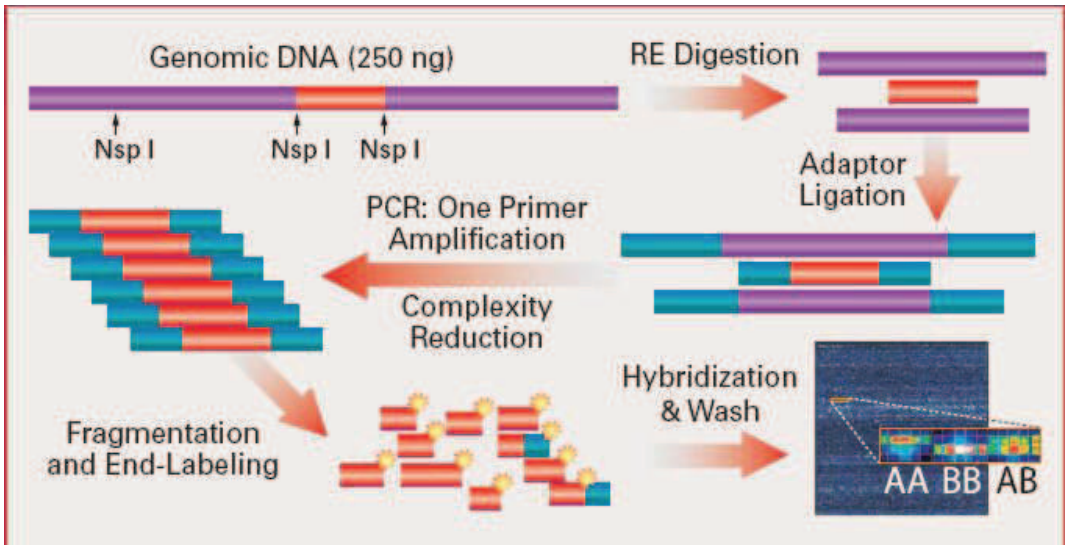

Figure 3.3: GeneChip mapping assay (GeneChip® Mapping 500K Assay Manual, Affymetrix)

- High throughput sequencing technologies

High throughput sequencing technologies enable researchers to perform massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison (Grada and Weinbrecht 2013). It includes a number of methods that can be grouped as template preparation, sequencing and imaging, and data analysis.

High throughput sequencing techniques in practice can be used for whole genome (WGS) or whole exome sequencing (WES). The initial sample preparation is identical for both WGS and WES. Genomic DNA is broken up into small fragments and sequence adaptors are added. These adaptors allow each fragment to be hybridised to the flowcell where the sequencing occurs. WES protocols proceed with the hybridization of the fragments to probes that are complimentary to all the known exons in the genome, which are then captured while the remaining DNA is washed away, leaving a pool of fragments containing exons. WGS requires no extra steps following the addition of adaptors and the library is ready to be sequenced at that point (Bras *et al*., 2012). WGS or WES generates an enormous amount of raw data requiring complex bioinformatic analyses to extract useful information (figure 3.4).

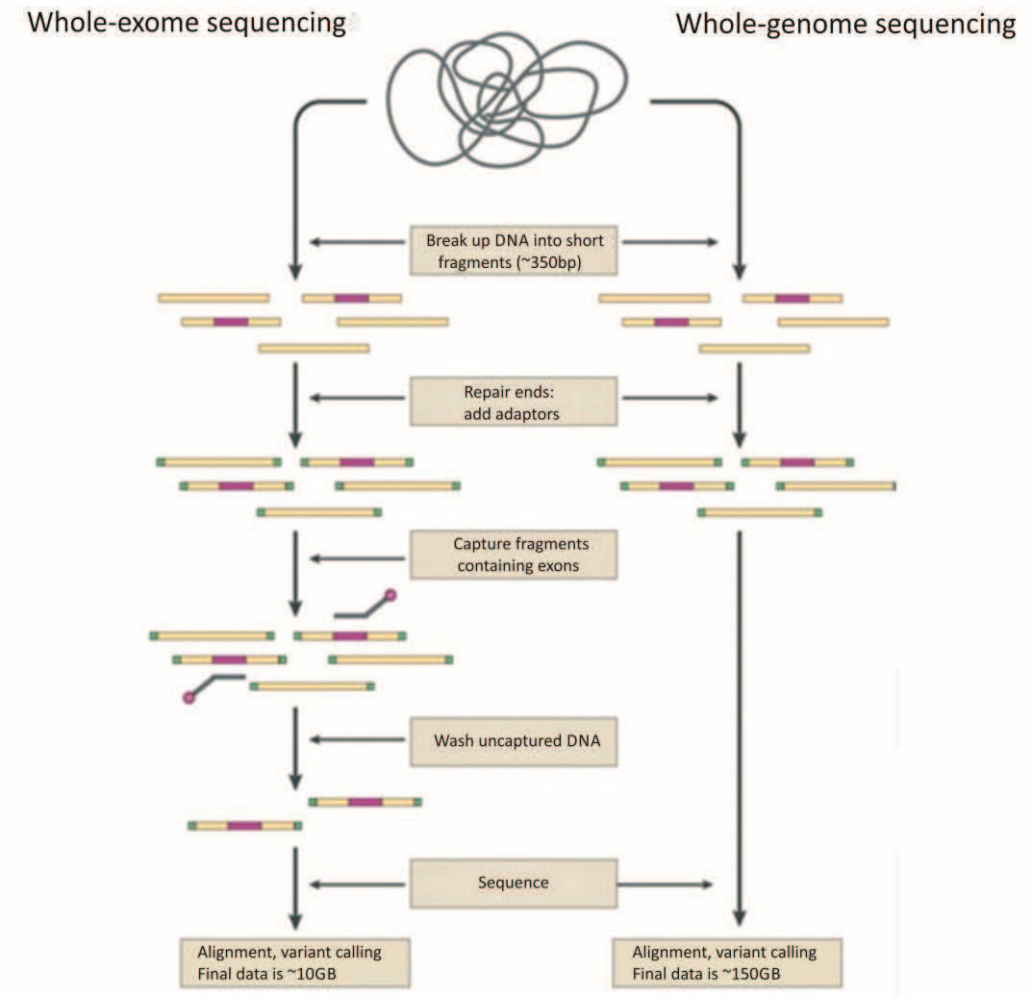

Figure 3.4: Whole exome versus whole genome sequencing protocol (adopted from Bras *et al*., 2012)

We prefer to apply WES in our actual studies. The exome represents just over 1% of the human genome (Grada and Weinbrecht 2013), but contains 85% of disease-causing mutations in Mendelian disorders (Rabbani 2014), which makes WES a cost-effective alternative to WGS. WES allows the identification of protein-coding mutations; which includes missense, nonsense, splice site, small deletion or insertion mutations. As all techniques, WES has some limitations; for instance coverage of exons never reach 100% (Bloch-Zupan *et al*., 2011*),* poor capture leads omission of a cusative gene, the mis-mapped reads or alignment errors could generate false variants, large deletions and duplications are difficult to detect and the causative gene could not be in the target definition since it is not yet referred in the Refseq database (El Inati *et al*., 2012).

Via WGS, one could have overview of possible disease-predisposing SNPs throughout the genome. Genome-wide association studies (GWAS) have been performed by different groups and a dozen gene polymorphisms have been proposed to be associated with impaired spermatogenesis (Aston and Carrel 2009, Aston *et al*., 2010, Hu *et al*., 2011, Hu *et al*., 2012, Kosova *et al*., 2012, Zhao *et al*., 2012, Xu *et al*., 2013, Zou *et al*., 2014, Qin *et al*., 2014, Hu *et al*., 2014). However, so far no overlapping genetic risk factors were identified and associations are generally weak; the correlation with male fertility in the diseased population of specific SNPs is generally less than 1% of the diseased population (Guerrero-Bosagna and Skinner 2014). Moreover, initial results are rarely confirmed by other studies, even replication study and meta-analysis of studies evaluating some genes have refuted the significance of the majority variants studied (Sato et al., 2013). Most striking example of confusing results is coming from recent studies of the Japanese group. They studied the association of four candidate SNPs identified previously, in fertile and infertile Japanese male

cohorts. The four SNPs in question have been found in no association with semen parameters in one study while three SNPs among four were significantly associated with the risk of male infertility in the second study (Sato *et al*., 2015a, 2015b).

The phenotypic heterogeneity of the study group is an important limitation, difficulties in phenotyping infertile men adds trouble to search for genes associated with infertility (Krausz and Carrel 2014). It is likely that rare SNPs and CNVs collectively contribute to explain a significant portion of male infertility that is currently classified as idiopathic even they are rare on an individual basis (Hotaling and Carrel 2014). Thus, well designed WGS studies with large cohorts of carefully phenotyped patients and controls are clearly needed.

Chapter IV

ASSISTED REPRODUCTIVE TECHNIQUES

Although various definitions have been used, originally assisted reproductive technologies (ART) refer to range of techniques and services to circumvent human infertility, performed to assist a couple to achieve the live birth of a healthy child. There are three stages of progressive interference: (i) ovulation induction with timed sexual intercourse (ii) ovulation induction combined with artificial insemination in which prepared sperm are transferred into the reproductive tract of female partner (iii) assisted fertilization where oocyte and sperm are joined outside the body prior to replacement of the embryo into the uterus. The latter one is the main subject of this chapter.

1- HISTORY of ART

The first documented application of artificial insemination in human was done in London in the 1770s by **John Hunter. Though, progress in the subject of in vitro ferilisation (IVF) had been slow,** IVF studies in animals' dates back as early as the 1890's. The first human IVF pregnancy was in 1973 reported by a team in Melbourne, Australia. However, it ended in an early embryo death (De Kretzer *et al*., 1973). Birth of Louise Brown in 1978, the first IVF baby in the world, was the start of a new era of ART and since then assisted fertilization is a well-established and accepted treatment for infertility.

Definition of the early embryo biopsy technique for genetic studies in 1988 was bringing hope for patients with a genetic disease risks (Wilton and Trounson 1989) and soon after the first report on biopsy taking from pre-implanted human embryos and sex detection by DNA amplification was published (Handyside *et al*., 1989). Immature oocyte retrieval followed by

in-vitro maturation (IVM) is a promising potential treatment option, especially for women who are infertile through polycystic ovarian syndrome (PCOS) (Cha *et al*., 1991). In 1992, a revolutionary procedure known as Intra-Cytoplasmic Sperm Injection (ICSI) in which sperm were micromanipulated directly into the egg was described (Palermo *et al*., 1992). Since its promising beginning, ICSI has become a routine treatment for male factor infertility around the world. Timeline of major ART milestones are described in figure 4.1A and 4.1B. By July 2012, an estimated 5 million "test tube babies" have been born worldwide, the figures were reported at the 28th annual meeting of European Society of Human Reproduction and Embryology (ESHRE).

2- LEGISLATION and DATA COLLECTION for ART

Laws and regulations governing ART services vary between different countries, from very restricted (Switzerland, Germany, Italy, Crotia) to very liberal (Spain, Greece, UK). In some countries such as USA and Australia, each state has its own legislation. Norway was the first country in the world to pass a law on ART on June 12th, 1987 (Norwegian law on Assisted Reproduction and Genetics) which is followed by UK (Human Fertilisation and Embryology Act**, 1990).** In France, legislation on ART is specific. The 1994, 2004 and 2011 laws of bioethics define ART and the conditions under which these techniques may be used, subject to rules of good practice.

Data collection is handled by the European IVF Monitoring (EIM) Consortium and more globally by international Committee on the Monitoring of ART (ICMART). Data from each participating country are sent to ESHRE once a year; a draft report is made and then scrutinized by all members.

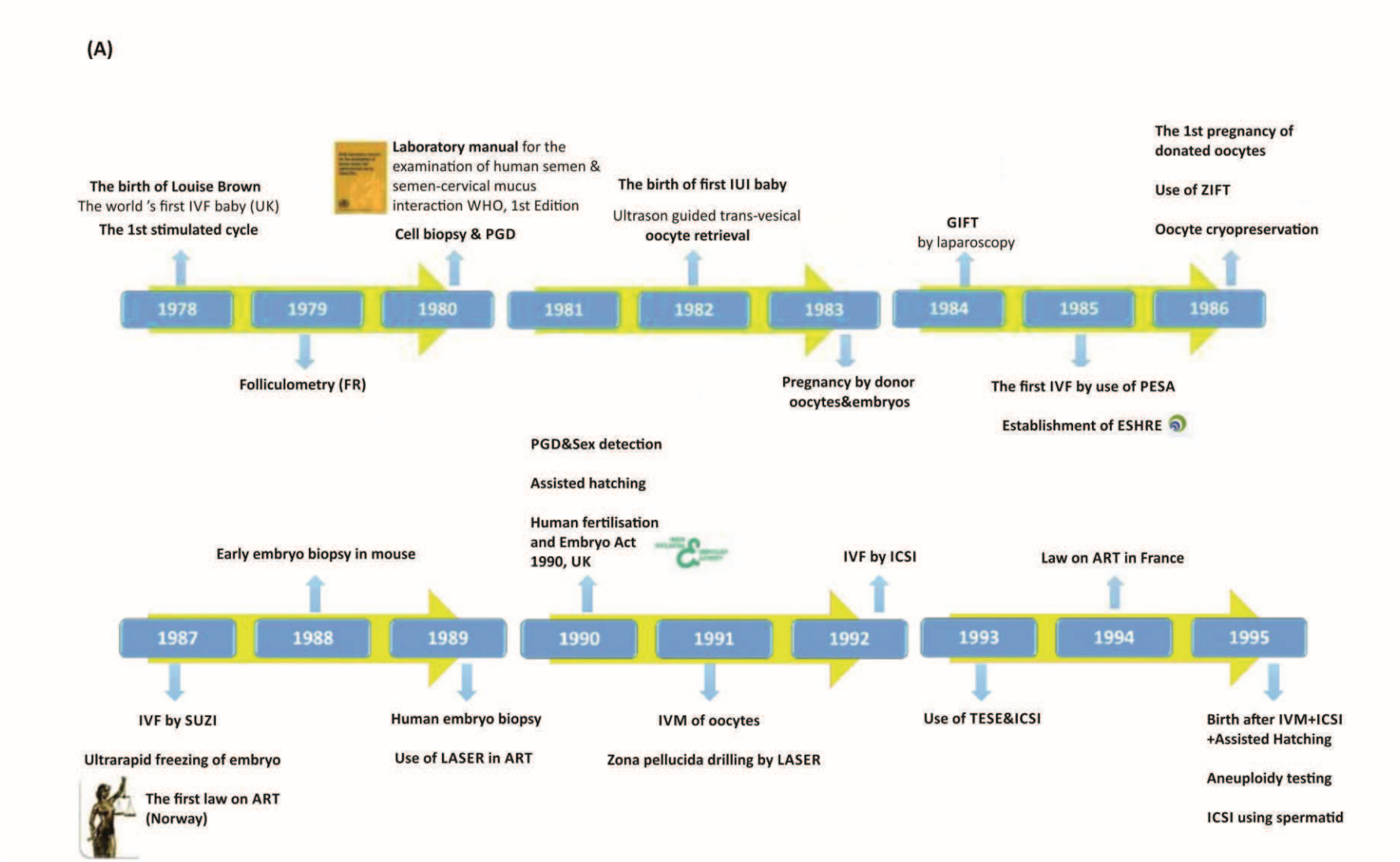

Figure 4.1A: Timeline of major ART milestones: Year 1978-1995 (Adopted from Kamel 2003)

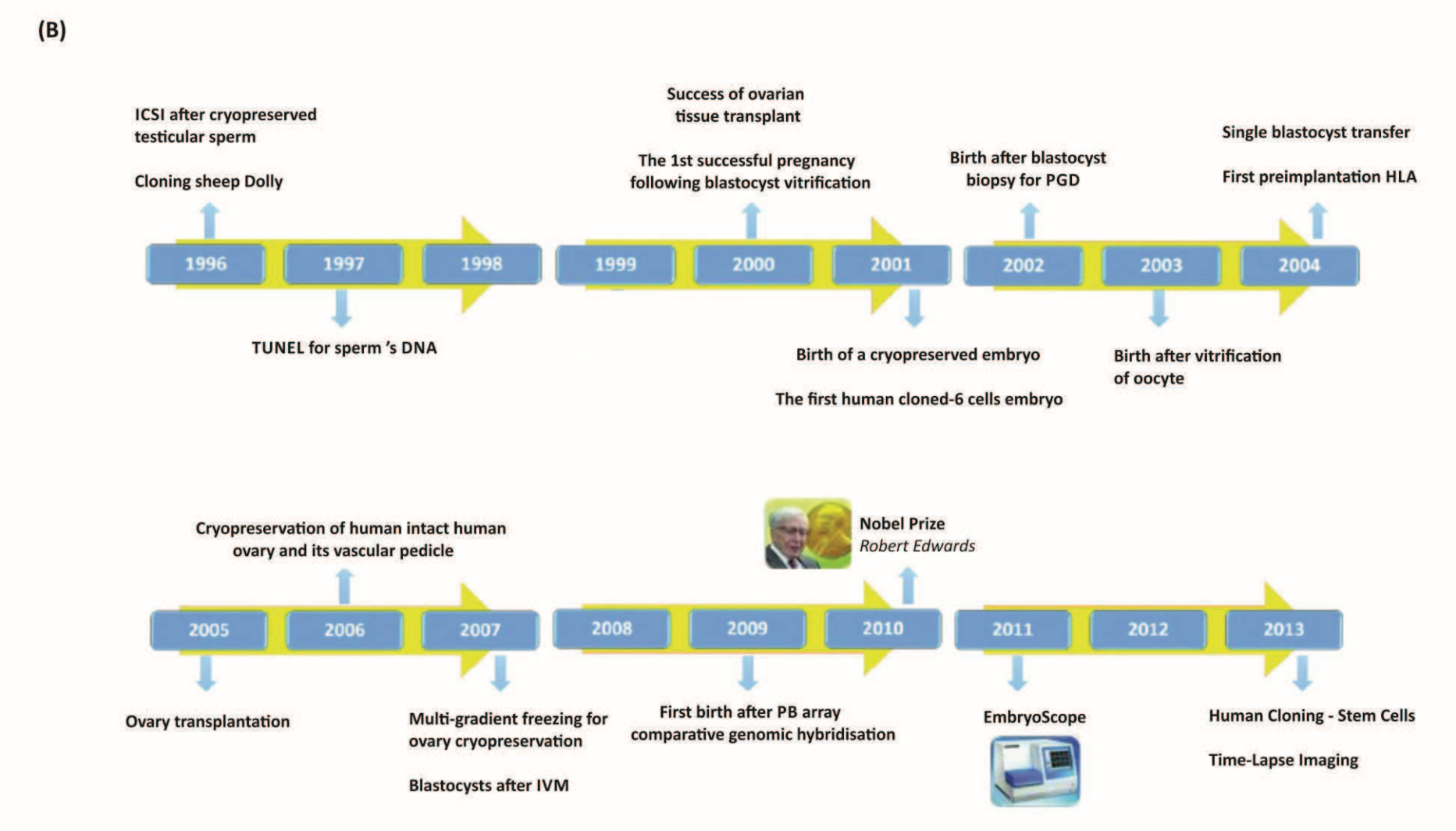

Figure 4.1B: Timeline of major ART milestones: Year 1996-2013 (Adopted from Kamel 2003)

3- ASSISTED FERTILIZATION PROCESS

The assisted fertilization process involves controlled ovarian hyperstimulation to promote multiple oocyte maturation, oocyte removal from the ovaries through aspiration of the follicules, sperm retrieval and preparation, fertilization in the laboratory, embryo culture and transfer.

a) Controlled ovarian hyperstimulation and oocytes collection

Although assisted fertilization can be performed in the unstimulated menstruation cycle, it has become widespread practice to carry out hormonal stimulation in order to obtain multiple oocytes available for the application. The most commonly used protocol consists of controlled ovarian hyper-stimulation (COH) with daily injections of recombinant human FSH (r-hFSH) to induce multiple follicle growth in the ovaries (Raju *et al*., 2013). Follicules are monitored using serum oestradiol levels and transvaginal ultrasound. When the follicles reach a sufficient size, human chorionic gonadotropin (HCG) is administered to induce maturation of the oocytes and collection is undertaken within 32 to 36 hours of administering HCG (Wang *et al*., 2011). Oocytes are cultured in a suitable medium for the subsequent applications (fig 4.2a).

b) Sperm retrieval and preperation

Generally, on the same day as the oocytes retrieval, semen can be collected for assisted fertilization by masturbation after a period of abstinence of 2–7 days, as recommended by the World Health Organization (WHO 2010) although frozen-thawed sperm can also be used. If the male partner is azoospermic, sperm may be retrieved through testicular biopsy. Main principles for testicular biopsy are explained in the following section.

Washing procedure of semen is required; because seminal plasma can exert toxic effects both on the sperm itself, as well as on the oocytes (Kanwar *et al*., 1979). Beside this, functional motile sperm need to be separated from those that are immotile, have poor morphology or are not capable to fertilize. Different separation techniques are available based on principles such as migration, filtration or density gradient centrifugation. Prepared sperm are incubated in a specialized medium until fertilization.

a) Fertilization

Two methods are currently used for fertilization; IVF and ICSI.

In the first one, namely IVF, collected oocytes are co-incubated with a defined number of motile spermatozoa in a culture medium, designed to mimic the tubal fluidic milieu. IVF was originally introduced for the treatment of tubal infertility caused either by occlusion of the Fallopian tubes or by impairment of their function (Edwards 1981). This technique is not suitable in case of low number of spermatozoa and bad sperm quality.

On the contrary, a single vital spermatozoon is enough to achieve fertilization for ICSI. Microinjection techniques have allowed men with severely compromised semen parameters to achieve fatherhood. Since its introduction, the use of ICSI has increased yearly. Today, ICSI is used in more than 70% of ART cycles in countries like Belgium, Greece, Spain and the percentage become higher in certain regions of the world such as southern Europe (Nyloe 2008, de Neubourg 2010).

For ICSI, retrieved oocytes are first separated from surrounding cumulus oophorus and corona radiate cells by mechanical and enzymatic process. Oocytes are then classified in order to identify mature ones suitable for fertilization. The position of the first polar body is

assumed to reflect the position of the maternal spindle (Lynch and Regueira 2015). Selected spermatozoon is first immobilized mechanically then is injected into the cytoplasm of an oocyte on a horizontal plane with the first polar body at the 12 or 6 o'clock position in order to avoid maternal spindle (fig 4.2b).

In both case, oocytes are inspected for fertilization after 16-18 hours, successfully fertilized oocytes, given the name zygote, and are cultured until the day of transfer.

b) Embryo culture and transfer

Different culture mediums are commercially available for the growing human preimplantation embryo. Physical parameters that can affect embryo development are also optimized in the special incubators providing a stable and appropriate culture environment for the embryo development. The duration of embryo culture can be varied, conferring different stages of embryogenesis at embryo transfer (fig 4.2 c-g). Embryo transfer can be performed either at the pronuclear stage, at cleavage stage (day 2 to 4 after co-incubation) or at the blastocyst stage (day 5 or 6 after co-incubation). Remaining good quality embryos can be cryopreserved for subsequent frozen-thawed embryo transfer in unstimulated treatment cycles. The pregnancy status is tested 16^{th}-18^{th} day of oocyte retrieval with a test which controls the level of beta human chorionic gonadotropin (*beta-hCG*) hormone in blood.

Multiple pregnancies are common outcome of ART as a result of the practice of transferring more than one embryo. Over the years, there has been a steady decline in the number of embryos transferred in a single cycle due to growing concerns about the risk and very serious health consequences of multiple pregnancies (Bryant *et al*., 2004).

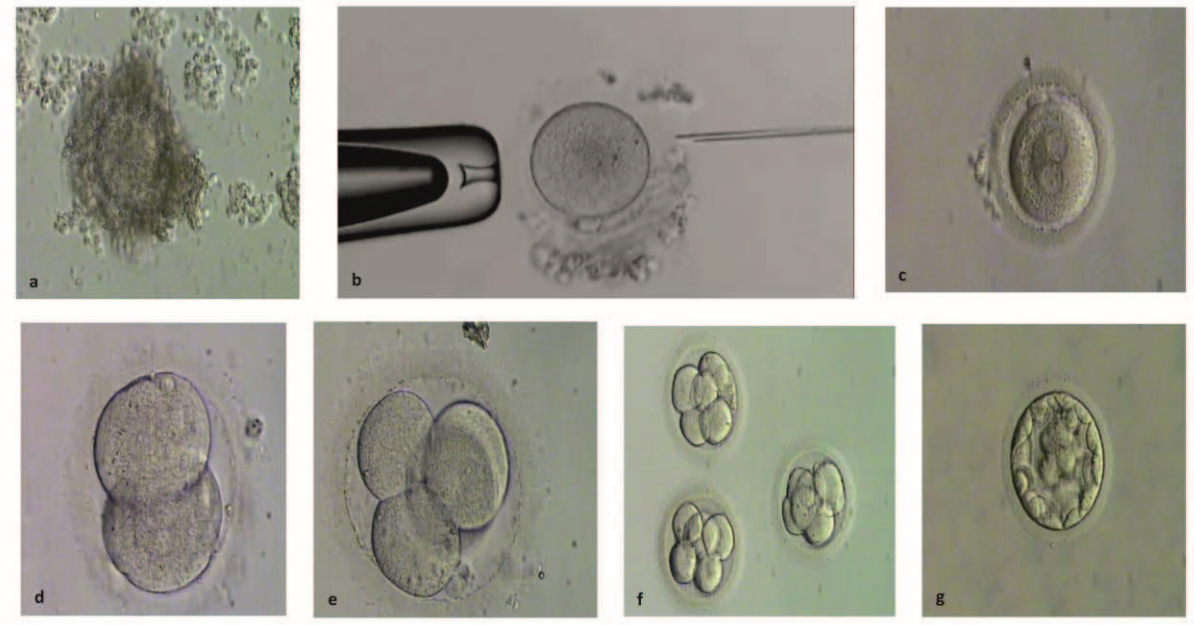

Figure 4.2: Human gametes and embryos (a) human oocyte with corona cells (b) application of ICSI (c-g) human embryos at different stages of embryogenesis; day 1 up to day 5 (photos were taken in embryology lab of Bahceci Clinic, Istanbul, TR).

4- SPERM RETRIEVAL IN AZOOSPERMIC MALES

For azoospermic men, sperm needs to be retrieved directly from the testis or epididymis. Azoospermia can be classified as obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). OA is the consequence of physical blockage to the male ductal system; it can be congenital (congenital absence of the vas deferens, idiopathic epididymal obstruction) or acquired (from infections, vasectomy, or other iatrogenic injuries to the male reproductive tract) (Wostnitzer 2014, Schlegel 2004). NOA, on the other hand, is defined as a complete absence of sperm in the ejaculate that is due to the absence of sperm production by the testes and not by an obstruction of the vas deferens. It accounts for approximately 60% of men with azoospermia and represents the most severe form of male factor infertility (Hamada *et al*., 2013).

The optimal management of NOA in clinical application is testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI). However in nearly half of the cases spermatozoa could not be found after testicular biopsies and so far, neither serum hormone assays, such as FSH and inhibin B, nor noninvasive assessments, such as testicular volume, could predict the probability of sperm retrieval sufficiently to direct clinical management (Carpi A *et al*., 2009), only testicular histopathology can be used as a predictor of successful sperm recovery.

In conventional TESE, testicular spermatozoa are isolated from testicular biopsy specimens obtained by open surgery under general or local anaesthesia from patients. On the contrary, microdissection testicular sperm extraction (micro-TESE) is far more complex type of a microsurgical method of sperm retrieval compared to a conventional TESE. The rationale of micro-TESE is to identify focal areas of sperm production within the testes, based on the size and appearance of the seminiferous tubules, with the aid of the operating microscope (Schlegel 1999). Spermatozoa can be retrieved from tubules that are dilated and this can be visualized with an operating microscope (Dohle *et al*., 2012). Recently, the successful use of the measurement of seminiferous tubule diameter for predicting successful sperm retrieval was published (Amer *et al*., 2008). Micro-TESE is offered as a better alternative to TESE because of increasing the chance of retrieving testicular sperm, decreasing the size of tissue removal therefore minimizing testicular damage (Esteves 2015).

Principally, removal of testicular tissue with the sole purpose of histopathology evaluation could potentially eliminate the rare foci of sperm production and thus jeopardize the chances of future retrieval attempts (Esteves 2015). Testicular biopsy prior to sperm retrieval is therefore not routinely recommended. Usually testicular biopsy performed same

day as oocytes retrieval, biopsy specimen is checked for the presence of spermatozoa in laboratory while a small sample retrieved from close area is sent for histological evaluation. Uncertainty of sperm acquisition, however, makes prognostic factors very desirable since failure to retrieve sperm resulting in an unnecessary ovarian stimulation cycle for the partner, may cause couples physical, psychological and financial distress (Tournaye *et al*., 1997).

- Histopathology and interpretation of biopsy specimen

Generally classification of biopsy specimens are based on five main histological patterns of spermatogenesis (Dohle *et al*., 2012): (i) absence of seminiferous tubules (tubular sclerosis); (ii) no germ cells within the seminiferous tubules (Sertoli cell only syndrome) (Figure 4.3a); (iii) incomplete spermatogenesis, not beyond the spermatocyte stage (spermatogenic arrest) (Figure 4.3b); (iv) all germ cell stages present including spermatozoa, but there is a distinct decline in the number of germ cells (hypospermatogenesis) (Figure 4.3c); and (v) normal spermatogenesis (Figure 4.3d). Sperm retrieval is usually good in men with hypospermatogenesis and limited in men with SCOs.

The mosaic appearance of the testis is well established; seminiferous tubules showing intact spermatogenesis could occur simultaneously with the ones showing spermatogenic arrest on different levels or even SCO in adjacent tubules within the same testis. This phenomenon is known as “mixed atrophy” (Sigg 1979). In these cases “score count” evaluation is appropriate when counseling infertile patients.

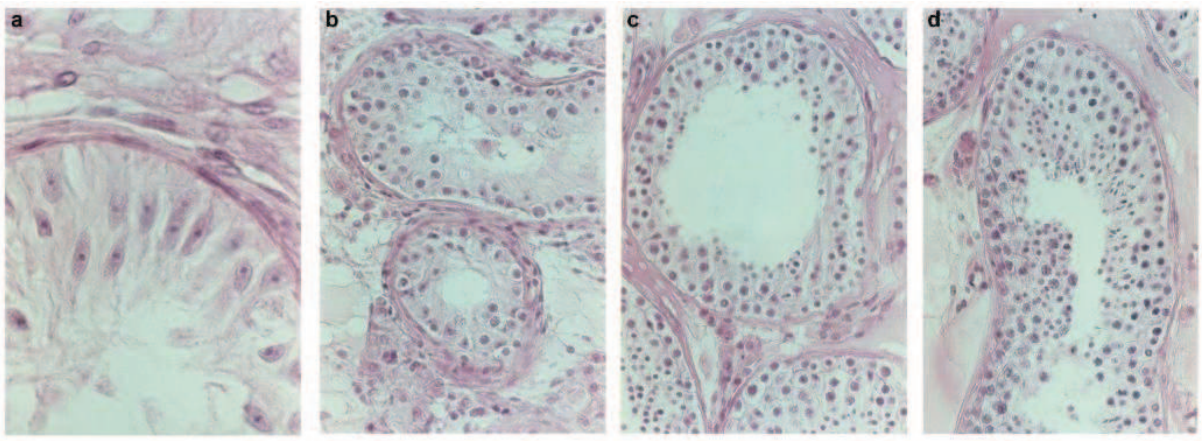

Figure 4.3: Biopsy specimens with different pathology (a) Sertoli cell only syndrome (b) Maturation arrests (c) Hypospermatogenesis (d) Normal spermatogenesis (adopted from Dohle *et al*., 2012).

In 1970, Johnsen proposed a scoring system for quantitatively describing spermatogenesis (Johnsen 1970). It was later modified by providing a score of every tubule within a given histological section (De Kretzer and Holstein 1976). In at least 100 seminiferous tubules, the level of sperm maturation is graded between 1 and 10 according to the most advanced germ cell in the profile; score 10 means normal spermatogenesis, while 5 shows spermatocytes as the most advanced cell and finally score 1 signifies no germ or Sertoli cells. In contrast, the score according to Bergmann and Kliesch is based on the percentage of tubules showing elongated spermatids (Bergmann and Kliesch 1998). Score 10 means 100% of tubules contain elongated spermatids, score 1 means 10% of tubules contain elongated spermatids.

The Johnsen score is still widely reported, but rarely according to the detail of its original description. Unfortunately the mean tubule score may not reflect the true status of spermatogenesis and scoring is quite labour-intensive; as a result it has little clinical utility in the current fertility practice.

Results

Nonsense mutations in *TEX15* and *MAGEB4* genes as an underlying cause of non-obstructive azoospermia and severe oligozoospermia

Non-obstructive azoospermia (NOA) and severe oligozoospermia (SO) are the most frequent causes of male infertility in man. Unfortunately, a significant number of cases remain idiopathic. The current estimate is that about 30 % of men seeking help at an infertility clinic are found to have oligozoospermia or azoospermia of unknown aetiology (Poongothai *et al*., 2009).

NOA is defined as a complete absence of ejaculate from the semen that is due to absence or marked reduction of sperm production by the testes. It affects 10% of infertile men and is diagnosed in 60% of azoospermic men. Testicular spermatozoa can be retrieved from some NOA men because of the existence of isolated foci of active spermatogenesis. However sperm are absent in the testis almost 50% of men with NOA, so far there are no absolute predictors of sperm yield for testicular biopsy. Active spermatogenesis regions could not be predicted in general; only testicular histopathology can be used as a predictor of successful sperm recovery. In many fertility clinics that perform intracytoplasmic sperm injection (ICSI) with freshly retrieved sperm, female partners of men with NOA who failed sperm retrieval may undergo unnecessary ovarian stimulation.

Although there is no consensus definition of SO, a sperm concentration of less than 5 million sperm/mL of ejaculate is commonly accepted as a limit for diagnosis and in the absence of clinical intervention, successful fertilization is close to zero. No treatment options are currently available for patients with SO except ICSI.

Considering the high predicted number of genes involved in male gametogenesis, it is likely that most 'idiopathic' forms of spermatogenic disturbances are caused by mutations or polymorphisms in spermatogenesis candidate genes (Nuti and Krausz 2008). However at

present, only few genes have been identified as a responsible of NOA and/or oligozoospermia in man.

Using exome sequencing, we identified two new genes, namely *TEX15* and *MAGEB4* involved in NOA and SO phenotype in consanguineous Turkish families. *TEX15* (Testis-expressed sequence 15) and *MAGEB4* (Melanoma Antigen Family B4) are expressed specifically in testis while silent in normal adult tissues, but re-expressed in selected tumor types. This expression pattern indicates both genes are belonging to a group of cancer-germline genes. They are potential target for immunotherapy, however normal functions of TEX15 and MAGEB4 are not clear.

TEX15 maps to chromosome 8, contains four exons, spans a genomic region of 59.06 Kb on the reverse strand and encodes a 2,789-amino acid protein. A homozygous single base substitution (c.2130T>G) was identified in the first family we have studied. The mutation should lead to early translational termination at the first exon of *TEX15,* it is co-segregated with the infertility phenotype and our data strongly suggest that it is the cause of spermatogenic defects in the family. All three affected brothers presented a phenotype reminiscent of the one observed in KO mice. In mice, disruption of the gene causes a drastic testis size reduction and meiotic arrest in the first wave of spermatogenesis in males. Further analysis revealed that in mice *Tex15* is required for chromosomal synapsis. It regulates the loading of DNA proteins onto sites of double-strand DNA breaks and thus its absence causes a failure in meiotic recombination (Yang *et al*., 2008). The data from our study suggested that the identified mutation correlates with a decrease in sperm count over time. A diagnostic test identifying the mutation in man could provide an indication of

spermatogenic failure and prompt patients to undertake sperm cryopreservation at an early age.

MAGE genes family encompasses more than 60 genes (Doyle *et al*., 2010), which can be classified in classes according to their expression pattern. Type I genes have an expression limited to germ cells and cancer cells, whereas type II genes are ubiquitously expressed. The type I MAGE genes are subdivided into three subfamilies namely *MAGE-A,-B,* and *-C,* all are located on the X chromosome. They are characterized by a large terminal exon encoding the entire protein.

MAGEB4 is a member of the *MAGEB* gene family, a type I member, which is clustered on chromosome Xp22-p21. Human *MAGEB4* has only one exon and encodes 346 amino acid protein. As in all MAGE proteins, it has a well conserved MAGE homology domain (MHD) located at the C-ter that can be traced back to protozoa (Barker and Salehi 2002). Murine Mageb4 was suggested to may have important functions in adult testis; for keeping the germ cell in an undifferentiated stage and involved in germ cell-specific mitosis (Osterlund *et al*., 2000). Though, mouse *Mageb4* does probably not corresponds to human MAGEB4 because of the presence of a repetitive region of 133 amino acids at the C-ter end.

The identified nonstop mutation in the family (c.1041A>T p.*347Cysext*24) substitutes the stop codon with cysteine residue, potentially adding 23 amino acids to the C-terminus of MAGEB4. Since nonstop mutations are rare and no consensus on their consequences is established, we designed some functional tests. We revealed that neither the cellular protein localization nor the mRNA stability is affected by the identified mutation. We also tested whether modifying the C-terminal part of MAGEB4 would modify its ability to interact with other proteins. Co-immunoprecipitation experiments demonstrated that MAGEB4

mutant as well as the wild-type form is able to interact with MAGEA3. We finally tested the ability of wild type and mutant MAGEB4 to self-interact, but we found no difference as control MAGEB4 interacts equally well with its wild type or mutant forms. Reciprocally, mutant MAGEB4 interacts with both wild type and mutant MAGEB4. However the modeling of the tertiary protein structure based on MAGEA3 model suggests that the addition of the 23 amino acids may affect the proper homo or heterodimerization of the protein. This study provides the first clue on the physiological function of a MAGE protein.

Both mutations were absent neither in data base nor in 107 (234 alleles) ethnically matched healthy fertile controls. 108 unrelated Turkish infertile patients were scanned for both identified mutations and no mutation was identified.

Chapter V

Exome sequencing reveals a nonsense mutation in *TEX15* causing spermatogenic failure in a Turkish family

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ORIGINAL ARTICLE

Exome sequencing reveals a nonsense mutation in *TEX15* causing spermatogenic failure in a Turkish family

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Abstract

Infertility is a global healthcare problem, and despite long years of assisted reproductive activities, a significant number of cases remain idiopathic. Our currently restricted understanding of basic mechanisms driving human gametogenesis severely limits the improvement of clinical care for infertile patients. Using exome sequencing, we identified a nonsense mutation leading to a premature stop in the *TEX15* locus (c.2130T>G, p.Y710*) in a consanguineous Turkish family comprising eight siblings in which three brothers were identified as infertile. *TEX15* displays testis-specific expression, maps to chromosome 8, contains four exons and encodes a 2789-amino acid protein with uncertain function. The mutation, which should lead to early translational termination at the first exon of *TEX15*, co-segregated with the infertility phenotype, and our data strongly suggest that it is the cause of spermatogenic defects in the family. All three affected brothers presented a phenotype reminiscent of the one observed in KO mice. Indeed, previously reported results demonstrated that disruption of the orthologous gene in mice caused a drastic reduction in testis size and meiotic arrest in the first wave of spermatogenesis in males while female KO mice were fertile. The data from our study of one Turkish family suggested that the identified mutation correlates with a decrease in sperm count over time. A diagnostic test identifying the mutation in man could provide an indication of spermatogenic failure and prompt patients to undertake sperm cryopreservation at an early age.

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Introduction

As attested by a recent World Health Organization (WHO) report, infertility is a global healthcare problem. Indeed, about 48.5 million couples suffer from infertility worldwide (1). The cause of infertility can be of female, male or mixed origin, each cause accounting for about one-third of all cases.

The definition of a male factor is generally based on abnormal semen parameters. Semen quality is usually assessed by sperm count in the ejaculate, percentage of motile sperm and morphology (2). Male infertility can then be defined as the absence of sperm in ejaculate (azoospermia), inability to produce spermatozoa in sufficient numbers (oligozoospermia, with diverse severity), inadequate motility (asthenozoospermia), abnormal morphology (teratozoospermia) or a combination of these defects. Non-obstructive azoospermia (NOA) and severe oligozoospermia (SO) are the most frequent causes of male infertility in man. Unfortunately, a significant number of cases remain idiopathic. The current estimate is that about 30% of men seeking help at an infertility clinic are found to have oligozoospermia or azoospermia of unknown aetiology (3). So far, the only available treatment is *in vitro* fertilization with an intra-cytoplasmic sperm injection (ICSI), which represents an empirical approach generally offered as a standard treatment option.

Spermatogenesis is an extremely complex process that involves highly specialized mechanisms. Considering the concerted action of more than 2000 genes in spermatogenesis, a mutation in any of these genes may be responsible for a spermatic defect (4), and therefore, it is likely that many 'idiopathic' forms may have a genetic origin.

Recent technological improvements enable researchers to implement whole genome approaches such as single nucleotide polymorphism (SNP) microarrays, whole genome or exome analysis for genetic testing studies. So far, the application of SNP microarrays and homozygosity mapping has successfully led to the identification of a few genes involved in infertility (5–9).

Genome-wide association studies (GWAS) have been performed by different groups, and a dozen gene polymorphisms have been proposed to be associated with impaired spermatogenesis (10–20). However, associations are generally weak, and initial results are rarely confirmed by other studies. The need to study a very large population (>10 000) displaying phenotypic homogeneity remains a major paradigm of GWAS (4). Therefore, the analysis of large families of well-documented male infertility with some degree of consanguinity provides an alternative approach to identify genes involved in infertility.

Following up on studies of family cases of teratozoospermia and the identification of two genes involved in globozoospermia (5,6,21), characterized by round-headed spermatozoa lacking an acrosome, we are now focussing our research on NOA and SO ($<1 \times 10^6$ spermatozoa/ml). We present here the analysis of a consanguineous Turkish family comprising six brothers; three infertile, two fertile, one of unknown fertility status and two fertile sisters. We revealed a nonsense mutation in *TEX15*, which co-segregated with the infertility phenotype, as the cause of spermatogenic defects in the family. Interestingly, the human phenotype is very similar to the one observed in the *Tex15* KO mouse. In mice, disruption of the gene causes a drastic testis size reduction, as observed for the patients described here, and meiotic arrest in the first wave of spermatogenesis in males.

Results

Infertility phenotype

A consanguineous Turkish family comprising six brothers, three infertile, two fertile, one of unknown fertility status and two fertile sisters (Fig. 1A), was identified at the Bahceci Health Group, Istanbul, Turkey. Parents were first-degree cousins. Two of the infertile brothers were diagnosed as NOA (IV:1 and IV:3; Fig. 1A), while the younger third infertile brother (IV:5; Fig. 1A) was SO with, in 2010, little sperm in the ejaculate. All affected brothers presented, considering an average normal size of 18–20 ml (22), a drastic testicular size reduction of more than 50% (Fig. 1B). Histopathology of the index patient (IV:3) identified a maturation arrest at the primary spermatocyte stage (Fig. 1C).

Brothers IV:1 and IV:3, with NOA, had a testicular sperm extraction (TESE) operation, a limited number of spermatozoa were detected and ICSI was performed. One embryo was transferred for both cases; transfer on Day 2 was realized for IV:1, while Day 3 transfer was chosen for IV:3. Transferred embryos were of poor quality, and no pregnancy was obtained (Supplementary Material, Table S1).

Two ICSI attempts were performed for patient IV:5, the first one with testicular sperm since no motile spermatozoa were found in the ejaculate. A pregnancy was achieved, but resulted in early abortion. Ejaculated sperm were used for the second attempt in 2012, and a healthy child was born. In 2014, a new semen analysis revealed azoospermia; no spermatozoon was detected even after concentration.

Whole exome sequencing and data analysis

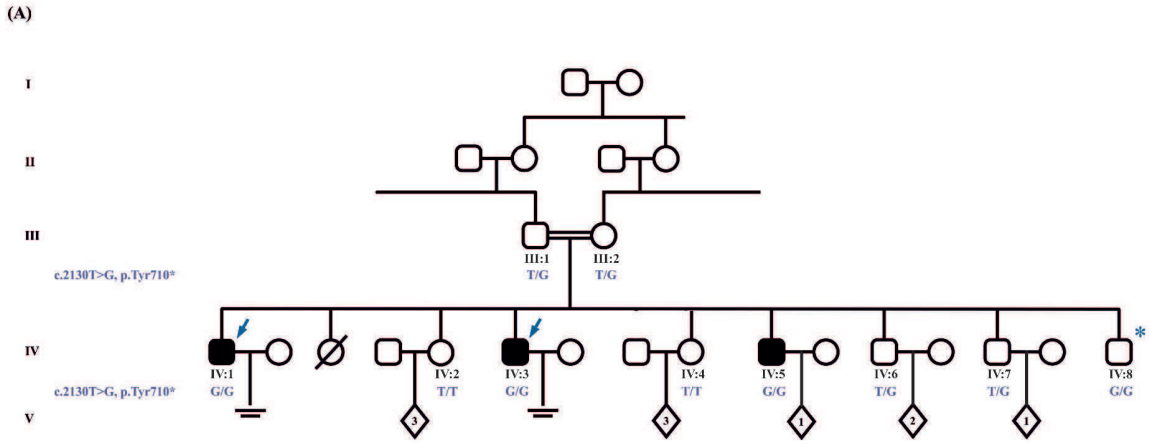
In order to identify the genetic cause of male infertility in the family, whole exome sequencing (WES) was applied for samples IV:1 and IV:3. For these samples, 6 and 6.7 GB DNA sequence were generated, respectively. In both the cases, >56% of the target exome was represented with >40-fold coverage (Supplementary Material, Table S2).

Given the known consanguinity in the family, we hypothesized that the disease should follow an autosomal recessive inheritance mode, and we thus tracked homozygous variants shared by both samples. We filtered variants according to coverage, minor allele frequency in populations (we filtered out when >1%), validation status of the reference SNP (not validated or validated by only one database) and novelty by comparing them with our in-house exome database.

Data analysis revealed 17 homozygous variations in 15 genes shared by the two samples (Supplementary Material, Table S3). Amazonia (http://amazonia.transcriptome.eu/), BioGPS (http://biogps.org/) and EMBL-EBI expression atlases (http://www.ebi.ac.uk/gxa/home) were scanned for all 15 genes that passed our filtering process. This revealed *TEX15* (MIM: 605795) as the most plausible candidate gene due to its specific expression in germ cells and the existence of a mouse KO model with a male sterility phenotype similar to the one observed in our patients. In particular, KO male mice presented a drastic testis size reduction and a meiotic arrest (23). The human *TEX15* maps to chromosome 8 and consists of four exons and spans a genomic region of 59.06 Kb on the reverse strand. The gene encodes a 2789-amino acid protein with only two predicted unknown domains named 'TEX15' (PF15326) according to the PFAM (24) and SMART (25) databases (Fig. 3C).

Mutation confirmation and screening

A homozygous single base substitution (NM_031271.3: c.2130T>G, p.Y710*) causes a premature stop at the first exon of *TEX15*, shared by two infertile brothers. The c.2130T>G variation, supported by 112 and 134 reads, respectively, for the samples sequenced, was not observed in any variation database. The



(B)

Patient code	Testis volume (ml)	
	Left	Right
IV :1	10	10
IV :3	12	12
IV :5	10	10.5

(C)

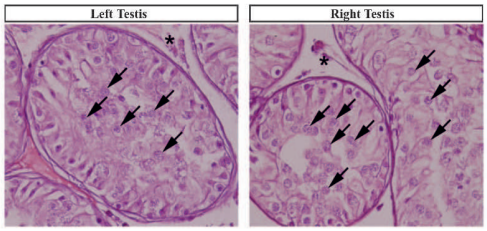


Figure 1. Consanguineous Turkish family with eight siblings. (**A**) Pedigree structure showing the segregation of c.2130T>G, p.Tyr710* mutation. Filled symbols indicate affected members, and clear symbols indicate unaffected members. One female sibling died at the age of 2, the cause of death is unknown. Asterisk (*) points out the family member for whom no information about semen analysis was available. Whole exome sequencing was performed on two affected brothers indicated with arrows. (**B**) Testicular volume of affected members in the family. Normal testicular volume for man is given as 18–20 ml[8]. (**C**) Testicular histology of patient IV:3. Representative sections of human left and right testes biopsies displaying maturation arrest pattern. Arrows indicate primary spermatocytes; asterisks indicate interstitial spaces. Bars represent 50 μm in length.

variant and the segregation in the family were confirmed through Sanger sequencing (Fig. 2). The mutation co-segregated with the infertility phenotype; the two NOA brothers and the oligozoospermic sibling were homozygous for the mutation, while parents were carriers as well as two fertile brothers. Fertile sisters were wild type; however, the youngest brother with an unknown fertility status was also homozygous for the mutation (Fig. 1).

The mutation was absent in 107 (214 chromosomes) fertile males of Turkish origin. In addition, all exons and intronic flanking sequences of *TEX15* were amplified and sequenced in order to validate variants and to look for other mutations in 85 unrelated individual NOA cases and 13 SO cases with similar phenotypes (primers listed in Supplementary Material, Table S4A). No mutations were found.

Expression of *TEX15*

In order to determine the expression profile of *TEX15* in man, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistology. Twenty-one total RNA samples from different organs, including testis, and 9 testicular tissues derived from 3 Sertoli cells only, 4 maturation arrests at different stages, one normal and one prepubescent patient were analysed by RT-PCR (primers listed in Supplementary Material, Table S4B). The results confirmed the restricted expression of *TEX15* to the testis (Fig. 3A) and very low levels of expression in Sertoli cells (not visible on the figure). *TEX15* expression was predominant in the germ line of the testis (Fig. 3B). This latter result was confirmed by immunohistology on the nine testicular tissues, which showed a high level of expression in the germ line and only little expression in Sertoli cells and interstitial cells (Fig. 4). These results are similar to those obtained in the mouse species (23). To reinforce the hypothesis that the function of the human and the mouse *Tex15* is similar, we compared both proteins and observed a high degree of amino acid identity over the entire sequence (52.46% identity; see Fig. 3C).

Case–control association study

Due to its known role during spermatogenesis, *TEX15* was a good candidate gene for infertility that led Aston *et al.* to test for a possible association between infertility and six non-synonymous SNPs located in the *TEX15* coding sequence (11). This case–control association study, performed in a cohort of men of European descent with NOA, SO and control individuals, revealed a marginal association between the major alleles of two SNPs of *TEX15*, rs323344 [NM_031271.3: c.4009T>G (p.L1337V)] and rs323345 [NM_031271.3: c.3932A>G (p.N1311S)], and infertility. A follow-up study conducted for these two SNPs in a European Balkan population failed to replicate this association (26). Chinese Han populations of NOA, SO and controls were also genotyped for all six non-synonymous SNPs of *TEX15*, and while no significant association signal was obtained for rs323344 [NM_031271.3: c.4009T>G (p.L1337V)] and rs323345 [NM_031271.3: c.3932A>G

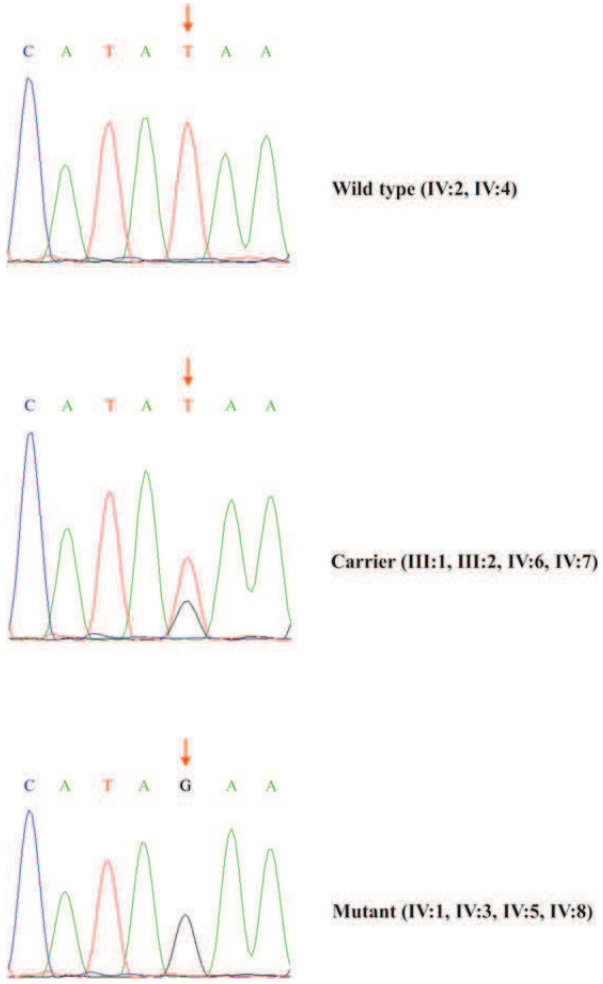


Figure 2. Plots of results from Sanger sequencing for mutant, carrier and wild-type samples. Allele in question is indicated by orange arrow. *TEX15* is in the reverse strand, and mutation causes tyrosine codon (TAT) change into stop codon (TAG).

(p.N1311S)], a slight association for the minor alleles of two other SNPs of *TEX15*, [NM_031271.3: c.3103A>G (p.I1035V)] and rs323347 [NM_031271.3:c. 310T>C (p.C104R)], was detected in SO patients (27). The major limitation of these three studies, as for all association studies performed so far in the field of infertility, resides in the phenotypic heterogeneity and the limited number of patients and controls analysed (4). In order to contribute to the analysis of the role of these SNPs in infertility, even if our cohort is also limited in number, we performed a case–control association study in Turkish males with NOA ($n = 85$) and control males ($n = 107$). For all four SNPs of *TEX15* tested, a slight increase in the frequency of the alleles previously associated was observed, but far from being significant, possibly due to the small size of the cohort (results summarized in Supplementary Material, Table S5).

Discussion

Studying a consanguineous Turkish family, we have identified, using WES, a nonsense mutation in *TEX15* leading in early translational termination in the first quarter of the protein and causing an infertility phenotype. Our data strongly suggest that the identified homozygous mutation in *TEX15* in three infertile brothers is the cause of a spermatogenic defect that is transmitted according to an autosomal recessive trait. Interestingly, the human phenotype is very similar to the one observed in the Tex15 KO mouse. In mice, disruption of the gene causes a drastic testis size reduction and meiotic arrest in the first wave of spermatogenesis in males. Further analysis revealed that in mice Tex15 is required for chromosomal synapsis. It regulates the loading of DNA proteins onto sites of double-strand DNA breaks, and thus its absence causes a failure in meiotic recombination (23). Interestingly, female KO mice were fertile. In man, we also observed a drastic testis size reduction and a meiotic maturation arrest. One major difference between the phenotype in man and in mice is that all male KO mice for *Tex15* are infertile, while in the family studied here two brothers were NOA while the third one was initially diagnosed as SO and became azoospermic with time. The youngest brother with an unknown fertility status was also homozygous for the mutation (Fig. 1). Despite a detailed explanation of the medical issue to the youngest brother, we were unable to convince him to have a spermiogram, which could have prompted him to cryopreserve some of his spermatozoa. Indeed, considering that the younger infertile mutated brother was SO and he became azoospermia with time, it is likely that the phenotype becomes more manifest with age.

In both NOA patients, some foci of spermatogenesis could be found via TESE. Therefore, it remains to be established whether mutations in *TEX15* correlate with a decrease in sperm count over time.

Although patient IV:3 displayed a maturation arrest, a very limited number of spermatozoa were extracted in the biopsy material, but no pregnancy could be achieved after ICSI. The mosaic appearance of the testis is well established, and a single small biopsy material may not always be representative of the entire testis picture (28). In a recent study, a mosaic pattern of spermatogenesis was found in 13.79% of men with maturation arrest (29). Mutation testing of *TEX15* could also be offered and possibly used as an indicator of sperm retrieval with a more detailed search in biopsy material for patients with maturation arrest.

Considering that the mutation introduced a premature stop codon, either it can lead to the production of a truncated protein missing 74.5% of its sequence including two predicted domains or the protein may be entirely absent due to a nonsense-mediated mRNA decay. Since TEX15 protein is testis-restricted, we could not, for ethical reasons, get access to testis biopsies performed only for research purposes in order to verify the production of a truncated form of TEX15 or its absence.

The identification and characterization of the genetic bases of male infertility have large implications not only for understanding the cause of infertility but also in determining the prognosis, selection of treatment options and management of such couples. It allows the development of a molecular diagnostic test for patients with a similar phenotype.

Materials and Methods

Patient recruitment

This project has been approved by the Comité de Protection de la Personne (CPP) of Strasbourg University Hospital, France (CPP 09/40—W AC-2008-438 1W DC-2009-I 002), as well as by the 'Istanbul University, Faculty of Medicine, Ethics committee for clinical research Faculty of Medicine' (2012/1671-1265). A written consent had been obtained for all participants before samples were collected.

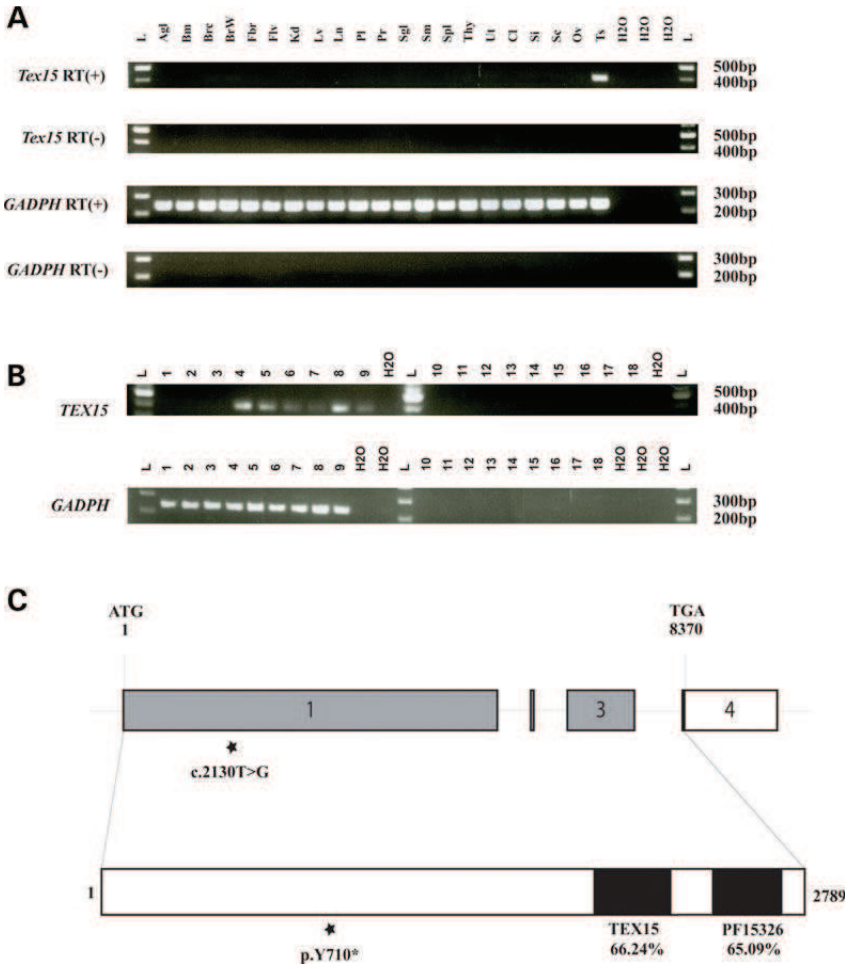


Figure 3. TEX15 expression studies at mRNA and protein levels. *GADPH* was used as internal control for RT-PCR, individual control without reverse transcriptase added to each sample and labelled as RT(–). (**A**) RT-PCR results of *TEX15* and *GADPH* for different human tissues. L: ladder, Agl: adrenal gland, Bm: bone marrow, CrC: brain cerebellum, BrW: brain-whole, FBr: foetal brain, Flv: foetal liver, Kd: kidney, Lv: liver, Ln: lung, Pl: placenta, Pr: prostate, Sgl: salivary gland, Sm: skeletal muscle, Spl: spleen, Thy: thymus, Ut: uterus, Cl: colon, Si: small intestine, Sc: spinal cord, Ov: ovary and Ts: testis. (**B**) RT-PCR results of *TEX15* and *GADPH* for human testicular samples with different histology. Wells 1–9: RT(+) and Wells 10–18: RT(–). Samples 1–3: Sertoli cell only, samples 4–7: maturation arrest at different stages, sample 8: normal fertile and sample 9: prepubescent. (**C**) Schematic representation of exons and protein sequence of *TEX15*. The exonic map of *TEX15* is shown in the upper part of the figure based on the RefSeq transcript (NM_031271.3). Within the four exons, the coding sequence is highlighted in grey. The protein sequence (Uniprot: Q9BXT5, TEX15_HUMAN) is represented in the lower part with the two PFAM domains as black rectangles (TEX15, PF15326). The human protein shares 52.46% identify with the mouse protein. The TEX15 domains share, respectively, 66.24 and 65.09% with the mouse protein. The nonsense mutation (c.2130T>G, p.Y710*) is highlighted in both the transcript and the protein sequence, respectively.

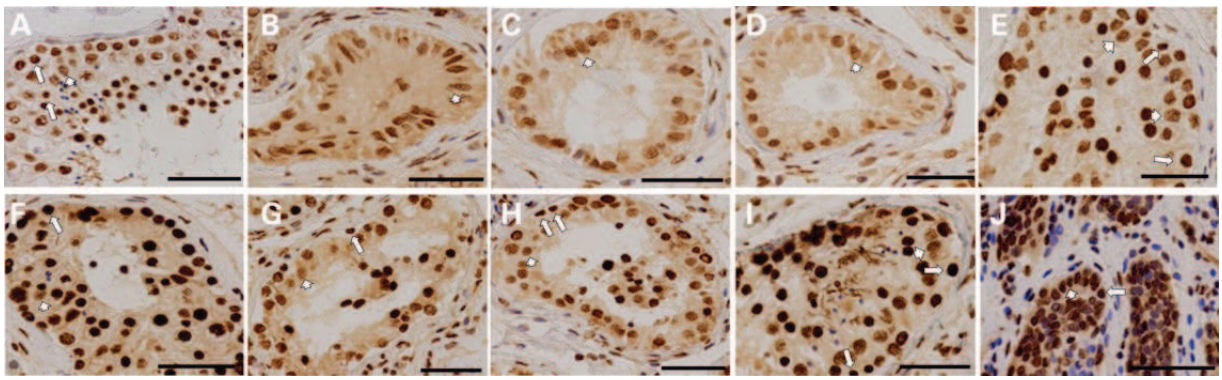


Figure 4. Immunohistochemistry for TEX15. (**A**) Positive control: mouse testis; insert: isotype control. (**B–D**) Testicular tissue from three Sertoli cell only patients. No germ cells are present. (**E–H**) Testicular tissue from four patients with maturation arrest. (**I**) Fertile adult testicular tissue. (**J**) Prepubescent testicular tissue. Germ cell nuclei are intensively stained, while most, but not all, somatic cells show faint expression. Scale bars: 50 µm. Arrow: germ cell; arrowhead: Sertoli cell.

The family comprised six brothers; three infertile, two fertile, one of unknown fertility status and two fertile sisters. All affected brothers had a normal karyotype, and no Y-chromosome microdeletions were found.

A total of 85 unrelated, Turkish individual NOA cases and 13 cases of SO were also collected. All patients were diagnosed through a complete andrological and urological diagnostic workflow for couple infertility, as they were seeking medical assistance for achieving pregnancy. NOA cases were selected based on two spermiograms, which were performed with at least a 15–20 days interval, and no spermatozoa were detected after concentration. Similarly, SO cases were selected with a threshold of $<1 \times 10^6$ spermatozoa/ml after semen analysis. NOA cases were sorted following histological scoring of testis biopsy. Exclusion criteria were patients with mono or bilateral cryptorchidism, varicocele, previous testis trauma, mixed azoospermia (obstructive associated with non-obstructive), recurrent infections, iatrogenic infertility, hypogonadotrophic hypogonadism, karyotype anomalies or Y chromosome micro-deletions. A total of 107 fertile Turkish males who fathered at least one child naturally were included in the study as a control group.

DNA extraction

As a DNA source, saliva samples from family members, all controls and SO cases were used, whereas blood samples were collected from individual NOA cases. Genomic DNA was extracted from saliva using Oragene DNA self-collection kit (DNA genotek, Ottowa, Canada) or from peripheral blood using IPrep™ Pure Link® gDNA Blood kit (Invitrogen, CA, USA), according to the manufacturer's instructions.

Whole exome sequencing

Whole exome sequencing of patients IV:1 and IV:3 (Fig. 1A) was performed by the Institute of Genetics and Molecular and Cellular Biology (IGBMC) microarray and sequencing platform, member of the 'France Génomique programme'. For this purpose, ~1 µg DNA was sheared to 150–200 bp with Covaris Sonolab v4.3.3 (Covaris, Woburn, MA, USA). Fragments were subjected to library preparation, and for each sample to be sequenced, an individual indexed library was prepared. DNA libraries were then enriched (SureSelectXT2 Target Enrichment System) and sequenced with Illumina Hiseq 2500 following Illumina's instructions.

Data processing and analysis

Image analysis and base calling were performed using CASAVA v1.8.2 (Illumina).

Bad-quality parts of reads (Phred score < 10) were trimmed off using SolexaQA (v.2.0) (30), and the reads were then mapped onto the reference genome GRCh37/hg19 using BWA (v0.7.5a) (31). Duplicate reads were marked using Picard (v1.68). Realignment around indels and base quality recalibration were performed using GATK (v2.5-2) (32) following developer's recommendations. Samtools (v0.1.19) (33) was used to exclude multi-mapped reads from downstream analysis. Variant calling was done using GATK UnifiedGenotyper (v2.5-2) (32). Variant quality score recalibration was done using GATK (v2.5-2) (32) to assign a well-calibrated probability to each variant call in a call set.

Detected variants were ranked by VaRank (34), which incorporates the annotations retrieved by the Alamut Batch software (Interactive Biosoftware, France) as well as allele frequency from our internal exome database. The annotation took into account the functional annotation and external data such as the HGVS nomenclature (genomic, cDNA and proteic), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), 1000Genomes (http://www.1000genomes.org/) and the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS/).

Mutation screening and case–control association study

The variant and the segregation in the family as well as the absence of variation in healthy matched Turkish controls were confirmed through Sanger sequencing. The identified mutation was submitted to ClinVar NCBI database (http://www.ncbi.nlm.nih.gov/clinvar/). All exons and exon/intron boundaries of *TEX15* (NM_031271.3) were amplified and sequenced for validating variants and excluding other mutations in a group of Turkish infertile men with similar phenotypes (primers listed in Supplementary Material, Table S4A). DNA amplicons were purified, and double-strand sequencing of each DNA fragment was performed by GATC (Cologne, Germany).

For association studies between *TEX15* SNPS and infertility, allelic distributions between control ($n = 107$) and infertile ($n = 85$) individuals were compared using Fisher's exact test (2BY2 Statistical Genetics Utility program, Rockfeller University, New York).

RT-PCR and immunohistology

Total RNA samples from different human tissues were purchased from Clontech (BD Biosciences Clontech, Palo Alto, CA); 20 RNA samples were included in Human Total RNA Master Panel II (Cat. 636643), and ovary RNA was obtained from Human Ovary Total RNA (Cat. 636555).

Human testicular tissue samples were obtained from adult patients undergoing a vasectomy reversal in the fertility centre of the UZ Brussels. During surgery, a testicular tissue sample measuring about 50 mm^3 was taken to evaluate the spermatogenic status. A small piece of this testicular sample was used for research purposes after written informed consent. The sample was transported to the laboratory on ice, washed to remove any residual blood and cryopreserved according to uncontrolled slow freezing (USF) method using 1.5 M dimethylsulphoxide (DMSO) and 0.15 M sucrose as cryoprotectant (35). One piece of tissue was fixed in acetic acid/formaldehyde/alcohol (AFA) (VWR, Belgium) for at least 1 h and transported to the anatomopathology department of the UZ Brussel for subsequent fixation and embedding in paraffin.

Prepubertal tissue was obtained from a patient who underwent testicular tissue banking as part of a fertility preservation programme. A maximum of 10% of the biopsied tissue was donated to research. One fragment of the prepubertal tissue was fixed in AFA, while the other fragments were cryopreserved.

Human testicular tissue was thawed as described before (35). RNA was extracted using the RNeasy Kit (Qiagen, Venlo, The Netherlands) in combination with the Qiagen Shredder Kit following the manufacturer's recommendations. On column, DNase treatment (RNase-free DNase Set; Qiagen) was performed for all the samples. The total amount of RNA was measured with a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE). The RNA was then stored at −80°C for later use.

cDNA synthesis was done according to the Invitrogen protocol starting from 1 µg RNA for pooled human tissue and 40 pg RNA for testicular tissue samples. DNase treatment was applied to each reaction in order to remove DNA contamination. Same samples were amplified in parallel with primers for the control

housekeeping gene *GADPH* (MIM 138400), and individual controls without reverse transcriptase for each sample were included in polymerase chain reaction (PCR) reactions (primers for RT-PCR listed in Supplementary Material, Table S4B). Thirty cycles of RT-PCR were followed by gel electrophoresis and ethidium bromide staining.

For immunohistochemical evaluation, paraffin-embedded samples were sectioned at 5 μm. Sections were deparaffinized in xylene (3 × 10 min) and subsequently rehydrated in a decreasing ethanol series (2 × 100, 90 and 70% isopropanol). After washing with phosphate-buffered saline (PBS; 70011-036; Life-technologies, Gent, Belgium) for 5 min, endogenous peroxidase activity was blocked by incubating the tissue sections with hydrogen peroxide (0.3% H_2O_2 in methanol). After washing in PBS, a heat-mediated antigen retrieval was performed by incubating the slides in 0.01 M citric acid in a water bath (95°C) for 75 min in order to break the methylene bridges, expose the antigenic sites and allow the antibodies to bind. In order to avoid non-specific background staining, sections were incubated with CAS BlockTM blocking agent (008120; Life-technologies).

After the blocking steps, TEX15 primary antibodies (1/200; HPA036800; Sigma) were applied to the sections and incubated in a humidified chamber. No primary antibody was applied to the negative control.

After three wash steps, sections were incubated with a peroxidase-labelled secondary antibody (Dako Real Envision Detection System; K5007; Dako, Heverlee, Belgium) for 1 h at room temperature. After washing and visualisation with 3, 3′-diaminobenzidine (DAB; Dako Real Envision™ Envision System), slides were counterstained with haematoxylin.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Chapter VI

A stop codon mutation in the X-linked *MAGEB4* associated with azoospermia and severe oligospermia

Article in preparation

Title: A stop codon mutation in the X-linked *MAGEB4* associated with azoospermia and severe oligozoospermia

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ABSTRACT

Infertility affects about 7% of men worldwide, and among these 40% of the cases remain idiopathic. It is likely that most 'idiopathic' human forms may have a genetic origin. So far only few human genes have been proposed as responsible, when mutated, of azoospermia or oligospermia. Studying a consanguineous Turkish family, we identified *MAGEB4* as a new X-linked gene involved in an inherited male infertility. Indeed, a nonstop mutation was found to co-segregate with the non-obstructive azoospermia and oligozoospermia phenotype in the family. This novel mutation (c.1041A>T, p.*347Cysext*24) substitutes the stop codon to a cysteine residue, potentially adding 23 amino acids to the C-terminus of the protein. *MAGEB4* is a type I member of the MAGE gene family, which is clustered on chromosome Xp22-p21. Human *MAGEB4* has only one exon and encodes a 346 amino acid protein. It is expressed specifically in the testis while silent in normal adult tissues, but re-expressed in selected tumor types. This expression pattern indicates *MAGEB4* is belonging to a group of cancer-germline genes. Although it is a potential target for immunotherapy, the normal function of MAGEB4 is not clear. We revealed that neither the cellular protein localization nor the mRNA stability is affected by the identified mutation. Co-immunoprecipitation experiments demonstrated that modifying the C-terminal part of MAGEB4 does not modify its ability to interact with other MAGE proteins. However the modeling of the tertiary protein structure suggests that the addition of the 23 amino acids may affect the proper homo or heterodimerization of the protein. This study provides the first clue on the physiological function of a MAGE protein.

INTRODUCTION

Infertility affects about 7% of men worldwide (Krausz *et al*., 2012); in which only for 60% an etiology is established (Chianese *et al*., 2014). Among infertile males 10 to 15 % will be diagnosed with azoospermia (Jarow *et al*., 1989) and 35% with oligoozoospermia (Baker, 2010).

Numerical and structural chromosomal abnormalities are known causes of azoospermic and oligospermic cases. Approximately 14% of azoospermic men and 5% of oligozoospermic men carry chromosomal abnormalities (Van Assche *et al.*, 1996). For a wide number of patients, no genetics diagnosis are possible and they remain classify as "idiopathic". The mouse model has showed up the complexity of spermatogenesis with concerted action of more than 2300 genes (Hotaling and Carrell, 2012). The analysis of the KO mutations of some of these genes in mice has shown their function in spermatogenesis, providing insight into molecular mechanisms involved in the appearance of azoospermia and oligospermia (Matzuk and Lamb, 2008). Therefore, it is likely that most 'idiopathic' human forms may have a genetic origin.

So far only few human genes have been proposed as responsible, when mutated, of azoospermia or oligospermia, such as *SOHLH1, NR5A1, Wt1, TAF4B, ZMYND15, TEX11 and TEX15* (Choi *et al*., 2010, Bashamboo *et al.*, 2010, Wang *et al.*, 2013*,* Ayhan *et al.*, 2014, Yatsenko *et al*., 2015, Okutman *et al*., 2015).

By analogy to the Y chromosome, the X chromosome is also interesting in view of studying male infertility. Indeed, at the beginning of the millennium, Wang and his team isolated 25 genes in mice that were expressed only in spermatogonia but not in somatic cells; 40% of them mapped to X chromosomes which suggested a predominant role in pre-meiotic stages

of mammalian spermatogenesis (Wang *et al.*, 2001). So far few genes on X chromosome have been studied in infertile men (Olesen *et al.*, 2003, Stouffs *et al.,* 2005, Ravel *et al.*, 2006, Stouffs *et al.*, 2006, Akinloye *et al.*, 2007, Stouffs *et al.*, 2008, Stouffs *et al.*, 2009, Lee *et al.*, 2009, Visser *et al.*, 2011). To date, no functional analysis have been performed for most of the variations and some of them were also observed in control fertile population, so it remains unsure whether these rare changes might be involved in male infertility (Stouffs and Lissens, 2012).

In a recent study, Yatsenko and his colleques have identified an X-linked gene, namely *TEX11*, for which mutations are responsible of an azoospermic phenotype (Yatsenko *et al.*, 2015). Interestingly, the phenotype observed in human is closed to the one observed in the KO mouse model (Yang *et al.*, 2008). With a frequency of 2.4% among azoospermic patients, mutations in *TEX11* are, so far, the most frequently found in this condition.

Studying a consanguineous Turkish family, we identified *MAGEB4* (*melanoma antigen family B4,* MIM: 300153) as a new X-linked gene involved in an inherited male infertility. Indeed, a nonstop mutation was found to co-segregate with the non obstructive azoospermia (NOA) and oligozoospermia phenotype in the family. This novel mutation (c.1041A>T, p.*347Cysext*24) substitutes the stop codon to a cysteine residue, potentially adding 23 amino acids to the C-terminus of *MAGEB4* since next in- frame stop codon is 69 nucleotides downstream. This study provides the first clue on the physiological function of a MAGE protein.

MATERIALS and METHODS

Patients and controls

The family contains male triplets; two of them were infertile as well as one younger infertile brother in addition to one more fertile brother. All affected brothers have a normal karyotype and no Y chromosome micro deletion was identified. The two fertile brothers fathered two and one child while two fertile sisters mothered four and five children respectively. The mother (III:2) has four siblings; two males and two females, all have children (figure 6.1).

A total of 108 unrelated, Turkish individual infertile men [88 NOA, 20 severe oligospermia (SO)] and 107 fertile Turkish males who fathered at least one child naturally were also included in the study. NOA cases were selected based on two spermiograms which were performed with at least a 15-20 days interval and no spermatozoa were detected after concentration. Similarly, SO cases were selected with a threshold of $<1x10^6$ spermatozoa/ml after semen analysis. Exclusion criteria were patients with mono or bilateral cryptorchidism, varicocele, previous testis trauma, mixed azoospermia (obstructive associated with non-obstructive), recurrent infections, iatrogenic infertility, hypogonadotrophic hypogonadism, karyotype anomalies or Y chromosome microdeletions.

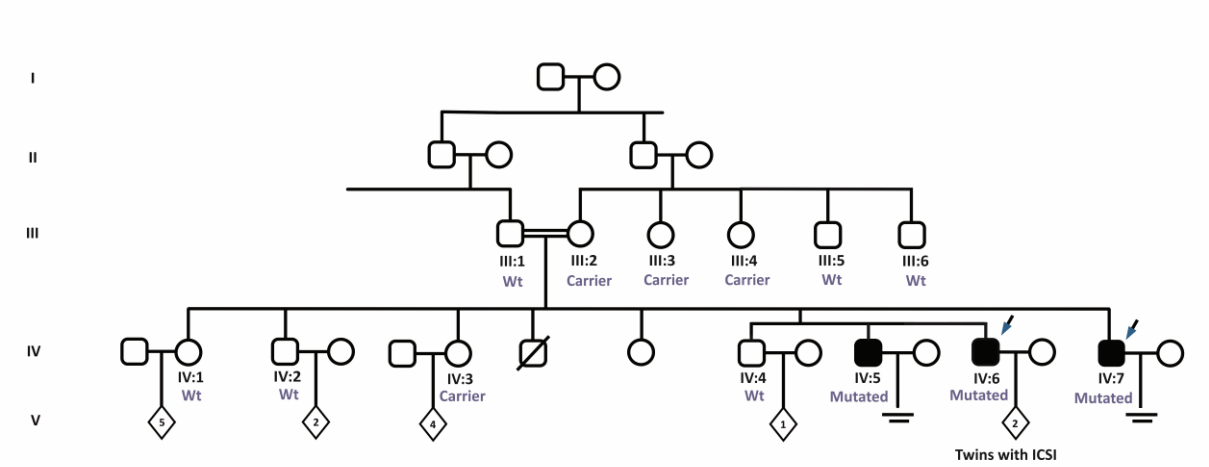

Figure 6.1: Consanguineous Turkish family with 8 siblings comprising male triplets. The segregation of mutation c.1041A>T, p.*347Cysext*24 in the family was indicated. Wt=wild type, A/A, Carrier = A/T, Mutated= T/T. Whole exome sequencing was performed on two affected brothers indicated with blue arrow.

DNA preparation

As a DNA source, saliva samples from family members, all controls and SO cases were used whereas blood samples were collected from individual NOA cases after obtaining written consent. Genomic DNA was extracted from saliva using Oragene DNA self collection kit (DNA genotek, Ottowa, Canada) or from peripheral blood using IPrep™ Pure Link® gDNA Blood kit (Invitrogen, CA, USA), according to the manufacturer's instructions.

This project has been approved by the Comité de Protection de la Personne (CPP) of Strasbourg University Hospital, France (CPP 09/40 - W AC-2008-438 1W DC-2009-I 002), as well as by the "Istanbul University, Faculty of Medicine, Ethics committee for clinical research Faculty of Medicine" (2012/1671-1265).

Array application and SNP analysis

Using 500K SNP Arrays (Affymetrix Inc., Santa Clara, CA), a genome-wide scan analysis was performed for parents and six siblings including the three infertile males, the two fertile males and one fertile female.

Samples and arrays were processed according to the Affymetrix "GeneChip Mapping 500K Assay Manual, Low throughput protocol", Cat Number 701930 Rev.3. Two hundred fifty nanogram of genomic DNA were used as starting material. Single restriction enzyme digestions were performed using Nsp I. Mapping 250K Nsp Arrays were hybridized at 49°C for 16hr at 60 rpm, washed and stained using a GeneChip Fluidics Station 450 and scanned using Affymetrix GCS3000-7G.

Raw images (CEL files) were processed using GeneChip Genotyping Analysis Software (GTYPE) version 4.1 to generate SNP allele calls. Homozygous regions shared between the patients were identified with the HomoSNP program, an in-house graphic interface that was set to identify regions of 35 or more consecutive homozygous SNPs

Defined homologous region were screened using the UCSC genome browser to list genes and identified genes were scanned for expression profiles through three different data bases; AmaZonia, BioGPS and EMBL-EMI Expression Atlas.

Selection criterias for candidate genes were based on predominant testis expression and possible role in spermatogenesis. Existence of a mouse KO model with a male infertility phenotype was also taken into account for candidate gene selection.

Whole exome sequencing

Whole exome sequencing of patients IV: 6 and IV: 7 (figure 1), was performed by the IGBMC microarray and sequencing platform, member of the "France Génomique programme". For this purpose, approximately 1µg DNA was sheared to 150-200bp with Covaris Sonolab v4.3.3 (Covaris, Woburn, MA, USA). Fragments were subjected to library preparation; for each sample to be sequenced, an individual indexed library was prepared. DNA libraries were then enriched (SureSelectXT2 Target Enrichment System) and sequenced with Illumina Hiseq 2500 following Illumina's instructions.

Data processing and analysis

Image analysis and base calling were performed using CASAVA v1.8.2 (Illumina).

Bad quality parts of reads (Phred score <10) were trimmed off using SolexaQA (v.2.0) (Cox *et al.,* 2010), reads were then mapped onto the reference genome GRCh37/hg19 using BWA (v0.7.5a) (Li and Durbin 2009). Duplicate reads were marked using Picard (v1.68). Realignment around indels and base quality recalibration were performed using GATK (v2.5-2) (DePristo *et al*., 2011) following developer's recommendations. Samtools (v0.1.19) (Li *et al*., 2009) was used to exclude multi-mapped reads from downstream analysis. Variant calling was done using GATK UnifiedGenotyper (v2.5-2) (DePristo *et al*., 2011). Variant quality score recalibration was done using GATK (v2.5-2) (DePristo *et al.,* 2011) to assign a well-calibrated probability to each variant call in a call set.

Detected variants were ranked by VaRank (Geoffroy *et al*., 2015) which incorporates the annotations retrieved by the Alamut Batch software (Interactive Biosoftware, France) as well as allele frequency from our internal exome database. The annotation took into account the functional annotation and external data such as the HGVS nomenclature (genomic, cDNA

and proteic), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), 1000Genomes (http://www.1000genomes.org/) and the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS/).

Mutation screening

All exons and intronic flanking sequences of nine candidate genes identified through linkage analysis were amplified and sequenced to verify a possible mutation in the infertile members of the family (primer sequences are available on Supplemantary data chapter VI, table S6.1). Identified variant from whole exome sequencing analysis and its segregation in the family were confirmed through polymerase chain reaction (PCR) and Sanger sequencing. The absence of variation in healthy matched Turkish controls was confirmed through Sanger sequencing. All exons and exon/intron boundaries of gene in question were sequenced for validating variants as well as excluding other mutations in a group of Turkish infertile men with similar phenotypes. Amplification conditions and all primers are listed in Supplemantary data chapter VI, table S6.2A. DNA amplicons were purified and double strand sequencing of each DNA fragment were performed by GATC (Cologne, Germany).

RT-PCR and immunohistology

Human total RNA samples including testis were purchased from Clontech (BD Biosciences Clontech, Palo Alto, CA) or prepared from surgical specimens from testicular tissues of affected individuals with different histology. There were total 9 testicular tissues derived from 3 Sertoli cells only, 4 maturation arrests at different stages, one normal and one prepubescent patient.

Human testicular tissue samples were obtained from adult patients undergoing a vasectomy reversal in the fertility centre of the UZ Brussels. A small piece of testicular sample was used

for research purposes after written informed consent. One fragment of the tissue was fixed in acetic acid/formaldehyde/alcohol (AFA) (VWR, Belgium), while the other fragments were cryopreserved according to uncontrolled slow freezing (USF) method using 1.5M dimethylsulphoxide (DMSO) and 0.15M sucrose as cryoprotectants (Baert et al., 2013). RNA extraction, cDNA synthesis and immunohistochemical evaluation were performed as described before (Okutman *et al*., 2015).

MAGEB4 primary antibodies (ab60048, ABCAM) were then applied to the sections and incubated in a humidified chamber. No primary antibody was applied to the negative control. Three washing steps followed by incubation with a peroxidase-labelled secondary antibody (Dako Real Envision Detection System; K5007; Dako, Heverlee, Belgium) for 1 hour at room temperature. After washing and visualisation with 3,3'- diaminobenzidine (DAB, Dako RealTM EnvisionTM Envision system), slides were counterstained with haematoxylin.

Construction of expression plasmids

DNA from III:1 and IV:7 was used as a template for wild-type and mutant construction by PCR. Three primers were designed for generation of wild-type and mutational constructs which were listed in Supplemantary data chapter VI, table S6.3. The PCR products were cloned into the Topo vector and the constructs were verified by DNA sequencing.

Wild type and mutant fragments were amplified through PCR by using forward and reverse primers listed in Supplemantary data chapter VI, table S6.4.

DsRed-Monomer N1 vector (BD Bioscience) was linearized by HindIII and AflII digestion. Amplified fragments were cloned into the linearized vector by one cycle of circular polymerase extension cloning (CPEC) reaction as explained recently (Quan and Tian 2009). Briefly 200ng of the linear vector was mixed with amplified fragments at equal molar ratio in

a 20µl volume containing Phusion High Fidelity reaction mixture (ThermoScientific). A short denaturation step (98°C, 30 seconds) was utilized to denature double stranded insert and linear vector, followed by annealing step at 55°C for 30 seconds and then polymerase extension was performed for 15 seconds per kb according to the length of longest piece. Reaction products were digested with DpnI and then mixture was transfected into XL-Blue strain for the protein production.

MAGEB4 wild type and mutant cDNAs were cloned in frame either with an HA- or a Flag N-terminal tag into pcDNA3. PCMV6 MAGEA3-Myc-Flag was purchased from Origene (Rockville, USA).

Cell cultures, transfections and treatments

COS7 cells were cultured in Dulbecco's modified Eagle Medium (DMEM), 10% fetal bovine serum and gentamicin at 37°C in 5% CO2. Cells were plated either in 6 well plate for immunoprecipitation or RNA stability assays or on glass coverslips in a 24 well plate for immunofluorescence and tranfected with the corresponding plasmids 24 hours after plating in DMEM + 0.1% fetal bovine serum using FugenHD (Roche) according manufacturer protocol. For RNA decay experiments, cells were incubated 1, 2 or 4 hours after transfection in actinomycin D at 5 ng/ml.

RNA isolation and RT-qPCR

Total RNA was isolated 24 hours after transfection using acidic phenol (Trizol reagent, Life Technologies) from transfected cells and cDNA synthesis reactions were carried out with Superscript II (Invitrogen). Real time PCRs were performed using the LightCycler 480 SYBR Green I Master kit (Roche) in a Lightcycler 480 (Roche), with 15 min at 94°C followed by 50 cycles of 15 sec at 94°C, 20 sec at 58°C and 20 sec at 72°C, using the forward primer

TAGCGCTACCGGACTCAGATC located into the plasmid and the reverse primer TAGGCTGACCAACCTTGAGATCC located within *MAGEB4.*

Immunofluoresence

Glass coverslips containing plated cells were fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 minutes and washed 3 times with PBS. Coverslips were incubated overnight in primary monoclonal mouse anti-HA antibody (1/200 dilution, clone 5B1D10, Pierce) at 4°C. Coverslips were washed twice with PBS before incubation with Rabbit anti mouse secondary antibody conjugated with Alexa Fluor 488 (1/500 dilution, Fisher) for 60 min, incubated for 10 minutes in PBS + DAPI (1/10 000 dilution) and rinsed twice prior to mounting in Pro-Long media (Molecular Probes). Slides were examined using a Leica DM4000 B confocal microscope, equipped with a Leica 100x HCX Plan Apo CS 1.40 objective, in 1 µm optical sections.

Immunoprecipitation and Western blotting analysis

Cells grown in 6 well plate were collected 24 hours after transfection and were incubated with lysis buffer (300mM NaCl, 10mM Tris [pH8.0], 1% Triton X-100) for 20 min followed by sonication on ice and centrifugation for 10min at 13 000 rpm at 4°C. Supernatant was incubated with 20µl of anti-HA magnetic beads (Pierce) with constant rotation for 2hours at 4°C. Beads were washed one time in lysis buffer and three times in 200mM KCl, 10mM tris [pH8.0] before resuspension in SDS loading buffer. For western blotting, proteins were denatured 3 min at 95°C, separated on 4-12% gradient gel (Nupage), transferred on nitrocellulose membranes (Whatman Protan), blocked with 5% non-fat dry milk (NFM) in Tris Buffer Saline buffer (TBS), incubated with mouse anti HA (clone 5B1D10, Pierce) or rabbit anti-Flag (8HCLC, Pierce) in TBS-5% NFM, washed 3 times and incubated with Donkey-anti-

rabbit or anti mouse Peroxidase antibody (Jackson Immunoresearch, 1:10000) 1 hour in TBS-5% NFM, followed by autoradiography using the ECL chemoluminescence system (ThermoFisher).

Alignment and structure prediction

The C-terminal end of MAGEA3 (sw:P43357) , wild type and mutant MAGEB4 (O15481) have been aligned with the sequence corresponding to the crystal structure of a MAGEA3 domain (PDB: 4V0P). The Jalview software (www.jalview.org) was used for sequence alignment and analysis of the sequence conservation. Secondary structure of the C-terminal end and mutant extension was predicted according to the software package PHD (https://npsa-prabi.ibcp.fr) (Rost and Sander, 1993, Rost and Sander, 1994). The crystal structures representations were drawn in the Pymol software. (*https://www.pymol.org)*

RESULTS

Infertility phenotype in the family

The family was identified at the Bahceci Health Group, Istanbul, Turkey. Saliva samples were collected from all family members with known fertility status and from siblings of the mother after signed consent form.

Our index patient is the youngest brother (IV:7) (figure 6.1), he had a microdissection testicular sperm extraction (micro-TESE) operation and histopathology report defined the biopsy material as Sertoli-cell only (figure 6.2). In December 2014, a new biopsy resulted in a retrieval of limited number of spermatozoa and they were frozen for further ICSI application.

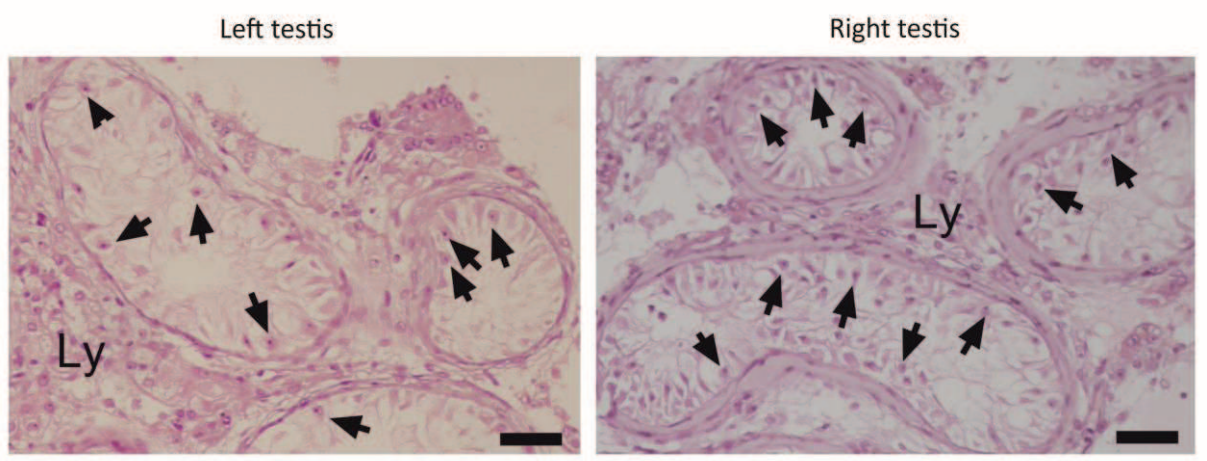

Figure 6.2: Testicular histology of patient IV:7. Representative sections of human left and right testis biopsies displaying absence of germinal cells without histological impairment of Sertoli and Leydig cells. Arrows indicate Sertoli cells; Ly Leydig cells. Bars represent 150 µm in length.

Patient IV:5 has severe oligospermia (< $1x10^6$ spermatozoa/ml) (figure 6.1); three attempts of ICSI were performed however no pregnancy was obtained. Sperm morphology and embryo quality were of poor quality. Patient IV:6 (figure 6.1), on the other hand, was diagnosed as non-obstructive azoospermia (NOA). A TESE allowed the recovery of sperm during the first IVF cycle. Biopsy material was frozen because of the poor hormonal response during the ovarian stimulation of the female partner. ICSI was applied at the next cycle with thawed sperm and pregnancy was achieved and healthy twins were born.

Linkage analysis

In a first instance, we performed a genome-wide scan analysis of the two parents (III:1 and III:2) and six siblings; the three infertile brothers (IV:5, IV:6 and IV:7), two fertile brothers (IV:2 and IV:4) and fertile sister (IV:1) (figure 6.1). At the time of array application, we did not have the DNA samples of other fertile sister (IV:3) and siblings of the mother (III:3, III:4, III:5, III:6). Array results revealed that among the triplets the two infertile brothers (IV:5, IV:6) are monozygotic twins. We first compared all the infertile brothers to the fertile

siblings. 29 homologous regions (Supplemantary data chapter VI, table S6.5) were identified including 628 genes which we checked expression profiles for the protein coding ones through three different databases. Thirty six genes showed specific testis expression according to at least one data base; five genes, namely GTSF1L, EPPIN, INSL6, TP53TG5 and SPINT3 were selected based on KO studies and/or their possible roles in male reproduction according to literature.

According to studies in mice, meiosis can be differently affected between male and female (Baker *et al*., 1995, Liu *et al*., 1998, Okunade *et al.*, 2004, Yang *et al*., 2008). Therefore fertile female sister (IV:1) could also be homozygous for the mutation causing male infertility without having fertility problem. Hence we performed a second screening by taking into account regions shared only in between infertile brothers and the fertile sister. Six different homologous regions (Supplemantary data chapter VI, table S6.6), were identified, in which twenty two genes showed specific testis expression at least in one database. Following the literature check four genes, namely SERPINA5, SPATA7, FAM181A and TDRD9 were selected. We sequenced the entire exons and intron/exon boundaries of total nine selected genes (primers are listed in Supplemantary data chapter VI, table S6.1). No mutation was identified.

Exome Sequencing Data analysis

Since analyzing of large number of genes would have been time consuming, we decided to perform whole exome sequencing on the DNA of two infertile brothers. Since IV:5 and IV:6 are monozygotic twins, we chosen to sequence IV:6 and IV:7.

A total of 9.8 GB and 9.2 GB of sequences were generated for samples IV: 6 and IV: 7 respectively. More than 74% of the target exome in both cases were represented with greater than 40-fold coverage (Supplementary data chapter VI, table S6.7).

Since we assumed a recessive inheritance, we tracked homozygous variants shared by both brothers through the whole exome. We filtered variants according to coverage (>10), minor allele frequency in populations (<1%), validation status of the reference SNP and novelty by comparing them to our in-house exome database.

Scanning through online expression databases (Amazonia, BioGPS, EMBL-EBI expression atlas) was applied to 36 genes that passed our filtering process. This revealed one gene from melanoma antigen family, *MAGEB4* as the most plausible candidate gene due to its specific expression to germ cells and suggested function for murine homolog in germ cell specific mitosis (Osterlund *et al*., 2000).

MAGEB4 is a type I member of the *MAGE* gene family, which is clustered on chromosome Xp22-p21. Human *MAGEB4* has only one exon, its expression is limited to testis and encodes a 346 amino acid protein. As in all MAGE proteins, it has a well conserved MAGE homology domain (MHD) located at the C-ter that can be traced back to protozoa (Barker and Salehi, 2002)

Mutation confirmation and segregation in the family

In the family, a single base substitution (c.1041A>T, p.*347Cysext*24) at the end of coding region of *MAGEB4* was identified in both NOA brothers (IV:6 and IV:7) with 22 and 48 reads respectively. The substitution leads to the stop codon to be turned to a cysteine codon (p.X347C) and cause loss of the normal translational termination. The next in-frame stop

codon is located 69 nucleotides downstream and according to GENESCAN (http://genes.mit.edu/GENSCAN.html) prediction, 907 base pair upstream to the polyA signal. Therefore, the mutation may eventually give rise to a MAGEB4 protein with 23 additional amino acids. Databases that collect human sequences and repertory polymorphisms among the human population i.e. Exome Aggregation Consortium (ExAC), Exome Variant Server (EVS), ensemble, dbSNP were scanned; our mutation was not listed in any of these databases. Family members were scanned through Sanger sequencing, the variation and its segregation with the pathology were confirmed. All three infertile males were homozygous for the mutation, the mother and two fertile sisters were carrier, the father and fertile brothers were wild-type (figure 6.3). The siblings of the mother were also checked; they are all fertile, her sisters (III:3, III:4) were carrier while brothers (III:5, III:6) were wild type. Carrier sisters and female siblings of the index patient have sons however; we could not convince them to provide us with a DNA sample.

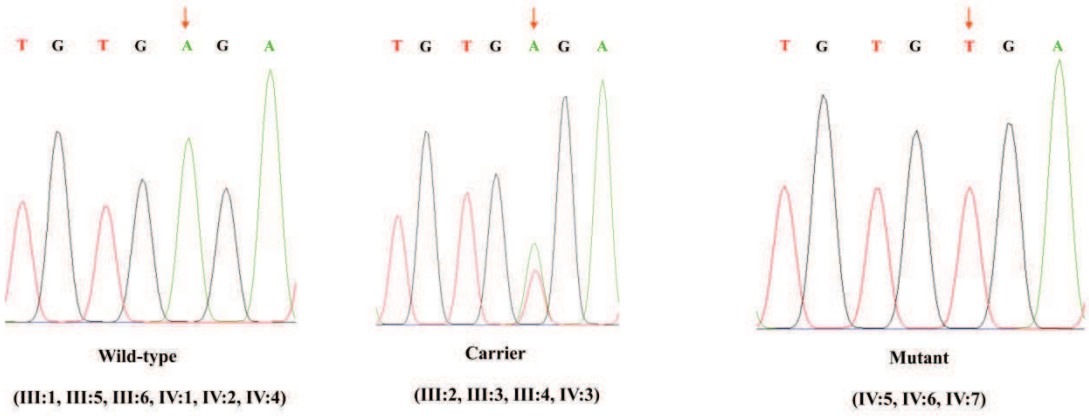

Figure 6.3: Electropherogram images of the identified mutation. Plots of results from Sanger sequencing for mutant, carrier and wild type samples. Allele in question is indicated by orange arrow. Mutation causes stop codon (TGA) change into codon for cysteine (TGT).

The mutation was not detected in 107 ethnically matched healthy fertile controls. In order to validate possible variants and to exclude other mutations, all exon and 5-UTR/3-UTR sites were sequenced in 108 unrelated Turkish infertile cases (88 NOA, 20 SO). No mutation was identified.

Expression of MAGEB4

Expression profile of MAGEB4 in human was studied at mRNA and protein levels. The results confirmed the restricted expression of MAGEB4 in the testis (figure 6.4A); MAGEB4 expression was detectable in all human testicular tissues except the pre-pubertal one (Figure 6.4B). Similarly, MAGEB4 was detected in differentiating germ cells and some Sertoli cells by immunohistochemistry on human adult tissue (Figure 6.4C).

Functional analysis

Nonstop mutations are rare and no consensus on their consequences is established. Such mutations may affect either the stability of the mRNA or the stability of the protein (Wallefeld *et al*., 2006, Cacciottolo *et al*., 2011, Oegema *et al.*, 2013, Gu *et al*., 2014, Seminara *et al*., 2003, Taniguchi *et al*., 1998). Recently a nonstop mutation in MSX gene was reported, the mutation had no effect on mRNA or protein stability but change the structure of the protein (Wong et al., 2014).

We first tested whether this mutation would have any effect on the stability of the messenger RNA. Quantitative RT-PCR of transfected MAGEB4 wild type and mutant indicated a similar expression of both RNA forms, but also a similar mRNA decay upon transcriptional inhibition through actinomycin treatment (Figure 6.5).

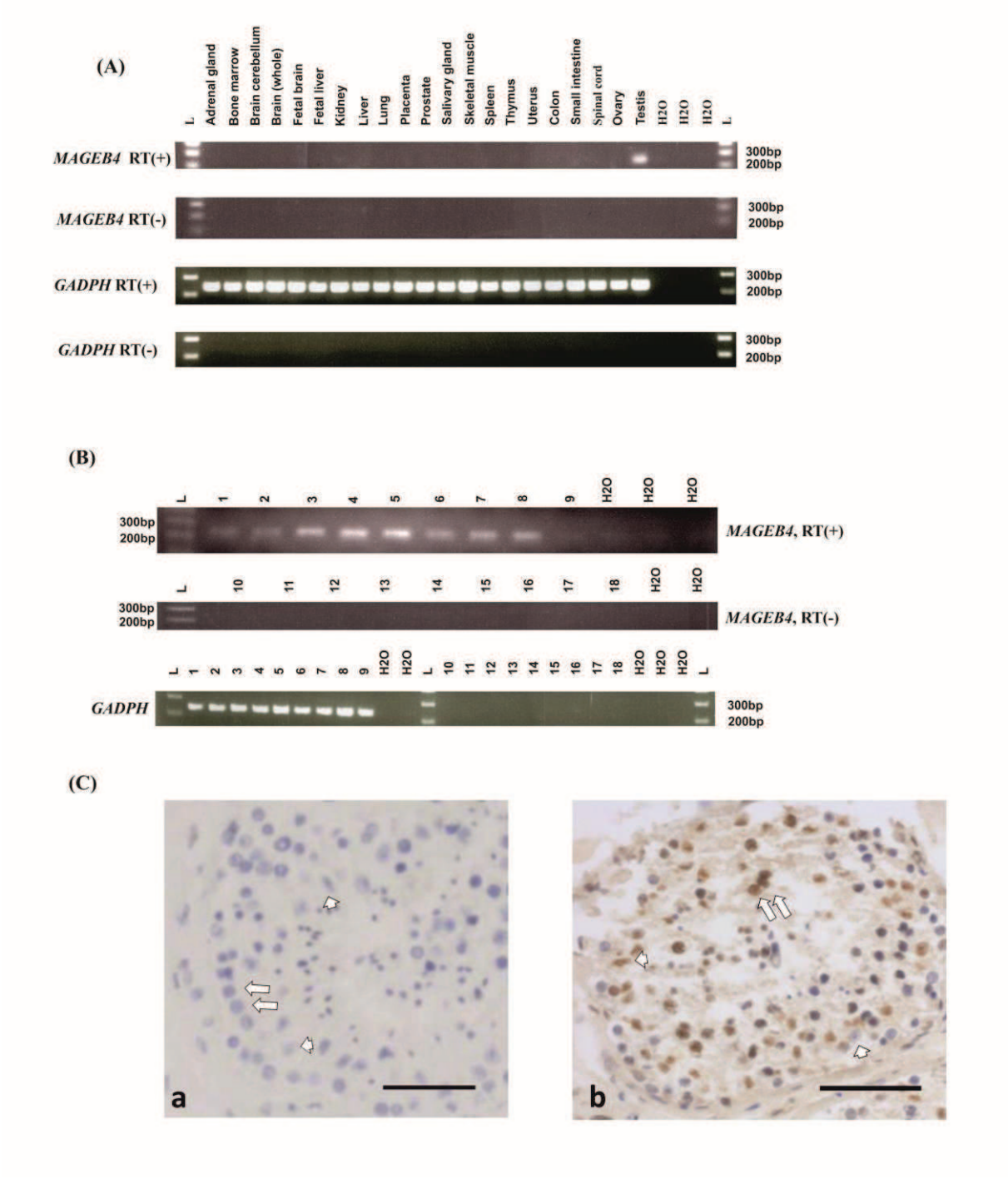

Figure 6.4: MAGEB4 Expression studies at mRNA and protein levels. GADPH was used as internal control for RT-PCR, individual control without reverse transcriptase added to each sample and labeled as RT(-)
(A) RT-PCR results of MAGEB4 and GADPH for different human tissues. (B)RT PCR results of MAGEB4 and GADPH for human testicular samples with different histology. Well # 1-9: RT(+), Well 10-18: RT(-). Sample 1-3: Sertoli cell only, sample 4-7: maturation arrest at different stages, sample 8: normal fertile, sample 9: prepubescent. (C) Immunohistochemistry for MageB4. (a) isotype control. (b) human adult testicular tissue. MageB4 is detected in differentiating germ cells and some Sertoli cells. Scale bars: 50 µm. Arrow: germ cell; Arrowhead: Sertoli cell

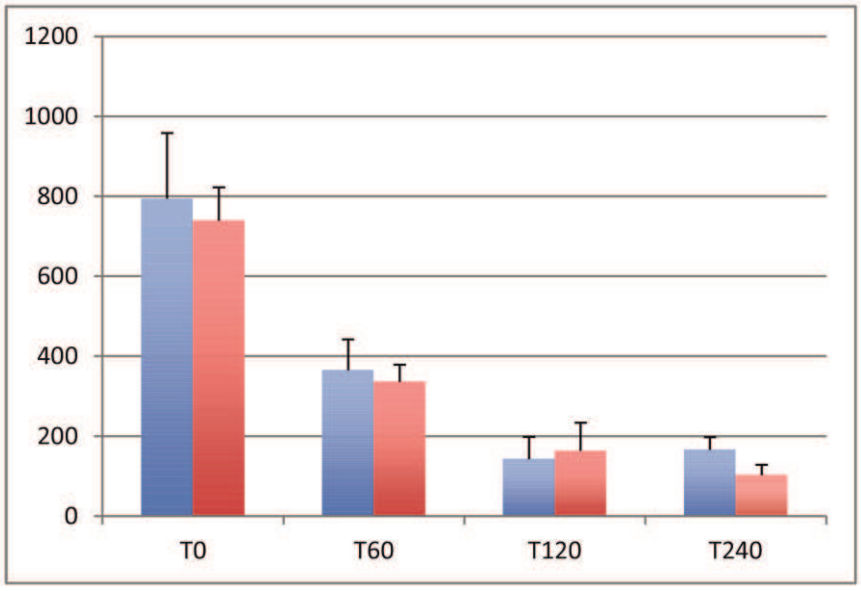

Figure 6.5: Quantitative RT-PCR of MAGEB4. Wild type or mutant MAGEB4 mRNA expressed in HeLa cells treated with actinomycin for 1, 2 or 4 hours.

Since mutation of MAGEB4 adds 23 novel amino acids to its C-terminal part, we investigated the cellular localization of HA-tagged wild type and mutant MAGEB4. In transfected cells both control and mutant proteins were localized at the cell membrane, in a pattern reminiscent of F-actin filopodia and lemellipodia structures, but with no difference between wild type and mutant MAGEB4 (figure 6.6). Also, we tested whether modifying the C-terminal part of MAGEB4 would modify its ability to interact with other proteins. However, co-immunoprecipitation experiments demonstrated that both wild type and mutant MAGEB4 interact similarly with MAGEA3 (Figure 6.7A). We finally tested the ability of wild type and mutant MAGEB4 to self-interact, but we found no difference as control MAGEB4 interacts equally well with its wild type or mutant forms. Reciprocally, mutant MAGEB4 interacts with both wild type and mutant MAGEB4 (Figure 6.7B).

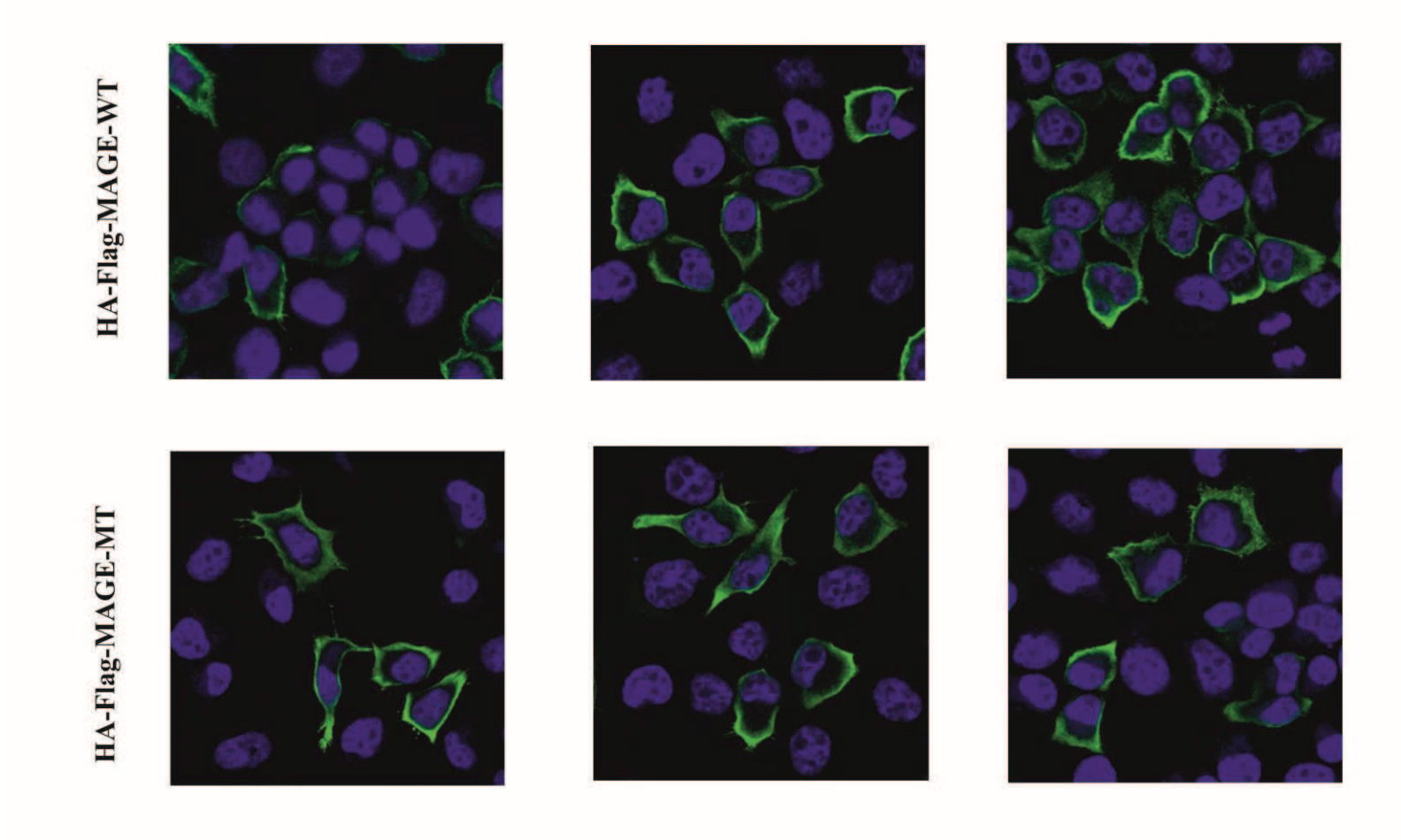

Figure 6.6: Confocal images of immunofluorescence labelling of HA-tagged wild type or mutant MAGEB4 transfected in HeLa cells. Nuclei are counterstained with DAPI. Magnification 400x.

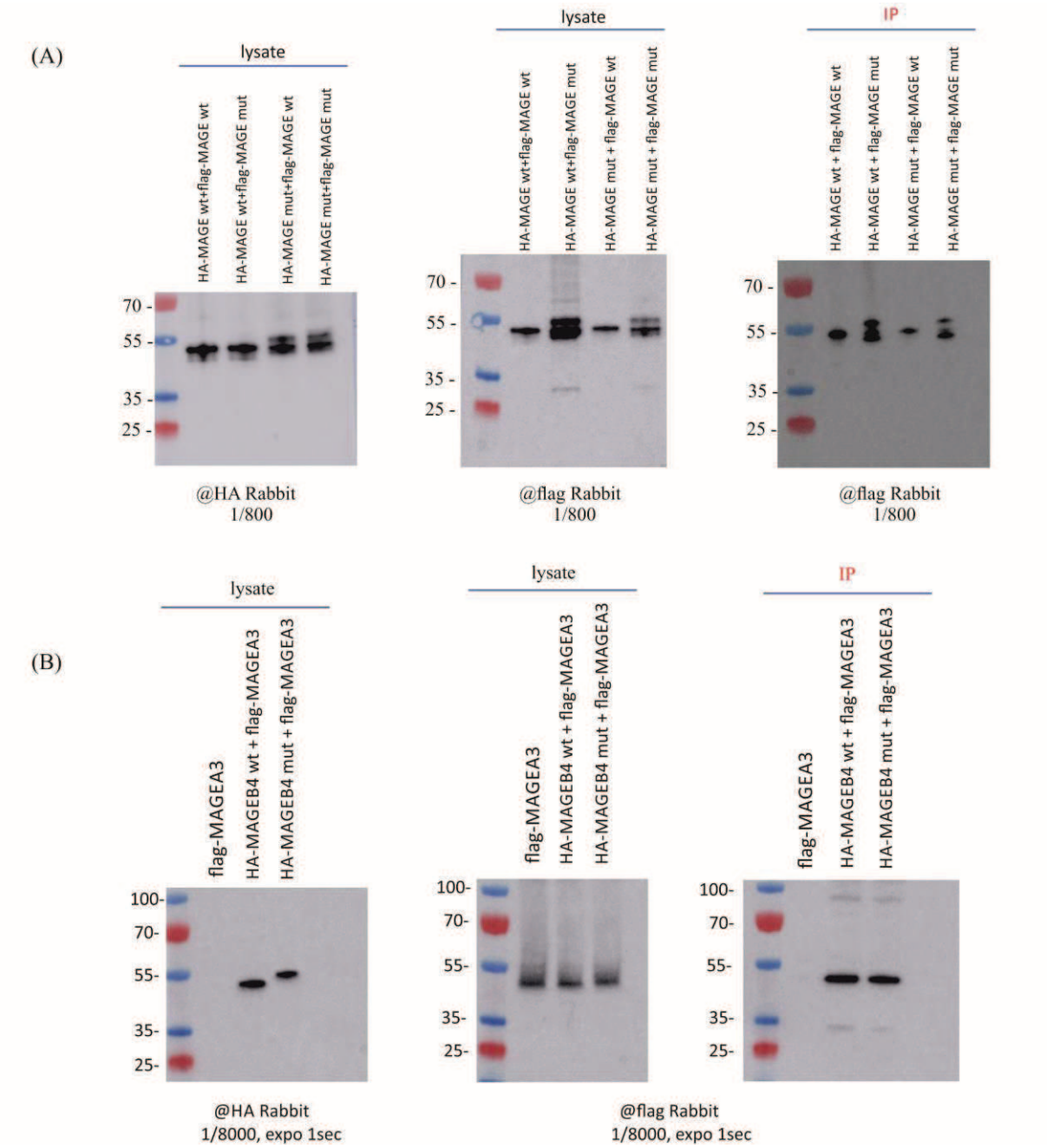

Figure 6.7: Interaction of MAGEB4 proteins with MAGEA3 (A) Immunoprecipitation of Flag-tagged MAGEA3 by wild type or mutant HA tagged MAGEB4. (B) Immunoprecipitation of Flag-tagged wild type or mutant MAGEB4 by wild type or mutant HA tagged MAGEB4.

Structure prediction

The protein sequences of MAGEB4 and MAGA3 have been aligned with the mutant sequence of MAGEB4 and the sequence corresponding to the crystal structure of a MAGEA3 domain encoding residues 102-310 (PDB: 4V0P) (figure 6.8). MAGEB4 is highly homologous to MAGEA3 on the MAGE domain but has an extension of 37 residues at the C-terminal end.

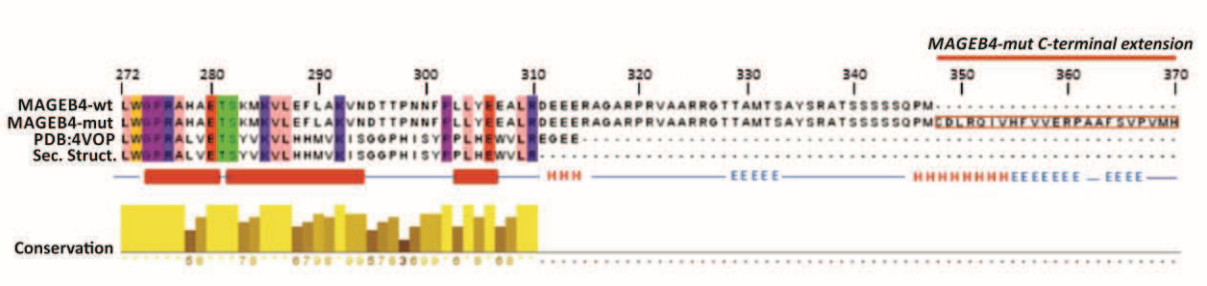

Figure 6.8: Sequence alignment of MAGEB4. The C-terminal end of MAGEB4 and MAGEA3 starting at residue 272 are aligned. Residues are coloured according to their physico-chemical properties using the Zappo coloring with 100% conservation threshold to display identities. Secondary structure elements are depicted as thick red bars for helices and red lines for loops of the crystal structure of MAGEA3.

Tertiary structure prediction for the C-terminal end and mutant extension of MAGEB4 indicates the presence of an α-helix preceded and followed by β-sheet regions (H= α-helix, E= β-sheet). The MAGE domain of the human MAGEA3 protein has been crystallized and its 3-dimensional structure has been determined and deposited in the protein databank with no related publication so far (PDB:4V0P). Since MAGEB4 is highly homologous to MAGEA3 throughout the conserved MAGE domain, the MAGEA3 model can be used to predict structural features for MAGEB4 (figure 9A and 9B).

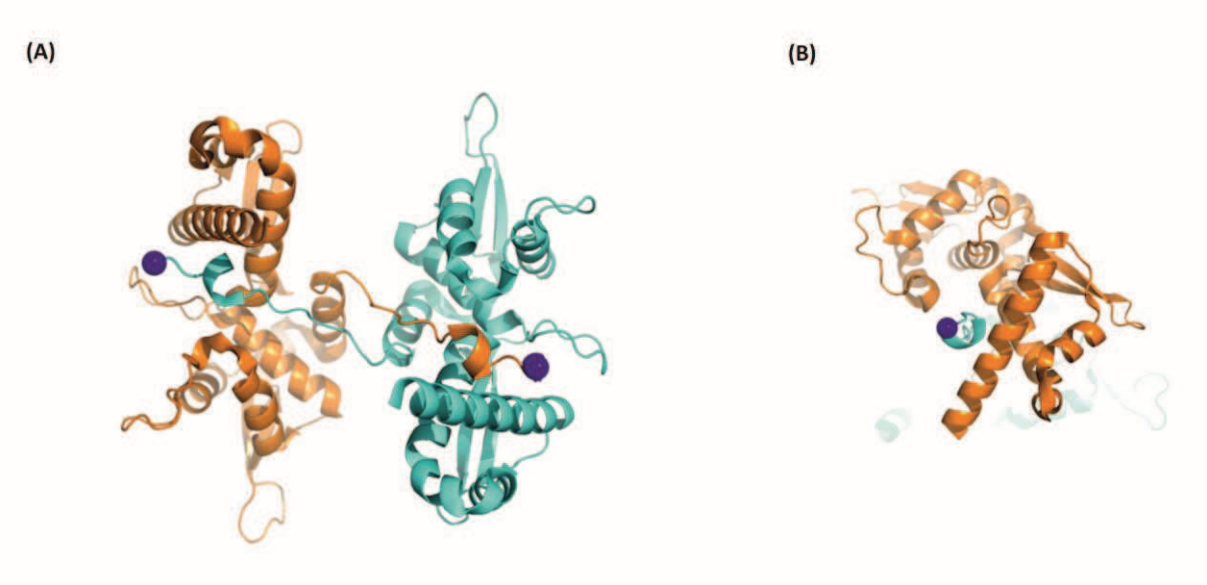

Figure 6.9: Structure prediction for MAGEB4 (A) Representation of the crystal structure of the conserved domain of MAGEA3 domain. In the crystal, the domain of MAGEA3 (PDB: 4V0P) comprising residues 102-310 (in cyan) is reaching through its C-terminal arm to a symmetry-related molecule (in orange). The last residue in the crystal structure Arg 310 is indicated by a blue sphere. (B) Perpendicular view of the dimer showing the C-terminal end of one monomer pointing through the other monomer.

DISCUSSION

Despite almost four decades of IVF practice, the human gametogenesis remains a black box. The absence of model considerably limits the understanding of the basic mechanism underlying human meiosis. The mouse model has brought, even if a large number of infertile mutant mice have been studied, only few lights to the human process (Teng *et al*., 2002, Miyamoto *et al*., 2003, Gianotten *et al*., 2004, Pirrello *et al*., 2005). Genome-wide association studies (GWAS) have not brought any consistent results (Krausz and Carrell, 2014).

So far, the only effective approach to identify human genes involved in human gametogenesis is the genetic approach. Indeed, during the last decade, studying family cases of infertility or group of infertile patients, the list of human genes has been growing up very quickly (Dam *et al.*, 2007, , Avenarius *et al.*, 2009, Koscinski *et al.*, 2011, , Ayhan *et al.*, 2014, Caburet *et al.*, 2014, Ramasamy *et al*., 2015, Okutman *et al.*, 2015).

We present here the analysis of a consanguineous Turkish family for which we identified, by whole exome sequencing, a single base substitution (c.1041 A>T, p.*347Cysext*24) in the X-linked *MAGEB4* that segregated with the non-obstructive azoospermia and oligospermia phenotype. The substitution changed the stop codon at position 356 to a codon for a cysteine. A new in frame stop codon is found 69bp downstream. So in theory, this mutation is susceptible to add 23 new amino acids to the C-terminus of MAGEB4.

The first members of the MAGE gene family were first described as only expressed by human melanoma and other cancer cells (De Plaen *et al*., 1994). Rapidly, it turns out that they were also expressed by male germ cells and thereby belong to the family of these genes classified as cancer testis antigens, being an excellent target to immunotherapy protocols (Daudi *et al*., 2014, Zhang *et al*., 2014).

MAGE genes family encompass more than 60 genes (Doyle *et al*., 2010), which can be classified in two classes according to their expression pattern. Type I MAGE proteins show restricted expression to the germline and trophoblast lineages, and are reactivated in cancer cells whereas the type II MAGE proteins are more ubiquitously expressed in a variety of somatic cells (Katsura and Satta, 2011). All MAGE family genes share a well conserved MAGE homology domain (MHD) that can be traced back to protozoa. It is a stretch of approximately 200 amino acids and usually located close to the COOH termini of the proteins except in the MAGED proteins, where it occupies a more central position. On average, all human MHDs are 46% conserved at the amino acid level (Doyle *et al.*, 2010) and when aligned, the MHDs can be divided into 5 distinct regions that represent distinct areas of conservation.

All type I genes are encoded by three X-linked clusters including more than 45 genes. Doyle and his team have been the first ones to define a biochemical and cellular function for MAGE. Indeed, they have shown that MAGE proteins formed complexes with E3 RING ubiquitin ligases (Doyle *et al.*, 2010). This binding takes place through the MAGE homology domain (MHD), which is also responsible to the specificity of the interaction.

MAGEB4 is a type I member of the MAGE gene family, which is clustered on chromosome Xp22-p21. So far, ten MAGEB genes have been identified; although they have different number of exons, in all except MAGEB18 last exon encodes the entire protein. Human *MAGEB4*, which has only one exon, is expressed only in testis and encodes a 346 amino acid protein. *MAGEB4* was present in the common ancestor of mammals; web search reveals that orthologs from mouse, rat, cow and dog were identified.

Murine mageb4 is expressed in the cytoplasm of premeiotic germ cells in adult testis. Expression was high in spermatogonia, the level then diminished during the transition into early meiosis, and no protein could be detected in cells that had entered the pachytene stage. It was suggested to have important functions in adult testis; for keeping the germ cell in an undifferentiated stage and involved in germ cell-specific mitosis (Osterlund *et al.*, 2000). However, mouse Mageb4 does probably not correspond to human MAGEB4 because of the presence of a repetitive region of 133 amino acids at the C-ter end.

Mutations which cause the loss of the normal translational termination (stop) codon are named as nonstop mutations. In fact “nonstop” mutations can alter protein functions by different ways. The mRNA can undergo a rapid decay, especially when no new stop codon is found before the polyA signal or over a certain distance, avoiding the production of the protein (Hamby *et al*., 2011). The addition at the C-ter of a stretch of amino-acid can affect

the stability of the protein, driving it to the proteasome (Taniguchi *et al.*, 1998, Seminara *et al.*, 2003, Wallefeld *et al.*, 2006, Cacciottolo *et al.*, 2011, Torres-Torronteras *et al.*, 2011, Oegema *et al.*, 2013, Gu *et al.*, 2014). Finally, it can conduct to the production of a stable protein with an altered structure (Wong *et al.*, 2014).

Since MAGEB4 protein is testis-restricted, we could not, for ethical reasons, get access to patient's testis samples to test these different possibilities. Therefore, we tested the effect of the mutation on MAGEB4 by in vitro cell transfection. We could show here that *MAGEB4* mRNA stability is not affected and that the protein can be produced, is stable and is localized to the same cytoplasmic compartment then the wild-type form. By immunoprecipitation, we also showed that mutant MAGEB4 as well as the wild-type form is able to interact with MAGEA3 and to form homodimers with it-self but also with wild-type MAGEB4.

Little information is available on oligomeric state or atomic structure of MAGE proteins. The crystal structure of the MHD of MAGEA3 protein indicates that the MAGE conserved core domain is forming a dimer with their respective C-terminal end stretching on the surface of the other monomer. Our results indicate that the dimer seen in the crystal might reflect the functional dimer observed in our co-transfection experiments and that the dimer is still present despite of the 37 aa-longer MAGEA3 C-terminal end. The non-stop codon mutation observed in this study adds another 23 residues to the C-terminus of the protein. We suggest that the MAGEB4 C-terminal tail and mutant extension would protrude from the position of Arg310 and extend outside the dimer interface. The mutant form with a 23 amino-acid extension of MAGEB4 does not affect the ability of the protein to homodimerize but could potentially alter post-translational modifications necessary for their regulation and/or association with cellular partners.

The mutation puts a novel cysteine residue which is not in the original protein. While free cysteine residues do occur in proteins, most are covalently bonded to other cysteine residues to form disulfide bonds. It is known that disulfide bonds not only play an important role in the stability of final protein structure, but also incorrect pairing of cycteine residues usually prevents the folding of protein into its native conformation (Sevier and Kaiser, 2002).

As a way to control the gene expression in the cell, eucaryotes have evolved ways to control the amount of normal mRNAs and also to maintain the fidelity in the production of such mRNAs. Through mRNA surveillance mechanisms, the cell distinguishes transcripts that are suitable for translation from those that are unsuitable (Klauer and van Hoof, 2012). Recently a new RNA surveillance pathway triggered by ribosome extension into the 3' untranslated region was reported. Transcripts that promote translation beyond the normal stop codon were degraded through ribosome-extension mediated decay (REMD). Mechanism of REMD was defined in a human alpha globin gene in which mutated transcripts are synthesized at levels similar to wild-type transcripts but are then rapidly cleared from terminally differentiating erythroblasts (Kong and Liebhaber, 2007). However, our study indicated the presence of mutant protein in transfected cells, so it is unlikely that mutant *MAGEB4* transcripts are degraded through REMD. On the other hand, it should be kept in mind that in testicular tissue, the behavior of *MAGEB4* transcripts and protein could be totally different.

This study represents the first indication of the physiological function of a MAGEB4 protein.

Chapter VII

Homozygosity mapping and whole exome sequencing in the quest of gene responsible for the infertility phenotype in a large Turkish family

ABSTRACT

Approximately 10 to 15% of infertile males are suffering from azoospermia. The optimal management of azoospermia in clinical application is testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI). However, so far there are no non-invasive predictors of sperm retrieval for TESE to direct clinical management.

In this part of the study we focus on azoospermia cases, our goal is to identify genes, when mutated, cause an azoospermia phenotype in men. To do so, we recruited a large family with 13 siblings comprising 3 well-defined azoospermic brothers. Our interest in the family relied on the fact that there were also four infertile sisters. We hypothesized that the same gene or two different genes could be the responsible of the infertility phenotype in the family. We used linkage analysis combined with whole exome sequencing in order to define candidate genes. So far, we sequenced the entire exons and intron/exon boundaries of total four selected genes however no mutation was identified. New studies with different strategies are planned. We anticipate that our work will contribute to a better understanding of gametogenesis biology and progress in this field will participate to the development of proper clinical care of azoospermic patients.

INTRODUCTION

Approximately 1% of all men and 10 to 15% of infertile males are suffering from azoospermia (Esteves 2015); this can be either classified as obstructive azoospermia (OA) or non-obstructive azoospermia (NOA). OA is the consequence of physical blockage of the male ductal system whereas NOA is caused by sperm production problem. NOA accounts for approximately 60% of men with azoospermia and represents the most severe form of male infertility (Hamada *et al.*, 2013). In clinical application testicular sperm extraction (TESE) and

intracytoplasmic sperm injection (ICSI) are offered for NOA patients. However in nearly half of the cases spermatozoa could not be found after testicular biopsies and pathology shows mostly either maturation arrest of spermatogenesis or a total absence of germ cells (Sertoli cell only, SCO) (Oates 2012). Based on studies of familial male infertility that often involves two or more siblings, most non-obstructive azoospermic men are believed to carry unknown autosomal mutations. However little is known about autosomal defects and infertility in humans (Nuti and Krausz 2008).

The goal of the following study is to improve our knowledge on human gametogenesis by identifying genes that, when mutated cause azoospermia. We anticipated that results will improve the clinical managements of infertile couples. For this reason, linkage analysis in combination with whole exome sequencing (WES) was performed on a large family originated from a small village in eastern part of Turkey including three azoospermic brothers and four infertile sisters (figure 7.1). As a principal, we work with consanguineous families; when parents are related, they are susceptible to harbor the same rare dormant recessive mutation they have inherited from a common ancestor. Their children thus have a risk to inherit the two copies of the mutation, therefore to develop the symptoms. Although there is no close consanguinity reported in this family, that region of Turkey has little or no migration which increase the possibility of far relation in between parents. We hypothesized that the mutation should follow an autosomal recessive inheritance mode and we thus tracked homozygous variants shared by infertile members. We also hypothesized that either same gene or two different genes are responsible for the infertility phenotypes observed in males and females in the family. Analysis was handled first to search homolog regions

shared by all infertile members then cases were analyzed by grouping males and females separately.

MATERIALS and METHODS

Patients

The family is consisted 13 siblings; 4 females and 3 males are infertile, the youngest female has unknown fertility status (figure 7.1). Infertile males were diagnosed as azoospermia; index patient (II:8) had a TESE operation and histopathology report revealed incomplete maturation arrest. Patients II:4 and II:9 were identified as azoospermia however no histopathology data was available. Three of infertile females (II: 1, II: 2 and II: 3) have a history of miscarriage at the beginning of their marriage. Other siblings were fertile with at least one child except II:13 who is unmarried. All affected siblings had a normal karyotype and no Y-chromosome micro-deletions were found for affected males.

According to oral information from patients, some members are unable to perceive odor (anosmia) (labeled with asterisk in the figure 7.1). When we got through the pedigree with the responsible doctor, problem matched with all of infertile females and one infertile male but none of the fertile ones, although the mother has mentioned about decreased ability to perceive odor (hypoanosmia).

Sampling and DNA preparation

Saliva samples were collected from all family members after signed consent form. DNA was extracted from saliva using the Oragene DNA Self-Collection kit (DNAGenotek, Ottawa, Canada) according to manufacturer's instructions, DNA quantity and quality was checked by nanodrop.

This project has been approved by the “Comité de Protection de la Personne (CPP) of Strasbourg University Hospital, France (CPP 09/40—WAC-2008-438 1W DC-2009-I 002)”, as well as by the “Istanbul University, Faculty of Medicine, Ethics committee for clinical research Faculty of Medicine (2012/1671-1265)”.

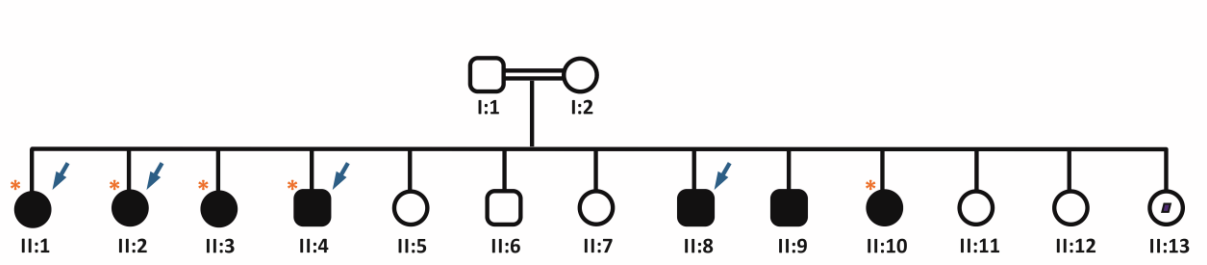

Figure 7.1: Pedigree of the Turkish family with 13 siblings. Filled symbols indicate affected members, clear symbols indicate unaffected members. Orange asterisks (*) points out the family members with anosmia or hypoanosmia. Blue rectangle (▮) indicates the member with unknown fertility status. Whole exome sequencing was performed on affected members indicated with blue arrows.

Mutation screening for Kallmann and related syndrome

It is known that Kallmann syndrome (KS) associates congenital hypogonadism due to gonadotropin-releasing hormone (GnRH) deficiency and an altered sense of smell; either totally absent (anosmia) or highly reduced (hyposmia) (Hanchate *et al*., 2012). In order to eliminate the possibility of a Kallmann and related syndromes, genes implicated in hypogonadotropic hypogonadism syndromic and non syndromic were scanned in laboratory of Dr Catherine Dodé (INSERM UMR745, Paris) (Supplemantary data chapter VII, table S7.1).

Whole genome analysis with SNP array

Genotyping microarray (Affymetrix 500K) was applied for eleven siblings (except II:3 and II:5), single nucleotide polymorphisms (SNP) obtained were analyzed with HomoSNP program which has been developed by the IGBMC Bioinformatics Department. The selection of regions was based on homozygosity of SNPs, threshold for the 500K array was

recommended by manufacturer as at least 35 consequitive SNPs. Defined homologous region were screened using the UCSC genome browser to list genes and identified genes were scanned for expression profiles through three different data bases; AmaZonia (http://amazonia.transcriptome.eu/), BioGPS (http://biogps.org/) and EMBL-EMI Expression Atlas (https://www.ebi.ac.uk/gxa/).

Considering a unique gene responsible from infertility phenotype in the family, a specific expression in ovary and testis were taken into account. When we refer to infertile males only, dominant testis expression and possible role in spermatogenesis were criterias for selection. Existence of a mouse KO model with a male sterility phenotype was also taken into account for determination of candidate genes. Oppositely, dominant oocyte and/ or ovary expression were selection criterias in case of female infertility.

Whole exome sequencing

A whole exome sequencing (WES) of patients II:1, II:2, II: 4 and II: 8 (figure 7.1), was performed by the IGBMC microarray and sequencing platform, member of the "France Génomique programme". For this purpose, approximately 1µg DNA was sheared to 150-200bp with Covaris Sonolab v4.3.3 (Covaris, Woburn, MA, USA). Fragments were subjected to library preparation; for each sample to be sequenced, an individual indexed library was prepared. DNA libraries were then enriched (SureSelectXT2 Target Enrichment System) and sequenced with Illumina Hiseq 2500 following Illumina's instructions.

Data processing and analysis of WES

Image analysis and base calling were performed using CASAVA v1.8.2 (Illumina). Reads are first mapped onto the reference genome GRCh37/hg19 using BWA (v0.7.5a) (Li and Durbin

2009). During comparisons, if a nucleotide is different from the reference genome in several reads at the same position then, a variation will be called. Variant calling was done using GATK UnifiedGenotyper (v2.5-2) (DePristo *et al*., 2011). Detected variants were ranked by VaRank (Geoffroy *et al*., 2015) which incorporates the annotations retrieved by the Alamut Batch software (Interactive Biosoftware, France) as well as allele frequency from our internal exome database. The annotation took into account the functional annotation and external data such as the HGVS nomenclature (genomic, cDNA and proteic), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), 1000Genomes (http://www.1000genomes.org/) and the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS/). Data were provided as lists of sequence variants relative to the reference genomes.

Results were analyzed in three steps. First, all samples were accepted as one group and homozygous variants shared by all samples filtered, and as a second step infertile males and infertile females were grouped separately. Third step was analyzing each sample separately in order to be able to see variations in badly covered genes.

PCR and DNA sequencing

When candidate genes were selected, mutation screening was performed by PCR and Sanger sequencing. All exons and exon/intron boundaries of the gene in question were sequenced for infertile patients while only mutated exon was sequenced for fertile controls. PCR primers were designed by using online tools (Primer3Plus: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and validated by online via BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and slicoPCR (https://genome.ucsc.edu/cgi-bin/hgPcr). DNA amplicons were purified and double strand sequencing of each DNA fragment were performed by GATC (Cologne, Germany).

RESULTS

In the quest of gene responsible for the azoospermia, linkage analysis in combination with WES was performed on a large Turkish family comprising 13 siblings.

Mutation screening for Kallmann and related syndrome

Kallmann syndrome is a form of hypogonadotropic hypogonadism (HH), characterized by delayed or absent puberty and an impaired sense of smell (anosmia/hypoanosmia)(Kotan *et al.*, 2014). To date at least sixteen different genes have so far been implicated in causing Kallmann syndrome or other forms of HH.

All exons and flanking splice sites of 16 genes were sequenced (Supplemantary data chapter VII, table S7.1); however no mutation has been identified. Same genes have been checked through whole exome datas from sequenced samples II:1, II:2, II:4 and II:8. All of them were well covered; known variations were detected though no mutation was identified.

Linkage analysis

A genome wide scan was performed on the DNA sample of 11 siblings using 500K SNP arrays (Affymetrix Genechip). There was not enough saliva sample from patient II:3 and sampling problem for the II:5 at the time of array application, we couldn't scan these two by SNP array however second sampling was done later for mutation screening of candidate genes. Since we supposed that either the same or different mutation could be responsible for infertility phenotypes in male and female members of the family; three strategies were used. Regions shared by all infertile, only infertile males and only infertile females were screened separately.

For the first analysis the selection of regions was based on homozygosity for at least 35 SNP. However, no homologous region was identified after first analysis with HomoSNP. Since there is no close consanguinity in the family, shared homologous regions could be very small, hence we made less strict analysis by decreasing the number of homozygous SNP to 10. Meanwhile, by the fact that meiosis can be differently affected between male and female (Baker *et al*., 1995, Liu *et al*., 1998, Okunade *et al*., 2004, Yang *et al*., 2008), fertile female sister could also be homozygous for the mutation causing male infertility. Therefore we performed a second screening by taking into account regions shared in between infertile brothers and the fertile sister. Same was applied for the female infertility version.

Second analysis did not reveal a region shared by all infertile members of the family. Though, 10 homologous regions were shared by all infertile males and 7 regions were shared by all infertile females (Supplementary datas Chapter VII, table S7.2). These regions contain 320 genes in total according to the UCSC Genome Browser.

Expression profile scan for all these genes would be time consuming, possible candidate genes could be in regions that are not covered by the array. We decided to perform whole exome sequencing on the genome of two infertile brothers and two infertile sisters, so we did not carry out data base scanning for expression and targeted sequencing.

High throughput exome sequencing

A whole exome sequencing of DNA obtained from two infertile brothers (II:4 and II:8) and two infertile females (II:1 and II:2) was performed.

In each case at least 8.4 GB of sequences were generated, more than 70% of the target exome were represented with greater than 40-fold coverage (Supplementary data Chapter VII, table S7.3).

We hypothesized that there is some degree of consanguinity in the family; the disease should follow an autosomal recessive inheritance mode. We thus tracked homozygous variants shared by infertile siblings. We filtered variants according to coverage, minor allele frequency in populations (we filtered out when >1%), validation status of the reference SNP (not validated or validated by only one database) and novelty by comparing them to our in-house exome database. Remaining variants were further filtered for homozygosity status in three steps.

Variants were assessed first for homozygous variations shared by all four infertile siblings, which revealed 21 homozygous variations in 19 genes. Later, we repeated the same by grouping infertile members as male and female separately; homozygous variations and categories are listed in table 7.1. Scanning through Amazonia (http://amazonia.transcriptome.eu/), BioGPS (http://biogps.org/) and EMBL-EBI expression atlas (http://www.ebi.ac.uk/gxa/home) were applied to all genes that passed our filtering process.

		All infertile	Only infertile male	Only infertile female
Homozygous variants with a coding effect		21	24	30
Variant category	**frame shift**	11	11	18
	nonsense	0	1	0
	missense	4	5	4
	synonymous	6	7	8

Table 7.1: Homozygous variations passing filtering process. Subset of filtered shared homozygous variants with a codig effect and relevant variant categories are given for three groups.

Expression scanning exposed no plausible candidate shared by all four samples. However, analysis revealed a homozygous frame shift mutation in OR2T35 (olfactory receptor, family 2, subfamily T, member 35) gene which is shared by all samples analyzed by WES. The variation is 7 base deletions, c.609_615del which exchange of amino acid cysteine for a stop

codon (p.Cys203*). The variation is listed neither in the Exome Aggregation Consortium (ExAC) database nor in EVS but it is defined in ensemble.org with the reference "rs143010547". So far, only nine genotypes were submitted with a deletion. There is no OMIM (*Online Mendelian Inheritance in Man)* entry for the gene, no available expression data and we couldn't gather information from SNP array since the gene is located in the telomere region. The OR2T35 gene encodes an olfactory receptor which interacts with oderant molecules in the nose to initiate a neuronal response that triggers the perception of a smell. Knowing the problem of perception of smell in five members of the family we decided to screen the variant in family members (primers were listed in the supplementary data Chapter VII, table S7.5a). However, results could not be confirmed on family members by Sanger sequencing; there are inconsistency between WES and Sanger sequencing results (supplementary data Chapter VII, table S7.5b).

Since two infertile females were identified as wild type with Sanger sequencing for the 7bp deletion in OR2T35 gene, we concluded that this deletion could not be the reason for the infertility phenotype in the family.

Predicted destructive effect of nonsense mutations on the protein function directed us to choose CPNE1 (Copine 1, MIM: 604205) as a candidate gene for infertile males, even though it has a broad tissue distribution as an expression profile including testis.

A homozygous substitution inducing a stop codon in exon 10 of CPNE1 gene was identified in two infertile brothers. Both have novel c.646C>T substitution which exchange of amino acid Q211 for a stop codon (p.Q211*). The family members were scanned for the variation (primers are listed in the supplementary data Chapter VII, table S7.6a); parents as well as some siblings were heterozygous for both substitutions while the youngest sibling with

unknown infertility status was homozygous (supplementary data Chapter VII, table S7.6b). However the third infertile male was identified as heterozygous as with two fertile male in the family. This results also confirmed by re-scan of HomoSNP; there was a homozygous region shared in between II:4, II:8 and II:9 but not with others (supplementary data Chapter VII, figure S7.1).

Since one infertile male was identified heterozygous for the substitution, it was concluded that the c.646C>T substitution in CPNE1 gene could not be the reason for the male infertility phenotype in the family.

Taking into consideration that the variation of interest could be in a badly or non-covered exon, we consider each sample separately and analyse data without filtering for the coverage. Two candidates were revealed; a frameshift variation on the TCEAL6 gene shared by all four members and a missense variation on the RGPD4 gene shared by two males but not covered at all for two females.

TCEAL6 (transcription elongation factor A (SII)-like 6, MIM: 158931) is located on the X chromosome, it belongs to a gene family coding for transcription factors. Its expression is specific to brain, testis and ovary. In four fertile members of the family, insertion of G causes a frameshift (c.523dup, p.Gln175Profs*27). The family members were scanned for the variation, the father has the mutation so it was concluded that the variation is not responsible for the infertility phenotype in the family (primers were listed in supplementary data Chapter VII, table S7.7).

RGPD4 (RANBP2-like and GRIP domain containing 4, MIM: 612707) maps to chromosome 2, belongs to a gene family with eight members, resulted from duplications of a region of

human chromosome 2 containing the RANBP2 (RAN binding protein 2, MIM:601181) and GCC2 (GRIP and coiled-coil domain containing 2, MIM:612711) genes. The expression profile is restricted to the testis, a missense variation (g.108479432G>A) was identified homozygously in two males and the substitution is predicted to be deleterious through SIFT and PolyPhen2. RGPD4 shows great homology (>98%) especially with RGPD2 and RGPD3 as well as with RANBP2 on chromosome 2. HomoSNP analysis shows no homology in corresponding region, however through checking the physical positions of SNPs in that region revealed there is no SNP covering the gene location of RGPD4 (supplementary data Chapter VII, figure S7.2). The region without SNP is approximately 115,2kb.

Given MAF value for the variation is 0.000, it is not listed in EVS database and in ensemble database only two genotypes were available, however the ExAC database reveals different results. The data set provided on this website spans over 60,000 unrelated individuals sequenced as part of various disease-specific and population genetic studies. Since the variation is too frequent according to the ExAC database (min >30% in every population), it was concluded that the identified RGDP4 variation could not be the underlying cause for the infertility phenotype in the family.

DISCUSSION

We performed linkage analysis combined with WES on the large family with 13 siblings comprising three azoospermic males and four infertile females (figure 7.1). Family was not consanguineous but from small village with a little or no migration, so there is a possibility of being far related. We supposed that may or may not be the same mutation for male and female, but some recessive mutation could be the cause.

There is handful of studies showing that male and female gametogenesis is different and one gene mutation can cause a male infertility whereas females are fertile. For example disruption of the *Tex15* gene in mice caused a drastic reduction in testis size and meiotic arrest in the first wave of spermatogenesis in males while female KO mice were fertile (Yang *et al*., 2008). Similarly, deletion of *Tex19.1* gene in mice leads reduction in fertility with a variable penetrance in males but females are fertile (Tarabay *et al.,* 2013).

On the other hand, there are also few examples in mice that same gene mutation causes infertility in male as well as in females. For instance, recently it was revealed that a recessive point mutation in a gene, encoding the regulatory protein bromo domain and WD repeat domain containing 1 (BRWD1), affects gametogenesis in both sexes of mice (Philipps *et al.* 2008). Although homozygotes for the found mutation are phenotypically normal, they are infertile; the females produce few oocytes that progress to metaphase II (MII), and none of them progress beyond the two pronuclei stage whereas the males produces few sperm. Apperantly, there is a common mechanism between spermiogenesis and oocyte maturation. There was no shared homolog region for all infertile members of the family according to our analysis, this discourage the hypothesis of the unique gene responsible from infertility for males and females in the family at a first look. On the other hand, it must be kept in mind that SNP arrays do not cover telomeric and centromeric regions of chromosomes.

Different approaches for analyzing WES data revealed four candidate genes; namely OR2T35, CPNE1, TCEAL6 and RGPD4. 7 base deletions on the exon 1 of OR2T35 gene could not be confirmed with Sanger sequencing, there was inconsistency in between WES and Sanger sequencing results. The olfactory receptor gene family is the largest in the genome with a great homology in terms of sequence (Gilad *et al.,* 2003). Although online software

tools showed specific amplification for the OR2T35 gene with designed primers, there could be possibility that we amplified the wrong gene. However according to the results we have so far, two infertile females were identified as wild type with Sanger sequencing for the 7bp deletion and this deletion could not be the reason for the infertility phenotype in the family. The c.646C>T substitution in exon 10 of CPNE1 induce a stop codon, leading early translational termination. However family scanning reveals the third infertile brother (II:9) also has the mutation heterozygously as two fertile males in the family so it could not be the reason for infertility phenotype. Similarly, a nonsense mutation in TCEAL6 gene was identified in the father (II:1) which removes the possibility of the being responsible from infertility phenotype in the family. On the other hand, missense variation in RGPD4 is too frequent according to the ExAC database, so it could not be the underlying cause for the infertility phenotype in the family.

CONCLUSION

The combination of SNP mapping and the whole exome sequencing approaches is relevant, however still have limitations and did not bring us advantages as we thought. Although SNP microarrays are very dense, fast as a technique, requires only a small amount of genomic DNA (250ng), genomic regions generated by homozygosity mapping are generally multiple mega bases in size and can contain multiple genes. Therefore, the identification of the mutated gene from a large number of candidates is expensive and time consuming. The fact that no information could be gathered for telomeric and centromeric regions is main limiting factors for the array studies. Although the coverage of exons never reaches 100%, so far WES is a better alternative for us. Besides, VaRank application and new filtering strategies

allow us to analyse variants all through the genome, we do not need any more to restrict analysis to a specific region defined by homozygosity mapping.

Up to date, no mutation has been identified as a responsible for infertility phenotype in the family. WES results reveal also variations for non-coding RNAs (ncRNAs). Recent studies have indicated several types of noncoding RNAs; including long noncoding RNAs (lncRNAs), microRNAs (miRNAs), endo-small interference RNAs (endo-siRNAs), and Piwi-interacting RNAs (piRNAs). They are expressed in the male germline and are required for spermatogenesis in animals, therefore constitute a novel regulatory target in spermatogenesis (Gou et al 2014, Luk et al 2014). Our next step will be to analyse variations in ncRNAs. Analysing for CNVs in homologous regions could also be alternative.

HomoSNP analysis revealed homologous regions shared by all infertile males or all infertile females. 320 genes were identified in these regions; next step could be checking the coverage for identified genes. There is a possibility some genes were not covered at all, then we could scanned expression profile for the uncovered genes in order to identify candidate genes.

We may change our strategy as well and search for heterozygous variations since there could be no known consanguinity in the family. The exome represents just over 1% of the human genome (Grada and Weinbrecht 2013); therefore whole genome sequencing (WGS) can be another alternative.

Conclusion and future perspectives

During my PhD, we identified two mutations that cause male infertility phenotype; a nonstop mutation in an X-linked gene and a stop-gained mutation in an autosomal gene. We have studied three families of well documented male infertility, comprising more than one man with azoospermia as well as one oligozoospermia and two or more unaffected men: (i) A family with 6 brothers; three of them were infertile and two fertile brother (ii) One family with triplets, two of them were infertile as well as one younger infertile brother (iii) A family of 13 siblings; 4 female and 3 male are infertile. There were some degree of consanguinity in the first two families whereas the third one was from very little city with no migration story in the family.

Using exome sequencing, we identified a nonsense mutation leading to a premature stop in the *TEX15* (c.2130T>G, p.Y710*) in the family comprising eight siblings in which three brothers were identified as infertile (Okutman *et al.,* 2015). *TEX15* displays testis specific expression, maps to chromosome 8, contains four exons and encodes a 2,789-amino acid protein with uncertain function. The mutation, which should lead to early translational termination at the first exon of *TEX15*, co-segregated with the infertility phenotype and our data strongly suggest that it is the cause of spermatogenic defects in the family. All three affected brothers presented a phenotype reminiscent of the one observed in KO mice. Indeed, previously reported results demonstrated that disruption of the orthologous gene in mice caused a drastic reduction in testis size and meiotic arrest in the first wave of spermatogenesis in males while female KO mice were fertile. The data from our study of one Turkish family suggested that the identified mutation correlates with a decrease in sperm count over time. A diagnostic test identifying the mutation in man could provide an

indication of spermatogenic failure and prompt patients to undertake sperm cryopreservation at an early age.

Studying second family, we identified *MAGEB4* as a new X-linked gene involved in an inherited male infertility. Indeed, a no-stop mutation was found to co-segregate with the NOA and oligozoospermia phenotype in the family. This novel mutation (c.1041A>T, p.*347Cysext*24) substitutes the stop codon with cysteine residue, potentially adding 23 amino acids to the C-terminus of MAGEB4. Beside few functional studies on cancer cells, nothing is known about the physiological role of the MAGE gene family. Murine homolog, Mage-b4, was suggested to may have important functions in adult testis; for keeping the germ cell in an undifferentiated stage and involved in germ cell-specific mitosis. Our study provides the first clue on the physiological function of a MAGE protein in human. The prediction of the MAGEB4 protein structure suggests that the addition of the 23 amino acids may affect the proper homo or heterodimerization of the protein, knowing that neither the cellular protein localization nor the mRNA stability is affected by the mutation.

Concerning the third family, no mutation was identified as an underlying cause of infertility phenotype in the family. The failure can be explained by the fact that either our hypothesis of third or more degree consanguinity in the family was wrong or mutation in question is not in the coding region of the genome.

Our strategy of combining linkage analysis with WES did not give us an advantage as expected. Although SNP microarrays are very dense, fast as a technique, requires only a small amount of genomic DNA (250ng), genomic regions generated by homozygosity mapping are generally multiple mega bases in size and can contain multiple genes. Therefore, the identification of the mutated gene from a large number of candidates is

expensive and time consuming. Moreover no information could be gathered for telomeric and centromeric regions, so we decided to change our strategy and use only WES for the future applications.

Our study has limited number of Turkish patients and controls. The aim of our next study is to scan two identified mutations in larger patient groups with different ethnicity. We have been able to collect 69 DNA from infertile French patients. Patients were divided into two groups; azoospermic and severe oligozoospermic. Samples will be screened first for the mutation site and then in case no mutations will be identified, all exons and exon/intron boundaries will be screened. We also try to collect more samples through our collaborators in different regions. For the third family, an analysis of ncRNAs and CNVs will be the next alternatives. Changing our strategies will also be in discussion; scanning heterozygous mutations instead of homozygous mutations could be an alternative. We also believe that recruiting other families will facilitate the analysis.

At the fundamental level, this project helps to identify new factors involved in important processes during human spermatogenesis, allowing the fine dissection of these mechanisms by defining causes that adversely affect gametes production. It also allows opening a research field to thwart these effects.

The first diagnostic step is important for the psyche of the patient, for adapting the best treatment and counsel not only to him but to his spouse and relatives. Results will help to guide individual management decisions and maximize ART treatment outcomes which are the keystone in the successful implementation of infertility care in low-resource settings. In the longer term, it will be possible to develop a diagnostic test and propose a molecular diagnosis to patients showing similar phenotype. For instance as in case of TEX15 mutation;

it is likely that the phenotype becomes more manifest with age. In the future, a diagnostic test identifying the *TEX15* mutation in man could provide an indication of spermatogenic failure and prompt patients to undertake sperm cryopreservation at an early age.

Results will also open the possibility to carry on more basic research on human spermatogenesis. Our work will create new collaborations with different R&D laboratories and results will be used for upcoming new R&D projects.

APPENDIX

French abstract

Résumé de thèse

État de la question

L'infertilité est définie par l'organisation mondiale de la santé (OMS) comme l'incapacité pour un couple de concevoir après 12 mois, ou plus, de rapports sexuels réguliers non protégés.

L'OMS estime qu'un couple sur quatre vivants dans des pays développés est confronté à un problème d'infertilité. Parmi les couples avec un projet parental, le facteur masculin d'infertilité est responsable d'environ 20% des cas.

Les causes connues de l'infertilité masculine sont multiples ; problèmes hormonaux, facteurs environnementaux, style de vie, infections ou facteurs génétiques. Malgré de longues années d'activités d'assistance médicale à la procréation, l'étiologie reste inconnue pour 40% des cas et donc sont classés comme idiopathiques. Considérant le nombre élevé des gènes potentiellement impliqués dans la gamétogenèse, il est fort probable que la majorité des formes 'idiopathiques' sont d'origines génétiques.

L'objectif de notre équipe est d'identifier des gènes, qui, lorsqu'ils sont mutés, sont responsables d'un phénotype d'infertilité masculine affectant la production de gamètes. Nous nous concentrons sur la production de spermatozoïdes ; à savoir l'azoospermie non obstructive (NOA, absence de spermatozoïdes lors de l'éjaculation sans obstruction du système de distribution) et l'oligozoospermie sévère (SO, $<1X10^6$ spermatozoïdes/ml dans l'éjaculat). Afin d'identifier des gènes uniquement impliqués dans des phénotypes d'infertilité, nous limitons notre approche à des cas non-syndromique d'infertilité.

Approches expérimentale

Afin d'identifier les gènes impliqués dans l'infertilité masculine nous avons décidé d'étudier des grandes familles consanguines avec des cas d'infertilité masculine bien identifiés.

D'un point de vue génétique, les mariages intrafamiliaux augmentent considérablement l'émergence de pathologies, du fait d'une transmission récessive. Quand le mari et la femme sont en relation familiale, ils sont successibles d'avoir hérité d'un ancêtre commun de la même mutation rare et dormante. Leurs enfants auront donc un risque de 25% d'hériter les deux copies de la mutation et donc de développer la pathologie. Puisque la mutation vient d'un ancêtre commun, la totalité de la région entourant cette mutation est identique, c'est pour cela que tous les marqueurs polymorphes de cette région sont homozygotes et peuvent être commodément détectés.

Trois familles ont été incluses dans cette étude : 1) une famille avec six frères dont trois sont infertiles ; 2) une famille avec huit enfants (trois filles et cinq garçons), dont des triplés, deux sont infertiles, ainsi qu'un plus jeune frère infertile et un frère fertiles ; 3) une famille avec 13 enfants dont 4 filles et 3 hommes sont infertiles (figure 1).

Nous avons utilisé comme une source d'ADN, des échantillons de salive pour les membres des familles, les contrôles et les cas de SO individuels, des prélèvements de sang pour les cas de NOA individuels. Pour l'ensemble des échantillons les consentements écrits ont été obtenus.

Ce projet a été approuvé par le Comité de Protection de la Personne (CPP) de l'hôpital universitaire de Strasbourg, France (CPP 09/40 - W AC-2008-438 1W DC-2009-I 002), ainsi que de "L'Université d'Istanbul, Faculté de Médecine, comité d'Éthique pour recherche clinique" (2012/1671-1265).

Dans un premier temps nous avons réalisé une analyse de génome total en utilisant une puce SNP. Faute de trouver le gène responsable nous avons opté pour une approche de séquençage complet de l'exome (WES). Nous avions envisagé de combiner les deux techniques afin de limiter l'analyse du WES aux régions homozygotes partagées par les patients.

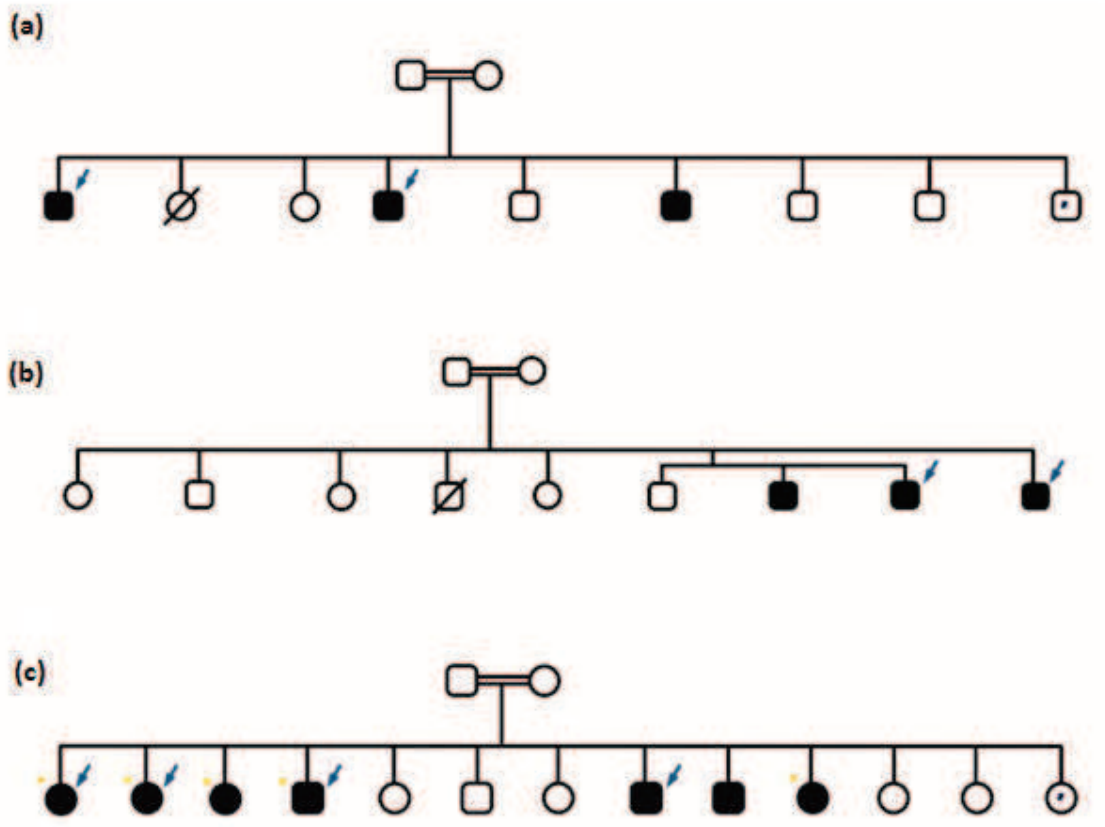

Figure 1: Pédigrée des familles identifiées. Les flèches bleues indiquent des échantillons pour lesquels une séquence d'exome complète a été appliquée. Les symboles remplis indiquent des membres affectés, les symboles vides indiquent des membres fertiles. Le rectangle bleu (▮) indique le membre avec le statut de fertilité inconnu. Des astérisques oranges (*) indiquent des membres avec l'anosmie ou hypoanosmie dans la troisième famille.

Résultats

Pour la famille 1, sept échantillons d'ADN (tous les hommes infertiles, 2 frères fertiles et les parents) ont été analysés par puce SNP. 5 régions homozygotes comprenant 23 gènes ont été identifiées; cependant ni le profil d'expression ni les informations dans la littérature n'étaient compatible avec un rôle de ces gènes dans l'infertilité. Les échantillons d'ADN de deux frères azoospermic (indiqué par des flèches bleues sur la figure 1a) ont été envoyés pour un WES.

6 GB et 6,7 GB de séquences d'ADN ont été générés respectivement pour deux frères atteints. Dans les deux cas, plus de 56% de l'exome ciblé a été représenté avec une couverture supérieure à 40 fois. Compte tenu de la consanguinité connue dans la famille, nous avons supposé que la maladie devait suivre un mode d'hérédité autosomique récessive et nous avons donc recherché les mutations homozygotes partagées par les deux frères affectés. Nous avons filtré les données en fonction de la couverture, de la fréquence faible des allèles dans les populations (> 1 %), de la validation des SNP de référence (non validés ou validés par une seule base de données) et la nouveauté en le comparant à notre base de données de l'exome en interne. L'analyse des données a révélé 17 variations homozygotes dans 15 gènes partagées par les deux échantillons (tableau 1).

	IV:1	IV:3
Read length	2x100	2x100
Total number of bases	6GB	6,7GB
Total number of reads	60,119,546	67,003,084
No. of mapped reads	54,512,034 (90.00%)	63,500,545 (94.00%)
Duplication rate	11%	10%
Nucleotide coverage of target regions: Bases >=5X coverage	97.81%	98.06%
Nucleotide coverage of target regions: Bases >=10X coverage	95.06%	95.99%
Nucleotide coverage of target regions: Bases >=20X coverage	84.96%	88.25%
Nucleotide coverage of target regions: Bases >=40X coverage	56.99%	63.73%
Total variants	56,908	55,894
Subset of filtered variants for coverage (>=10, %:>=15)	43,869	44,928
Subset of filtered variants for rs validation (max1)	5,223	5,507
Subset of filtered variants for minor allele frequency (<0,01)	4,802	5,043
Subset of filtered variants for prediction (SIFT and PolyPhen-2)	4,613	4,830
Subset of filtered homozygous variants	981	1,064
Subset of filtered shared homozygous variants with a coding effect	17	
Variant category nonsense	1	
missense	5	
synonymous	11	

Tableau 1 : Traitement et analyse de données WES pour la famille 1.

L'analyse a révélé une mutation non-sens dans le gène *TEX15* conduisant à la production d'une protéine tronquée. Le gène *TEX15* en humain est sur chromosome 8 et consiste en quatre exons at couvre une région génomique de 59.06 Kb. Le gène code pour une protéine à 2789 acides aminés avec deux domaines conservé inconnus nommés 'TEX15'et 'PF15326' selon les bases de données PFAM et SMART (figure 2).

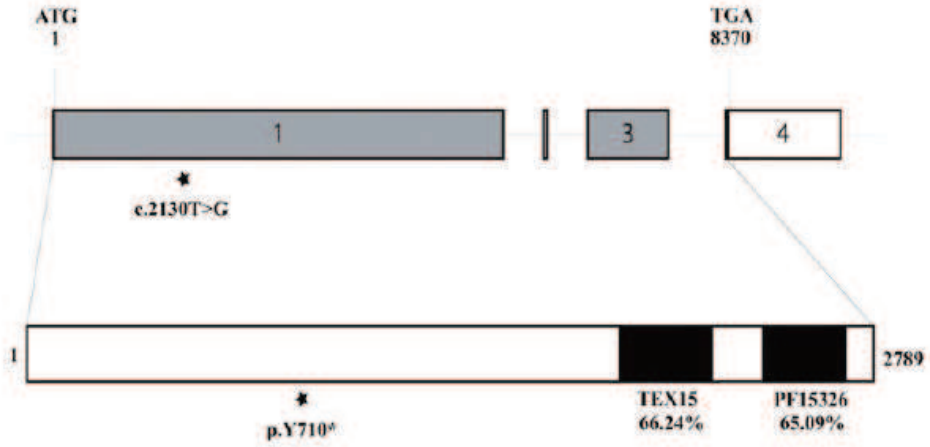

Figure 2: Représentation schématique des exons et de la séquence de la protéine TEX15. La carte exonique de TEX15 est représentée dans la partie supérieure de la figure basée sur la transcription RefSeq (NM_031271.3). Pour les quatre exons, la séquence codante est présentée en surligné avec le gris.La séquence de la protéine (Uniprot : Q9BXT5, TEX15_HUMAN) est représenté dans la partie inférieure avec les deux domaines de PFAM présentés sous forme de rectangle noirs (TEX15, PFAM15326). Ces domaines de la protéine humaine, présentent respectivement, 66.24 et 65.09% de similarité avec protéine de souris. La mutation non-sens (c.2130T > G, p.Y710 *) est mis en surligné à la fois sur le transcrit et la séquence protéique, respectivement.

La mutation est retrouvée à l'état homozygote chez l'ensemble des hommes infertiles de la famille, les membres fertiles sont soi hétérozygote ou de type sauvage (figure 3).

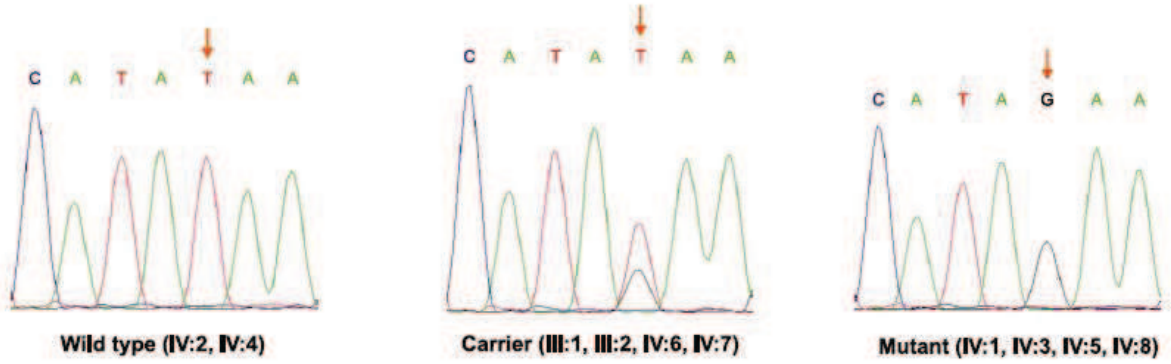

Figure 3: Résultats du séquençage d'ADN par la méthode de Sanger pour les patients mutants, porteur et normaux. L'allèle en question est indiqué par la flèche orange. La mutation induit le remplacement d'un codon codant pour une tyrosine (TAT) par un codon-stop (TAG).

Deux des frères infertiles ont été diagnostiqués comme NOA tandis que le troisième frère infertile plus jeune était SO avec, en 2010, peu de spermatozoïdes dans l'éjaculat. En 2014, une nouvelle analyse de sperme a révélé un azoospermia pour ce patient; aucun

spermatozoïde n'a été détecté même après concentration. Il est important de noté que le phénotype humain est très semblable à celui observé dans les souris KO pour *Tex15*. En effet, la mutation de *Tex15* chez la souris conduit, chez les mâles, à un arrêt de la spermatogenèse par blocage de la méiose dès la première vague de spermatogenèses tandis que les souris KO femelles sont fertiles. De plus, comme observé chez les souris mutantes, les trois frères affectés présentent une réduction drastique de la taille des testicules (tableau 2).

Patient code	Testis volume (ml)	
	Left	Right
IV :1	10	10
IV :3	12	12
IV :5	10	10.5

Tableau 2: Volume testiculaire des membres affectés de la famille. Le volume testiculaire normal pour l'homme est de 18-20ml (Condorelli *et al.,* 2013)

Dans la famille 2, deux frères infertiles ont été diagnostiqués comme NOA tandis que le troisième était SO. 8 échantillons d'ADN; tous les hommes infertiles, 2 frères fertiles et une sœur fertile ainsi que les deux parents, ont été analysés par puce SNP. Ceci a permis d'identifié 628 gènes dans les régions homozygotes partagées uniquement par les hommes infertiles. Les profils d'expression de l'ensemble de ces gènes ont été vérifiés via trois bases de données différentes.

Trente-six gènes ont montré une expression spécifique du testicule selon au moins une base de données ; cinq gènes, à savoir GTSF1L, EPPIN, INSL6, TP53TG5 et SPINT3 ont été sélectionnés sur la base des études KO et / ou de leurs rôles possibles dans la reproduction masculine selon la littérature.

Dans un deuxième temps, nous avons considéré les régions homozygotes partagées entre tous les hommes infertiles sans éliminer les régions partagées avec la sœur fertile. En effet, la méiose se déroulant différemment entre les hommes et les femmes, celle-ci peut être porteuse de la mutation sans présenter une infertilité. Six régions homozygotes différentes ont été ainsi identifiées. Nous avons concentré notre analyse sur les gènes présentant une expression spécifique dans le testicule et rechercher dans la littérature une éventuelle implication de ceux-ci dans l'infertilité. Vingt-deux gènes ont montré une expression spécifique des testicules au moins dans une base de données. Suite à la vérification de la littérature quatre gènes, à savoir SERPINA5, SPATA7, FAM181A et TDRD9 ont été sélectionnés. Au total, nous avons séquencé l'ensemble des exons et des frontière exon/intron de neuf gènes, sans identifier de mutation.

Nous avons ensuite réalisé un WES sur l'ADN de deux frères infertiles (indiqué par des flèches bleues sur la figure 1b). Un total de 9,8 Gb et 9,2 Gb de séquences ont été générés pour les échantillons des deux frères atteints respectivement. Dans les deux cas, plus de 74 % de l'exome ciblé étaient représentés avec une couverture supérieure à 40 fois. Les mêmes critères ont été appliqués comme pour la première famille pour le traitement et l'analyse des données (tableau 3).

	IV :6	IV :7
Read length	2x100	2x100
Total number of bases	9,2GB	9,8GB
Total number of reads	92,226,364	98,302,546
No.of mapped reads	86,168,510 (93.00%)	92,420,352 (94.00%)
Duplication rate	7%	6%
Nucleotide coverage of target regions: Bases >=20X coverage	92.68%	92.32%
Nucleotide coverage of target regions: Bases >=40X coverage	74.34%	76.87%
Total variants	59,253	58,680
Subset of filtered variants for coverage (>=10, %:>=15)	50,794	51,385
Subset of filtered variants for rs validation (max1)	9,872	9,819
Subset of filtered variants for minor allele frequency (<0,01)	5,579	5,422
Subset of filtered variants for prediction (SIFT and PolyPhen-2)	5,356	5,182
Subset of filtered homozygous variants	631	631
Subset of filtered shared homozygous variants with a coding effect	38	
Variant category nonsense	1	
frameshift	16	
missense	8	
synonymous	13	

Tableau 3: Traitement et analyse de données WES pour la famille 2.

L'analyse des bases de données d'expression en ligne (L'Amazonia, BioGPS, EMBL-EBI expression atlas) a été appliquée à 36 gènes qui ont passé notre processus de filtrage. Ceci révèle MAGEB4 comme le gène du candidat

Une substitution d'une *base* (c.1041 A > T) à la fin la région codante de gène *MAGEB4* a été identifiée chez les deux frères NOA. Cette substitution transforme le codon stop en un codon correspondant à une cystéine (p.X347C) et élimine la terminaison normale de *la* traduction, ajoutant potentiellement 23 acides aminés au C-terminal du gène *MAGEB4*.

MAGEB4 est un membre de la famille de type I des gènes MAGE, qui est regroupée sur le chromosome Xp22 - p21. MAGEB4 humain, qui a un seul exon, est exprimé uniquement dans les testicules et code pour une protéine de 346 acides aminés. Comme tous les autres gènes

de la famille MAGE, il a un domaine d'homologie MAGE bien conservé (MHD) qui peut être retrouvé jusque chez les protozoaires.

La mutation et la ségrégation dans la famille ont été confirmées par séquençage Sanger (figure 4).

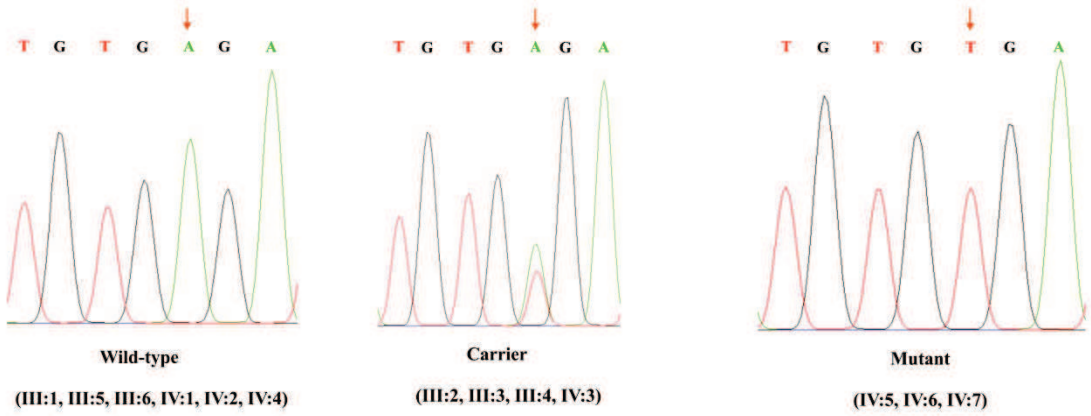

Figure 4: Images d'électrophérogramme de la mutation identifiée. Résultats du séquençage Sanger pour les patients mutants, porteurs et normaux. L'allèle en question est indiqué par la flèche orange. La mutation induit le changement d'un codon stop(TGA) en codon pour une cystéine (TGT).

La stabilité de l'ARN messager (ARNm), la localisation cellulaire et la dimérisation de la protéine pour des formes types sauvage (Wt) et mutantes ont été comparées, aucune différence n'a été notée (figure 5-7).

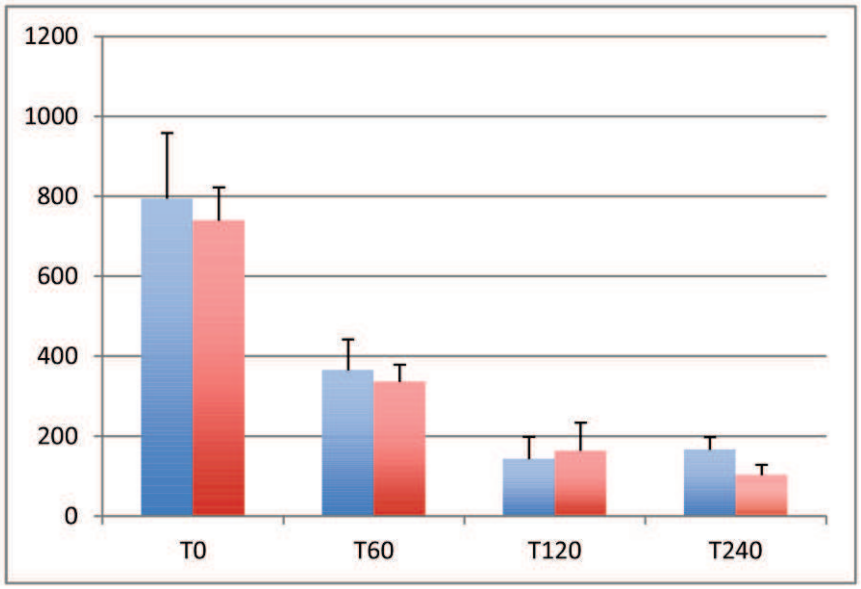

Figure 5: RT-PCR quantitative de MAGEB4. Dosage des ARNm de MAGEB4 de type sauvage ou mutant exprimé dans des cellules HeLa traitées avec l'actinomycine pour 1, 2 ou 4 heures.

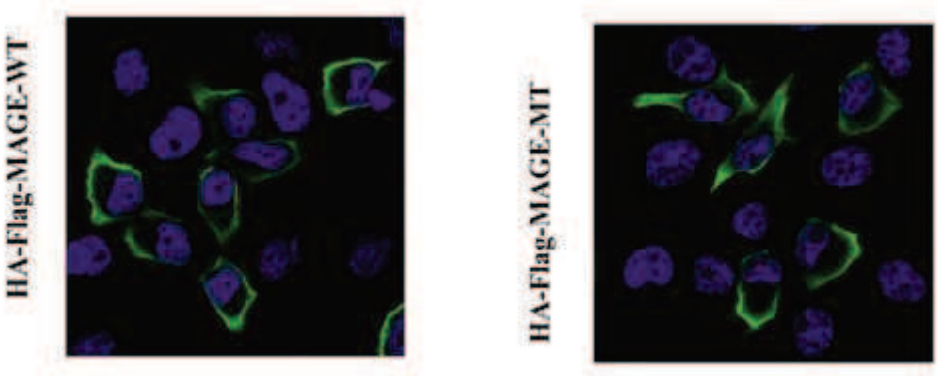

Figure 6: Images confocale de marquage en immunofluorescence de MAGEB4 type sauvage ou mutant HA-étiqueté transfectées dans des cellules HeLa. Les noyaux sont contre-colorés au DAPI. Grossissement 400x .

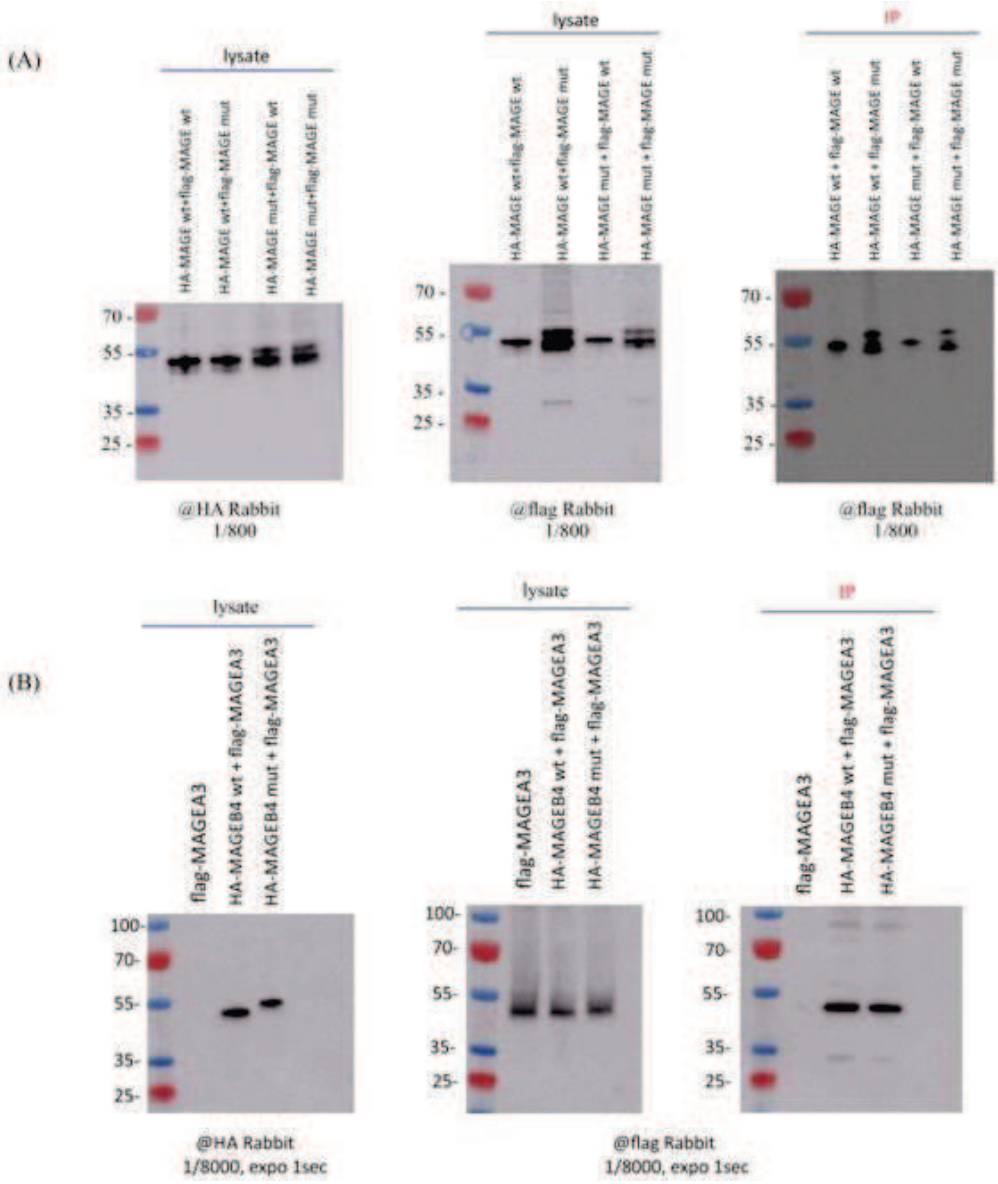

Figure 7: Interaction des protéines MAGEB4 avec MAGEA3 (A) Immunoprécipitation de MAGEA3 étiqueté d'un Flag par le type sauvage ou mutant de MAGEB4 étiqueté d'un HA. (B) Immunoprécipitation du sauvage ou mutant de MAGEB4 étiqueté d'un Flag par le type sauvage ou mutant de MAGEB4 étiqueté d'un HA.

Puisque MAGEB4 est hautement homologue à MAGEA3 au niveau du domaine MAGE conservé, le modèle de MAGEA3 peut être utilisé pour prévoir les caractéristiques structurelles de MAGEB4 (figure 8).

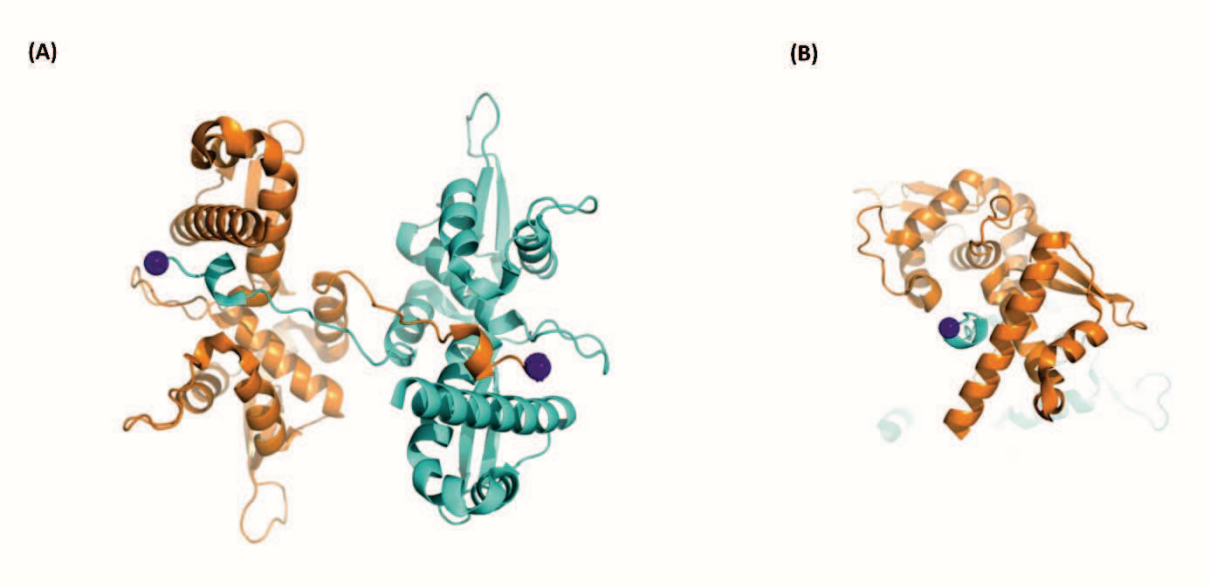

Figure 8: Prédiction de la structure de MAGEB4. (A) Représentation de la structure cristalline du domaine conservé de MAGEA3. Dans le cristal, le domaine de MAGEA3 (PDB : 4V0P) comprenant les résidus 102 à 310 (en cyan) s'étend par son bras C-terminal à une molécule associée par symétrie (en orange). Le dernier résidu dans la structure cristal, Arg 310, est indiqué par une sphère bleue. (B) Vue perpendiculaire du dimer montrant l'extrémité C-terminale d'un monomère pointant vers l'autre monomère.

L'absence des deux mutations a été évaluée chez 107 contrôles fertiles d'origine turc. Une analyse d'une cohorte *de* 108 patients infertiles turcs présentant un phénotype similaire n'a pas permis de mettre en évidence des mutations dans les gènes *MAGEB4* et *TEX15.*

Afin de déterminer le profil d'expression de MAGEB4 et TEX15 chez l'homme, nous avons réalisé des expériences de RT-PCR et d'*i*mmunohistochimie. Les résultats ont confirmé l'expression limitée au testicule des deux gènes (figure 9).

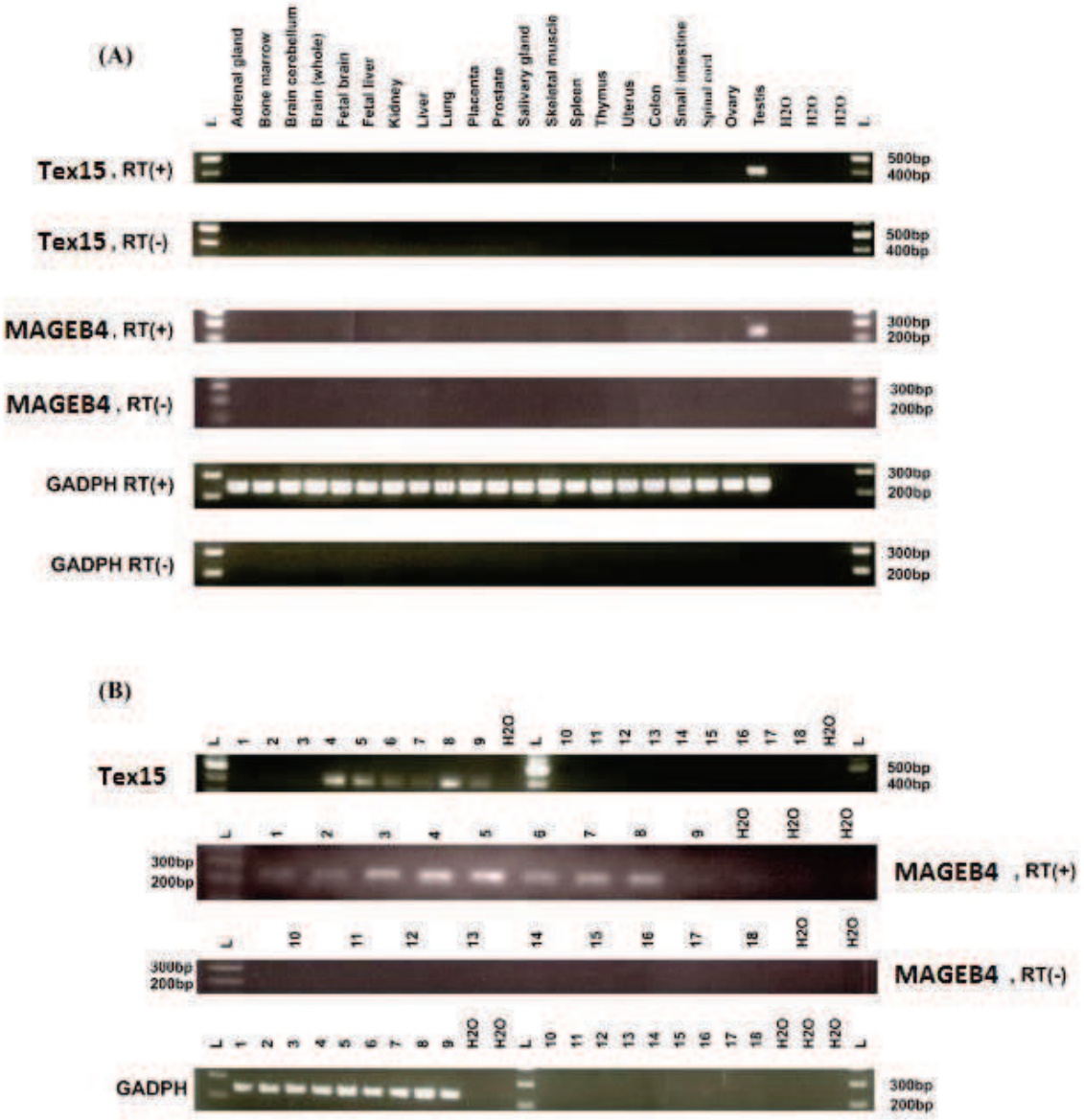

Figure9: Les études d'expression pour les deux gènes identifiés aux niveaux d'ARNm. GADPH a été utilisé comme contrôle interne pour la RT -PCR , le contrôle individuel sans transcriptase inverse ajouté à chaque échantillon et étiqueté comme RT (-) L : marqueur (A) résultats de RT-PCR sur différents tissus humains (B) résultats de RT-PCR sur l'homme échantillons testiculaires avec histologie différente. Echantillon 1-3 : cellules de Sertoli uniquement, l'échantillon 4-7 : arrêt de la maturation à des stades différents, échantillon 8 : homme fertile, échantillon 9 normale : pré pubère.

Pour la grande famille 3, selon des informations orales des patients, en plus des problèmes d'infertilité, quelques membres de la famille présenteraient une anosmie. Une telle association infertilité, anosmie est compatible avec un syndrome de Kallmann. Afin d'éliminer cette hypothèse, avec la collaboration du Dr Dodé (Institut Cochin, Paris), l'ensemble des gènes impliqués dans diverses formes du syndrome de Kallman ont été analysée, sans qu'aucune mutation ne soit trouvée (tableau 4).

Gene	Syndrome	Exon #
KAL1	KS1	14
FGFR1	KS2	18
PROKR1	KS3	3
PROK2	KS4	3
CHD7	KS5	38
FGF8	KS6	5
SOX10	KS	4
GNRHR	HH7	3
KISS1R	HH8	5
TAC3	HH10	7
TACR3	HH11	5
GNRH1	HH12	3
KISS1	HH13	3
WDR11	HH14	29
HS6ST1	HH15	2
SEMA3A	HH16	17

Tableau 4 : Liste des gènes scannés pour des syndromes liés à Kallmann. KS : syndrome de Kallmann HH: hypogonadisme hypogonadotrophique.

L'ADN de l'ensemble des enfants de cette famille a été analysé par puce à SNP; 5 régions homozygotes partagées par tous les hommes infertiles et 4 régions partagées par toutes les femmes infertiles ont été identifiées. Aucune région partagée par tous les membres infertiles de la famille n'a été identifié. Le WES d'ADN obtenu de 2 hommes infertiles et 2 femmes infertiles a été réalisé.

L’analyse d’expression n’a révélé aucun candidat gène plausible partagé par les quatre échantillons. Cependant, l’analyse a révélé une mutation homozygote de frame-shift dans OR2T35 (olfactory receptor, family 2, subfamily T, member 35) le gène est retrouvé chez tous les échantillons analysés par WES. La variation est une délétion de 7 bases, c.609_615del, qui remplace un codon codant pour une cystéine par un codon-stop (p.Cys203*). Cependant, les résultats n’ont pu être confirmés chez les membres de la famille par séquençage Sanger ; il y a une incohérence entre les résultats de WES et Sanger. Puisque deux femmes infertiles ont été identifiées de type sauvage pour la mutation avec le séquençage Sanger, nous avons conclu que cette délétion ne pouvait pas être la raison du phénotype d’infertilité dans la famille.

Effet destructeur prédit de la mutation non-sens sur la fonction de la protéine nous a dirigés à choisir CPNE1 (Copine 1, MIM: 604205) comme un gène candidat pour les hommes infertiles. Une substitution homozygote introduisant un codon stop dans l'exon 10 du gène a été identifié dans CPNE1 chez deux frères infertiles. Cependant, le troisième frère infertile a été identifié hétérozygote pour la substitution par un séquençage Sanger, il a été conclu que la c.646C > T substitution dans CPNE1 gène ne peut pas être la raison de la stérilité mâle phénotype dans la famille.

Prenant en considération le fait que la variation d'intérêt pouvait être dans un exon mal ou non - couvert, nous avons considéré chaque échantillon séparément et analysé les données sans filtrage pour la couverture. Deux candidats ont été identifiés ; une variation du cadre de lecture du gène TCEAL6 sur chromosome X et une variation de faux-sens sur le gène RGPD4.

Les membres de la famille ont été analysés pour la variation TCEAL6, le père a la mutation de sorte qu'il a été conclu que la variation n’est pas responsable du phénotype d’infertilité dans

la famille. La variation dans RGDP4 est trop fréquente dans la base de données ExAC (min 30% dans chaque population), on a conclu que la variation RGDP4 identifiée ne pouvait pas être raison pour le phénotype d'infertilité dans la famille.

Conclusion et Perspectives

L'étude de trois familles turques consanguines, nous a permis d'identifier deux nouveaux gènes impliqués dans une infertilité masculine.

Dans famille 1, tous les mâles affecté, présente une mutation à l'état homozygote dans le gène *TEX15*, mutation stop responsable soit de l'absence de la protéine ou au mieux de la production d'une protéine tronquée des 2/3. La mutation identifiée sur le gène *TEX15* est un nouvel exemple de défauts autosomique récessif dans l'infertilité masculine. Nos données suggèrent que la mutation identifiée puisse corréler avec une diminution du nombre de spermatozoïdes au fil du temps. Un test diagnostique identifiant la mutation chez un patient pourrait fournir une indication d'organiser au plus tôt une cryopréservation du sperme.

Dans la famille 2, tous les mâles affectés présentent une mutation non-sens dans le gène *MAGEB4* lié au chromosome X, mutation responsable de la perte de la terminaison normale de traduction, menant, très probablement, à une extension de 23 acides aminés de la protéine. Concernant MAGEB4, aucune information n'est disponible dans la littérature sur son éventuelle fonction. De plus, les données sur les mutations non-stop sont rares et très variées. Elles peuvent conduire à une instabilité de l'ARNm ou de la protéine ou à la production effective d'une protéine avec une adjonction d'acides aminés.

Nous avons montré ici que la stabilité de l'ARNm de *MAGEB4* n'est pas modifiée at que la protéine peut être produite, qu'elle est stable et est localisée dans le même compartiment

cytoplasmique que la protéine sauvage. Par immunoprécipitation, nous avons aussi montré que MAGEB4 mutant, aussi bien que le type sauvage, peut interagir avec MAGEA3 et former homo ou hétéro-dimer avec lui, mais aussi avec MAGEB4 type sauvage. D'autre part, il convient de garder à l'esprit que dans le tissu testiculaire, le comportement des transcriptions de MAGEB4 et protéine pourrait être totalement différente. La modélisation de la structure tertiaire de la protéine indique que l'addition des 23 acides aminés peut affecter la bonne homo ou hétérodimérisation de la protéine. Cette étude fournit la première indication sur la fonction physiologique d'une protéine MAGE.

Jusqu'ici, l'analyse n'a pas permis d'identifier une mutation responsable du phénotype d'infertilité dans la famille 3.

Les résultats de WES relèvent aussi des variations pour des ARN non codants (ncARNs). Des études récentes ont indiqué plusieurs types d'ARN non codants ; incluant des longs ARN non codants (lncARNs), micro ARNs (miARNs), les petits ARN interférents endogènes (endo-siARNs) et les Piwi-interacting RNA (piARN). Ils sont exprimés dans la lignée germinale mâle et sont nécessaires pour la spermatogenèse chez les animaux, constituent donc une cible réglementaire roman dans la spermatogenèse (Gou et al 2014, Luk et al 2014). Notre prochaine étape sera d'analyser les variations dans ncARNs. Analyser les CNV dans les régions homologues pourrait également être une alternative. L'analyse de méthylation et séquençage du génome entier seront également considérés.

Notre stratégie de combiner la puce SNP avec le WES ne nous a pas donné d'avantage réel. Des régions produites par la cartographie d'homozygotie sont généralement très grande, de plusieurs méga bases et peuvent contenir des centaines de gènes. Au bout du compte l'identification du gène muté est longue est coûteuse. De plus, les régions télomériques et

centromériques sont mal voir pas couverte et le chromosome X est exclu de l'analyse. La stratégie du WES seul a maintenant notre préférence.

Notre étude comprend un nombre encore trop limité de patients et de contrôles turcs. Le dépistage de mutations sera continué sur un plus grands de patients infertiles, mais aussi chez des hommes d'autres origines ethniques. Nous avons pu recueillir 69 l'ADN de patients français infertiles. Les patients ont été divisés en deux groupes ; azoospermic et oligozoospermic sévère. Les échantillons seront examinés d'abord pour le site des mutations et ensuite dans le cas où aucune mutation sera tous les exons et les limites exon / intron seront examinées.

De plus, nous poursuivons notre travail de recrutement de nouvelles familles de façon a identifié de nouveaux gènes responsables d'infertilité masculine.

Notre compréhension des bases génétiques de l'infertilité masculine a de grandes implications pour comprendre les causes d'infertilité. Ces études devraient permettre de nouvelles approches dans la prise en charge des patients, déjà en leur offrant un diagnostic, mais éventuellement en modifiant aussi leur prise en charge. Ceci est très bien illustré par le travail réalisé sur *TEX15* qui doit nous inciter à proposer au plus vite une cryopréservation spermatique. Cette étude est une illustration d'un continuum entre la recherche fondamentale et des partenaires cliniques.

Supplementary data Chapter V

Exome sequencing reveals a nonsense mutation in *TEX15* causing spermatogenic failure in a Turkish family

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Supplemental Data

Exome sequencing reveals a nonsense mutation in Tex15 causing spermatogenic failure in a Turkish family

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Table S1: Results of IVF attempt for the two NOA brothers (IV:1 and IV:3)

	IV:1	**IV:3**
#spermatozoa obtained after TESE	1 motile, 4 immotile	5 motile
# retrieved oocyte	13	6
# mature oocyte	5	5
# 2pn	3	4
Day of embryo transfer	D2	D3
# of transferred embryo	1	1
Clinical pregnancy	No	No

Table S2: Exome sequencing and data pipeline statistics

	IV:1	IV:3
Read length	2x100	2x100
Total number of bases	6GB	6,7GB
Total number of reads	60,119,546	67,003,084
No.of mapped reads	54,512,034 (90.00%)	63,500,545 (94.00%)
Duplication rate	11%	10%
Nucleotide coverage of target regions: Bases >=5X coverage	97.81%	98.06%
Nucleotide coverage of target regions: Bases >=10X coverage	95.06%	95.99%
Nucleotide coverage of target regions: Bases >=20X coverage	84.96%	88.25%
Nucleotide coverage of target regions: Bases >=40X coverage	56.99%	63.73%

Table S3: Summary of variant filtering process

	IV:1	IV:3
Total variants	56,908	55,894
Subset of filtered variants for coverage (>=10, :>=15)	43,869	44,928
Subset of filtered variants for rs validation (max1)	5,223	5,507
Subset of filtered variants for minor allele frequency (<0,01)	4,802	5,043
Subset of filtered variants for prediction (SIFT and PolyPhen-2)	4,613	4,830
Subset of filtered homozygous variants	981	1,064
Subset of filtered shared homozygous variants with a coding effect	17	
Variant category nonsense	1	
missense	5	
synonymous	11	

Table S4: Primer sequences used for the study. (A) Primers for *TEX15* exons and exon/intron boundries. PCR reactions were carried out with Taq DNA polymerase (Roche diagnostics GmbH, Mannheim, Germany), 1.5mM Mg concentration, 35 cycles and in total 30µl volume. (B) Primer sequences used in RT-PCR. Qiagen Multiplex PCR kit (Qiagen, Venlo, The Netherlands) was used for the amplification in total 25µl as a reaction volume and 30 cycles.

(A)

Exons	Primers	Forward	Reverse	Annealing temperature (°C)
Exon 1	1a	ctgtcacctttgtgcatttca	gactcctgttgggctctctg	61.8
	1b	cagggcaatgtaaggaccaa	atttctttggcctggggtat	61.8
	1c	tggaactgaaattggggaaa	tgacactgcattatcttctctgc	58.2
	1d	ggagacaaaaagcaggattctc	gcatgttttcatggctatgtg	58.2
	Mut	cagagcattgaattggaaagtg	tcactattctcccttcctgtgg	64
	1e	cgtatcagttgggaaggtctg	tcgtccttgggataatgaaga	60.8
	1f	tccagggatcagaacatacca	gagtggtctggggaaaacag	60.8
	1g	cctccacatcccaaagtacaa	cagttcctgatgctacatccaa	59.4
	1h	gcagagctgtagagaaaaagagc	gcttccacaaataaagggagaa	59.4
	1i	tcatacggcccttatagctaatc	cacgtactgcctcgttgttc	58.2
	1j	tccaggaaaacaggaccatc	tcactttccaaaggcaaaca	61.8
	1k	ggtttcgaggtatgctttgg	acagcatttggcataggaaa	58.2
	1L	tggcccacattaggaaagtc	aatccacgtccttccaaaaa	61.8
Exon 2	2	gatgatggcagaacaagcaat	tccttagcttactctcatccccta	61.8
Exon 3	3a	gccaatagaatgggaaaacg	cagaaaggcaggatttttgg	60.8
	3b	gctgcttttgcaatttgtga	tgccattgctgttgctgta	60.8
	3c	cagtctgcatgtaacccaaca	ccaatccattggcaattcta	60.8
Exon 4	4a	gggcaaataagcactttgatct	tcttctggaaatggctcttg	64
	4b	gcataatctcagtgtttccttgag	ctgcatggaccaaatagaaatg	64
	4c	agggaaggtcaagcaaagaac	gcctggcccatatttatgct	64

(B)

Primers	Sequence 5' to 3'	Annealing temperature(°C)
TEX15-cDNA-ex1F	tggcccacattaggaaagtc	60
TEX15-cDNA-ex2R	cttggatgcttgaatgttgc	
GADPH-F	gtggacctgacctgccgtct	
GADPH-R	ctgtagccaaattcgttgtc	

Table S5: Case-control association study performed for *TEX15* SNPs in Turkish (TK) males with non-obstructive azoospermia (NOA) and control individuals (CONT).

rsID	SNP (GRCh37)	Association with infertility already reported	Allele Frequencies				Genotype Frequencies		
			TK-CONT (n=214)	TK-NOA (n=170)	NOA vs CONT OR [95 CI]	P	TK-CONT (n=107)	HWE P	TK-NOA (n=85)
rs323344	Chr8:g.30702525A>C	with major allele A (p=0.008)[11]	f(C)= 0.070 f(A)= 0.930	f(C)= 0.059 f(A)= 0.941	allele A 1.206 [0,528-2,757]	0.409	f(CC)= 0 f(CA)= 0.140 f(AA)= 0.860	NS	f(CC)= 0.035 f(CA)= 0.047 f(AA)= 0.918
rs323345	Chr8:g.30702602T>C	with major allele T (p= 0.012)[11]	f(C)= 0.070 f(T)= 0.930	f(C)= 0.053 f(T)= 0.947	allele T 1.348 [0,575-3,162]	0.319	f(CC)= 0 f(CT)= 0.140 f(TT)= 0.860	NS	f(CC)= 0.035 f(CT)= 0.035 f(TT)= 0.930
rs323346	Chr8:g.30703431T>C	with minor allele C (**p=0.037**; **OR=1.474 [1,031-2,108]**)[26]	f(C)= 0.084 f(T)= 0.916	f(C)= 0.112 f(T)= 0.888	allele C 1.370 [0,695-2,701]	0.23	f(CC)= 0 f(CT)= 0.168 f(TT)= 0.832	NS	f(CC)= 0.035 f(CT)= 0.153 f(TT)= 0.812
rs323347	Chr8:g.30706224A>G	with minor allele A (**p=0,037**; **OR=1.462 [1.022-2.092]**)[26]	f(G)= 0.107 f(A)= 0.893	f(G)= 0.124 f(A)= 0.876	allele A 1.170 [0.624-2.196]	0.37	f(GG)= 0 f(GA)= 0.215 f(AA)= 0.785	NS	f(GG)= 0.035 f(GA)= 0.176 f(AA)= 0.789

[11]Aston et al., 2010 [26]Ruan et al., 2012 ; NS: not significant ; P: p-value obtained by Fischer's exact test; OR: odd-ratio; CI: confident interval; HWE: Hardy Weinberg Equilibrium; NS: not significant; in bold: significant p-value

Supplementary data Chapter VI

A stop codon mutation in the X-linked *MAGEB4* associated with azoospermia and severe oligospermia

Supplemental Data

Chapter VI

A stop codon mutation in the X-linked *MAGEB4* associated with azoospermia and severe oligospermia.

Table S6.1: Primer sequences of nine candidate genes identified through linkage analysis. Annealing temperatures and PCR product size are indicated for each pairs.

Gene name	Exon	Forward	Reverse	Annealing Temperature (°C)	Product size (bp)
GTSF1L	1a	ctcccaagcagtgcacaata	acagcctcatgttcctccag	63	606
	1b	atcgtgcaggagaaagaacc	ttgtcaaattgccctctgtg	63	644
EPPIN	1	gtgcagcctaggggtgtaag	tgggctaagatggtggaaag	61.8	417
	2	gccctgaggaaaagaactcc	tctcccaagtccagcagttt	61.8	397
	3	gggtctccttgcaaatgtgt	cttttgccaggtgcattctt	61.8	494
	4a	cccaaagcaaacccactact	cccttgcctaaaaagggttt	61.8	631
	4b	aggcttctggtgacatcattc	ccatcaacgataggctggat	61.8	751
	4c	taacggcttccagcttcatc	agctttcccatcactggaga	61.8	655
INSL6	1	ggaattccgcagtgtttgtg	ggcacaaattccacttcctg	63	505
	2a	agtttcacaaccctgcgaat	tgatgcccccaaaacaaa	63	517
	2b	tcacccctttggtaagacaaga	ggaaaacaacagggtcacca	63	461
TP53TG5	1	cctcacctctgccacataca	tttccttttctcccatgctg	64	349
	2-3	ggcatctcaaactcctgacc	cacctcttcagcctcagctc	64	695
	4a	gctcatcacacctcctcaca	attgcggtagggcttaatga	64	492
	4b	caaggacatcgagggatgat	cactaccagccacacaccac	64	410
	5	gcttaaagggaggcttttcc	cgctggcttctttggttcta	64	422
SPINT3	1	tgtgctttggctgaacactc	gaaatgggctctgtttgtgc	61.8	330
	2	agagcacagccattgtggat	tctcccagtgggttaagcag	61.8	619
SERPINA5	1-2	agctggtggtgtaaggcttg	cagttgtggcaaccaaatgt	61.8	599
	3a	gtgccatcccttctctttga	gttcctgaaggagctgctga	61.8	498
	3b	gtccagcacaaagatgcaga	caacaggcacccaaaagaac	61.8	493
	4	ctggggctgccataaagat	caggacttggatgtcacagg	61.8	549
	5	aggagccataaccaccattg	tggagtggagttgagtggaa	64.8	408
	6a	ctccatggctcagttgaaca	tctttgccactaccatgctg	61.8	602
	6b	tggggaaatgtggagaacat	agccaggcccatcagattat	61.8	584
	6c	cactcaagtgccattcatcc	agtggtgcctttcttcatgg	61.8	367

SPATA7	0	ccccagggaatatttcacaa	gtccctgcctccctttatc	61.8	387
	1	ccaaaagttcacggactgct	actcagccaggggtaggaag	61.8	666
	2	cgcaatcacctttaggaaa	cacatccagggcacaataat	61.8	410
	3	cattggcattatcagtgcaag	ttaatgagaagtgaagctggaggt	61.8	331
	4	aggatcttgtgttttccatcg	gcacaggaatttcagttttgc	61.8	367
	5	ttaggcttggccagtttgtc	ttgtgacagaggcctgaaca	61.8	630
	6a	tgtaaacccttgaggctatcat	ttatccaaaagatccccactg	61.8	413
	6b	gagctctggagccctgtatg	tacgcataaggtgcttgacc	61.8	483
	7	gatgggtctcattcttttgag	gcagaccttctgtatactggcta	61.8	321
	8	gcgctaccactgcttctaca	gaggacttgtcttggggaaa	61.8	800
	9	ccaaattctgaaacacttctgg	tgttcgagatgatggatatgct	61.8	403
	10	ccttgcataatttgagaaggtagac	ctcccaagtggtgaacttcc	61.8	464
	11	aacaggaaaggattagtcttcagc	ttcacttctcccaccaccac	61.8	351
	12a	gtggtggtgggagaagtgaa	ccccttctgaatgctttgat	61.8	410
	12b	gttattgtcggcaccaaagg	gtgagacccaggacagagga	61.8	602
FAM181A	1	tcctgtgattctgacctgga	gctgtaaagaagggctggtg	63	384
	2	gaatacggctcttggcacat	ctccctgctgcctactgtgt	63	522
	3a	tttagggctgagaggtgctg	cttttcctcatgggcacct	64.7	601
	3b	caaggagaaggtgctgagga	ctgggctcttcctccaaag	63	455
	3c	ctgccccttccagtaccat	ctatggtctgcacagggaca	63	510
	3d	agtgacccttcagccttgc	ctcctcaccctccttccttt	63	445

Table S6.2: Amplifying conditions and primers used in PCR rections. (A) MAGEB4 exon is 2142 bp in length, it was divided into three parts that superimposed about 70base. PCR reactions were carried out with Taq DNA polymerase (Roche diagnostics GmbH, Mannheim, Germany), 1.5mM Mg concentration, 35 cycles and in total 30µl volume. (B) Primer sequences used in RT-PCR. Qiagen Multiplex PCR kit (Qiagen, Venlo, The Netherlands) was used for the amplification in total 25µl as a reaction volume and 30 cycles.

(A)

Primer name	**Forward**	**Reverse**	**Annealing temperature (°C)**	**Product size (bp)**
MG1	gagccttgttgtcacccagt	tcccagatttcctcttcacg	65	953
MG2	acgatggaaaccagagcagt	caggcctccacattcttga	61.8	959
MG3	gttgctttattcgtggtttgtc	caggagccctccaaagtaca	65	841

(B)

Primer name	Forward	Reverse	Annealing temperature (°C)	Product size (bp)
MAGEB4-RT	ctgataaaggcgacgagagc	gtgcgctgagagactttcct	60	235
GADPH	gtggacctgacctgccgtct	ctgtagccaaattcgttgtc	60	216

Table S6.3 : Primers used for PCR in MAGEB4 constructs

Primers	Forward	Reverse
MAGEB4-whole exon	agaatgactaagaacaatagccca	ctacttcctggtcccaccaac
MAGEB4-Wt-coding	atgcctcggggtcagaagag	tcacatgggttgggaagagcta
MAGEB4-Mt-Coding	atgcctcggggtcagaag	ttattcatgcatcacaggaacag

Table S6.4: Primers used for cloning wild type and mutant form of MAGEB4 into vector

Forward

(5'-CTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTAGAATGACTAAGAACAATAGCCCAGT-3')

Reverse

(5'-CGCTTACAATTTACGCCTTAAGATACATTGATCTACTTCCTGGTCCCACCAACTTT-3')

Table S6.5: Homolog regions shared only by infertile males.

CHR	Start	End
2	179395764	180971826
	182019839	184590546
	185113825	188672873
	189518105	190064442
	191611878	192701461
	195663362	196622528
3	88326686	89293974
	95516434	96534999
	97999687	99948725
	101473064	104133713
	104421434	104989599

5	39880493	40253602
	64351549	64894645
	71387753	72469980
	72851572	73261254
7	10344828	10901891
	15473314	15804265
	48331310	48844298
8	121089053	122042130
9	4036564	6099080
	6513056	9985882
	10359368	10870887
	11266172	11433473
	11701459	15359878
	16053191	18420609
10	5588603	6022583
15	34477531	34833829
	34955596	36654501
20	40111844	46358273

Table S6.6: Homolog regions shared by all infertile males and the fertile sister

CHR	Start	End	# of genes in the region
11	82856844	83256927	21
	94713908	96450510	35
14	73033136	73829054	34
	88928501	97569409	188
	97840986	106010961	409
15	22589585	23315478	27

Table S6.7: Exome sequencing and data pipeline statistics together with summary of variant filtering process

	IV :6	IV :7
Read length	2x100	2x100
Total number of bases	9,2GB	9,8GB
Total number of reads	92,226,364	98,302,546
No.of mapped reads	86,168,510 (93.00%)	92,420,352 (94.00%)
Duplication rate	7%	6%
Nucleotide coverage of target regions: Bases >=20X coverage	92.68%	92.32%
Nucleotide coverage of target regions: Bases >=40X coverage	74.34%	76.87%
Total variants	59,253	58,680
Subset of filtered variants for coverage (>=10, :>=15)	50,794	51,385
Subset of filtered variants for rs validation (max1)	9,872	9,819
Subset of filtered variants for minor allele frequency (<0,01)	5,579	5,422
Subset of filtered variants for prediction (SIFT and PolyPhen-2)	5,356	5,182
Subset of filtered homozygous variants	631	631
Subset of filtered shared homozygous variants with a coding effect	38	
Variant category nonsense	1	
frameshift	16	
missense	8	
synonymous	13	

Supplementary data Chapter VII

Homozygosity mapping and whole exome sequencing in the quest of gene responsible for the infertility phenotype in large Turkish family

Supplementary data

Chapter VII

Homozygosity mapping and whole exome sequencing in the quest of gene responsible for the infertility phenotype in large Turkish family

Table S7.1: Genes that were scanned for Kallmann related syndromes. KS: Kallmann syndrome HH: hypogonadotropic hypogonadism

Gene	Syndrome	Exon #
KAL1	KS1	14
FGFR1	KS2	18
PROKR1	KS3	3
PROK2	KS4	3
CHD7	KS5	38
FGF8	KS6	5
SOX10	KS	4
GNRHR	HH7	3
KISS1R	HH8	5
TAC3	HH10	7
TACR3	HH11	5
GNRH1	HH12	3
KISS1	HH13	3
WDR11	HH14	29
HS6ST1	HH15	2
SEMA3A	HH16	17

Table S7.2: Homolog regions shared (a) only by infertile males and (b) only by infertile females.

(a)

For infertile males			
CHR	**START**	**END**	**Number of genes in the region**
6	136520887	136785134	6
	158594164	158913155	12
7	35503161	36090817	15
	41486337	41680390	1
	54625897	54872385	3
11	9268610	10392327	28
	111587219	112011283	19
15	79975318	80314940	9
22	35619815	37629994	73
	38335970	41358425	108

(b)

For infertile females			
CHR	**START**	**END**	**Number of genes in the region**
4	111711818	112363120	14
	42656122	43020957	5
	43394499	43622591	4
7	7310575	7761009	10
9	3879264	4036569	2
12	102745260	102978172	3
15	92037412	92422259	8

Table S7.3: Exome sequencing and data pipeline statistics

	II :1	**II :2**	**II :4**	**II :8**
Read length	2x100	2x100	2x100	2x100
Total number of bases	8.4 GB	10 GB	8.8 GB	9.4 GB
Total number of reads	84 658 466	100 233 990	88 738 858	94 202 994
Number of mapped reads	79 281 223 (93%)	95 246 211 (95%)	79 081 736 (89%)	83 832 436 (88%)
Duplication rate	6%	6%	15%	13%
Bases >=5X coverage	98.3%	98.6%	93.05%	93.01%
Bases >=10X coverage	96.92%	97.55%	89.99%	89.88%
Bases >=20X coverage	91.96%	93.6%	84.37%	84.3%
Bases >=40X coverage	73.44%	77.55%	70.57%	71.7%

Table S7.5 (a) Primer sequences for screening the frameshift mutation in OR2T35 gene (b) Results of OR2T35 mutation screening in all family members .

(a)

Primer	**Forward**	**Reverse**	**Annealing temperature (°C)**
OR2T-mut	CAGTTCAAATCTTCTACCTGACCC	AGGAACACGTAGCAAAGGCT	64

(b)

Sample	Fertility status/sex	Anosmia	WES result	Sanger result
I :1	Fertile male			Wt
I :2	Fertile female			Wt
II :1	Infertile female	*	Homo del	Wt
II :2	Infertile female	*	Homo del	Wt
II :3	Infertile female	*		Wt
II :4	Infertile male	*	Homo del	Hetero del
II :5	Fertile female			Wt
II :6	Fertile male			Wt
II :7	Fertile female			Wt
II :8	Infertile male		Homo del	Hetero del
II :9	Infertile male			Hetero del
II :10	Infertile female	*		Hetero del
II :11	Fertile female			Hetero del
II :12	Fertile female			Wt
II :13	Unknown, female			Hetero del

Table S7.6: (a) Primers to sequence CPNE1 on family members (b) Results of screening CPNE1 on family members

(a)

EXON	Forward	Reverse	Annealing temperature (°C)
1	AGGGACTGAATATCCCCCTCT	GTCCTCCTTGCATCCTCTCAG	64
2	TGTGTGGGATTTTGTTACGG	TGTGTGGGATTTTGTTACGG	65
3	TTGCCTGTGATAGCTTGAGG	GGCAGTTTCAAAACGCCTAC	64
4-5	CCAGGGTTGGTTGTATCTGC	TCAGGATAACAGGCAGGGTTA	64
6-8	CTAGGGGGTGCTGAGTGTTC	GCTGCCACACTTCTCTACCC	64
9-11 (mut)	GGCCTGTGACTAGCAAGGTT	AAATGAAACCAGGGTCATGC	62
12-14	TGTCAAGATTTGTCGGGTGA	AGGAATCCAACCCTGCATCT	65
15	GCAAGTGCTCAGGACTCCAT	CTCGAACCCAGTAGGCAGAG	65
16	CCCTGGTTTACAAGGGTGAA	AGGTGCAGGTTGCAGTGAG	64.7
17	CTGCCTTAGCCTCCCAAAG	AACAGCTGCCCAAAGTCTGT	64.7
18	CCCTTTCACCTTTCCCTGAT	TTTGAGTGGGGCTTTACACC	64

(b)

Patient code	Infertility status/sex	Allele status
I:1	Fertile male	heterozygous
I:2	Fertile female	heterozygous
II:1	Infertile female	heterozygous
II:2	Infertile female	heterozygous
II:3	Infertile female	Wt
II:4	Infertile male	**homozygous**
II:5	Fertile female	heterozygous
II:6	Fertile male	heterozygous
II:7	Fertile female	Wt
II:8	Infertile male	**homozygous**
II:9	Infertile male	heterozygous
II:10	Infertile female	heterozygous
II:11	Fertile female	heterozygous
II:12	Fertile female	heterozygous
II:13	Female, unknown fertility	**homozygous**

Table S7.7: Primers to sequence TCEAL6 mutation on family members

Primer	Forward	Reverse	Annealing temperature (°C)
TCEAL6-mt	CCACGTGAACACACACCTTAC	TTTATTGCCTTTGTAAGCGCAC	58.2

Figure S7.1: Visualisation of homozygous regions via HomoSNP program for CPNE1 gene location. Dark blue rectangles show homology >44SNPs while light blue rectangles shows homozygosity between 40-44 SNPs according to used microarray. II:4 and II:8 are infertile males, II:13 is a female with unknown fertility status. Infertile sisters (II:1 and II:2) are not homozygous for CPNE1 gene region

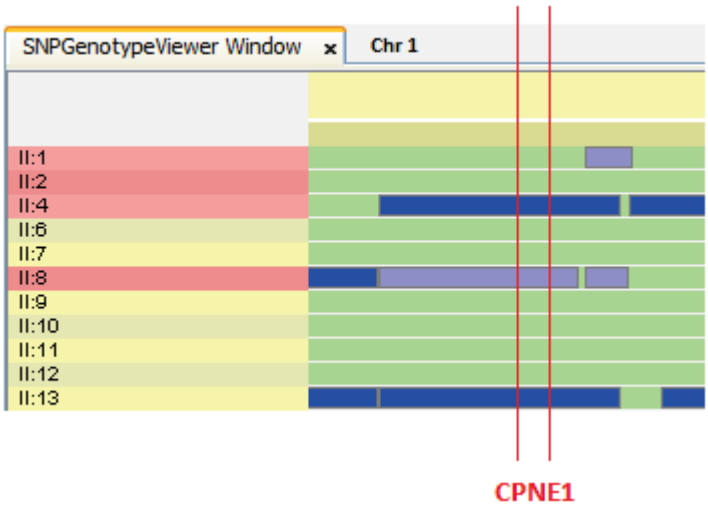
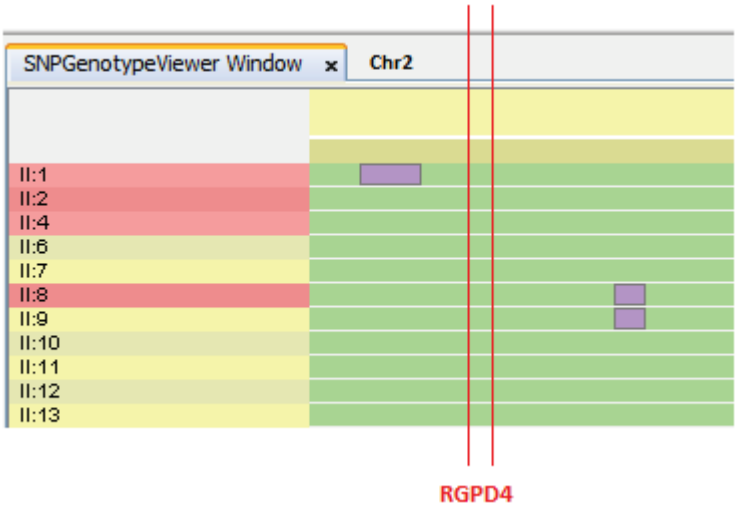


Figure S7.2: (a)Visualisation of homozygous regions via HomoSNP program for RGPD4 gene location. (b) SNP coverage around the gene RGPD4. For SNP array hg18 was used as a reference genome, RGPD4 gene location is 107,826,937-107,890,841.

(a)



(b)

SNP ID	Chromosome	Physical Position	dbSNP RS ID
SNP_A-1902230	2	107775181	rs860551
SNP_A-2281856	2	107778231	rs831969
SNP_A-2161619	2	107792241	rs17269251
SNP_A-1846596	2	107907419	rs6716554
SNP_A-1827469	2	107909534	rs2052819
SNP_A-1841212	2	107910514	rs1346328

RGPD4

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Özlem OKUTMAN

Genetics of male infertility

Genes implicated in non-obstructive azoospermia and oligozoospermia

Parmi les couples avec un projet parental, le facteur masculin d'infertilité est responsable d'environ 20%. Malgré de longues années d'activités d'assistance médicale à la procréation, un nombre important de cas reste idiopathiques. Considérant le nombre élevé des gènes potentiellement impliqués dans la gamétogenèse, il est fort probable que la majorité des formes 'idiopathiques' sont d'origine génétique. Dans l'étude présente, nous avons d'identifier deux nouveaux gènes impliqués dans une infertilité masculine. Nos données suggèrent que la mutation dans *TEX15* puisse corréler avec une diminution du nombre de spermatozoïdes au fil du temps. Un test diagnostique identifiant la mutation chez un patient pourrait fournir une indication d'organiser au plus tôt une *cryopréservation* du *sperme.* On a aussi identifié MAGEB4 liées à l'X comme un nouveau gène impliqué dans une infertilité masculine héritée. Cette étude fournit le premier indice sur la fonction physiologique d'une protéine MAGE.

Mots-clés: l'infertilité masculine, séquençage complet de l'exome, TEX15, MAGEB4, mutation non-sens

Among couples with a desire for a child, male factor is responsible approximately 20%. Despite long years of assisted reproductive activities, a significant number of cases remain idiopathic. Considering the high predicted number of genes involved in male gametogenesis, it is likely that most 'idiopathic' forms may have a genetic origin. In the present study, we have defined two new genes implicated in male infertility. Our data suggested that a nonsense mutation in TEX15 correlates with a decrease in sperm count over time. A diagnostic test identifying the mutation in man could provide an indication of spermatogenic failure and prompt patients to undertake sperm cryopreservation at an early age. We also identified *MAGEB4* as a new X-linked gene involved in an inherited male infertility. This study provides the first clue on the physiological function of a MAGE protein.

Keywords: male infertility, whole exome sequencing, TEX15, MAGEB4, nonsense mutations