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ARTIFICIAL TESTIS TO STUDY EARLY GONADAL DEVELOPMENT AND GERM CELL DIFFERENTIATION

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Artificial Testis to Study Early Gonadal Development and Germ Cell Differentiation

Thesis for doctoral degree (Ph.D.)

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"*Verily We created man from a product of wet earth; then placed him as a drop (of seed) in a safe lodging; then We fashioned the drop into a clot, then We fashioned the clot into a little lump, then We fashioned the little lump into bones, then clothed the bones with flesh, and then produced it another creation*." [23:12-14]

Holy Quran, 14 centuries ago, long before any ultrasound machine existed

To my parents and my lovely wife

ABSTRACT

Studying the early germ cell development is of great significance, as germ cells transfer the genetic material between generations. However, in humans, this type of studies *in vivo* is not applicable, for obvious ethical reasons. Interestingly, differentiating human pluripotent stem cells (hPS cells) towards germ cells *in vitro* was recently reported. Therefore, studying differentiation of hPS cells towards germ cells is intriguing. On the other hand, due to the advancement in cancer therapy, the survival rates of the patients receiving chemotherapy and radiotherapy are improving dramatically. However, the improved survival rates increased the demand on fertility preservation, since most of the cancer treatments are gonadotoxic. In case of pubertal and post-pubertal male patients, semen cryopreservation offers a feasible and efficient option. Meanwhile, pre-pubertal patients do not have this option. Thus, finding an option for or such patients is absolutely encouraged.

Hence, we aimed in this thesis at studying the early development and differentiation of the male germ cells *in vitro*, in order to find a robust protocol for fertility preservation for pre-pubertal male patients. Specifically, we investigated whether the culture conditions and gene expression profile could be used to predict the differentiation potential of human embryonic stem (hES) cells towards male germ cells, tried to optimize the culture conditions for rat germ cell differentiation using a three-dimensional (3D) culture system, investigated the possibility of differentiating rat germ cells *in vitro* from immature testicular tissue, and studied the effects of long term *in vitro* culture on testicular tissues from pre-pubertal patients.

We have found that undifferentiated hES cell lines exhibit different gene expression profiles. Furthermore, the suspension culture method resulted in downregulation of the pluripotency markers *NANOG* and *POU5F1*, compared to the culture on feeders. In addition, BMP7 stimulation resulted in an upregulation in the germ cell markers *KIT* and *DDX4* and somatic cell markers *FSHR* and *HS3BD1*. To study the optimal culture conditions for germ cell differentiation *in vitro*, rat testicular cells were cultured in a 3D culture system. We found that the choice of medium has an effect on Leydig cell functionality. In addition, the germ cells were migrating outwards the cell aggregations, making the conditions unfavorable for germ cell differentiation. Exploiting a reported method for murine germ cell differentiation *in vitro* to other species, we have obtained round spermatids expressing Crem and Acrosin (post-meiotic markers) from rat undifferentiated spermatogonia, using the organ culture system and MEMα medium + 10% KSR. Interestingly, when we cultured human pre-pubertal testicular tissue for long term *in vitro* using an organ culture system, we have found that the Leydig and Sertoli cells showed viability and functionality for up to 42 days and 21 days respectively.

In conclusion, we have assessed the effect of culture conditions on the differentiation potential of hES cells towards male germ cells *in vitro*. We have also investigated the culture conditions suitable for rat germ cell differentiation *in vitro* using a 3D culture system or an organ culture setup. In addition, we have also studied the effect of long term culture of human pre-pubertal testicular tissue *in vitro*.

LIST OF SCIENTIFIC PAPERS

I. Kristín Rós Kjartansdóttir, Ahmed Reda, Sarita Panula, Kelly Day, Kjell Hultenby, Olle Söder, Outi Hovatta, Jan-Bernd Stukenborg
A combination of culture conditions and gene expression analysis can be used to investigate and predict hES cell differentiation potential towards male gonadal cells
PLoS ONE, 2015, 10, 12

II. Ahmed Reda, Mi Hou, Luise Landreh, Kristín Rós Kjartansdóttir, Konstantin Svechnikov, Olle Söder, Jan-Bernd Stukenborg
In vitro spermatogenesis – optimal culture conditions for testicular cell survival, germ cell differentiation, and steroidogenesis in rats
Frontiers in Endocrinology, 2014, 5, 21

III. Ahmed Reda, Mi Hou, Timothy R Winton, Robert E Chapin, Olle Söder, Jan-Bernd Stukenborg
In vitro differentiation of rat spermatogonia into round spermatids in tissue culture
Published (Epub ahead of print) in MHR, 2016,
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IV. Jan-Bernd Stukenborg*, Ahmed Reda*, Joao Pedro Alves-Lopes*, Victoria Keros, Virpi Töhönen, Ragnar Bjarnason, Patrik Romerius, Michael Sundin, Ulrika Norén Nyström, Cecilia Langenskiöld, Rod T. Mitchell, Olle Söder, Kirsi Jahnukainen, Cecilia Petersen
Long-term culture of human testicular tissue from pre-pubertal boys subjected to gonadotoxic treatment regimens
Manuscript

(* these authors contributed equally to the work)

ADDITIONAL PUBLICATIONS NOT INCLUDED IN THE THESIS

1- Kristín Rós Kjartansdóttir, Anette Gabrielsen, Ahmed Reda, Olle Söder, Rosita Bergström-Tengzelius, Claus Yding Andersen, Outi Hovatta, Jan-Bernd Stukenborg *, Jens Fedder*
Differentiation of stem cells upon deprivation of exogenous FGF2: a general approach to study spontaneous differentiation of hESCs in vitro
Systems Biology in Reproductive Medicine, 2012, 58: 330–338

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Male germ cell development in humans
Horm Res Paediatr, 2014; 81:2–12

3- Maha Al-Asmakh*, Jan-Bernd Stukenborg*, Ahmed Reda, Farhana Anuar, Mona-Lisa Strand, Lars Hedin, Sven Pettersson, Olle Söder
The gut microbiota and developmental programming of the testis in mice
PLoS ONE, 2014, 9, 8

(* these authors contributed equally to the work)

CONTENTS

LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
AA	Amino acids
Acr	Acrosin
ACTB	Beta actin
ALL	Acute lymphoblastic leukemia
AMH	Anti-Müllerian hormone
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AR	Androgen receptor
ART	Assisted reproductive techniques
Bcl2	B-Cell CLL/Lymphoma 2
BDNF	Brain-derived neurotrophic factor
BLIMP1	B lymphocyte-induced maturation protein 1
BMP	Bone Morphogenetic Protein
BSA	Bovine serum albumin
BTB	Blood testis barrier
cAMP	Cyclic adenosine monophosphate
c-Myc	V-Myc myelocytomatosis avian viral oncogene homolog
Crem	cAMP responsive element modulator
CSF1	Colony stimulating factor 1
CTNNB1	Catenin Beta 1
DAB	3, 3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole
DAZ	Deleted in azoospermia
DAZL	Deleted in azoospermia-like
DDX4	DEAD Box Protein 4
DMSO	Dimethyl sluphoxide
DNA	Deoxyribonucleic Acid

d*pc*	Days *post coitum*
d*pp*	Days *postpartum*
DTM	Decellularized testicular matrix
EBs	Embryoid bodies
ECM	Extracellular matrix
EEF1A1	Eukaryotic Translation Elongation Factor 1 Alpha 1
ELISA	Enzyme Linked Immunosorbent Assay
Erk	Extracellular signal-regulated kinase
ES	Embryonic stem
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor
GABRB3	Gamma-Aminobutyric Acid Type A Receptor Beta3 Subunit
GAL	Galanin And GMAP Prepropeptide
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDF3	Growth Differentiation Factor 3
GDNF	Glial Cell Derived Neurotrophic Factor
GFP	Green fluorescent protein
GFRα1	Glial cell line-derived neurotrophic factor family receptor-α1
GnRH	Gonadotropin releasing hormone
GSK3	Glycogen synthase kinase
Gy	Gray
hCG	Human chorionic gonadotropin
HDRBT	High-dose-rate brachytherapy
hEG	Human embryonic germ
hES	Human embryonic stem
hFFs	Human foreskin fibroblasts
hiPS	Human induced pluripotent stem
HL	Hodgkin’s lymphoma
hPGCLCs	Human primordial germ cell-like cells

hPS	Human pluripotent stem
HRP	Horseradish peroxidase
HS3BD1	3β-Hydroxysteroid dehydrogenase
HSCT	Hemopoietic stem cell transplantation
ICM	Inner Cell Mass
IgGs	Immunoglobulins
IL1α	Interleukin 1 α
INSL3	Insulin like 3
IU/L	International units per liter
IVF	*In vitro* fertilization
IVS	*In vitro* spermatogenesis
JMML	Juvenile myelomonocytic leukemia
JNK	C-Jun N-terminal kinase
KIT	V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene-Like Protein
Klf4	Krueppel-like factor 4
KO-DMEM	Knockout Dulbecco Modified Eagle's Medium
KS	Klinefelter syndrome
KSR	Knockout Serum Replacement
LH	Luteinizing Hormone
LHCGR	Luteinizing Hormone/Choriogonadotropin Receptor
LIF	Leukemia Inhibitory Factor
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplatic syndromes
MEF	Mouse embryonic fibroblast
MEK	MAPK/Erk kinase
MEMα	Minimum Essential Medium Alpha
mEpiS	Epiblast derived mouse stem
mRNA	Messenger Ribonucleic Acid
MSCs	Mesenchymal stem cells
NEAA	Non-essential amino acids

NEUROD1	Neuronal Differentiation 1
NHL	Non-Hodgkin’s lymphoma
NT3	Neurotrophin-3
OLIG2	Oligodendrocyte Lineage Transcription Factor 2
PAX6	Paired Box 6
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
Pen/Strep	Penicillin-streptomycin
PFA	Paraformaldehyde
PGC	Primordial germ cell
PI3K- AKT	Phosphatidylinositol-3-Kinase and Protein Kinase B
Plzf	Promyelocytic Leukemia Zinc Finger
PMCs	Peritubular myoid cells
POU5F1	POU domain, class 5, transcription factor 1
PRDM	Positive regulatory domain zinc finger protein
PRM	Protamine
RA	Retinoic acid
RBPs	RNA binding proteins
RIA	Radioimmunoassay
ROCKi	Rho-associated coiled-coil kinase inhibitor
RT-PCRs	Reverse transcriptase-polymerase chain reactions
SCF	Stem cell factor
SCID	Severe combined immune-deficient
SCNT	Somatic cell nuclear transfer
SCOS	Sertoli cell only syndrome
SMAD	Contraction of Sma and Mad (Mothers against decapentaplegic)
SOX	SRY box
SRY	Sex determining region Y
SSCs	Spermatogonial stem cells
SSEA	Stage-specific embryonic antigen
STAR	Steroidogenic acute regulatory protein

STRA8	Stimulated by retinoic acid gene 8
SYCP3	Synaptonemal complex protein 3
TBI	Total body irradiation
TBS	Tris buffered saline
TDGF1	Teratocarcinoma-derived growth factor 1
TEM	Transmission electron microscopy
TESE	Testicular sperm extraction
Tfap2c	Transcription factor AP-2 gamma
TGFβ	Transforming growth factor β
TLDAs	Taqman Low-Density Arrays
TNFα	Tumor necrosis factor α
TRA	Tumor-related antigen
Tspo	Translocator protein
TUNEL	Terminal deoxynucleotidyl transferase dutp nick end-labeling
UCHL1	Ubiquitin c-terminal hydrolase l1
UCS	Umbilical cords stem
VIM	Vimentin
WNT3A	Wingless-type MMTV integration site family member 3 A
WT-1	Wilms tumor gene 1
ZBTB16	Zinc finger and BTB domain containing 16

1 INTRODUCTION

Among multicellular organisms, the cell is considered the building block, where each cell type is specialized for a certain function. Germ cells are a unique type of cells, specialized in transferring the genetic material from one generation to the next. Based on that, the presence of healthy germ cells is extremely important for each organism to ensure passing its genetic material to the next generation. In humans, as they are not an exception, germ cells are the means by which humans can parent their own biological children. Hence, studying the germ cell development is of a great importance to humans. However, germ cell development begins at the early fetal life, which makes this type of studies *in vivo* extremely difficult, due to obvious ethical reasons. In order to study the early germ cell development *in vivo*, one option is to study other mammals (e.g. rats or mice) and extrapolate the observations to have an idea about the early germ cell development in humans. As an alternative, hES cells can be differentiated towards germ cells, allowing the researchers to study closely the early stages of germ cell development.

On the other hand, germ cell differentiation *in vitro* is a promising tool that can help a lot of infertile people or people who are at high risk for infertility. However, understanding the spermatogenesis process *in vivo* is crucial to develop a robust system for *in vitro* germ cell differentiation. Therefore, germ cell differentiation *in vivo* will be discussed in details.

1.1 HUMAN STEM CELL DERIVED GERM CELLS

Notably, hES cells can differentiate into different cell lineages (1-4). One of the cell lineages they can differentiate to is the germ cell lineage. By differentiating hES cells towards germ cells, it gives us a unique opportunity to study the early germ cell development in humans.

1.1.1 Human Pluripotent stem cells (hPS cells)

The pluripotent term means the ability of a cell to give rise to other different cell types, while stem cell indicates the one-cell origin of different cell types (5). Thus, pluripotent stem cells are the cells that give rise to different types of cells. We will discuss here the origin, properties, and characterization of stem cells.

1.1.1.1 Origin and potency of stem cells

In mammals, after fertilization of the oocyte by a sperm, the zygote cell is formed. This zygote cell divides into 2, 4, 8, and 16 cells, which is called then the morula (6,7). Up to this stage, the cells are called balstomeres. Blastomeres are characterized at this stage by being totipotent, which means that they can give rise to the embryonic and the extraembryonic tissues (7). After 3 more cell divisions, the 128 cells formed are called blastula. In the blastula stage, the cells differentiate to give two different structures; an extraembryonic outer layer called trophoplast, and an inner mass of cells called inner cell mass (ICM). By this differentiation, the blastula is called blastocyst (7). The trophoplast cells will contribute to the placenta and will not contribute to the forming fetus, while the cells in the ICM are described

as pluripotent cells, which means that they can give rise to any of the cell types forming the fetus (8,9). Upon gastrulation, these cells differentiate to the three different germ layers (endoderm, mesoderm and ectoderm) which will form the fetus later. After this stage, the potent cells in the embryo can give rise to either many cell lineages, but not all cell lineages (multipotent) or to one cell lineage only (unipotent) (10).

1.1.1.2 Properties of stem cells

Pluripotent stem cells are characterized by two main properties; the ability to self-renew and the ability to differentiate towards the three different germ layers (endoderm, mesoderm and ectoderm) (1,11). Normal cells (non-stem cells) have a limited capacity of self-renewal. The chromosomes in these cells have terminal Deoxyribonucleic Acid (DNA) segments called telomeres. Upon DNA replication by DNA polymerases, each replication cycle implies deletion of DNA sequence from the telomeres, which means shorter chromosomes (12). In normal cells, this process can occur few times, after which the whole telomere is deleted and the chromosome starts to lose basic segments of its DNA. The normal cell cannot tolerate such loss, leading to cell death (13). On the contrary, pluripotent stem cells express uniquely an enzyme called telomerase. This enzyme is responsible for maintaining the telomere stable in each cell cycle, leading to a prolonged lifetime for the stem cells, implying so many cell divisions (12,14).

Stem cells divide either by symmetric division or asymmetric division. In case replenishing of cells is needed, stem cells divide symmetrically to give two identical daughter stem cells. When stem cells are giving rise to other lineage, they divide asymmetrically to give one daughter stem cell and one daughter cell from the new cell lineage (15).

1.1.1.3 Characterization of stem cells

Discovery of the different sources of stem cells necessitated the characterization of these cells, in order to recognize them (16). This characterization is based on morphological basis, expression of specific cell markers, and differentiation potential *in vivo* and *in vitro* (17).

Morphologically, hPS cells are characterized by high nucleus to cytoplasm ratio with a prominent nucleolus. When cultured *in vitro*, they form flat compact colonies with distinct borders (1,17). On the other hand, certain markers are expressed solely by hPS cells. These markers can be used to recognize stem cells from other types of cells. The markers include surface markers; such as stage-specific embryonic antigen 3 (SSEA3), SSEA4, tumor-related antigen (TRA)-1-60, TRA-1-81, and alkaline phosphatase (AP) (1,16,18), and transcription factors; such as POU class 5 homeobox 1 (POU5F1 or OCT4), NANOG and SRY (sex determining region Y) box 2 (SOX2), which are needed for pluripotency (16,19). As the cells become more differentiated, they lose the ability to differentiate towards different cell types and to self-renew (20). Hence, the expression of the pluripotency markers is inversely proportional with the expression of differentiation markers. The differentiation potential of stem cells can be evaluated *in vivo* and *in vitro* (21). The *in vivo* teratoma assay depends on the idea that injecting the stem cells into severe combined immune-deficient (SCID) mice

results in the formation of teratoma, which contains cells from the three germ layers (endoderm, mesoderm and ectoderm) (1). Meanwhile, the *in vitro* assay is based on the ability of stem cells to form spontaneously differentiated embryoid bodies (EBs), containing cells from the three germ layers (22).

1.1.2 Sources of hPS cells

There are three main sources of hPS cells; from embryos (embryonic stem cells, hES cells), from the adult tissues (adult or somatic stem cells), or by induction (human induced pluripotent stem cells, hiPS cells). Other sources include carcinoma stem cells (23), embryonic germ cells (hEG cells) (24), and umbilical cords stem cells (UCS cells) (25).

The first embryonic stem (ES) cell line derived from mouse was reported in 1981 (11,26). Seventeen years later, the first human ES (hES) cell line was isolated. Using the leftover embryos from *in vitro* fertilization (IVF) clinics, cells from the ICM could be isolated, either mechanically or enzymatically, and placed in culture conditions supporting the undifferentiated state, as shown in Figure 1 (1,27). Nowadays, the advancement in the stem cell technology allowed researchers to derive hES cell lines from isolated single blastomeres at the morula stage (28,29). On the other hand, in each tissue of the adult or juvenile human body, there are some cells that are responsible for the regeneration of that tissue. These cells are called somatic (from the body tissues) or adult (could be found in the adult body) stem cells. They have the ability for self-renewal and the ability to differentiate towards a limited number of cell lineages *in vivo*, depending on the tissue (30,31). However, the plasticity of these cells increases *in vitro* and they become able to differentiate towards many cell lineages, even if they do not share the same germ layer (32-34). The importance of the adult stem cells as a clinical tool arises from the fact that they are much less controversial ethically than hES cells to use. Examples of the adult stem cells include the hemopoietic stem cells (35), the mesenchymal stem cells (MSCs) (36), the neural stem cells (37), and the spermatogonial stem cells (SSCs) (38,39).

Another source for hPS cells is the hiPS cells. By this technique, the normal somatic cells are induced by forcing the expression of certain pluripotency genes to become pluripotent. The first iPS cells produced from mouse fibroblasts was reported in 2006, by the forced expression of four transcription factors; *Pou5f1*, *Sox2*, Krueppel-like factor 4 (*Klf4*), and v-Myc myelocytomatosis avian viral oncogene homolog (*c-Myc*) (40). One year later, the first iPS cell line was produced from human fibroblasts, using the same four factors (41). Meanwhile, another group has reported similar success with different transcription factors (*POU5F1*, *SOX2*, *LIN28* and *NANOG*) (42). In these reports, they used retroviral and lentiviral transfection as a method of inducing the pluripotency gene expression. However, this method implies the risk of genomic instability, due to the integration of exogenous genes (43). Thus, the use of non-integrating viral vectors (Sendai virus) and episomal plasmids are becoming more popular (44,45), as they do not imply integration of exogenous DNA.

Recently, the production of hiPS cells by somatic cell nuclear transfer (SCNT) has been reported as well (46). Moreover, using proteins and messenger Ribonucleic Acid (mRNA) directly to produce hiPS cells has been also reported (47,48). Another problem facing the clinical use is using a feeder-free culture condition to derive hiPS cells. However, it was possible to achieve such derivation using Matrigel and fibronectin, albeit not xeno-free (49-52). Although the production of hiPS cells in xeno-free conditions on laminin was recently reported (53), it is still a challenge to derive hiPS cells in a combination of feeder-free, xeno-free, chemically defined, and integration-free conditions.

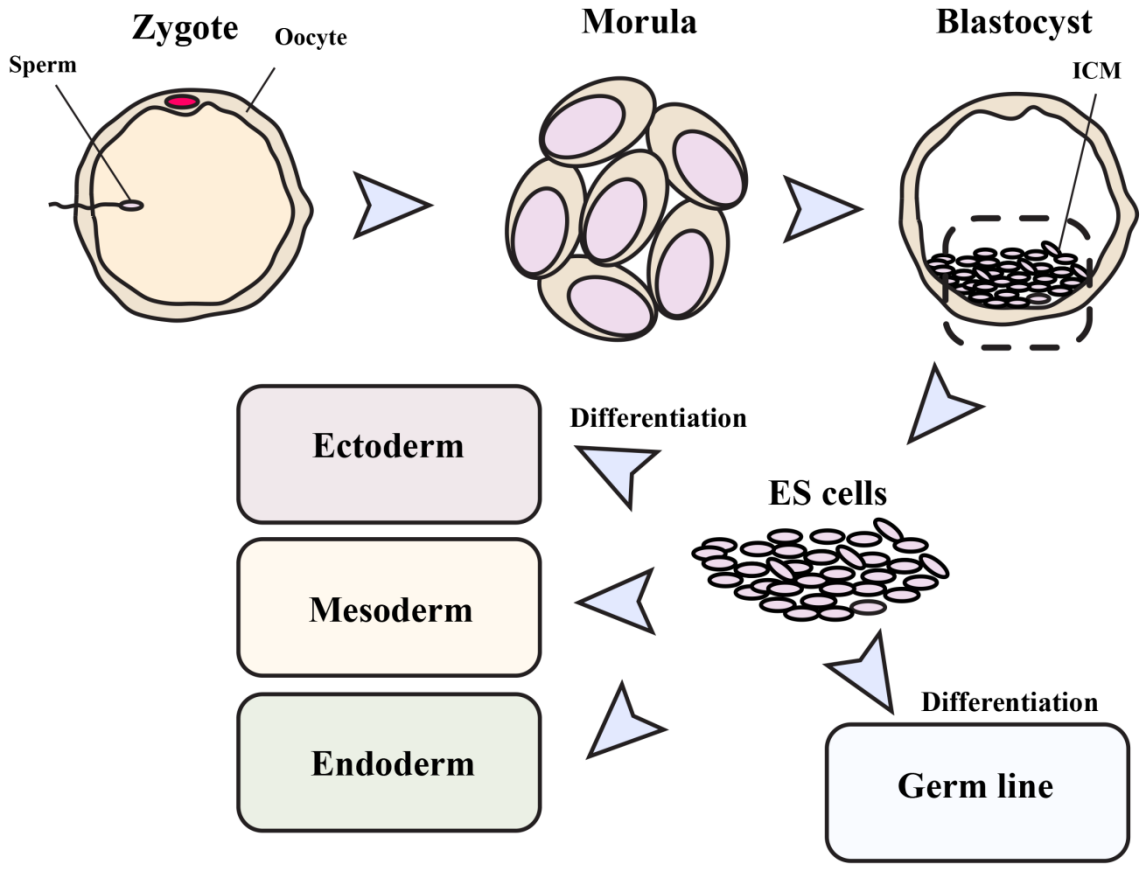

Figure 1. The embryonic stem (ES) cells. The zygote is formed when a sperm fertilizes an oocyte. This zygote (one cell) develops to the morula (16 cells) which develops to the blastocyst. The ES cells are derived by isolating the inner cell mass (ICM) from blastocysts. The ES cells have the differentiation potential towards the three germ layers; ectoderm, mesoderm, and endoderm, as well as the germ line.

1.1.3 Culture conditions for hPS cells

As discussed earlier, hES cells are undifferentiated cells that are not specialized for any specific function. They can differentiate towards any cell lineage from the three germ layers. Hence, when the hES cells cultured *in vitro*, they need to be kept under certain conditions that allow their propagation but inhibit their differentiation. It has been shown that hES cells need

basic fibroblast growth factor (FGF2) to keep their pluripotency, through the Phosphatidylinositol-3-Kinase and Protein Kinase B (PI3K-AKT) and MEK (MAPK (Mitogen-activated protein kinase)/Erk kinase)-ERK (Extracellular signal-regulated kinase) pathways (54-59). Moreover, Transforming growth factor β (TGFβ) signaling through the Contraction of Sma and Mad (Mothers against decapentaplegic) SMAD2,3 is also required to maintain the pluripotency of the hES cells (60,61). The first hES cell line was derived on a mouse embryonic fibroblast (MEF) feeder layer using serum conditioned medium. The feeder layer was essential to support the attachment of the cells and the maintenance of pluripotency for the hES cells, by providing FGF2 and TGFβ1 (1). However, the serum can vary from one batch to another, and as the animal-derived component can have the risk of viral contamination. Besides, if the clinical use is intended, the culture condition should be chemically defined and xeno-free as much as possible (62). Recently, researchers aim at finding the optimal culture conditions to keep the hES cells undifferentiated in culture using feeder-free, xeno-free, serum-free, and chemically defined medium. In that context, the mouse fibroblast could be replaced by human feeder cell layer, in order to minimize the animal-derived substances in the culture (63-65). Later on, the feeder layer could be replaced by Matrigel, which is a mouse tumor extract, fibronectin and laminin (66-69), and the serum has been replaced by knockout serum replacement (KSR) (70,71). In another attempt to keep the hES cells undifferentiated in a feeder-free culture, the suspension method, which implies culturing of the hES cells in cell aggregates in low-adhesion plates, has been reported (72). The passage of the hES cells can be performed enzymatically (e.g. collagenase and trypsin) or mechanically (1,64,73). Both methods showed a very similar genomic profile for the hES cells after passaging (74). However, hES cells tend to have a poor survival after dissociation into single-cell level (75). This survival was much improved using Rho-associated coiled-coil kinase inhibitor (ROCKi) in the culture medium (76,77). Using laminin-521/E-cadherin and xeno-free Nutristem medium, it was possible to dissociate the hES cells to a single cell level with improved survival, without using ROCKi (28). In addition, it has been reported that 3D culture system was used successfully to culture hES cells (78-80). The 3D culture system in these studies helped the ES cells to form vascular structure, epithelial structure and insulin-producing islets, as these structures necessitate a spatial support to be formed.

The accumulating evidences are indicating that the hES cells resemble more the epiblast derived mouse stem cells (mEpiS cells) in culture requirements and gene expression profile than the mouse ES cells (mES cells) (81). Therefore, it is believed that there are two developmental states of stem cells; naïve (mES cells) and primed (mEpiS cells and hES cells) (61). The naïve state of mES cells requires Leukemia Inhibitory Factor (LIF) signaling and dual inhibition of MAPK/Erk kinase (MEK) and glycogen synthase kinase (GSK3) (82). Hence, mEpiS cells could be converted into mES cells using this dual inhibition (2i) (83-86). Since then, many researchers tend now to get the hES cells into the naïve state first prior to differentiation, using the (2i/LIF) culture conditions (87-92). However, this naïve state of the hES cells needs to be more characterized.

1.1.4 Differentiation potential of hPS cells

hPS cells can differentiate towards various cell lineages which enables them to be a prospective clinical tool in regenerative medicine. Depending on the culture conditions, researchers could differentiate hPS cells towards different cell lineages, as shown in Figure 1. However, it has been reported that the different hPS cell lines exhibit different gene expression profiles and hence different potentialities to differentiate towards the different cell lineages (16,93). Recently, hPS cells have been differentiated successfully towards neural cells (94,95), cardio muscular cells (96-98), hepatocytes (99), prostate cells (100), insulin-producing endocrine cells (101,102), and retinal epithelial cells (103). The source of the hPS cells is crucial to the application they are intended to be used for. Although hES cells are easily derived compared to the hiPS cells, however, the ethical issues and the risk for immune-rejection related to the hES cells makes hiPS cells more prone to clinical use. Nonetheless, generating hiPS cells takes a lot of time and effort. Moreover, it implies sometimes (depending on the transfection method) a risk for gene insertion or deletion due to exogenous DNA integration, which makes the use of hiPS cells still not fully exploited. Besides, the produced hiPS cells could retain some epigenetic modifications from its original somatic state (104). In that context, using the adult or somatic stem cells (e.g. hemopoietic stem cells, MSCs, and SSCs) as a clinical option is a promising strategy. They can be used directly as multipotent cells (e.g. hemopoietic stem cells) (105), de-differentiated first to more naïve stage by culturing *in vitro* and utilize them later as pluripotent cells (e.g. SSCs) (106,107), or transdifferentiate them to another cell lineage directly (e.g. SSCs (39) and MSCs (36)).

1.1.5 Differentiation of hPS cells towards male germ cells

The main aim for differentiating hPS cells towards male germ cells is to study the early human germ cell development. However, another aspect is to model human male infertility *in vitro*. Many groups have reported that hES cells differentiated towards male germ cells (108-114). In addition, others have reported similar results using hiPS cells (115-117). However, the efficiency of such method was very poor, making the improvement of such protocol inevitable. One of the important growth factors, Bone Morphogenetic Protein 4 (BMP4), has been reported to induce the differentiation of hPS cells towards the male germ cell lineage (108,113,118). Moreover, V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene-Like Protein (KIT) signaling and Wingless-type MMTV integration site family member 3 A (WNT3A) signaling were shown to induce this differentiation (108,118). It has been also reported that overexpression of the Deleted in azoospermia (*DAZ)* family genes, DEAD Box Protein 4 (*DDX4)* and Deleted in azoospermia-like (*DAZL)* induce the production of haploid cells from hPS cells (116,119,120). One more factor found to induce the differentiation was the Retinoic Acid (RA) (121). However, more recent protocols are available now to for differentiation of hPS cells towards male germ cells (122,123). The culture conditions used in such differentiation varied from utilizing feeder fibroblast layer (108,110,122) to EBs formation (112,120).

As mentioned earlier, hES cells resemble more the mEpiS cells (primed state) rather than the mES cells (naïve state). Knowing that the differentiation potential of mEpiS cells towards the germ cell lineage is less than the mES cells, it has been suggested that culturing the hES cells in a more naïve state would allow them to differentiate towards the germ cell lineage (124). Using four kinases inhibition (MAPK, GSK3, p38, and c-Jun N-terminal kinase [JNK]), it was possible to culture hPS cells under naïve state (90). These four kinases inhibition enabled one group to differentiate hPS cells under the naïve conditions towards human primordial germ cell (PGC)-like cells (hPGCLCs) with much improved efficiency (125). In another report, hiPS cells were differentiated towards hPGCLCs with similar efficiency while cultured in primed state (126).

Moreover, it has been shown that ES cells and iPS cells can differentiate *in vitro* to germ cell (127,128). These *in vitro* produced germ cells have been reported to differentiate to haploid cells in culture, which could be thought of as a clinical option for infertile males with Sertoli cell only syndrome (SCOS) (115,116). The putative germ cells produced by hPS cells differentiation could be isolated by different surface markers, such as glial cell line-derived neurotrophic factor family receptor-α1 (GFRα1), KIT, and TRA-1-81. However, these markers are not germ cell specific, which makes the use of other intracellular markers (e.g.DDX4) more appropriate (120). Besides, it has been recently reported that hPS cells could be differentiated towards haploid post-meiotic cells, though in a small population of cells (117-120,122,123,129). In these reports, DNA content analysis was performed using flow cytometry, along with examining the protein expression of some post meiotic markers; e.g. ACROSIN and PROTAMINE 1.

1.1.6 Molecular basis for stem cells differentiation to germ cells

In the undifferentiated pluripotent state, hPS cells express the pluripotency related genes, such as *POU5F1*, *SOX2*, and *NANOG*. Upon differentiation, the pluripotency genes are generally downregulated, while the differentiation and development genes are upregulated. However, when it comes to the germ cell lineage, this might not be the case. The early stages of germ cell development share the expression of some pluripotency related genes (e.g. *POU5F1*) with stem cells. Thus, the downregulation of such genes is not expected to coincide with the differentiation of hPS cells towards the germ cell lineage. However, *SOX2* was shown to be downregulated by the BMP4 signaling during the human germ cell specification (126,130). This comes in contrast to the mouse model, where *Sox2* is essential for the mouse germ cell specification and development (131-133). Another difference to the mouse model is the role of *SOX17* in human germ cell specification. Recently, *SOX17*, a known marker for endoderm development (134), has been shown to be essential for human germ cell specification (125). In the same context, B lymphocyte-induced maturation protein 1; *BLIMP1* (also known as positive regulatory domain zinc finger protein 1; *PRDM1*), which is a downstream of *SOX17*, was shown to stabilize the genes related to the germ cell lineage specification and to repress the neural differentiation (126). *BLIMP1* was also reported to inhibit the mesoderm and endoderm development (125).

On the other hand, FGF2 stimulates its receptor in the hPS cells undifferentiated state and the downstream MAPK-AKT and PI3K-AKT pathways, which inhibits the BMP signaling pathway and hence the differentiation (56,135). Then, upon stimulation with BMPs, differentiation of the cells is induced through the activation of the SMAD signaling (136). A member of the TGFβ family, ACTIVIN, was found to be important, along with NODAl, for self-renewal of the hPS cells (58,137). However, the NODAL/ACTIVIN pathway was shown to be important in the early PGCs development, as it ensures maintaining the cells undifferentiated, hence, not entering meiosis (138-140).

1.1.7 Ethical issues related to hPS cells

The ethical controversy of hPS cells usage in medicine and research depends on a great extent on the source of these stem cells, rather than the intended use itself. In case of hES cells, their derivation from the human embryos brings them to the frontline of this controversy. While the derivation of these cells is restricted in research to leftover embryos from IVF clinics at the first12 weeks of gestation, the ethical dilemma arises from the debate about whether to consider the embryo as a human being from the first day of conception or after a certain period of development (e.g. 120 days), in other words, it is about when life actually starts (141). The opposing argument for stem cell use is usually associated with the religious and ethical debate opposing abortion. In the USA, it was banned in 2001 to derive novel hES cell lines, despite accepting the already existing ones as a response for this controversy. However, no such limitations were imposed in the majority of the European countries (142). On the other hand, usage of the adult (or somatic) stem cells in medicine and research is not of a big controversy like the hES cells, as they can be derived from the body without any loss of life (143). Similarly, usage of the hiPS cells in clinical use can be considered much less controversial than hES cells, as their derivation does not imply destruction of any human embryos (141). However, for research purposes, ethical issue could evoke regarding the use of such cells in certain uses without consent from the donor, which would break the donor's confidentiality (144). This issue can be avoided by the proper consent from the donor. One more option to produce human pluripotent cells is by SCNT. The ethical issues related to such technique are even more controversial, starting from the creation of human embryo for the sake of research, passing through the usage of the SCNT technique *per se*, and maybe ending with the use of animal oocytes with human nuclei to produce the embryos (141,145).

Another issue to add to the ethical burden in this thesis was the usage of such stem cells to produce germ cells. These germ cells, once produced, could be used later in producing human beings, which would be of a great controversy. However, the main aim of germ cell derivation from hES cells was to study the early germ cell development, which implies the restriction to the early stages only, with no intention of using the produced germ cell in producing human beings. Moreover, not similar to the use of stem cells in clinical trial, producing germ cells does not include introduction of stem cells in the human body, which can imply some risk for the patient (141,146).

From one perspective, deriving the hES cells from the leftover embryos at IVF clinics is the important point. As the produced embryos need to be destroyed if not used for clinical purpose, it would be a waste to lose such material and not using it in research, especially if the production of a human being is not intended with this research. The usage of hES cells is of great importance in research, as it provides us with an unlimited supply of information about the early stages of development. Hence, it could be considered that using hES cells in research is acceptable ethically; based on the current method of derivation and the benefits and uses they are exploited for.

1.2 TESTIS

In mammalian males, the testis is a unique specialized organ characterized by the ability to produce androgens and gametes. These gametes are capable of transferring the genetic material to the next generation. Herein, we will discuss the structure, morphology and origin of testis in human and rodents, along with the germ cell development.

1.2.1 Structure and morphology

In human and rodents, the testes are found in the scrotum, which is located under the penis and formed of smooth muscles and skin. The function of the scrotum is to give about 2-3°C lower to the testes than the body temperature, which is 37°C. This lower temperature is important for proper spermatogenesis (147). The size of a healthy human testis ranges from 12 to 30 ml, with an average of 18 ml. Measuring the testis size is performed either by the orchidometer or by ultrasound measuring, which is proven to be more accurate (148). In rats, the average of testis size is about 18 ml, which is quite large in relation to the size of the body when compared to other species (149,150).

In all mammals, each testis is an oval structure composed of convoluted seminiferous tubules wrapped in a connective tissue sheath called tunica albuginea. This sheath makes some invaginations in the testis, forming lobules. Meanwhile, the seminiferous tubules divide the testis into two compartments; the intra-tubular compartment and the interstitial compartment. The intra-tubular compartment contains the germ cells, Sertoli cells lying on the basement membrane, and peritubular myoid cells (PMCs) surrounding the tubules, while the interstitial compartment contains Leydig cells, immune cells, blood vessels, and connective tissue (as shown in Figure 2). Within the seminiferous tubules, tight junctions between the Sertoli cells form the blood testis barrier (BTB). This BTB is responsible for protecting the haploid germ cells in the intra-tubular compartment from the immune system (151). The germ cells are differentiated in the seminiferous tubules, giving rise eventually to haploid cells called spermatozoa. These spermatozoa travel through the seminiferous tubules to the rete testis and then through the efferent ducts to the epididymis, where they get fully matured. The epididymis is formed mainly of three parts; the head (caput), the body (corpus) and the tail (cauda). The main function of the epididymis is to get mature the spermatozoa by gaining the ability to move and fertilize, while being kept in the tail of the epididymis. Afterwards, sperm pass through the vas deferens to the seminal vesicle, where they are stored till ejaculation through the urethra (152).

1.2.2 Origin and development

Due to the scarcity of the human material at the very early embryonic stages, most of the knowledge available about the testis development in humans is extrapolated from studying this development in other species (e.g. rodents). In mammals, the testis is derived from the mesoderm as an undifferentiated gonad, which has the potential to develop towards both types of gonads; testis and ovary. However, the decision to differentiate towards a specific type of gonad is pre-determined from the moment of fertilization, using the XY specification

model (XY for testis and XX for ovary). In other words, the expression of the *SRY* gene (located on the Y chromosome) is responsible for the sex determination (153).

In humans, the *SRY* expression begins at week 6 of gestation, leading to the expression of *SOX9* in some cells at the genital ridge. The *SOX9* expression results in the differentiation of these cells to immature Sertoli cells, through the FGF9 signaling pathway (154). These immature Sertoli cells produce then the Anti-Müllerian hormone (AMH) which is responsible for the regression and degeneration of the Müllerian duct. The other mesenchymal cells in the differentiating gonad develop, due to the *SOX9* expression in the differentiating Sertoli cells, to PMCs and fetal Leydig cells. The fetal Leydig cells produce testosterone, resulting in the development of the Wolffian duct, and dihydrotestosterone, resulting in the development of the external genitalia. However, the fetal Leydig cells persist in humans till the age of 6 months postnatally, afterwards, they disappear. Around puberty, the adult Leydig cells emerge once more, resuming the testosterone production (Figure 2). Hence, there is a peak of serum testosterone level around birth and up to 6 months of age, during which the descending of the testes into the scrotum takes place, followed by a quiescent period till puberty, where the testosterone levels rise once more (155,156). Meanwhile, descending of the testis into the scrotum takes place by the 7^{th} month of gestation or shortly before birth. In the differentiating gonad, the Sertoli cells aggregate with the PGCs, originating from outside the gonad, to form the testis cords, which are the precursors for the seminiferous tubules. The appearance of the testis cords at gestational week 7-8 is the first sign of the gonadal differentiation towards a testis (153,157). Regarding the immature Sertoli cells, they have two phases of proliferation in humans; the first starts from fetal life till the first year postnatally, and the second starts before puberty and persist till the age of 15 years (158,159). This proliferation is under the control of the gonadotropin; follicle stimulating hormone (FSH), while the maturation is stimulated by the presence of thyroid hormone (158). Sertoli cell proliferation is an important step, as the total number of Sertoli cells is quite relevant to the number of germ cells they can nurse, leading to a relevant number of sperm the testis can produce after puberty (160). Around 15 years of age, Sertoli cells stop proliferating and start showing evidences of maturation (Figure 2); such as the changes in morphology (polarization and tri-forked nuclei), secretion of Inhibin B, and expression of certain maturation markers (e.g. $P27^{KIP1}$) (158).

In the rat, the *Sry* expression starts around 11.5 days *post coitum* (d*pc*) for one day and then shuts off, in contrary to the situation in humans where this expression persists. However, the cascade of events following the *Sry* expression is quite similar in both species. The *Sry* expression stimulates the expression of *Sox9*, resulting in the testis cord formation around 13.5 d*pc*. These testis cords are formed of the migrating PGCs and the immature Sertoli cells (161). Postnatally, the rat testis differs from the human testis in the time frame they develop through. While in humans there is a lag phase between the neonatal period and puberty, these two overlap in the rat, leading to a continuous development with no quiescent period (158,162-164). The rat fetal Leydig cells increase in number at the first week postnatally, followed by the proliferation of the adult Leydig cells precursors, which differentiate to the mature adult Leydig cells eventually around puberty or 60 days *postpartum* (d*pp*) (165,166).

Once fully matured, the adult Leydig cells stop proliferating (167). As for the immature Sertoli cells, they start proliferating in the fetal life without interruption and quit only around 15-21 days postnatally, where they become mature (Figure 2). Coincident with this maturation is the formation of the BTB as tight junctions between the Sertoli cells (158,166).

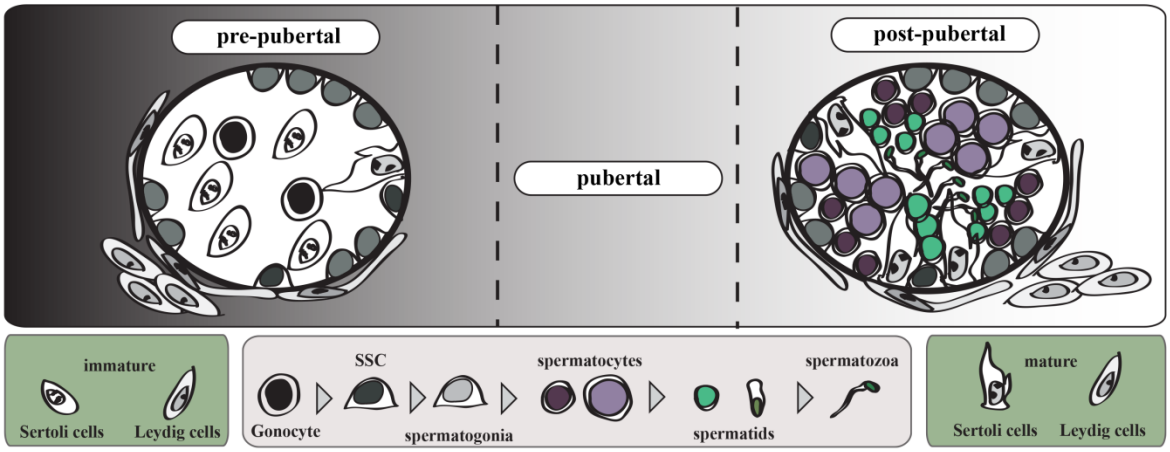

Figure 2. Pre-pubertal and post-pubertal testis. A transverse section of a pre-pubertal testis shows a testis cord surrounded by immature peritubular myoid cells (PMCs) and fetal Leydig cells in the interstitial tissue. The testis cord contains gonocytes in the middle, spermatogonial stem cells (SSCs) on the basement membrane, immature Sertoli cells and a few differentiating Sertoli cells. On the other side, a transverse section in a post-pubertal testis shows a seminiferous tubule surrounded by mature PMCs and adult Leydig cells in the interstitial tissue. The seminiferous tubule contains mature polarized Sertoli cells on the basement membrane and germ cells at different stages of development; SSCs on the basement membrane, spermatocytes, spermatids, and spermatozoa in the luminal compartment. The bottom of the figure shows the development of the somatic cells from immature to mature cells (Sertoli and Leydig cells) and the different developmental stages of the germ cells (gonocytes, SSCs, spermatogonia, spermatocytes, spermatids, and spermatozoa).

1.2.3 Germ cell development

In humans, PGCs, which are the precursors for germ cells, are specified during the week 3-4 of gestation at the junction between the allantois and the yolk sac outside the developing gonad, in response to BMPs signaling (168,169). The BMP signaling stimulates the expression of *PRDM14* and *PRDM1* (also known as *BLIMP1*), which in turns stimulates the expression of *AP-2 gamma*. The expression of these three gens is important for germ cell specification process (130,170-173). These PGCs are characterized by being round large cells with round nucleus positive for AP (174,175). At week 5 of gestation, they start migration from the hindgut through the dorsal mesentery till they reach their destination at the genital ridge (which is still undifferentiated gonad yet) by the end of the 6th week of gestation (176-180). This migration is thought to be through passive and active movements under the control

of the KIT signaling, as the migrating PGCs express the KIT receptor (178,181-184). During their migration and after colonization till gestational week 9, the PGCs proliferate in number from about 1000 to 150 000 cell (157). However, the PGCs are still bi-potential till this step; they can differentiate to either male or female germ cells, depending on the signaling from the somatic cells in the differentiating gonad (185). Once the PGCs are enclosed inside the testis cords with the immature Sertoli cells they are called gonocytes, where they are located at the center of the cords (157,186,187). These gonocytes remain mitotically active, even after the sex determination decision on the germ cell level around 11-12 weeks of gestation. This sex determination decision to the male side is mediated through FGF9 signaling and RA inhibition, both of which inhibit the cells from entering meiosis (185,187,188). However, they stop proliferation in the third trimester and erasure of the DNA methylation occurs (189,190). Then, they enter mitotic arrest at G0 till after birth, where they resume their proliferation till 6 months of age postnatally (191). At this age, they start to reside on the basement membrane and change morphologically and on gene expression level to what we call SSCs (Figure 2) (157). These SSCs stop expressing the pluripotency genes expressed earlier by the gonocytes; such as *POU5F1, NANOG*, Transcription factor AP-2 gamma *(TFAP2C)* and *KIT* (192).

In the rodents, a very similar process of germ cell specification takes place, with a different time frame. The specification of the PGCs starts at 6.25 d*pc* (168,169). Around 40 cells positive for AP are recognized at the yolk sac junction with the allantois at 7.25 d*pc*, which are identified as PGCs (174-176). At the age of 8.5 d*pc*, the PGCs start migrating towards the genital ridge, where they colonize before the age of 11.5 d*pc* (178). The sex cords are formed around 13.5 d*pc*, and the PGCs are called then gonocytes, located at the center of the cords (193). These gonocytes remain mitotically active for 2-3 days after colonization, followed by mitotic arrest at G0 till birth, where they resume their proliferation at 4 d*pp* (194). Meanwhile, they start heading to the basement membrane where they reside and hence called SSCs around 4-6 d*pp* (Figure 2) (193). In contrast to the situation in humans, the SSCs in rodents do not lose their expression of the pluripotency markers; such as *Pou5f1, Nanog*, *Tfap2c* and *Kit* (171,195-197).

Nevertheless, the germ cells are characterized by the presence of some unique RNA binding proteins (RBPs). These RBPs can be used as markers for germ cells through the different stages of development (198,199). For instance, *NANOS2* is expressed in humans from week 14 of gestation in the gonocytes, while in the adult testis it is expressed in the spermatogonia and some spermatocytes and round spermatids (200,201). Besides, *Nanos2* is expressed in the rodents at 13.5 - 17.5 d*pc* and in the adult testis in the SSCs (202,203). The function of NANOS2 is thought to be inhibiting the entrance to meiosis by suppressing Stimulated by retinoic acid gene 8 (STRA8), DAZL, and synaptonemal complex protein 3 (SYCP3) (203,204). In addition, *NANOS3* is expressed in the fetal testis at around 8 weeks of gestation in the gonocytes and continues to be expressed in the adult testis the spermatogonia, spermatocytes, and round spermatids (200,205,206). In the rodents, *Nanos3* is expressed in the fetal testis from 7.35 d*pc* in the PGCs and in the adult testis in the undifferentiated

spermatogonia (207,208). The suggested role of NANOS3 is to suppress apoptosis in the migrating PGCs (209,210) and to maintain the SSCs population postnatally (207,208).

Other examples for the germ cell specific RBPs include *DAZL*, *BOULE* and *DDX4*. Both *DAZL* and *BOULE* are members of the *DAZ* (deleted in azoospermia) gene family. *BOULE* is expressed in the germ cells at the meiotic phase (211). Meanwhile, *DAZL* is expressed in the germ cells from the fetal life in the first trimester and throughout the adult life in spermatogonia, spermatocytes and post-meiotic cells (211-215). The possible role of DAZL is to bind to DDX4 and SYCP3 to promote their translation (216,217). In addition, *DDX4* is expressed in humans in the migratory PGCs after 7 weeks of gestations and continues in the adult life in the spermatogonia, spermatocytes and spermatids (214,218,219). Although *DDX4* is essential for spermatogenesis, its exact role still needs to be elucidated (219). In rodents, *Boule*, *Dazl*, and *Ddx4* have a very similar expression pattern to humans. *Dazl* is expressed in the migratory PGCs in the fetal testis and continues to be expressed exclusively in the adult testis in the germ cells (211,213,220-222). Meanwhile, *Ddx4* is expressed in the fetal testis at 11.5 d*pc* in the PGCs and continues to be expressed exclusively in the germ cells in the adult testis (223,224).

1.3 SPERMATOGENESIS

Spermatogenesis is the process by which diploid SSCs differentiate to haploid spermatozoa. This process is a complex process occurs *in vivo* in the testis, necessitating a lot of factors and cues both intrinsically and extrinsically (225). In this section, stages and the cycle of spermatogenesis will be discussed, along with the somatic cell environment and hormonal control of spermatogenesis.

1.3.1 Stages

In mammals, the same three stages of spermatogenesis are conserved; spermatocytogenesis, spermatidogenesis, and spermiogenesis. The whole spermatogenesis process takes about 74 days in humans (226) and 56 days in rats (152,227). It starts around puberty in humans and at 9 d*pp* in rats (228). However, production of spermatozoa in rats is only evident around puberty. Starting from puberty, production of spermatozoa continues throughout the whole adult life, although it tends to decline while the age advances (229).

1.3.1.1 Spermatocytogenesis (the mitotic phase)

It is the process by which the spermatogonia proliferate and mitotically divide to give primary spermatocytes (Figure 2). Interestingly, there is no known specific marker for SSCs so far. However, in an attempt to find a marker for the SSCs, there are some suggested markers, which included Zinc Finger And BTB Domain Containing 16 (ZBTB16), Ubiquitin C-Terminal Hydrolase L1 (UCHL1), and GFRA1 (187,230,231).

In humans, there are two types of undifferentiated spermatogonia; A_{dark} and A_{pale} spermatogonia. These terms are based on the difference in the intensity of the nucleus when stained with hematoxylin (232). The A_{dark} spermatogonia are quiescent and do not usually undergo any mitosis, unless there is some kind of injury to the testis. Hence they are known as the reserve spermatogonia or the real stem cells (233). Meanwhile, A_{pale} spermatogonia divide mitotically to give a self-renewed cell and type B spermatogonia. These B spermatogonia divide mitotically to give two primary spermatocytes which enter meiosis (234). Due to the limited number of replication prior to meiosis in humans, the total number of spermatozoa obtained from one SSC cannot exceed 32, which is extremely low compared to the situation in rodents where the number of spermatozoa can reach thousands (235).

In rodents, the undifferentiated spermatogonia include A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia. Meanwhile, the differentiating spermatogonia include 4 types of A spermatogonia (A1-A4), one type of Intermediate spermatogonia (Int) and one type of B spermatogonia (236). The A_{single} spermatgonia divide to give two A_{paired} cells. These cells divide to give 4-16 cells of $A_{aligned}$ cells. The division goes on to give the A1-A4 spermatogonia, Int spermatogonia and B spermatogonia. The B spermatogonia divide to give primary spermatocytes (152,228).

It is worth mentioning that physiological apoptosis of spermatogonia is a part of the normal regulatory mechanism of spermatogenesis (237,238). Increased or reduced apoptotic activity results in impaired spermatogenesis (239,240).

1.3.1.2 Spermatidogenesis (the meiotic phase)

In this stage, each diploid primary spermatocyte divides by meiosis to give four haploid spermatids (Figure 2). In humans and rodents, the process is quite similar. First, the primary spermatocyte undergoes one round of DNA replication in the pre-leptotene phase (before entering the prophase I of the first meiosis or meiosis I). Then, it enters the prophase I, which is formed of 4 subsequent steps; leptotene (where the meiosis proteins attach to the chromosomes), zygotene (where the chromosomes are paired), pachytene (where the crossover and recombination of DNA take place), and diplotene (where the chromosomes separate from each other). This stage (prophase I) takes the longest time in meiosis, which lasts 24 days in humans, 18 days in rats, and 12 days in mice (152,227,241). Later on, the cell finishes meiosis I, enters prophase II and finishes meiosis II within 1-2 days, forming the haploid round spermatids (234,242).

One of the markers that can be used to show if the germ cells have entered meiosis is the SYCP3 protein. The SYCP3 and SYCP2 bind to a pair of homologous chromatids in the leptotene phase. In the zygotene phase, the two chromatids attach to each other by the SYCP1 protein (243). The crossover between the two homologous chromatids takes place in the pachytene phase, followed by degradation of the synaptonemal complex (SC) protein in the diplotene phase (244). Another marker for the late meiotic stage is the cyclic adenosine monophosphate (cAMP) responsive element modulator (CREM) protein. The CREM protein is expressed in mammalian male germ cells mainly from the late pachytene spermatocyte stage and on. However, it has more than one isoform; the repressor form is expressed and dominates in the early pre-meiotic stage, while the activator form is expressed and dominates in the meiotic stage. Generally, it is highly expressed in the meiotic stage compared to the early pre-meiotic stage, and that is why it is mainly detected in the late meiotic stage. Nevertheless, CREM has a pivotal role in activating the genes that encode structural proteins needed for spermatozoa differentiation (245-247).

1.3.1.3 Spermiogenesis (the post-meiotic phase)

During this stage in mammals, the haploid round spermatids produced by meiosis differentiate to the mature spermatozoa with no change in the number of cells (226). However, there is a dramatic change in morphology that takes place in four steps (Figure 2) (234). First, in the Golgi phase, the proacrosomal granules are formed and attached to one pole of the nucleus. Second, in the cap phase, the acrosomal granules aggregate, fuse and form the acrosomal cap, while the cell becomes more elongated. Third, in the acrosomal phase, the histone proteins are replaced by protamine in the nucleus, which becomes more condensed. Meanwhile, the flagellum starts to mature and be visible. And finally, in the maturation phase, the cytoplasm shrinks in volume, due to the extrusion of residual bodies,

which are phagocytosed by the Sertoli cells (242,248). Once the spermatozoa are mature, they are released to the lumen. Then they move by the seminiferous tubule contractions to the epididymis, where they get fully matured by gaining the ability to swim and fertilize the female gametes (249).

The markers used usually to recognize the germ cells in the post-meiotic stage include Protamine 1 (PRM1) and ACROSIN. The protein PRM1 is one of two forms of protamine (PRM1 and PRM2) that replaces the histone proteins in the chromatin during spermiogenesis in the maturing spermatozoa. In mice and humans, the two forms of protamine (PRM1 and PRM2) are present (250). However, in rats, only one form of protamine (Prm1) is present, even though the *Prm2* gene is available, due to inefficient transcription (251). ACROSIN, which is a serine protease in the acrosome of the post-meiotic haploid male germ cells, is expressed as an inactive zymogen, proacrosin. Hence, it is stored in the acrosome till it is needed in fertilization (252,253). ACROSIN is co-existent in the acrosome with another enzyme important for fertilization, which is the hyaluronidase enzyme. While hyaluronidase is important to disperse the *cumulus oophorus* (a cluster of cells surrounding the oocyte), ACROSIN is important to penetrate the *zona pellucida* (a glycoprotein layer surrounding the oocyte between the oocyte and the *cumulus oophorus*) (254-256). However, it was reported that ACROSIN is not essential for fertilization, in humans (257) or in mice (258). In rats, the *Acr* (acrosin) gene is transcribed in the diploid stage, while it stays under translational control to be first seen at protein level in the post-meiotic haploid round spermatid stage (252,254).

1.3.2 Spermatogenic cycle

In the adult mammalian testis, spermatogenesis process through the seminiferous tubules actually resembles a wave. When a germ cell advances in development, it gets closer to the lumen and further from the basement membrane. Hence, at any point of the seminiferous tubule, there is more than one germ cell above each other in different stages of development. Interestingly, these germ cells develop together in a synchronized pattern. These synchronized patterns are found in sequence along the seminiferous tubule, making the development through the seminiferous tubule look like a wave (259). Thus, the different germ cells from different developmental stages are locked together in one tubular stage. This tubular stage (which is a group of developmental stages) is repeated along the seminiferous tubule every certain number of tubular stages in a cyclic pattern, which makes the stages looks more like a cycle. This cycle is called ‘the spermatogenic cycle’. The length of the spermatogenic cycle and the number of stages in the cycle is characteristic for each species (260-262). Many researchers have investigated the spermatogenic cycle, trying to quantify the length of the cycle and determine the number of cycle stages in the different species. There are different methods used to determine the cycle length. The first one was to use X-ray irradiated testis to investigate the rate of germ cell disappearance due to the destruction of the SSCs (263-265). The second method was to label the developing germ cells with radioactive elements and trace these cells through their development (266-268). For instance, the length of the cycle in the rat was estimated to be 12.9 days (269), while the length of the

cycle in humans was estimated to be 16 days (270). A lot of effort has been invested in an attempt to determine the stages of the cycle in the different species. In the rat, Leblond and Clermont have divided the seminiferous cycle into 14 stages, despite of the presence of previous attempts for staging resulted in dividing the cycle into less number of stages, ranging from 6 to 12 stages of the cycle (262). Leblond and Clermont have used the different developmental stages of the spermatid to the mature spermatozoa as markers for the cycle stages. Hence, the first developmental stage of the spermatid represents the first stage of the cycle (262). In humans, similar efforts have been done for staging the cycle, which divided the seminiferous cycle into 6 consecutive stages (226,271).

One characteristic difference between humans and rodents regarding the spermatogenic cycle is that one cross section of the adult testis can show histologically more than one stage of the cycle in humans, while it shows only one stage of the cycle in rodents. The reason behind this fact is that the number of mitotic divisions of the germ cells prior to meiosis is very small in humans compared to the rodents. This leads to the presence of a small number of germ cells in each developmental stage in humans compared to rodents, which might have thousands of cells in the same stage. This in turn results in a smaller area of the seminiferous tubule occupied by these cells in humans compared to rodents. Hence, one cross section of the seminiferous tubule in an adult human testis can show more than one cycle stage, while it is not the case in rodents (260). It is worth mentioning that the spermatogenic cycle is different from the seminiferous epithelium wave. While the spermatogenic cycle is the time taken by a specific stage of the cycle to re-appear at the same spot of the seminiferous tubule, the seminiferous epithelium wave describes more the distance between two successive spots of the seminiferous tubule with the same stage of the cycle (260).

1.3.3 Hormonal regulation of spermatogenesis

In mammals, the beginning of spermatogenesis coincides with the onset of puberty. The onset of puberty in humans comes after a lag period in the infancy period, where the testis seems quiescent. On the contrary, the onset of puberty in the rodents is overlapping with the infancy period, which suggests that the quiescent period is missing in rodents (158). Interestingly, the initiation and maintenance of spermatogenesis is under the control of the hypothalamus-pituitary-gonadal endocrine axis (234,272). Around puberty, kisspeptin, which is released in the brain, stimulates the secretion of the gonadotropin releasing hormone (GnRH) from the hypothalamus (273). The GnRH stimulates in turn the pituitary gland to secrete the gonadotropins; FSH and the luteinizing hormone (LH).

Actually, FSH exerts its effect on the Sertoli cells through the FSH receptor (FSHR). This receptor starts to be expressed in the Sertoli cells at 14.5 d*pc* in the rat testis, and continues to be expressed throughout the whole adult life. However, it becomes fully active only at 16.5 d*pc* in the rat (274). Stimulation of the FSHR in the Sertoli cell results in two different effects, depending on the level of maturation of the Sertoli cell. In the immature Sertoli cells, stimulation of the FSHR results in an increased proliferation rate (275,276). Meanwhile, in the fully mature Sertoli cells, stimulation of the FSHR results in an increased metabolic and

secretory activity of the Sertoli cell (277). However, the expression of the FSHR is not changed in both cases (278). The stimulation of the FSHR leads to a cascade of events resulting in the increase of cAMP. However, this effect is also dependent on the maturity of the Sertoli cells; the immature Sertoli cells have a much profound and enhanced effect compared to the mature ones (279). Notably, FSH secretion from the pituitary gland is under the control of the negative feedback from the testis. Sertoli cells produce inhibin in response to the FSH stimulation, which in turns reduce the pituitary secretion of FSH (280). Although the role of FSH in the Sertoli cell proliferation and maturation is well-established, the role in spermatogenesis is still controversial (281). Interestingly, some reports have shown that FSH is not needed for restoration or maintenance of spermatogenesis in adult rats (282-284). However, other reports showed the importance of FSH in spermatogenesis (285). The suggested effect of FSH on its receptor in the mature Sertoli cell, although the cAMP production is not enhanced as in the immature cell, can be attributed to another signal transduction pathway like the Ca^{++} signaling (281).

On the other hand, LH exerts its effect on the Leydig cells through the Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR). The LHCGR receptor is expressed in both the fetal and adult Leydig cells. In the fetal cells, the receptor is expressed from 16 d*pc* in rodents. The LH main two actions are to stimulate the differentiation of the progenitor Leydig cells to the adult Leydig cells and to stimulate the Leydig cells to produce testosterone and dihydrotestosterone. However, it was reported that LHCGR receptor is not needed for testosterone production by the fetal Leydig cells (286). The effect of LH on Leydig cells is mediated through the induction of the Steroidogenic Acute Regulatory Protein (STAR). This protein is responsible for transferring cholesterol into the mitochondria, which potentiate the biosynthesis of testosterone (287). The testosterone produced by the Leydig cells has a negative feedback on the pituitary gland LH secretion. The feedback is achieved through the metabolites of testosterone; estradiol and dihydrotestosterone (288). The testosterone produced by the Leydig cells act through the androgen receptors (AR), which are found in Sertoli cells, Leydig cells and PMCs. Interestingly, there are neither FSHRs nor ARs in the germ cells at any developmental stage. Therefore, the effect of FSH and testosterone on spermatogenesis is mediated through the Sertoli cells (289). However, the existence of estrogen receptors in germ cells has been reported, which suggests a possible effect of estradiol on spermatogenesis (mediated by the positive effect on germ cell survival) (290-292).

Other hormones with a suspected role in spermatogenesis include osteocalcin, thyroxin, glucocorticoids, and prolactin. Osteocalcin was reported to enhance the testosterone production by Leydig cells (293). Meanwhile, the thyroid hormone is responsible for the maturation and cessation of proliferation of the Sertoli cells (294). Glucocorticoids inhibit the testosterone production in the Leydig cells by suppressing the steroidogenic enzymes (295). Finally, prolactin has been shown to support the LH mediated testosterone production in the Leydig cells (296).

1.3.4 Somatic cell environment

The presence of a healthy somatic cell environment in the mammalian testis surrounding the germ cells is extremely important in spermatogenesis. Germ cells are surrounded by 4 types of cells; Sertoli cells, Leydig cells, PMCs, and immune cells, with each type of cells having a specific role in spermatogenesis.

The Leydig cells produce testosterone in response to the LH signaling. Then, the produced testosterone, acts on three types of cells which have the ARs in the testis. These types of cells include Sertoli cells, PMCs and Leydig cells. The AR signaling in Leydig cells is a sort of feedback mechanism, which enhances the Leydig cell proliferation and differentiation (297,298). Leydig cells produce also colony stimulating factor 1 (CSF1), which influence the proliferation of SSCs through the CSF receptors on the surface of the SSCs (299,300).

In the PMCs, the AR signaling is important for the formation of the basement membrane and for the cross talk with Sertoli cells. The PMC specific AR knockout mice showed impaired spermatogenesis and Sertoli cell function, showing the importance of the AR signaling in the PMCs (301). Another signaling pathway in the PMCs is through the platelet derived growth factor (PDGF) signaling. Notably, Sertoli cells produce PDGF, which acts on the PMCs through the PDGF receptors (302). The direct effect of PMCs on germ cells can be attributed to the Glial Cell Derived Neurotrophic Factor (GDNF) and LIF secreted by the PMCs, which can act on germ cells through the GFRα receptors and LIF receptors found on SSCs (303). However, GDNF secretion is to some extent under the control of the PDGF signaling coming from Sertoli cells (302).

Since mature Sertoli cells have FSHRs and ARs, they receive two important signals by two hormones; FSH, and testosterone. The FSH signaling stimulates the proliferation of SSCs, mediated by GDNF, FGF2, and c-KIT ligand or stem cell factor (SCF) (304-306). Meanwhile, the AR signaling in Sertoli cells is important for entering the meiotic phase of spermatogenesis, through the production of RA, which is important for the germ cells to enter meiosis (306). Both FSH signaling and testosterone signaling in the Sertoli cell are crucial in the spermiogenesis stage and to complete the spermatogenesis process (233,289). In addition to the classical genomic pathway through the AR, testosterone exerts its effect on Sertoli cells through a non-genomic pathway, where the testosterone induces Ca^{++} influx and stimulates Src tyrosine kinase. This non-classical pathway is important in the Sertoli cell-germ cell attachment and the release of the mature spermatozoa into the lumen (307). Moreover, Sertoli cells produce also transferrin, activing A and BMP4. The GDNF produced by Sertoli cells acts on the SSCs through the GFRα and c-RET receptors. The main role of GDNF is to regulate the self-renewal of the SSCs through the AKT signaling pathway and the Src family kinase signaling pathway (308-311). In addition, Sertoli cells produce FGF2, which regulates the self-renewal of the SSCs as well, by enhancing the self-renewal genes (312). Another factor produced by the Sertoli cells is the SCF, which stimulate the differentiation of the spermatogonia through the c-KIT signaling. It is worth mentioning that in the undifferentiated spermatogonia the c-KIT expression is inhibited by the ZBTB16 expression (313).

Meanwhile, the RA produced by Sertoli cells is important for the germ cells to enter meiosis through the STRA8 signaling pathway (314,315). Transferrin produced by Sertoli cells, on the other hand is responsible for shuttling iron to the developing germ cells (316). Finally, Sertoli cells produce activing A and BMP4, which induce the differentiation of SSCs through the SMAD signaling pathway (317,318).

The immune cells surrounding the germ cells include macrophages, leukocytes, and mast cells. These cells produce a group of factors called cytokines; such as interleukin 1 α (IL1α), IL1β, IL6, interferon γ, tumor necrosis factor α (TNFα) and TGFβ. These factors have a direct effect on the proliferation and differentiation of the germ cells and somatic cells in the testis (319,320). In addition, TNFα and TGFβ initiate the disassembly of the BTB during the movement of germ cells across the BTB. This disassembly is important to allow the germ cells to pass the BTB (321). Afterwards, the BTB re-assemble once more under testosterone effect, dividing the intra-tubular compartment into two regions; one for the haploid post-meiotic cells with no access to blood components, and one for the diploid pre-meiotic cells exposed to the blood components (322,323). Macrophages produce also CSF1 and RA, which are important for SSCs self-renewal and entry of meiosis as discussed earlier. The significance of macrophages in spermatogenesis was shown by their ablation in the testis, which caused a disturbance in spermatogenesis (324).

1.4 FERTILITY PRESERVATION IN MALES

Fertility preservation is the measures and procedures taken to save, retain or restore the fertility for males who are at high risk to develop infertility. In males, this infertility is defined as the inability of a person to reproduce with a natural sexual intercourse due to reduced qualitative or quantitative parameters of the semen. Therefore, the indications for fertility preservation (in particular, the cancer treatments) and the current and future options for fertility preservation in males (in particular, *in vitro* spermatogenesis) will be discussed.

1.4.1 Indications for fertility preservation in males

The indications for fertility preservation in males include the syndromes or diseases that can eventually lead to infertility, or the exposure to specific treatments that are used to treat some syndromes or diseases.

1.4.1.1 Syndromes and conditions causing infertility

These conditions include Klinefelter syndrome and cryptorchidism (325). Klinefelter syndrome (KS) is a chromosomal disorder with a prevalence of 0.2% in males, which results usually from the existence of 3 sex chromosomes; a pair of X chromosomes and one Y chromosome (326). Individuals with KS show normal onset of puberty and a brief testicular growth. However, this growth declines shortly after the onset of puberty, with elevated gonadotropins and reduced testosterone levels in serum (hypergonadotropic hypogonadism). In addition, the germ cells at the spermatogonial stage proceed to apoptosis instead of entering meiosis, leading to meiotic arrest and loss of germ cells (327-329). The fertility preservation recommendation for such condition is to cryopreserve the retrieved sperm or semen for adolescents or to cryopreserve the testicular tissue for pre-pubertal boys (330-333). However, it is rare to find pre-pubertal boys with KS, as it is usually diagnosed after the onset of puberty. Notably, SSC transplantation is not recommended for such individuals, as the deterioration of the seminiferous tubules occurs later on in life, which will make the restoration of a normal spermatogenesis impossible (334,335).

On the other hand, cryptorchidism is a congenital condition where one testis or both testes remain retracted in the abdomen without descending into the scrotum at birth or shortly after birth within 3 months. The hyper-thermal conditions the germ cells face at this early developmental stage causes a progressive loss of germ cells. Its prevalence is approximately 1-3% of the newborn males, with a serious prognosis of prospected infertility in the adulthood and high risk to develop germ cell tumors (336,337). Surgical intervention is indicated in such condition as early as at the age of 6 months, to minimize the undesirable consequences (338). As the bilateral orchidopexy has a worse prognosis than the unilateral one, it is a technical routine to take a testicular tissue biopsy during the surgical operation, to investigate whether the germ cells are still present or not (339). In that context, cryopreserving the testicular tissue for further fertility preservation options if needed was suggested (325,340).

1.4.1.2 Gonadotoxic treatments

Some treatments are highly effective against the diseases and conditions they are intended to cure. However, these treatments have a major side effect; which is the gonadotoxic effect. The gonadotoxic effect is defined as any deleterious effect to the gonads; causing subfertility or infertility. The conditions or diseases treated by such gonadotoxic treatments include systemic lupus erythematosus, some renal diseases, sickle cell anemia, Fanconi anemia, multiple sclerosis, and, most important, cancer (341,342).

Systemic lupus erythematosus, multiple sclerosis and some renal diseases necessitate cyclophosphamide as a treatment, which is known for its gonadotoxicity (343-347). In addition, sickle cell anemia and Fanconi anemia are two conditions that are treated mainly by hemopoietic stem cell transplantation (HSCT) (348,349). This HSCT needs some sort of preparation before the transplantation called conditioning, which implies total body irradiation (TBI) and the usage of cyclophosphamide or busulfan; both of which are known to cause gonadotoxicity (350,351).

1.4.2 Effect of cancer therapy on testis

Cancer therapy is by far the major indication for fertility preservation. Cancer is defined as a group of diseases characterized by abnormal cell growth, which has the ability to spread or invade other tissues. Hence, treating cancer depends in the first place on inhibiting cell growth and multiplication. To achieve such goal, there are two main components in most of the cancer treatment protocols; chemotherapy and radiotherapy. Chemotherapy is a term describes a group of medications that possess a toxic effect on the proliferating cells. These medications are called collectively cytotoxic agents, as they inhibit cell proliferation by different mechanisms, classifying them to different classes. These classes include mainly two classes; the alkylating agents (e.g. cyclophosphamide, busulfan, and ifosfamide), which cause damage to the DNA and induce apoptosis, and antimetabolites (e.g. methotrexate), which inhibit DNA and RNA synthesis. Meanwhile, radiotherapy is based on the deleterious effect of the ionizing radiation on DNA, leading to cell death by apoptosis (325,352).

The effect of the treatment on testis, and therefore fertility, is extremely harmful. In general, A_{dark} spermatogonia in humans are the reserve SSCs, which do not proliferate in the normal healthy testis, while the A_{pale} spermatogonia proliferate regularly to produce differentiated B spermatogonia. However, the A_{dark} spermatogonia do proliferate if the A_{pale} spermatogonia are depleted. Thus, A_{dark} spermatogonia are more resistant to damage by gonadotoxic treatments than other types of spermatogonia (353,354). In addition, the repeated exposure or exposure to very high doses of gonadotoxic treatments can deplete the pool of the A_{dark} spermatogonia and cause damage to the somatic cell environment in the testis (354,355). The effect of chemotherapy or radiation depends on the age of the patient and the type, dose and duration of treatment (356). In childhood, all of the testicular cells have a high turnover rate, which makes them more sensitive than the adult testicular cells (355,357). Moreover, childhood malignancies *per se* may have a deleterious effect on the testis (358-361)

1.4.2.1 Effect of chemotherapy on testis

Not all chemotherapeutic agent cause the patients to develop the same risk for infertility, some agents imply more risk than the others. Hence, there are some agents with high risk (the alkylating agents) and some agents with low risk (the antimetabolite agents) (355). This is because alkylating agents deplete the spermatogonial pool by damaging directly the DNA, which induces apoptosis (362). For instance, cyclophosphamide with a dose of 9 g/m^2 for pre-pubertal patients and a dose of 10 g/m^2 for post-pubertal patient cause prolonged azoospermia (363-365). However, a recovery of spermatogenesis occurred in many cases with these doses, suggesting higher doses of cyclophosphamide are required to develop a permanent azoospermia (366). Busulfan on the other hand requires a dose of 600 mg/kg to exhibit the same effect (367). Thus, a low dose of alkylating agents can cause depletion of the A_{pale} spermatogonia with a recovery after a while, and a reversible azoospermia in adults. Meanwhile, a high dose of alkylating agents can cause permanent infertility in all ages (355). It is also worth mentioning that repeated low doses of cyclophosphamide implies more risk for infertility than single high dose, as the repeated doses can deplete the reserve A_{dark} spermatogonia (368).

The effect of chemotherapy on Leydig cells seems attenuated if compared to the effect on germ cells. However, the exposure to alkylating agents can cause Leydig cell damage, characterized by increased LH levels as a compensatory mechanism, which results usually in normal testosterone levels (354,369). Therefore, neither pre-pubertal nor post pubertal patients exhibit any signs of Leydig cell damage (e.g. delayed secondary sexual development for pre-pubertal and low testosterone levels for post-pubertal) after the exposure to low dose of alkylating agents (370). However, higher doses of alkylating agents can cause severe damage to the Leydig cell, leading to apparent dysfunction (371).

1.4.2.2 Effect of radiotherapy on testis

Effect of radiotherapy on the testicular functions depends on the field, dose and fractionation of radiation (372,373). A low total dose of 0.1 Gray (Gy) can cause disturbance of spermatogenesis for a short time, while higher doses of 2-3 Gy can cause a prolonged azoospermia. A high total dose of radiation (more than 6 Gy), on the other hand, can cause permanent azoospermia, leading to infertility (374-376). Fractionation of the radiation might lead to more severe toxicity to the testis. Thus, a total fractionated dose of 1.2 Gy can cause permanent infertility (377,378). On the other hand, a recent study presents different data, where the fractionated dose had a more tolerable effect than the single dose of irradiation (379). The field of radiation is a major factor upon which the gonadotoxic effect depends. In case of TBI, needed before HSCT, a total dose of 12 Gy can cause permanent infertility (380,381). Meanwhile cranial irradiation with a dose of 30-50 Gy in brain tumors can lead to secondary infertility, due to disturbance in the hypothalamic-pituitary-gonadal axis (382-385). Testicular irradiation (in pre-pubertal patients for testicular leukemia or in post-pubertal patients for testicular cancer) is usually at a dose of 20-24 Gy, which is sufficient to cause permanent infertility, due to germ cell depletion (374,386).

Leydig cells, albeit less sensitive than germ cells to radiotherapy, can be affected by the testicular irradiation at a dose of 24 Gy, leading to impaired testosterone production and delayed puberty, which necessitates hormonal therapy (373,387). However, adults who receive a dose of 20 Gy of testicular irradiation or less could exhibit normal testosterone production (372,381,388). Nevertheless, using new radiotherapy techniques can cause less damage to the testis, such as the high-dose-rate brachytherapy (HDRBT) given neoadjuvantly for patients with rectal cancer (389).

1.4.3 Fertility preservation options in males

In the few past decades, cancer treatment protocols have improved a lot, leading to a dramatic increase in the survival rates, reaching about 80% in childhood cancers (390,391). Meanwhile, leukemia represents 30% of all pediatric malignancies, with acute lymphoblastic leukemia (ALL) constituting two thirds of the leukemia cases (392). However, the advancement in cancer treatments did not guarantee better adverse effects. As the increased risk for infertility is one of the main adverse effects, it was not an exception (393-395). Thus, about 46% of the childhood cancer survivors and more than 40% of the ALL survivors have reduced fertility (396-399). However, these figures tend to improve with time after treatment (370). In addition, using antimetabolites instead of the high dose alkylating agents improved a lot the fertility outcome for the survivors (368,400). On the other hand, Hodgkin's lymphoma (HL) patients treated with alkylating agents had much worse fertility parameters compared to the HL patients who did not receive alkylating agents in their treatment protocol, or even compared to the ALL patients (401-406). Meanwhile, non-Hodgkin's lymphoma (NHL) treatment protocol leaves the patients with a risk of infertility similar to the ALL patients. In case of relapse (about 30% of the cases), these patients require an HSCT operation, which necessitates conditioning with TBI and high dose of cyclophosphamide, rendering the patients with high risk to develop infertility (379-381,407).

As the survival rate after childhood cancer increased, the demand on fertility preservation increased as well. Hence, it was important to investigate the fertility preservation options for such patients. Herein, the current options for fertility preservation in males and the experimental techniques that could be used in the near future will be discussed.

1.4.3.1 Current options for fertility preservation in males

The options depend to large extent on the pubertal stage of the patient. If the male patient is post-pubertal or pubertal, the safest and easiest option is to cryopreserve a semen sample form the patient, obtained mainly by masturbation (408). However, electro-ejaculation, vibratory stimulation and testicular sperm extraction (TESE) can be used as well. Later on, these samples can be thawed and the sperm used by assisted reproductive techniques (ART) to achieve pregnancy (325,367,409). Thus, semen cryopreservation should be offered to all patients with fertility preservation indication, regardless to the risk to develop infertility, once they can deliver semen sample. However, if the male patient is pre-pubertal or cannot deliver semen sample, there is no current proved clinical method for fertility preservation (Figure 3).

1.4.3.2 Experimental techniques for fertility preservation in males

As semen cryopreservation is not always feasible, there are some techniques under investigations as options for fertility preservation. These techniques are intended for pre-pubertal patients as well as azoospermic pubertal patients due to pre-treatment or the malignancy itself (403,410). These techniques include gonadal protection, testicular cryopreservation, autologous transplantation (SSCs or tissue), xeno-grafting of testicular tissue, and *in vitro* germ cell maturation (325,355,367).

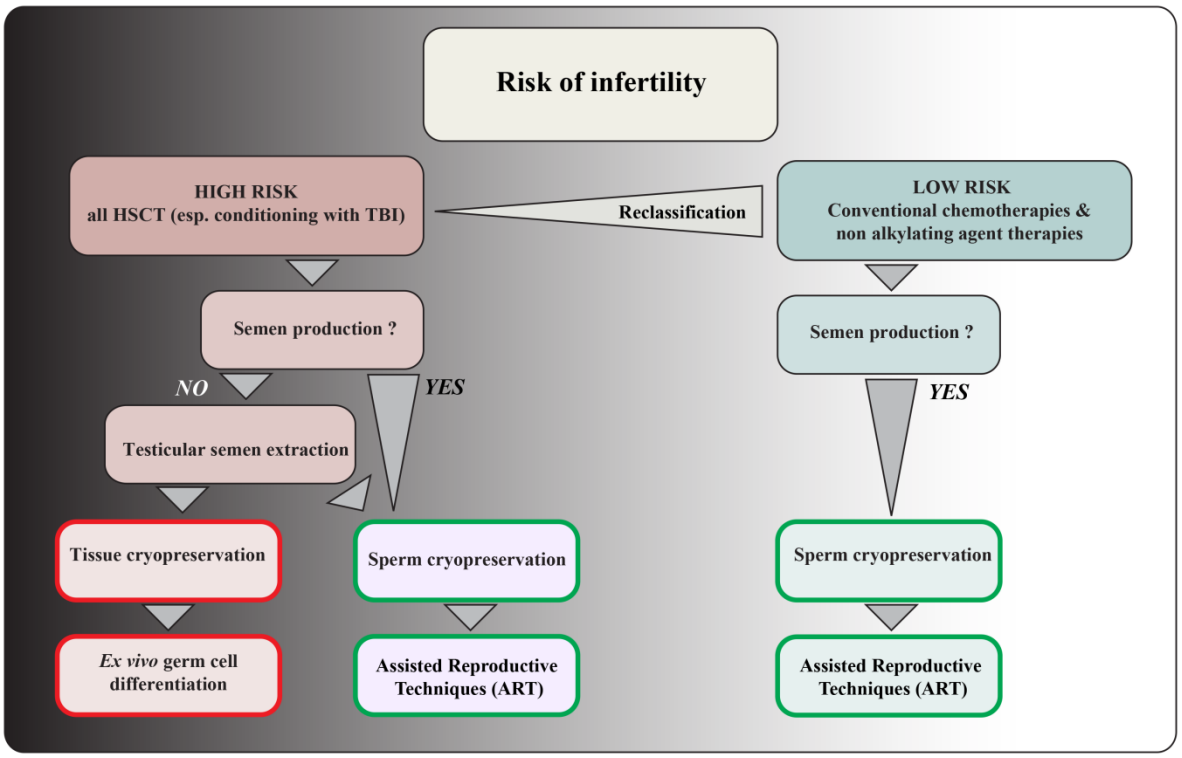

Figure 3. Fertility preservation options in males. The risk for infertility is assessed. In case of low risk, while a semen sample can be obtained, sperm cryopreservation is performed. The cryopreserved sperm can be used later exploiting assisted reproductive techniques (ART) to produce pregnancy. In case of high risk (hemopoietic stem cell transplantation HSCT with total body irradiation TBI), while a semen sample can be obtained, sperm cryopreservation is performed as well, followed by ART when required. In case of high risk, while a semen sample is not feasible, a biopsy of testicular tissue is obtained instead. If it is possible to get sperm through testicular sperm extraction (TESE), cryopreservation for the sperm is performed. If it is not possible to extract sperm, the testicular tissue is cryopreserved for later *ex vivo* germ cell differentiation if possible. The green boxes indicate a current fertility preservation option, while the red boxes indicate an experimental technique, not a guideline yet.

Gonadal protection is a technique aiming at suppressing the hypothalamic-pituitary-gonadal axis by GnRH agonist or antagonist, leading to gonadal quiescence. This quiescence should turn the germ cells less sensitive to chemotherapy and radiation (411). Gonadal protection has shown some promising results in rats more than in mice (412-417). However, no such encouraging results could be obtained in humans or non-human primates (NHP) (418-420).

Testicular cryopreservation is a technique designed for storing the testicular tissue for long term at a very low temperature (liquid nitrogen temperature, -196°C). Hence, when the male patient is pre-pubertal or cannot deliver semen samples, the risk to develop infertility due to the treatment is evaluated. If the risk is high, the patient should undergo an invasive surgical operation to obtain a testicular biopsy and cryopreserve it. Testicular biopsy should be taken ideally before introducing any treatment to the patient. Testicular tissue cryopreservation is considered as the cornerstone for the experimental techniques of fertility preservation, as the germ cells can be stored by this preservation and they could be used later in the different techniques; autologous transplantation, xeno-grafting, and *in vitro* germ cell maturation. So far, there are two methods for cryopreservation; slow freezing and vitrification. In the slow freezing method, the tissue is stored with a cryoprotective agent, which can be diffusible (e.g. ethylene glycol and dimethyl sluphoxide or DMSO) or non-diffusible (e.g. polymers). The role of the cryoprotective agent is to inhibit the cytotoxicity due to the ice crystal formation. On the other hand, in vitrification, the tissue is frozen in an extremely short time, in order to inhibit the formation of ice crystals and to avoid the relevant cytotoxicity. Recently, there is an advancement in the testicular tissue cryopreservation protocols, which will enable researchers to focus on the way the germ cell can mature *ex vivo* (395,421-426). Noteworthy, cryopreservation of single cell suspension instead of the tissue could be easier. However, this suspension necessitates enzymatic and mechanical digestion, which will compromise the cell survival (427).

Autologous transplantation is a technique aiming at transplanting the testicular tissue or SSCs, with or without cryopreservation, back into the donor's testis. Transplantation of SSCs in mice has been reported earlier, where spermatogenesis could be re-established in germ cell ablated seminiferous tubules (428,429). After mating, these mice could reproduce and the offspring was fertile as well (430). Notably, neither the donor's age nor cryopreservation of the SSCs did affect the outcomes (431-433). In addition, it has been reported that transplantation of SSC of rhesus monkey could re-establish spermatogenesis in the testis of busulfan-treated and X-ray treated rhesus monkey recipients (434,435). Using the same technique, SSCs could be transplanted in human cadaver testes as well (436). Since the number of SSCs in the testis is limited, a step of propagating the SSCs *in vitro* prior to the transplantation might be required (437). This step has been reported to be feasible recently (438,439). On the other hand, the outcome of testicular tissue transplantation depends on the age of the donor; where the testicular tissue from immature animals had better survival and ability to re-establish spermatogenesis than the testicular tissue from adult ones (440-442). Using this grafting technique, spermatogenesis could be established after grafting neonatal testicular tissue from different species into nude mice, producing an offspring from the

obtained mature spermatozoa (443-446). Autologous grafting for NHP testicular tissue in orthotopic and ectopic positions has been reported earlier, with a success in producing mature spermatozoa using fresh and cryopreserved tissue (447-449). Noteworthy, testicular tissue grafting produced more efficient spermatogenesis in mice than transplantation of SSCs (450). Although autologous transplantation, for tissue or SSCs, seems promising technique, it is still a research tool more than a clinical option. The reason behind this is that in case of many pediatric malignancies (e.g. ALL), testicular tissue might be a infiltrated with cancerous cells (451). Hence, grafting testicular tissue may imply the risk for recurrence of cancer, as a few cancer cells are required to re-establish the disease (452). In that context, transplantation of SSCs might look better, as the testicular cells are dissociated and the isolated cells should be only the SSCs. However, finding a de-contaminating protocol for the SSCs from the leukemic cells seems challenging, rendering SSC transplantation as a research tool only (453-456). This is mainly due to the difficulty to find a specific surface marker for SSCs (457). Nonetheless, a recent study showed that a full de-contamination protocol could be achievable, which might be promising (456).

Xeno-grafting of testicular tissue describes transplanting testicular tissue from one species (e.g. human) into another species (e.g. nude mice). So far, spermatogenesis could be established by grafting monkey testicular tissue (NHP) in nude mice (458). However, complete spermatogenesis from human xeno-grafting in nude mice has not been reported yet, although meiotic activity could be observed (459-462). Unequivocally, considering xeno-grafting as a clinical tool is quite far, due to the risk of zoonosis. However, it can be used as a tool to detect any contamination with leukemic cells in the testicular graft, as it will reproduce the cancer in the recipient animal (463).

1.4.4 *In vitro* germ cell maturation

As it was discussed previously, the experimental techniques for fertility preservation are a bit far from the clinic in the time being. Hence, the only technique that avoids the setbacks for the other techniques is the *in vitro* germ cell maturation or *in vitro* spermatogenesis (IVS) (464). It aims at culturing *in vitro* the testicular tissue, taken from the pre-pubertal patients at high risk to develop infertility before starting treatment, in order to get mature germ cells. These mature germ cells can be, in principle, used later with ART to achieve pregnancy when desired. Hence, this method avoids the risk for cancer recurrence (as in autologous transplantation) and the risk for zoonosis (as in the xeno-grafting) (325,367,465).

Studies on IVS started a century ago (466). Meanwhile a lot of efforts in IVS were performed in the 60's by Steinberger (467,468). However, these studies showed the progression of spermatogenesis till meiosis, followed by a meiotic arrest. Since then, studies on IVS revealed that IVS depends on a lot of factors; such as the pH, the temperature (467,469,470), the cell-cell contact (471,472), the oxygen level (230), the culture method, the composition of the culture medium, and presence or absence of serum, hormones and some growth factors (473,474). There are 3 major methods for IVS; conventional two-dimensional cell culture, 3D culture, and organ culture.

1.4.4.1 Conventional two-dimensional (2D) culture system

In the conventional 2D cell culture, germ cells are cultured in liquid culture medium, with or without other types of cells. Interestingly, studies on IVS using 2D culture showed the progress of spermatogenesis from meiotic stage to the post meiotic stage (475-477). In these studies, spermatocytes and round spermatids were obtained from testicular biopsies of non-obstructive azoospermia patients and co-cultured with Vero cells or Sertoli cells. The co-cultured germ cells could develop to elongated spermatids and spermatozoa. Moreover, these elongated spermatids had normal blastocyst formation rates when used to fertilize oocytes in vitro. The studies showed also that FSH and testosterone, when added simultaneously to the culture, showed improved differentiation and meiosis index. In addition, studies on IVS using germ cells at earlier stages of development have been reported (478,479). In these studies, SSCs were isolated from obstructive azoospermia and non-obstructive azoospermia patients, as CD49f+ cells, or from cryptorchid patients. These SSCs were co-cultured with Sertoli cells and developed *in vitro* to post-meiotic round spermatids. The round spermatids had normal fertilization potential when used in fertilizing oocytes. However, no full spermatogenesis has been reported using this 2D culture system, probably due to the loss of the spatial arrangement of the testicular cells *in vitro* by this method, which has been shown to be important to achieve germ cell maturation (480).

1.4.4.2 Three-dimensional (3D) culture system

In an attempt to keep the spatial arrangement of the germ cells with the other testicular cell types *in vitro*, the use of agarose or methylcellulose to form a 3D semi-solid culture system was investigated (481). The use of extracellular matrix (ECM) in cell culture started earlier to establish clonogenic assays for hemopoietic stem cells (HSCs) (482). In their study, Stukenborg and colleagues obtained a single cell suspension from testes of 10 d*pp* mice. Using magnetic-activated cell sorting (MACS), the cells were separated into two fractions, depending on the GFRα expression; SSCs enriched and SSCs depleted. The two fractions were inoculated into two layers of a 3D matrix, formed of agarose and culture medium. This study showed the importance of functional somatic cells to IVS, since the fraction with SSCs co-cultured with somatic cells had Crem and Boule positive cells. In another study, the same group showed that the murine SSCs could develop to elongated spermatids, expressing protamine on protein level. They have also shown gonadotropins were essential for germ cell differentiation *in vitro*. In additions, the nature of the matrix, being agarose or methyl cellulose, did not have an effect on the culture system. However, the efficiency of this 3D culture system to produce mature germ cells was low (480). Other groups have earlier reported using the same concept of 3D culture system to differentiate germ cells from other species (e.g. rat and human) *in vitro*. In one study, Lee and colleagues isolated single cell suspension from testes of 18 d*pp* rats. This suspension was inoculated into monolayer culture, collagen matrix culture, or collagen + Matrigel. The group has shown that the 3D culture system support the differentiation of the germ cells to the post-meiotic stage. The same group has cultured spermatocytes from testicular biopsies of non-obstructive azoospermia patients in a 3D collagen culture system. The results showed that these spermatocytes could

differentiate to haploid cells *in vitro*, using the 3D culture system (483,484). However, the starting material that they used did not contain only SSCs, but more developmentally advanced germ cells were included in the culture. Thus, a full spermatogenesis *in vitro* could not be claimed.

1.4.4.3 Organ culture system

Usage of the organ culture for IVS has started decades earlier (468). In their studies, Steinberger and colleagues utilized the organ culture method, which was developed previously by Trowell (485). Interestingly, the tissue was cultured at the interface between the liquid phase and the gaseous phase, permitting the tissue to have access to the nutrients in the liquid phase and oxygen in the gaseous phase. The privilege in using the organ culture technique is that the spatial arrangement for the testicular cells is preserved i*n vitro*, keeping testicular architecture with the same type of surrounding cells as *in vivo*. However, for decades, it was not possible to show full spermatogenesis *in vitro* in any species. Some reports showed human germ cell differentiation *in vitro* using the organ culture method, but still it was not full spermatogenesis (486,487). In his study, Tesarik and colleagues isolated seminiferous tubules from testicular biopsies of obstructive azoospermia patient. The isolated tubules were cultured *in vitro* in an organ culture setup, resulting in post-meiotic sperm cell differentiation. Moreover, the *in vitro* developed spermatids could be used in fertilizing oocytes, which indicates to the functionality of these spermatids.

In 2010, Gohbara and his group could show full spermatogenesis *in vitro* in mice, using organ culture technique (488). The group has cultured testicular tissue form transgenic mice at 4.5-14.5 d*pp* in an organ culture setup. The transgenic mice were expressing green fluorescent protein (GFP) exclusively in the germ cells at the mid-meiotic stage (*Acr-GFP*) and the end-meiotic stage (*Gsg2-GFP*). The group has cultured the testicular tissues on agarose stands, to be at the interface between the gaseous phase and the liquid medium phase. The cultured tissues resulted in the presence of post-meiotic round spermatids. One year later, the same group has proved the functionality of the produced mature germ cells, by producing healthy offspring that was fertile as well (489). Later on, they used SSCs, adult testicular tissue, and cryopreserved neonatal tissue instead of the fresh neonatal testicular tissue, in order to prove the concept (490-493). One important modification the group has made was introducing Minimum Essential Medium alpha (MEMα) and KSR to the culture medium instead of the normal fetal bovine serum (FBS). Using FBS in IVS earlier usually resulted in meiotic arrest. This KSR was intended to be used for stem cell propagation, keeping their stemness and preventing their differentiation (494). Surprisingly, using KSR in murine testicular tissue culture induced germ cell differentiation. Using the organ culture technique, other groups have reported male germ cell differentiation as well (495-497). In these studies, the authors have used the organ culture technique to assess the effect of retinol and the cryopreservation temperature on murine germ cell differentiation *in vitro*. However, full spermatogenesis *in vitro* has never been reported in humans. The reason behind the difficulty in human germ cell differentiation *in vitro* is still not fully understood. However, the

differences in the hormonal regulation of spermatogenesis between the different species, with some differences in the spermatogenesis process itself, might explain this difficulty.

In an attempt to explore the ECM in the testis, it has been reported that there are more than 100 proteins present in the ECM after decellularizing human testis, which opens the door for studying the role of ECM in spermatogenesis *in vivo* (498). More important, it could be of great implication to use the decellularized testicular matrix (DTM) as a scaffold to culture testicular cells *in vitro*.

Certainly, production of the mature spermatozoa from the immature germ cells is the objective of IVS. However, the ultimate goal is to use the produced mature germ cells to produce offspring. Although it has been shown in mice that the produced offspring by IVS is healthy and fertile, it is still not the case in humans. First, the production of mature spermatozoa from immature testicular tissue has not been shown in humans yet. Second, as we should be more cautious when it comes to humans, even if the mature human spermatozoa are produced by IVS, it is not the end of the story. Still, we need to investigate the genetic and epigenetic stability of the produced haploid cells before using them in ART to produce pregnancy, since the genetic and epigenetic status of the cells can differ after culture. In the same context, effect of the long-term culture on SSCs genetic and epigenetic profiles has been evaluated (499-501).

It is noteworthy that *in vitro* germ cell maturation will addressed in detail in the results and discussion section of the separate studies included in the thesis, as this is the main aim of the thesis.

2 AIM OF THE THESIS

The overall aim of this thesis was to elucidate the mechanisms involved in the male germ cell development *in vitro*, with the ultimate goal of establishing an *in vitro* model for human spermatogenesis as a clinical tool in future.

The specific aims of the four projects were:

1. To investigate whether the culture conditions and gene expression profile could be used to predict the differentiation potential of hES cells *in vitro* towards male germ cells.

2. To optimize the culture conditions for rat male germ cell differentiation *in vitro* using a three-dimensional culture system.

3. To investigate the possibility of producing mature rat male germ cells *in vitro* from immature pre-pubertal testicular tissue, exploiting the optimal organ culture conditions that were published for mouse previously.

4. To study the effects of long term *in vitro* culture on testicular tissues from pre-pubertal patients, prior to or during gonadotoxic therapy, using the previously published organ culture technique.

3 MATERIAL AND METHODS

3.1 ETHICS

3.1.1 Human embryonic stem cells (hES cellss)

In paper I, the hES cell lines HS207 (XY), HS360 (XY) and HS401 (XY) were derived at Karolinska Institutet hospital, Huddinge, Stockholm, Sweden, under the ethical approval obtained from the Ethics Board of Karolinska Institutet and the Regional Ethics Board in Stockholm with the number Dr no 454/02.

3.1.2 Animals

In the paper II and III, the Sprague-Dawley rats were used under the ethical approvals obtained from the Ethics Board of Karolinska Institutet and the Regional Ethics Board in Stockholm with the numbers N489/11 and N280/14.

3.1.3 Human subjects

In paper IV, testicular tissues were collected from the patients under the ethical approvals obtained from the Ethics Board of Karolinska Institutet and the Regional Ethics Board in Stockholm (Dnr 2013-2129-31-3) and the National Ethics Board of Iceland, Reykjavik (VSN 15-002). Due to the fact that the patients were minors, parents of all patients gave a written and informed consent and the study was performed according to the amended Declaration of Helsinki.

3.2 HUMAN AND ANIMAL CELLS AND TISSUES

3.2.1 Human embryonic stem cells (hES cells)

The ES cell lines used in paper I, with normal XY karyotyping (as we were intending to differentiate the cells towards male germ cells), were derived from ICM either by immunosurgery (HS207), using 10mg/ml protease pronase E solution (Sigma Aldrich, St. Louis, USA), 20% anti-human whole serum, and 20% guinea pig complement (Sigma Aldrich), or mechanically (HS360 and HS401) using flexible metal needles. Subsequently, cells were plated on mitotically inactivated feeders (64,65,502,503). While immunosurgery gives cleaner separation for the ICM, the mechanical separation has the advantage of being xeno-free.

3.2.2 Animals

The animals used in paper II and III were Sprague-Dawely rats, purchased with their mothers from Charles River (Sulzfeld, Germany), transferred to Karolinska Institutet, handled, and sacrificed by decapitation at the age of 5-7 d*pp* under the ethical approvals obtained previously at Karolinska Institutet. The age of 5-7 d*pp* was chosen so that it represents the same stage of development in testis as in the pre-pubertal patients.

3.2.3 Human subjects

In paper IV, twelve pre-pubertal patients with different diagnoses (acute myeloid leukemia (AML), juvenile myelomonocytic leukemia (JMML), Wilms' tumor, high risk hepatoblastoma, myelodysplatic syndromes (MDS), sickle cell anemia, Fanconi anemia, and thalassemia major), from nine months of age and up to 11.5 years of age, were recruited in the study. The exclusion criteria were mainly testis volume more than 10ml and increased risk of bleeding or other complications.

3.3 CELL AND TISSUE CULTURE

3.3.1 Stem cell culture and differentiation

In paper I, the hES cell lines were cultured on mitotically inactivated human foreskin fibroblasts (hFFs; CRL-2429; ATCC, VA, USA) in Knockout Dulbecco´s Modified Eagle´s medium (KO-DMEM, 10829018, Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 20% KSR (10828028, Gibco), 0.5% penicillin-streptomycin (Pen/Strep, 15140122, Gibco), 2 mM L-Glutamax (35050038, Gibco), 1% non-essential amino acids (NEAA, 11140035, Gibco), 0.5 mM 2-mercaptoethanol (31350010 Gibco) and 8 ng/ml rFGF2 (234-FSE/CF, R&D Systems, MN, USA) as described previously (Figure 4) (93,109). The medium was changed 6 times per week and the cells were passaged mechanically every 4-6 days using a surgical scalpel.

To obtain the spheres, the hES cell colonies were scraped off the feeder layer with a surgical scalpel and transferred to 24-well ultra-low-attachment-surface plates (3473, Corning Inc., NY, USA) in Neurobasal medium (21103049, Gibco) supplemented with 14% KSR, 2 mM Glutamax, 0.5% Pen/Strep, 1% NEAA, 20 ng/ml rFGF2, 25 ng/ml rActivin A (120-14E, Peprotech, NJ, USA), 1.0 µg/ml human fibronectin (354008, BD Biosciences, NJ, USA), 0.5 µg human placental laminin/ml (L6274, Sigma Aldrich), 0.001% porcine gelatin (G2625, Sigma Aldrich), 10 ng/ml each of recombinant brain-derived neurotrophic factor (rBDNF, 450–02, Peprotech), recombinant neurotrophin-3 (rNT3, 450–03, Peprotech) and recombinant neurotrophin-4 (rNT4, 450–04, Peprotech), and 1 × Nutridoma (11011375001, Roche, Basel, Switzerland) as described earlier (Figure 4) (72). The medium was changed every other day and the spheres were passaged mechanically into 4-6 pieces by a surgical scalpel at intervals of 8–10 days.

For differentiation of the cells, they were differentiated on a feeder layer without rFGF2, in the presence and absence of 10 ng/ml BMP7 (354-BP-010, R&D Systems). The cells were not passaged during differentiation and the medium was changed every other day. For differentiation of spheres, they were differentiated for 2 weeks either in normal non-stimulated conditions in 50% KO-DMEM (10829018, Gibco) and 50% F12 (21127, Gibco) or with stimulation by adding 10 ng/ml BMP7. The spheres were not passaged during differentiation and the medium was changed every other day. Afterwards, cells were stimulated by 5 IU/L (international units per liter) each of human chorionic gonadotropin (hCG, Pregnyl 5000IE, Merck Sharpe & Dohme, NJ, USA) and recombinant FSH (rFSH,

Gonal F 75IE, Merck KGaA, Darmstadt, Germany) for 2 days. hCG has the same action of LH on the same receptor, but it is used widely instead of LH in research, as it is easier and cheaper to get.

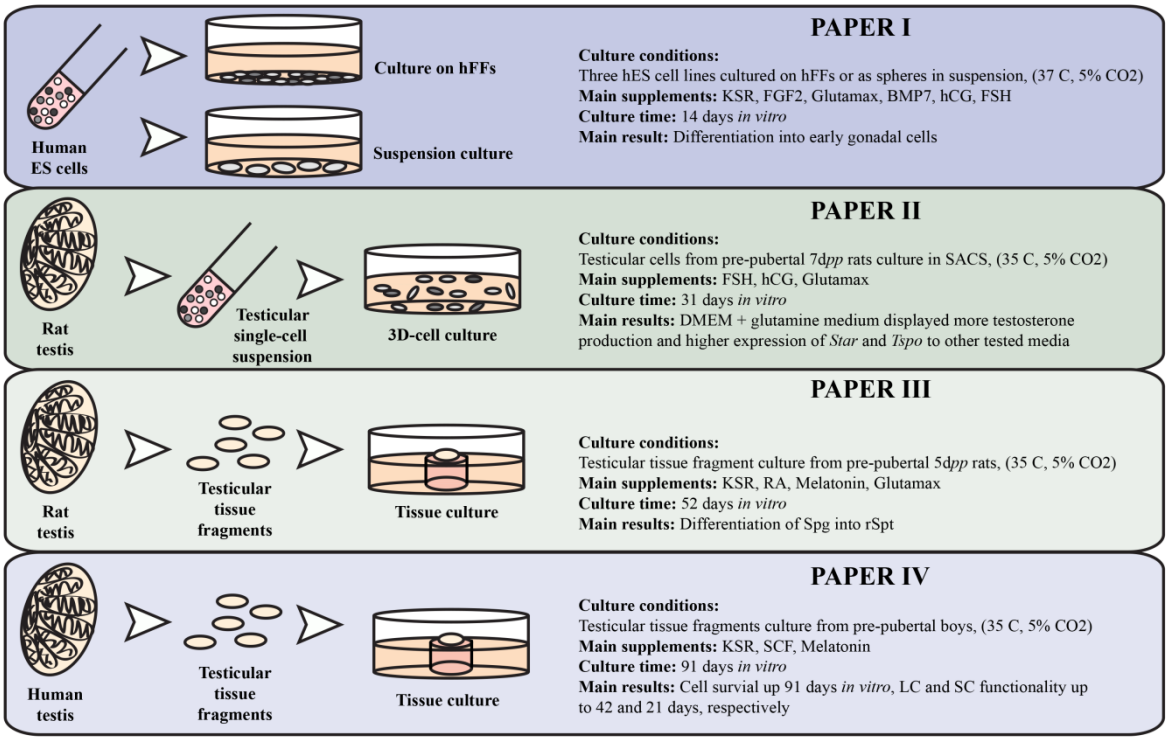

Figure 4. Human embryonic stem (ES) cell and testicular cell and tissue culture. In paper I, three hES cell lines (HS207, HS360, and HS401) where cultured either on feeder cells (human foreskin fibroblasts; hFFs) or in suspension culture as spheres. The main supplements were knockout serum replacement (KSR), basic fibroblast growth factor (FGF2), Glutamax, human chorionic gonadotropin (hCG) and follicle stimulating hormone (FSH). The culture ran for 14 days at 37°C and 5% CO_2. In paper II, rat testicular single cell suspension was obtained by enzymatic digestion of 7d*pp* rat testes. The single cell suspension was cultured in a three-dimensional (3D) culture system, formed of culture medium Dulbecco modified essential medium (DMEM) and agarose 0.7% in 1:1 ratio. The main supplements were Glutamax, FSH, and hCG. The culture ran for 31 days at 35°C and 5% CO_2. In paper III, rat testicular tissue fragments were obtained from 5d*pp* rat testes and culture using the organ culture setup, on agarose stands and using Minimum Essential Medium alpha (MEMα). The main supplements included Glutamax, KSR, melatonin, and retinoic acid (RA). The culture ran for 52 days at 35°C and 5% CO_2. In paper IV, testicular fragments from pre-pubertal boys at high risk to develop infertility were obtained by surgical intervention, and cultured in an organ culture system on agarose stands using NutriStem® hESC XF Medium and MEMα medium. The main supplements included KSR, melatonin and stem cell factor (SCF). The culture ran for 91 days at 35°C and 5% CO_2.

3.3.2 Three dimensional cell culture (3D)

In paper II, after decapitation, testes obtained from pre-pubertal rats 7d*pp* were de-capsulated in DMEM containing glutamine (41966, Gibco) and supplemented with1 % pen/strep. To obtain single cell suspension, testicular tissue was digested by three-step enzymatic digestion using collagenase/Dispase (269638, Roche; final concentration: 0.04/0.32U/ml), DNAse (104159, Roche; final concentrations: 48U/ml), and Collagenase IV (C-1889, Sigma-Aldrich; final concentrations: 50 U/ml) as described previously (453). The cells were counted in a Bürker chamber, and examined for viability by trypan blue staining (15250061, Gibco; 1:20 dilution).

The testicular single cell suspension was cultured in agarose-medium matrix, prepared by mixing autoclaved 0.7% SeaKem® LE agarose (50004, Lonza, Basel, Switzerland) or 0.7% LMP agarose (15517022, Invitrogen, Thermo Fisher Scientific) with the relevant culture medium (supplemented with 1% pen/strep) at a ratio of 1:1 to give a final agarose concentration of 0.35% agarose, as previously described (Figure 4) (481). The culture media investigated in this study included DMEM + glutamine or without glutamine (DMEM-glutamine; 21969, Gibco), DMEM + Glutamax (31966, Gibco), DMEM/F12 (21331, Gibco), F12 (21765, Gibco), and MEM (21430, Gibco). For stimulation, rFSH (Gonal F 75 IE, Merck; final concentration: 5 IU/L) and hCG (Pregnyl 5000 IE, Merck Sharpe and Dohme; final concentration: 5 IU/L) were used. To investigate their effect on testosterone production, amino acids (AA; 11130-036, Gibco) or NEAA (11140-035, Gibco) were added separately to F12 medium at a final concentration of 4%, similar to their concentrations in DMEM. The single cell suspension was inoculated in the agarose medium matrix with a concentration of 1.0×10^6 cells/ml. All cell cultures were maintained at 35°C under 5% CO_2 and performed in triplicates.

3.3.3 Animal tissue culture

In paper III, the organ culture technique, as described previously by Sato (489), was used in culturing the rat testicular tissue. Agarose stands were prepared by mixing autoclaved 0.7% SeaKem® LE agarose with the relevant medium at a ratio of 1:1 to give a final agarose concentration of 0.35% agarose. These stands were placed in 6-well plates, with the culture medium placed to the level of the top of the stand. Rat testicular tissue was obtained from 5 d*pp* rats after decapitation and de-capsulation of the testes in MEMα (22561-021, Gibco) culture medium supplemented with 1% pen/strep. Subsequently, testes were cut with sterile forceps and scissors into small pieces (≈$1mm^3$ in size for each) and placed on top of the agarose stands (Figure 4). In some wells, rat epididymal fat from 5 d*pp* rats was cut with sterile forceps and scissors into small pieces (≈$1mm^3$ in size for each) and placed on top of the agarose stand in direct contact with the testicular tissue. The culture medium consisted of either MEMα without Glutamax (22561-021, Gibco) or MEMα with Glutamax (32561-029, Gibco); both were supplemented with 10% KSR (10828-028, Gibco) and 1% pen/strep. Melatonin (M5250, Sigma Aldrich, final concentration 10^{-7} M) or RA (R2625, Sigma Aldrich, final concentration 10^{-6} M) or a combination of both was added to the culture

medium, depending on the composition intended to investigate if they have an additional benefit for germ cell differentiation. The rat testicular tissue was cultured at 34.5°C and 5% CO for up to 52 days, and the culture medium was replaced once per week.

3.3.4 Human tissue culture

In paper IV, the same organ culture technique, as described previously in the animal tissue culture (section 3.3.3), was exploited in culturing the human testicular tissues. In brief, testicular tissue was obtained from the patients by an open surgical operation and 20% of one testis (testis volume below 10ml) was taken and divided into two pieces; 2/3 of the material was further processed for cryopreservation, according to Keros *et al* 2007 (424), whereas the remaining 1/3 of the fresh tissue material was directly used for tissue culture experiments described in this study. The tissues were transferred in NutriStem® hESC XF Medium (05-100-1A, Biological Industries, CT, USA) supplemented with 1% penicillin/streptomycin (Pen/Strep; 15140-122, Gibco) on ice within 24 hours after the surgical operation to the lab in Karolinska Institutet Hospital in Solna, Sweden. The biopsies were then cut with a sterile forceps and a small pair of scissors into small pieces (≈1mm3 in size for each) and processed as exactly described in the animal tissue culture (section 3.3.3) as shown in Figure 4. Two different culture media, supplemented with 1% Pen/Strep, were used in culturing the testicular tissues; MEMα (22561-021, Gibco) and NutriStem® hESC XF Medium. The different supplements used included 10% CTS™ KnockOut™ Serum Replacement XenoFree Medium (KSR-XF; 12618013, Gibco), melatonin (M5250, Sigma Aldrich, final concentration 10^{-7} M), RA (R2625, Sigma Aldrich, final concentration 10^{-6} M), rFSH (Gonal-f® 75 IE-IU, Merck, final concentration 5 IU/L), testosterone (T1500, Sigma Aldrich, final concentration 20ng/ml), and SCF (AF-300-07-10UG, Peprotech, NJ, USA, final concentration100ng/ml). The testicular tissues were cultured at 35°C and 5% CO_2 for up to 91 days, as it is expected to take longer time for germ cell differentiation in human than in rodents (271), and the culture medium was replaced once per week.

3.4 STAINING

3.4.1 Embedding and sectioning

Samples were fixated either in paraformaldehyde (PFA) 4% in phosphate buffered saline (02176, Histolab, Gothenburg, Sweden) or in Bouin's solution (HT10132, Sigma-Aldrich) overnight at 4°C, followed by gradual dehydration in ascending ethanol concentrations 30, 50, and 70%; each at room temperature overnight. Bouin's solution gives better fixation for soft tissue, such as the testis, as it preserves the structure much better than PFA does (152). However, PFA is preferred over Bouin's solution in case immunostaining is desired, as many primary antibodies do not work well with Bouin's solution fixated samples. Later on, samples were dehydrated in ascending ethanol concentrations (80%, 96% and 99.6% ethanol), each at room temperature for at least 6 hours. Samples were then transferred in butyl acetate (45860, Sigma-Aldrich) overnight at room temperature followed by embedding in melted paraffin (ParaplastX-TRAR, P3808, Sigma-Aldrich) at 61°C overnight. Paraffin-

embedded samples were cut into 5 μm sections after cooling down, using a Biocut sectioning machine (Reichert-Jung, NY, USA), and sections were placed on microscope slides (10143352, Superfrost Plus, Thermo Scientific, Thermo Fisher Scientific) and dried at 37°C overnight.

For transmission electron microscopy, samples were fixated in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. Afterwards, samples were rinsed with the same buffer, post-fixed in 2% osmium tetroxide in this buffer at 4°C for 2 hours, dehydrated in ethanol and then acetone and embedded in LX-112 (Ladd, Burlington, Vermont, USA). Subsequently, Leica ultracut UCT (Leica, Wien, Austria) was used to obtain ultra-thin sections (approximately 40–50 nm thick).

3.4.2 Periodic acid Schiff staining (PAS)

PAS staining is used to detect carbohydrates, glycoproteins, and glycolipids in the tissue. Periodic acid is an oxidizing agent that oxidizes the glycols groups in sugars to aldehyde groups, which condenses with Schiff's reagent to form a red purple color (504). Sections of PFA fixated or Bouin's solution fixated samples were de-paraffinized in xylene and re-hydrated in descending ethanol concentration (99.6, 96, and 70%). Afterwards, PAS kit (101646, Merck) was used to stain the sections according to the manufacturer's protocol. After rinsing in distilled water, sections were incubated in periodic acid for 5 minutes, followed by rinsing again in distilled water. Later on, sections were incubated in Schiff's reagent for 15 minutes, followed by rinsing in distilled water. Sections were then counterstained in hematoxylin (Mayer's Hemalaun solution, 1092491000, Merck) for 2 minutes, followed by rinsing under tap water for 3 minutes. Subsequently, sections were de-hydrated in ascending ethanol concentrations (70, 96, and 99.6%) followed by mounting in Entellan® new (1079610100, Merck). When counterstaining was not desired, sections proceeded directly to mounting without counterstaining.

3.4.3 Immunohistochemistry

In this thesis, beside the regular immunohistochemistry, staining of the apoptotic cells using a kit special for apoptosis detection was performed.

3.4.3.1 Regular immunohistochemistry

Sections of PFA fixated samples were de-paraffinized in xylene and re-hydrated in descending ethanol concentration (99.6, 96, and 70%). Endogenous peroxidase was blocked using 0.3% H_2O_2 in ethanol 96% for 10 minutes at room temperature. After washing in washing buffer (phosphate buffered saline (PBS) or Tris buffered saline (TBS)), antigen retrieval was performed on the sections, using 0.1% citrate buffer 0.1% TritonX for 8 minutes at room temperature, 0.1M citrate buffer (pH 6.0) in the microwave at 600W for 15 minutes, or 0.01M citrate buffer 0.05% Tween20 (pH 6.0) for 30 minutes at 95°C. After cooling down for 30 minutes and washing again, sections were blocked for non-specific protein binding with a blocking buffer, formed of 5-10% normal serum (donkey, horse or goat; depending on

the secondary antibody used) and 0.1-1% Bovine serum albumin (BSA) in PBS or TBS, for 20-30 minutes at room temperature. Sections were then incubated with the primary antibodies or immunoglobulins (IgGs; from the same species as the primary antibody) for negative control, diluted in the corresponding blocking buffer at 4°C overnight (all the primary antibodies used are listed in Table 1). After washing, sections were incubated with the biotinylated secondary antibodies diluted in the same blocking buffer at room temperature for 1-2 hours (all the secondary antibodies and sera used are listed in Table 2). Afterwards, sections were washed and Vectastain ABC elite Standard kit (PK-6100, Vector labs, CA, USA) was applied on the sections according to the manufacturer's protocol. After washing, ImPACT™ DAB (3, 3'-Diaminobenzidine) kit (SK-4105, Vector labs) was used for detection according to the manufacturer's protocol. Sections were then counterstained with hematoxylin (Mayer's Hemalaun solution, 1092491000, Merck) for 5 seconds and washed in running tap water for 2 minutes. Later on, sections were dehydrated using ascending ethanol concentrations followed by xylene, and mounted finally in Entellan® new (1079610100, Merck).

3.4.3.2 Staining of the apoptotic cells

The Terminal deoxynucleotidyl transferase dUTP nick end-labeling TUNEL Assay kit (DeadEnd™ Colorimetric Tunel System, G7130, Promega, WI, USA) was used to detect the apoptotic cells according to the manufacturer's protocol. The kit is specified to detect the fragmented DNA, which is the end result of apoptosis (505). In brief, sections of PFA fixated samples were de-paraffinized and re-hydrated as previously mentioned in the immunohistochemistry section (3.4.3). Later on, sections were treated with proteinase-k and then incubated with biotinylated nucleotide mix + rTDT enzyme + enzyme buffer at 37°C for one hour. Afterwards, sections were washed, incubated with streptavidin-HRP (horseradish peroxidase) complex, and detected with DAB. Sections were then counterstained with hematoxylin, dehydrated in ascending ethanol concentrations and mounted in Entellan® new mounting medium.

Table 1. A list of all the primary antibodies used in the thesis.

	Antibody	Origin	Concentration in µg/ml	Catalogue and company	Paper
1	Ddx4	Rabbit polyclonal	2-10	Ab13840, Abcam, Cambridge, UK	I, II and III
2	Ddx4	Mouse monoclonal	2	Ab27591, Abcam	IV
3	Vimentin	Rabbit polyclonal	50	Ab8545, Abcam	I
4	Vimentin	Rabbit monoclonal	2-5	Ab92547, Abcam	II
5	3βHsd	Mouse monoclonal	2	Sc-100466, Santa Cruz, Texas, USA	I
6	3βHsd	Rabbit polyclonal	1	Sc-28206, Santa Cruz	II
7	AMH	Rabbit polyclonal	5	Ab103233, Abcam	I
8	SOX9	Rabbit polyclonal	2-5	AB5535, Millipore, Billerica, Massachusetts, USA	I and IV
9	WT-1	Rabbit polyclonal	2-5	Ab89901, Abcam	I and IV
10	INSL3	Rabbit polyclonal	4	Sc-134586	I

11	AP2γ	Rabbit polyclonal	2	Sc-8977, Santa Cruz	II
12	Crem	Rabbit polyclonal	0.5-1	Sc-440, Santa Cruz	III
13	Acrosin	Rabbit polyclonal	4	NBP2-14260, Novus Biologicals, Minneapolis, USA	III
14	Ki67	Rabbit polyclonal	5	Ab16667, Abcam	II and III
15	POU5F1 (OCT4)	Goat polyclonal	4	Sc-8629, Santa Cruz	I
16	IgGs	Rabbit	Depending on the primary antibody	Ab27478, Abcam	I, II, III and IV
17	IgGs	Rabbit	Depending on the primary antibody	Sc-2027, Santa Cruz	I and II
18	IgGs	Mouse	Depending on the primary antibody	Sc-2025, Santa Cruz	I and IV
19	IgGs	Goat	Depending on the primary antibody	Sc-2028, Santa Cruz	I

3.4.4 Immunofluorescence

In this thesis, we have performed single immunofluorescence and double immunofluorescence.

3.4.4.1 Single immunofluorescence

Sections of PFA fixated samples were treated as previously described in the immunohistochemistry section (3.4.3), till the incubation with the primary antibodies. Subsequently, sections were incubated with either Cy3- or Alexa Fluor 488-conjugated secondary antibodies, diluted in the blocking buffer used in blocking of the non-specific protein binding, for 30-60 minutes at room temperature (all secondary antibodies and sera are listed in Table 2). After washing, sections were mounted in VECTASHIELD mounting medium with DAPI (4',6-Diamidino-2-Phenylindole; H-1500, Vector labs).

3.4.4.2 Double immunostaining

The protocol developed by van den Driesche and colleagues (506) was exploited, as it has the privilege of using two primary antibodies simultaneously from the same species. As previously described in the immunohistochemistry section (3.4.3), sections of PFA fixated samples were treated till the antigen retrieval step. After antigen retrieval, sections were blocked for endogenous peroxidase using 3% H_2O_2 in methanol for 30 minutes at room temperature. Afterwards, blocking of non-specific protein binding was performed using 20% normal chicken serum 5% BSA in TBS for 30 minutes at room temperature. Sections were then incubated with the primary antibodies or IgGs for negative control at 4°C overnight. After washing, sections were incubated with HRP-conjugated chicken secondary antibodies against the same species from the primary antibodies for 30 minutes at room temperature. Later on, sections were washed and the TSA™ Plus Fluorescein System (NEL741001KT, Perkin Elmer Life Sciences, Waltham, Massachusetts, USA) was applied according to the manufacturer's protocol. Antigen retrieval was repeated once more and sections were incubated with 3% H_2O_2 in TBS-Tween 0.01% for 30 minutes at room temperature. Later on, sections were incubated again as previously mentioned with the primary antibodies or IgGs for negative control, washed, incubated with the HRP-conjugated chicken secondary antibodies. After washing, TSA™ Plus Cyanine 3 System (NEL744001KT, Perkin Elmer Life Sciences) was applied according to the manufacturer's protocol and the sections were mounted in VECTASHIELD mounting medium with DAPI (H-1500, Vector labs).

Table 2. A list of all the secondary antibodies listed in the thesis.

	Secondary antibody origin	Against	Conjugation	Concentration in µg/ml	Catalogue, company	Paper
1	Goat	Rabbit IgG	Biotin	5	BA1000, Vector labs	I
2	Horse	Goat IgG	Biotin	5	BA9500, Vector labs	I
3	Horse	Mouse IgG	Biotin	5	BA200, Vector labs	I
4	Chicken	Rabbit IgG	HRP	2	Sc-2963, Santa Cruz	I, II and III
5	Donkey	Rabbit IgG	Cy3	2.5-5	711-166-152, Jackson Immunoresearch, PA, USA	I, II, III and IV
6	Donkey	Mouse IgG	Alexa Fluor 488	4.6	715-546-150, Jackson Immunoresearch	IV
7	Goat	Rabbit IgG	Biotin	5	Ab64256, Abcam	II and III

3.5 MICROSCOPY, MORPHOLOGIC, AND HISTOLOGICAL EVALUATION

3.5.1 Transmission electron microscopy (TEM)

After preparation of the ultrathin sections, they were contrasted by uranyl acetate and then lead citrate and examined under a Tecnai 12 Spirit Bio TWIN transmission electron microscope (FEI Company, Eindhoven, Netherlands) at 100 kV. Capturing of the digital images was done using a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). For identification of the different cell shape, locations, and size; the criteria formulated by Sathananthan and colleagues were followed (507).

3.5.2 Microscopy for immunostaining and PAS staining

All stained sections were examined under the microscope (Eclipse E800; Nikon, Tokyo, Japan) with a 12.5 million-pixel, cooled digital color camera (Olympus DP70, Olympus Optical, Tokyo, Japan). The different cell types were identified on the basis of size, shape and location, according to Russel and colleagues (152).

3.5.3 Time lapse

Time lapse was taken using an Eclipse TE200 (Nikon) microscope, with the pictures taken with an Infinity1 camera (Lumenera, Ontario, Canada). Frames for the time lapse were taken every one second and represented in the videos with the real-time pace.

3.5.4 hES cells photography

hES cells photography was performed using a Nikon SMZ-U microscope (Nikon) with an Infinity 1 camera (Lumenera).

3.5.5 Morphologic and histological evaluation

In paper I, evaluation of DDX4- and SOX9-expressing cell colonies was done by manual examination of at least 45 cell colonies from each cell line.

In paper II, the apoptotic rate was calculated as a ratio of the TUNEL-positive cells (apoptotic cells) to the total number of cells in a section. At least, 500 cells for each sample were counted, and the rate was expressed relative to the rate on the first day of culturing, in order to avoid the effect of the different culture techniques.

In paper III, the evaluation of Ddx4/Ki67 double staining was done by counting the number of tubules containing Ddx4 positive/Ki67 positive cells per the total number of tubules in a section (germ cell proliferation index) and was compared in two culture conditions (MEMα + 10% KSR ± Glutamax). Then, 60 to 80 tubules were counted for each culture condition. For the evaluation of Ddx4/Crem staining, the number of tubules containing Ddx4 positive/Crem positive cells per the total number of tubules in a section was counted and compared across the different culture conditions. A total of 100 to 450 tubules were counted for each condition. For the evaluation of Acrosin staining, the number of tubules containing Acrosin positive cells per the total number of tubules in a section was counted and compared within

the different culture conditions. A total of 100 to 450 tubules were counted for each setting. For the morphologic evaluation, the number of tubules containing primary spermatocytes or round spermatids per the total number of tubules in a section was used as an index for the germ cell differentiation progress. The total number of tubules counted per culture condition varied between 80 and 180 tubules. For the gross morphologic evaluation, 4X pictures of the PAS stained sections were taken and the necrotic region in a section was identified and quantified relative to the total surface area of the same section (as a percentage) using the ImageJ software (National Institutes of Health, USA). A number of 3 to 9 biological replicates were recruited from each culture condition for statistical analysis, and the percentage of necrotic area was compared between the different conditions.

In paper IV, the necrotic area calculation was performed by taking 4X pictures of the PAS stained sections, and then, surface area of the necrotic region in a section was identified and quantified in relation to the total surface area of the same section (as a percentage) using the ImageJ software (National Institutes of Health, USA).

3.6 GENE EXPRESSION ANALYSIS

3.6.1 RNA extraction and cDNA amplification

Samples taken were snap frozen in dry ice and stored at -80°C till RNA extraction was performed. For RNA extraction, TRIzol® Reagent (15596-018, Invitrogen, Thermo Fisher Scientific) and RNeasy Mini Kit (74104, Qiagen, Venlo, Netherlands) were utilized according to the manufacturer's protocol. The TRIzol® reagent depends on guanidine/isothiocyanate/phenol/chloroform extraction method, which separates the RNA from DNA and proteins (508). The RNA isolated was treated with DNase1 Amplification Grade (AMPD1, Sigma-Aldrich) to eliminate the genomic DNA contamination. For cDNA amplification, the IScript cDNA synthesis kit (170-8891, Bio-Rad, CA, USA) was applied according to the manufacturer's protocol.

3.6.2 Reverse transcriptase-polymerase chain reactions (RT-PCRs)

In this technique, the mRNA of interest is reverse transcribed by reverse transcriptase into cDNA, which is amplified by PCR and then detected as a band by gel electrophoresis (509). The presence of the band means that the gene is expressed, while the absence of the band means the gene is not expressed. In order to perform RT-PCR analysis, the Expand High Fidelity PCR System (11759078001, Roche) was applied, using the 2710 Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific). Primers specific for messenger RNAs considered to be consensus markers for undifferentiated hES cells were used, along with *ACTB* (beta actin) as an endogenous control. A list of all the primers used for gene expression analysis by the RT-PCR technique is included in Table 3, including *GABRB3* (Gamma-Aminobutyric Acid Type A Receptor Beta3 Subunit).

Table 3. A list of all the primers used in gene expression analysis by RT-PCR technique.

Fw: forward, Rev: reverse.

	Gene	Species	Sequence 5´→3	Paper
1	*NANOG*	Human	Fw: CAAAGGCAAACAACCCACTT Rev: CTGGATGTTCTGGGTCTGGT	I
2	*POU5F1*	Human	Fw: GACAACAATGAAAATCTTCAGGAGA Rev: TTCTGGCGCCGGTTACAGAACCA	I
3	*TDGF1*	Human	Fw: AGCACAGTAAGGAGCTAAACA Rev: CAGTTCCGTCCGTAGAAGGAG	I
4	*GDF3*	Human	Fw: GTACTTCGCTTTCTCCCAGAC Rev: GCCAATGTCAACTGTTCCCTT	I
5	*GABRB3*	Human	Fw: CAAGCTGTTGAAAGGCTACGA Rev: ACTTCGGAAACCATGTCGATG	I
6	*ACTB*	Human	Fw: CCTGGCACCCAGCACAAT Rev: GGGCCGGACTCGTCATAC	I

3.6.3 Quantitative PCR (qPCR)

The iCycler iQ™ multicolor RT-PCR detection system (Bio-Rad) was utilized to perform the qPCR, while the iCycler iQ® ver.3.1 software (Bio-Rad) was used in data analysis. Two main technologies were used in this thesis to perform qPCR; the TaqMan technology (Applied Biosystems) and SYBR® green technology (Bio-Rad). The TaqMan technology is based on a pre-designed probe for a specific gene. This probe has a reporter dye at its 5' end and a quencher at its 3' end. When the probe is at rest, the quencher is close enough from the dye that it is not detected, while when the probe binds to the template and amplification process starts, the reporter dye becomes free and then it can be detected. The more DNA amplified, the more reporter dye detected (510). For the SYBR® green technology, it depends on the binding of a fluorescent dye to the double stranded DNA. The dye can only be detected once bound to the DNA. The more DNA amplified, the more fluorescent dye detected (511). For these two technologies, the TaqMan Gene Expression Master Mix (4369510, Applied Biosystems) and the iQ SYBR® Green Super mix (170–8882, Bio-Rad) were used respectively. All primers and probes of the genes used in gene expression analysis by the SYBR® green technology and by the TaqMan technology are listed in Table 4 and Table 5 respectively. As an endogenous control, Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH)* was used in paper I for the ES cells, while *Actb* was used in paper II and III for the rat testicular cells. The comparative ddCt (threshold cycle) method was used in expressing he fold of change in gene expression (2^{-ddCt}).

Table 4. A list of all the primers used in gene expression analysis by qPCR using SYBR® green technology. Fw: forward, Rev: reverse.

	Gene	Species	Sequence 5´→3	Paper
1	*POU5F1*	Human	Fw: GACAACAATGAAAATCTTCAGGAGA Rev: TTCTGGCGCCGGTTACAGAACCA	I
2	*KIT*	Human	Fw: GGCATGCTCCAATGTGTGG Rev: GGTGTGGGGATGGATTTGC	I
3	*GAPDH*	Human	Fw: GAAGGTGAAGGTCGGAGTCAAC Rev: CAGAGTTAAAAGCAGCCCTGG	I
4	*Star*	Rat	Fw: CTGCTAGACCAGCCCATGGAC Rev: TGATTTCCTTGACATTTGGGT	II
5	*Tspo*	Rat	Fw: GCTATGGTTCCCTTGGGTCT Rev: GGCCAGGTAAGGATACAGCA	II
6	*Actb*	Rat	Fw: TGAAGATCAAGATCATTGCTC Rev: ACTCATCGTACTCCTGCTTGC	II

3.6.4 TaqMan Low-Density Arrays (TLDAs)

TLDA technology is based on a microfluidic card, pre-designed to have 90 probes, based on the TaqMan technology, distributed in 90 wells to detect genes specific for undifferentiated ES cells or their differentiated derivatives, along with 6 endogenous control genes (512). In paper I, TLDA cards (4385344, Applied Biosystems), designed for International Stem Cell Initiative (16), were used to evaluate the gene expression profile of the three cell lines cultured on feeders or in spheres. A total of 90 genes, in addition to 6 control genes, where investigated. The control genes used were *GAPDH, ACTB, RAF1, CTNNB1* (Catenin Beta 1), *EEF1A1* (Eukaryotic Translation Elongation Factor 1 Alpha 1) and *18S*, as these genes were suggested to be the most stable ones when analyzing gene expression in hES cells (16). Mean values of the replicates were used for heat maps and clustering analysis (Euclidean distance with complete linkage) using GENE-E software.

(http://www.broadinstitute.org/cancer/software/GENE-E/index.html).

Table 5. A list of all the probes used in gene expression analysis by qPCR using TaqMan® technology.

	Gene	Species	Assay ID	Paper
1	*NANOG*	Human	Hs02387400_g1	I
2	*DDX4*	Human	Hs00251833_m1	I
3	*FSHR*	Human	Hs00174865_m1	I
4	*VIM*	Human	Hs00185584_m1	I
5	*HSD3B1*	Human	Hs04194787_g1	I
6	*INSL3*	Human	Hs01394273_m1	I
7	*GAPDH*	Human	Hs02758991_g1	I
8	*Acr*	Rat	Rn01407524_m1	III
9	*Crem*	Rat	Rn01538528_m1	II and III
10	*Prm1*	Rat	Rn02345725_g1	II and III
11	*Sycp3*	Rat	Rn01648688_m1	III
12	*Boll*	Rat	Rn01441407_m1	II and III
13	*Kit*	Rat	Rn00573942_m1	II and III
14	*Zbtb16*	Rat	Rn01418644_m1	II and III
15	*Dazl*	Rat	Rn01757162_m1	II and III
16	*Ddx4*	Rat	Rn01489814_m1	III
17	*Actb*	Rat	Rn00667869_m1	II and III

3.7 HORMONAL ASSAY

In order to investigate the somatic cell environment functionality *in vitro* (e.g. Leydig cells and Sertoli cells) we have performed hormonal assays to evaluate the testosterone and Inhibin B production.

3.7.1 Testosterone assay

To evaluate the testosterone production, testosterone was extracted from the tissue and medium samples using ethyl acetate. Steroids (e.g. testosterone) diffuse form the aqueous phase into the organic phase (ethyl acetate). After separation of the two phases and evaporation of the ethyl acetate, the residue was re-suspended in PBS and testosterone was evaluated by either radioimmunoassay or enzyme linked immunosorbent assay.

3.7.1.1 Testosterone radioimmunoassay (RIA)

The RIA kit for testosterone evaluation is based on using tubes pre-coated with testosterone antibodies. When testosterone form in samples is incubated with the I^{125} testosterone, both types of testosterone compete on these antibodies. The higher the testosterone concentration in the sample, the lower the radioactivity bound to the coated tube. By measuring the radioactivity in the tube, we can determine the concentration of testosterone, in relation to a standard curve (513). The COAT-A-COUNT1 kits (TKTT2, Siemens, Munich, Germany) was used in accordance with the manufacturer's protocol to evaluate testosterone production. The extracted testosterone was incubated with I^{125} testosterone at 37°C for 3 hours in special coated tubes. After decantation of the mixture, the radioactivity in the tube was counted in a Gamma counter (1470 Wizard Wallac, GMI, Ramsey, MN, USA) for one min. The standards provided with the kits (0–55 nmol/L) were used for calibration.

3.7.1.2 Testosterone Enzyme Linked Immunosorbent Assay (ELISA)

In this assay, the testosterone in the sample competes with testosterone-HRP conjugate supplied with the kit on binding to the plates that were pre-coated with testosterone antibody. Hence, the higher the testosterone concentration, the lower the color detected. The testosterone ELISA kit (EIA-1559, DRG instruments, Marburg, Germany) with an intra-assay CV < 5% and inter-assay CV < 10% was used according to the manufacturer's protocol to perform the assay. The microplate reader Fluostar Omega (BMG LabTech, Ortenberg, Germany) was used for reading the absorbance at 450nm, while the standards provided with the kit were used for generating a standard curve.

3.7.2 Inhibin B Gen II ELISA

Inhibin B is composed of 2 subunits; subunit β_B and subunit α. Inhibin B shares with inhibin A the subunit α, while they differ in the subunit β (β_B for inhibin B and β_A for inhibin A). On the other hand, inhibin B shares with activin B the subunit β_B. Hence, to evaluate inhibin B production, a sandwich ELISA is applied to detect the inhibin B (514). The ELISA kits for Inhibin B Gen II (A81303, Beckman Coulter, CA, USA) with an intra-assay CV < 5% and

inter-assay CV < 10% was used to evaluate inhibin B levels, utilizing calibrators and controls (A81304, Beckman Coulter) in accordance with the manufacturer's recommendations. Samples are incubated with plates pre-coated with antibody for the β_B subunit. Then, the plates are incubated with biotinylated antibody for the subunit α. Later on, streptavidin-HRP conjugate is applied and the color is developed with a substrate and the absorbance is measured at 450 nm.

3.8 STATISTICS

In paper I, the unpaired t-test was used with a two-tailed *p* value (Prism 6, GraphPad Software, Inc., CA, USA) was used in TLDA array statistical analysis, while one way ANOVA (analysis of variance) was used with the Bonferroni multiple comparison test (Prism 6, GraphPad Software, Inc., CA, USA) in comparing all cell lines and culture conditions. To compare values under the different experimental conditions, One-Way RM ANOVA (SigmaPlot 11.0; Systat Software Inc., CA, USA) was used. Following the Shapiro–Wilk test for normality, pair-wise multiple comparisons were performed with Holm–Sidak or Dunnett's procedure (SigmaPlot 11.0; Systat Software Inc.; CA, USA), as indicated in the figure legends. A *p*-value of ≤ 0.05 was considered statistically significant.

In paper II, III and IV, Student's t -test, One-way ANOVA, One-way RM ANOVA and ANOVA on ranks were used to compare the differences between experimental conditions (SigmaPlot11.0; Systat Software Inc., CA, USA). Pairwise multiple comparisons were performed with the Holm–Sidak procedure following the Shapiro–Wilk test for normality, as stated in the Figure legends (SigmaPlot 11.0; Systat Software Inc.). A difference was considered to be statistically significant if the *p* value was ≤ 0.05.

4 RESULTS AND DISCUSSION

4.1 DIFFERENTIATION POTENTIAL OF HUMAN ES CELLS TOWARDS MALE GERM CELLS (PAPER I)

It has been reported that the different hES cell lines have different tendencies regarding differentiation potential towards the germ cell lineage (93). Since hES cell lines do not share an indistinguishable gene expression profile (16), we aimed in this study to investigate the possibility of using the gene expression profile of the hES cell line to predict its differentiation potential towards the germ cell lineage. The hES cells were cultured in conventional feeder layer culture and in suspension. The reason the suspension culture was investigated is that it has been reported before to support the differentiation of ES cells to the neural cell lineages, as it gives the spatial support to the differentiated cells (72). Hence, whether the sphere culture supports the differentiation of the ES cells to germ cells needs to be investigated.

4.1.1 Undifferentiated hES cell lines exhibit different gene expression profiles in the different culture conditions

The three hES cell lines used in this study (HS207, HS360 and HS401) were cultured either on hFFs or as spheres in suspension culture. All of the cell lines expressed the pluripotency consensus markers in both culture conditions. Using the TLDA cards, the expression of 90 genes, related to pluripotency and differentiation assessment, were investigated for the three cell lines cultured in both conditions. The samples exhibited clustering in favor of the culture condition rather than the cell line, indicating that the gene expression profile is affected mainly by the different culture conditions, rather than by the cell line. When the three hES cell lines were cultured as spheres in suspension culture, 17 genes were significantly downregulated and 23 genes were significantly upregulated, compared to the three cell lines when cultured on feeders. Among the upregulated genes, we have found the stem cell markers *SOX2, KIT, FGF4,* and *LIN28* and the endodermal marker *SOX17*, while among the downregulated ones, we have found *FGF5* and Galanin and GMAP Prepropeptide (*GAL)*. Some ectoderm markers (Paired Box 6 (*PAX6)*, Oligodendrocyte Lineage Transcription Factor 2 (*OLIG2)*, and Neuronal Differentiation 1 (*NEUROD1*)) were upregulated in the suspension culture compared to the culture on feeders, which could be due to supplementing the suspension culture with neurotrophic factors (rBDNF and rNT3), while we could not detect any of the ectoderm markers to be downregulated in the suspension culture compared to the culture on feeders.

One of the important signaling pathways in germ cell specification we investigated was the NODAL/ACTIVIN pathway. When the two culture conditions were compared in each cell line separately, *EBAF* and *NODAL* showed significant lower expression in the suspension culture compared to the culture on feeders in the cell lines HS207 and HS401, while *GDF3* (Growth Differentiation Factor 3) showed significant lower expression in the suspension culture compared to the culture on feeders in the cell lines HS360 and HS401. Nevertheless, *LEFTB* was significantly downregulated in the suspension culture compared to the culture on

feeders in all cell lines, whereas Teratocarcinoma-Derived Growth Factor 1 (*TDGF1)* was significantly upregulated in suspension culture in HS207 cell line, and significantly downregulated in suspension culture in HS401 cell line, both compared to the culture on feeders. The early germ cell markers (*SOX2*, *KIT* and *LIN28*) were significantly upregulated in all cell lines in the suspension culture compared to the culture on feeders. Meanwhile, *FGF4* was significantly upregulated in the suspension culture in the cell lines HS360 and HS401, and *SOX17* was significantly upregulated in the suspension culture in the HS270 cell line, when both were compared with the culture on feeders. In other words, the HS360 had the most stable NODAL/ACTIVIN pathway compared to the other two cell lines, while keeping an upregulated germ cell markers expression.

4.1.2 Absence of FGF2 in hES cell culture medium has different effects on gene expression profile depending on the culture condition

We have used two culture methods to achieve spontaneous differentiation of the three hES cell lines; differentiation on a feeder layer and differentiation in spheres. Since spontaneous differentiation on feeders was reported previously (93,111), this culture method was used as a control for the suspension method. The growth factor FGF2 was reported previously to be important for maintaining the pluripotency of hES cells, and the deprivation of pluripotent cells from this factor resulted in spontaneous differentiation of the hES cells (54,93). Hence, we have used this technique to achieve a spontaneous differentiation of the three hES cell lines used. However, depriving the cells cultured on feeders from FGF2 did not reduce significantly the expression of the pluripotency markers *NANOG* and *POU5F1* in any of the three cell lines used, which indicated that the differentiation was partly unsuccessful. A plausible explanation for that could be the FGF2 and Activin produced by the hFF cells, both of which has a supportive role in the expression of pluripotency genes (63,135,515). On the other hand, deprivation of the three cell lines cultured on feeders from FGF2 resulted in upregulation of *DDX4* expression in the cell lines HS360 and HS401, upregulation of *KIT* in the HS207 cell line, and downregulation of *KIT* in the HS360 cell line. Meanwhile, the expression of the Sertoli cell markers *FSHR* and vimentin (*VIM)* was not changed after the spontaneous differentiation.

Spontaneous differentiation in the suspension culture was achieved by culturing the hES cell spheres from the three cell lines in un-supplemented medium without passaging. Herein, the expression of *POU5F1* was downregulated in the three cell lines, whereas the expression of *NANOG* was downregulated only in the HS360 cell line. Surprisingly, the expression of the germ cell marker *DDX4*, the Sertoli cell markers *VIM* and *FSHR*, and the Leydig cell markers Insulin-Like 3 (*INSL3)* and 3β-Hydroxysteroid dehydrogenase (*HS3BD1)* was not altered in any of the three cell line after the spontaneous differentiation in suspension culture. Using TEM, the morphologic characteristics of the hES cells after spontaneous differentiation in suspension were not uniform; some cells exhibited the characteristics of undifferentiated hES cells and others exhibited the characteristics of differentiated hES cells.

4.1.3 BMP7 stimulates differentiation of hES cells in suspension towards putative male gonadal cells

It has been reported previously that BMPs promote the differentiation of the pluripotent cells towards the germ cell lineage (109,113,128). Thus, we tried to induce the differentiation of the three cell lines with BMP7 in both culture methods; on feeders and in suspension. When the three cell lines were cultured on feeders, BMP7 stimulation did not result in downregulation of the pluripotency markers *NANOG* and *POU5F1* in any of the three cell lines. We also found that the expression of *DDX4* was upregulated in the HS360 and HS401 cell lines, while the expression of *KIT* was upregulated in the HS207 and HS401 cell lines. As for the Sertoli cell markers, *FSHR* expression was significantly upregulated in the HS207 cell line, whereas *VIM* was downregulated in the cell lines HS207 and HS401.

When the spheres were differentiated in suspension culture, BMB7 stimulation resulted in significant downregulation in the expression of the pluripotency markers *NANOG* and *POU5F1* in the three cell lines. In addition, *DDX4* expression was significantly increased in the HS360 and HS401 cell lines, whereas *KIT* expression was significantly increased in the HS360 cell line. As for the Sertoli cell markers, *FSHR* was significantly upregulated in the HS360 cell line, while *VIM* was significantly upregulated in the HS207 and HS401 cell lines. In regards to the Leydig cell markers, *INSL3* was significantly upregulated in the HS207 cell line, and the *HS3BD1* was significantly upregulated in the HS360 cell line. The downregulation of the pluripotency genes, along with the upregulation in the gonadal cells markers, could be an indication that BMP7 induces the differentiation towards gonadal cells in suspension culture, which was not the case with the hES cells cultured on hFFs.

4.1.4 Assessment of protein expression in suspension culture after spontaneous differentiation and BMP7 stimulation

When the three cell lines were cultured in suspension, they were either spontaneously differentiated or BMP7 stimulated. To compare between both methods, assessment of protein expression of certain markers by immunofluorescence was performed. The expression of SOX9, a Sertoli cell marker, showed a characteristic pattern of cell colonies surrounded by cytoplasmic SOX9 positive cells. With spontaneous differentiation, only the HS401 cell line had 5.0% of colonies positive for SOX9, while with BMP7 stimulation, the HS207 cell line had (14.9%), the HS401 cell line had (11.8%), and the HS360 cell line had (1.5%) of colonies positive for SOX9. When the expression of Wilms tumor gene 1 (WT-1), another Sertoli cell marker, was evaluated, it revealed a very similar pattern for the SOX9 expression (edges of colonies with both methods of differentiation). However, WT-1 expression was nuclear and occurred in all cell lines, regardless to the BMP7 stimulation.

DDX4 expression, the germ cell marker, was observed in all cell lines after both methods of differentiation in suspension; spontaneous differentiation and BMP7 stimulation. However, the HS360 cell line was the only cell line that showed an increased percentage of the DDX4-positive cells in the colonies expressing DDX4 after BMP stimulation. Thus, the cell line HS360 was investigated more by immunohistochemistry for some markers. We found that

POU5F1, DDX4, VIMENTIN, AMH, INSL3 and HS3BD1 were expressed in the spheres of HS360 cell line after differentiation using BMP7 stimulation.

4.1.5 Putative Sertoli and Leydig cells do not secrete hormones at this stage of differentiation

To assess the functionality of the cells assumed to be Sertoli cell-like or Leydig cell-like cells, testosterone and inhibin B production in the culture medium was evaluated. The results revealed that the testosterone could be detected at an extremely low level (3.82 ± 0.63 pmol/L), while inhibin B could not be detected (limit of detection was 10 ng /L).

4.1.6 Discussion

The reason behind using hES cells is that it is crucial to study the early germ cell developmental stages in order to establish a robust system to differentiate the human germ cells *in vitro* to mature gametes. However, the difficulties in performing such studies, along with the differentiation potential of hES cells towards the different cell lineages, encouraged the researchers to study the hES cells differentiation towards germ cells *in vitro* as a model for the early germ cell development.

Differentiation of stem cells, albeit in low efficiency, towards germ cells has been reported earlier (93,108,111,114). It has also been reported that the suspension culture can maintain the pluripotent state of the hES cells (72). Moreover, the suspension culture supported the differentiation towards the neural cell lineage; due to the spatial 3D support it gives to the culture. Hence, we tried here to investigate the effect of using different cell lines of hES cells and different culture conditions (on hFF feeders or in suspension) on the gene expression profile and the differentiation potential of the hES cells. When three different hES cell lines were examined, the gene expression profile for each one of them was, albeit similar, not identical to the other in the same culture condition, and the gene expression profile was also different for the same cell line when cultured in two different conditions. This indicated that the culture conditions could have an effect on the gene expression profile of hES cells, and hence, could have an effect on its differentiation potential.

On the other hand, NODAL/ACTIVIN signaling pathway has been reported earlier to be of great importance to germ cell lineage specification (138-140,516,517). In the early gonadal development, NODAL/ACTIVIN signaling ensures that the PGCs do not enter meiosis and stay undifferentiated to develop towards the male gonocytes (138). Meanwhile, *EBAF* and *LEFTB* inhibit *NODAL*, whereas *TDGF1* and *GDF3* activate this pathway (517-519). In this study, the pivotal genes involved in this pathway (*EBAF, TDGF1, LEFTB, NODAL* and *GDF3*) were found to be differently expressed between the hES cell lines and the different culture conditions. When the cell lines HS207 and HS401 were cultured in suspension, the NODAL/ACTIVIN pathway seemed mainly downregulated, while when the HS360 cell line was cultured under the same conditions, the NODAL/ACTIVIN pathway was more stable. Thus this cell line was expected to be the most promising among the three cell lines to differentiate towards male gonadal cells. In the same context, other genes, important for male

germ cell development, were found to be upregulated in the HS360 cell line, such as *LIN28, KIT* and *SOX2* (520-523).

Surprisingly, depriving the hES cells from exogenous FGF2 did not result in a reduction the pluripotency genes expression when the cells were cultured on feeders, while it could reduce the expression of such genes when the cells were cultured in suspension. This could be due to the fact that the hFF cells secrete interleukin 6 (IL6) and FGF2, which were reported earlier to be enough for keeping the hES cell pluripotent in culture (63,515).

Although *DDX4* gene expression was detected in the undifferentiated state, which was also reported earlier (16,93,108,110,113), DDX4 protein was only detected in the BMP7 stimulated differentiated cells. In general, the suspension culture, with or without BMP stimulation resulted in an upregulation of the genes specific for Sertoli cell and Leydig cell. Sertoli cells have an important role in sex determination and germ cell development (524,525). Two of the main markers for Sertoli cells have been detected on protein level after differentiation in this study; SOX9 and WT-1. Both markers are expressed in the immature Sertoli cells and are important for Sertoli cell differentiation (526,527). Other Sertoli cell markers that have been detected on protein levels with immunohistochemical staining included VIM and AMH (158,528). Moreover, Leydig cell markers, INSL3 and HSD3B1 (187,529,530) have been detected on protein level in the differentiated cells as well. When the functionality of these putative cells was investigated by evaluating the testosterone and inhibin B production, there was extremely low levels of testosterone and non-detectable levels of inhibin B, this could be due to that the cells did not secrete testosterone and inhibin B, or there were too few functional cells.

Taken together, the gene expression profile of hES cells depends on the cell line and the culture conditions. In addition, BMP7 stimulation resulted in upregulation in the germ cell markers *KIT* and *DDX4* and somatic cell markers *FSHR* and *HS3BD1*, with a downregulation in the pluripotency markers *NANOG* and *POU5F1*.

4.2 EFFECT OF CULTURE CONDITIONS ON RAT GERM CELL MATURATION *IN VITRO* IN 3D CULTURE (PAPER II)

The effect of the culture medium in 3D culture conditions on rat germ cell maturation *in vitro* has not studied yet. Thus, we have employed the 3D agarose culture system we used earlier with mouse testicular cell culture (480) to culture the rat pre-pubertal testicular cells, aiming at optimizing the culture conditions, in regards to the culture medium and gonadotropin (hCG and FSH) stimulation.

4.2.1 Effect of the culture media and gonadotropins on testosterone production by rat Leydig cells in a 3D culture system

When we used gonadotropins in stimulating the rat pre-pubertal testicular cells in a 3D culture system, it resulted as expected in an increased production of testosterone after 24 hours. However, the DMEM + glutamine culture medium *per se* had a significant higher level of testosterone production compared to the other culture media (F12, DMEM/F12, and MEM). The DMEM + glutamine culture medium contains higher levels of amino acids in comparison to the other culture media used, as stated by the supplier. By adding either AA or NEAA to F12 culture medium, the difference in testosterone production could not be compensated.

The effect of adding glutamine or Glutamax, a substituent for glutamine, to the culture medium has been studied as well. When the DMEM + glutamine culture medium was compared to the DMEM – glutamine or DMEM + Glutamax culture media, DMEM + glutamine had a significantly higher level of testosterone production compared to the other two culture media after 24 hour of stimulation with gonadotropins. This difference disappeared after 7 and 14 days of culture. However, the DMEM + Glutamax culture medium had the highest testosterone production among the three culture media without any stimulation by gonadotropins for up to 14 days.

4.2.2 Influence of the various culture media on the expression of steroidogenic genes by Leydig cells in a 3D culture system

The qPCR technique was utilized to assess the effect of culture medium on the gene expression profile for the steroidogenic enzymes *Star* and Translocator Protein (*Tspo)*. After 24 hours of gonadotropins stimulation of the rat testicular cells in the 3D culture system, the DMEM + glutamine resulted in a significantly higher expression of *Star* compared to F12, F12 + AA, F12 + NEAA or DMEM/F12, and a significantly higher expression of *Tspo* compared to F12 + AA, DMEM/F12 or MEM.

4.2.3 Medium-related effects on the differentiation of pre-pubertal rat male germ cells *in vitro*

After culturing rat pre-pubertal testicular cells in 3D culture system using three culture media, DMEM + glutamine, DMEM + Glutamax and F12, the expression of six genes specific for germ cell differentiation was evaluated by qPCR. Using the DMEM + glutamine medium, the expression of *Zbtb16* (also known as Promyelocytic Leukemia Zinc Finger; *Plzf*) was

significantly upregulated after 21 days of culture. On the other hand, *Kit* and *Dazl* were significantly downregulated after 21 days of culture using DMEM + glutamine or DMEM + Glutamax culture medium, while *Crem* was significantly downregulated after 21 days of culture using DMEM + glutamx medium. The expression of *Boll* or *Protamine* was not detected under any of the experimental conditions.

4.2.4 Gonadotropins protect rat testicular cells in the different culture media from apoptosis

The TUNEL assay was used to evaluate the effect of gonadotropins stimulation on apoptosis. Using three different culture media, DMEM + glutamine, DMEM + Glutamax, and F12, rat pre-pubertal testicular cells were cultured in a 3D culture system for 7 days, with or without gonadotropins stimulation. The results revealed that gonadotropins stimulation, compared to no stimulation at all, resulted in significantly less apoptotic cells (%) using DMEM + glutamine or DMEM + Glutamax culture medium. Moreover, when the three culture media were used without gonadotropins stimulation, the DMEM + glutamine culture medium had significantly less apoptotic cells (%) compared to DMEM + Glutamax or F12 culture medium, while there was no such difference when the three culture media were used with gonadotropins stimulation.

4.2.5 Discussion

Our group has previously used the 3D agarose culture system in producing post meiotic germ cells from mouse *in vitro* (480,481). Considering a few culture media were used earlier in the germ cell culture (489,531), the effect of choosing a specific culture medium has not been studied yet. Moreover, the important role of gonadotropins (hCG and FSH) and functional somatic environment of the testis (Leydig and Sertoli cells) in spermatogenesis has been well established (230,473,481,532). Hence, we wanted to exploit the 3D culture system, our group used previously in culturing murine testicular cells, to culture rat pre-pubertal testicular cells. Our aim was to investigate the optimal culture condition (choice of culture medium and gonadotropin stimulation) for germ cell maturation *in vitro*. Herein, we have studied the effect of culture medium and gonadotropins stimulation on Leydig cell functionality, as an important player in the somatic environment, by evaluating the testosterone production, and on the expression of steroidogenic enzymes and the genes related to germ cell differentiation. Moreover, the effect on cell viability *in vitro* has been studied as well. In that context, gonadotropins stimulation had a significant impact on cell survival, which has been reported earlier (237,533,534). The exact mechanism by which gonadotropins exert the anti-apoptotic effect is still unclear. However, the same effect on rat granulosa cells has been elucidated to be through the stimulation of a B-Cell CLL/Lymphoma 2 (Bcl2) anti-apoptotic family member protein Mcl-1 (535).

Gonadotropins stimulation had a significant effect on testosterone production, which is expected. Nevertheless, the DMEM + glutamine culture medium had the most pronounced effect on testosterone production among the media tested, which indicates the importance of

glutamine to testosterone production. This positive effect of glutamine was exhibited by the upregulation of the steroidogenic enzymes *Star* and *Tspo*; both of which are important regulatory enzymes in testosterone synthesis, responsible for transferring cholesterol through the mitochondrial membrane (536-539). Glutamine, in general, is an important source of energy, as well as a precursor for protein biosynthesis (540-543), which could explain the positive effect, by stimulating the production of the essential enzymes in steroidogenesis. Glutamax, on the other hand, is a dipeptide of alanine-glutamine. This dipeptide is used to release glutamine over a long time of culture, so that the cells are not damaged by the high pH produced by glutamine decomposition in culture (544). Adding AA and NEAA to F12 could not upregulate the steroidogenic enzymes or elevate the level of testosterone production to the level produced by the DMEM + glutamine medium, which could be a result of the difference in ingredients, other than the amino-acids, between the different culture media.

The gene expression analysis for germ cell differentiation related genes revealed an overall downregulation of these genes, irrespective to the culture medium used, which indicates a non-robust system for germ cell differentiation *in vitro*. The qPCR results were confirmed by the morphologic and immunohistologic evaluation, which showed germ cells emerging from the colonies containing other testicular cells, with no differentiated germ cells could be observed. Thus there was no spatial support for the germ cells by Sertoli cells, as it is the case *in vivo* (532,545,546), which could explain the poor efficiency of the system.

Taken together, the culture conditions (choice of culture medium *per se*) have an effect on the Leydig cell functionality, but not the germ cell differentiation *in vitro* using 3D culture conditions. Moreover, the germ cells are migrating outwards the colonies in the 3D culture, leading to unfavorable conditions for germ cell differentiation, as they lose contact with the Sertoli cells.

4.3 RAT GERM CELL MATURATION *IN VITRO* USING ORGAN CULTURE SYSTEM (PAPER III)

The organ culture technique has been reported recently to support murine germ cell differentiation *in vitro* (489). Hence, we have exploited the same culture system to investigate the possibility of pre-pubertal rat male germ cells cultured for 52 days to differentiate *in vitro*, using different culture conditions; plain MEMα culture medium, with or without Glutamax, and with or without supplementation with melatonin and RA. Since Glutamax has shown a better cell viability profile *in vitro* than glutamine in the second study of the thesis, it was used in this third study of the thesis instead of glutamine. The effect of co-culture of the rat testicular tissue with epididymal fat was also investigated. In addition, germ cell differentiation has been assessed by immunohistologic and morphologic evaluation, as well as on the level of gene expression.

4.3.1 Effect of culture medium on rat male germ cell proliferation *in vitro*

When the rat pre-pubertal testicular tissue was cultured in an organ culture setup for 52 days *in vitro*, using MEMα culture medium with or without Glutamax, there was no significant difference between the two culture media regarding the proliferation index. This index was calculated as the percentage of tubules containing Ddx4-positive/Ki67-positive cells compared to the total number of tubules in a section.

4.3.2 Rat male germ cell differentiation *in vitro* using organ culture system

After staining the rat testicular tissues cultured for 52 day *in vitro* in the different conditions with PAS staining, the morphologic evaluation revealed the presence of cells exhibiting the morphologic characteristics of spermatocytes and round spermatids in the cultured tissues. However, there was no difference between the different culture conditions in the number (%) of the tubules containing differentiated germ cells. In order to confirm the morphologic observations, gene expression and protein expression of the genes related to germ cell differentiation were evaluated by qPCR and immunostaining respectively.

4.3.2.1 Gene expression

When the genes of interest (*Acr*, *Crem*, *Prm1*, *Sycp3*, *Boll*, *Kit*, *Zbtb16*, *Dazl*, and *Ddx4*) were investigated by qPCR, the results showed a slight overall upregulation for all of the genes in the cultured tissues compared to the control pre-pubertal tissues. However, this upregulation did not reach significance, due to the high variance between the biological replicates. One exception was *Sycp3*, where the plain medium without supplementation had a significant higher expression than the control pre-pubertal tissues. In addition, the adult (60 d*pp*) control testicular tissue showed a significant higher expression profile for all of the genes, except for *Kit* and *Zbtb16*, compared to the pre-pubertal tissues and the cultured tissues. As for *Sycp3*, the cultured tissue in the plain medium, with no supplementation, was not significantly different from the adult control testicular tissue. Finally, comparing the different culture conditions with each other resulted in no significant difference, except for the plain medium without supplementation, where *Sycp3* was significantly upregulated.

4.3.2.2 Protein expression

To investigate the protein expression, immunostaining for Crem and Acrosin was performed on the cultured tissues. The results showed the presence of cells positive for Crem and Acrosin in the cultured rat testicular tissues. The Crem positive cells had a uniform intensity profile; a strong nuclear Crem positive staining, while the Acrosin positive cells had either one of two intensity profiles; dim cytoplasmic or bright perinuclear. Interestingly, there was no significant difference between the different culture conditions in the number (%) of the tubules containing Crem positive or Acrosin positive (for any of the two intensity profiles) cells. However, when the two intensity profiles of Acrosin were pooled together, the plain non supplemented medium had a significant higher number (%) of tubules containing Acrosin positive cells compared to the other culture conditions.

4.3.3 Functionality of rat Leydig and peritubular cells *in vitro* using organ culture system

Evaluation of testosterone production was used as a functionality assessment for the rat Leydig cells cultured for up to 52 days *in vitro*. Thus, intra-testicular testosterone was extracted and evaluated using an ELISA kit specific for testosterone. The results showed that the testosterone production after 3 days of culture was significantly higher than the production after 10 and 52 days of culture. Moreover, when the different culture conditions were compared together, there was no significant difference between the different culture conditions, in regards to testosterone production, after culturing the rat testicular tissues for 52 days *in vitro* in the different conditions.

After 21 days of culture, the rat testicular tissues co-cultured with epididymal fat showed spontaneous contractility lasted till the end of the culture after 52 days, which indicates the functionality of the peritubular cells. This contractility was not observed in the rat testicular tissues cultured alone with no fat. However, this positive effect on PMCs was not translated to appositive effect on germ cell differentiation, as it was not observed.

4.3.4 Discussion

Recently, the first full *in vitro* maturation of murine male germ cells into functional gametes using the organ culture technique has been reported by Sato and colleagues (489). Since then, other researchers have reported similar achievements (495-497). As it could be of a great importance to translate this success into a clinical option, it is crucial to repeat the same success in other species. Hence, we have used here the rat model, as a step forward from the mouse model to clinics. Rats take for a full spermatogenesis *in vivo* a period of time that is longer than mice but shorter than humans (547). Moreover, they have a longer spermatogenesis recovery time after irradiation compared to mice, but still they have a shorter recovery period compared to humans (548). In addition, a recent trial to differentiate the rat male germ cells *in vitro* has been reported (549). However, in that study, the authors did not investigate the expression of post meiotic germ cell markers on protein level. Our group has used earlier the 3D culture system for rat and mouse male germ cell maturation *in*

vitro (480,550). However, the 3D culture system showed a very poor efficiency, due to the dis-orientation of the germ cells in relation to the Sertoli cells, as it was discussed previously in the second paper (section 4.2.5). Therefore, we have tried here to differentiate immature rat male germ cells *in vitro*, exploiting the organ culture technique used earlier by Sato, under different culture conditions, while focusing on the investigation of the post-meiotic markers; Crem and Acrosin, on protein level.

In rats, Crem is expressed from the late pachytene spermatocyte stage to the released spermatid (247). Meanwhile, the *Acr* gene is transcribed in the diploid stage, but its translation to the Acrosin protein is suppressed till the post-meiotic haploid round spermatid stage (252,254). Then, Acrosin is expressed as an inactive zymogen (proacrosin), which is stored in the acrosome till fertilization (253). The usage of Crem and Acrosin as markers for post-meiotic germ cells has been reported earlier (480,489,490). Thus, we have investigated here the protein expression of Crem and Acrosin. Using immunostaining, Crem was found to be expressed in the nucleus, which is in accordance with what has been reported earlier (551). Meanwhile, two intensity profiles of Acrosin have been noticed; dim cytoplasmic and bright perinuclear, in contrast with the previous reports, where the Acrosin expression was only bright perinuclear (252,254). However, it has been reported earlier in mice that Acrosin has a faint cytoplasmic expression in the pachytene spermatocyte stage, followed by condensation of the Acrosin in the post-meiotic haploid stage in the acrosome to give a bright perinuclear expression (552). Consequently, we have proposed that Acrosin in rats first expressed faintly in the cytoplasm in the late pachytene spermatocyte stage, followed by concentration of the Acrosin in the acrosome in the post-meiotic haploid stage.

The rat testicular tissues were cultured for 52 days, which represents the time needed for full spermatogenesis *in vivo* for rats (269,553), whereas the plain culture medium used here was MEMα + 10% KSR; the optimal culture medium used by Sato and colleagues previously (489). Indeed, we have confirmed the possibility of immature rat male germ cell differentiation *in vitro* using the organ culture system. However, we have noticed that the efficiency of the organ culture system in rat male germ cell differentiation *in vitro* into post-meiotic cells was very low, compared to the efficiency of the same system in mouse (497). This differentiation was confirmed by the gene expression analysis, which showed an overall upregulation of the genes related to germ cell differentiation after 52 days of culture. The upregulation of these genes did not reach significance, due to the scarcity of the differentiated cells.

In order to optimize the culture conditions for rat germ cell differentiation *in vitro*, we intended to add different supplements to the culture medium; Glutamax, melatonin and RA. Glutamax was shown earlier to have a positive effect on cell viability (550). Meanwhile, melatonin, which is produced mainly by the pineal gland (but also by the testis) (554), has an anti-cytotoxic effect, through scavenging the reactive oxygen species (ROS) (555-562). In addition to being essential for germ cell differentiation *in vivo* (563-569), RA was also shown to have a positive effect on stem cell differentiation towards male germ cell *in vitro* (570,571)

and on the male germ cell differentiation *in vitro* (478,572). Unfortunately, none of the previous supplements could improve the efficiency of the organ culture system in differentiating the rat male germ cells *in vitro*, which is consistent with what Sato and colleagues have reported previously, that DMEM + 10% KSR was enough to differentiate the mouse male germ cells *in vitro* (489). In the same context, epididymal fat was co-cultured with the rat testicular tissue to improve the efficiency of the culture system, as it was reported earlier to be important for spermatogenesis (573,574). However, no positive effect for the epididymal fat on germ cell differentiation *in vitro.* In addition, no positive effect on testosterone production could be observed, which matches with what has been reported earlier (574). On the other hand, epididymal fat did stimulate the PMCs to show spontaneous contractility.

Taken together, rat undifferentiated spermatogonia could be differentiated *in vitro* to round spermatids showing Acrosin and Crem on protein level as post-meiotic markers, using the organ culture conditions previously described for murine germ cell differentiation *in vitro*. However, the supplementation with melatonin, RA, or Glutamax did not enhance the germ cell differentiation *in vitro*.

4.4 HUMAN PRE-PUBERTAL TESTICULAR ORGAN CULTURE (PAPER IV)

Maturation of human male germ cells *in vitro* turned to be of a great importance to solve various male infertility problems. One of the compelling indications is young boys undergoing gonadotoxic therapy, as they do not have sperm that can be cryopreserved for later use (325,367). Recently, Sato and colleagues reported for the first time a full mouse spermatogenesis *in vitro*, using an organ culture system (489). This was followed by us showing the differentiation of rat germ cells *in vitro* to round spermatids, using the same organ culture system. Hence, we aimed in this study at culturing for long term (91 days) human pre-pubertal testicular tissue *in vitro*, taken from patients undergoing gonadotoxic therapy, using an organ culture system and different culture conditions (Nutristem and 10% KSR-xf supplemented with different combinations of RA, SCF, rFSH, melatonin, and testosterone). The ultimate goal was to establish a robust protocol to differentiate the human immature male germ cells *in vitro*.

4.4.1 Effect of size of the cultured tissue on the necrotic area

When human testicular tissues were cultured *in vitro* using an organ culture system, they showed a characteristic pattern of a central necrotic region and a peripheral healthier ring. The surface area of the necrotic region was measured by ImageJ software and the percentage of this area to the total surface area of the tissue section was calculated.

By comparing the necrotic area (%) of the tissue sections between the different culture conditions and different time points, there was no significant difference due to the different culture conditions or due to the period of culture, although there was a trend of increased necrotic area (%) after 77 days of culture in all culture conditions. However, there was a clear trend of increased necrotic area (%) as the radius of the tissue piece decreased less than 300 μm, irrespective to the culture condition or the period of culture.

4.4.2 Viability and functionality of Sertoli cells and Leydig cells *in vitro* using organ culture system

Functional somatic environment (Sertoli and Leydig cells) are crucial for germ cell differentiation. Hence, investigating the viability and functionality of these cells is of great importance to establish an *in vitro* system for germ cell differentiation. Testosterone and inhibin B production in the culture medium was used to assess the viability and functionality of Leydig and Sertoli cells *in vitro* respectively. Evaluation of the testosterone produced revealed an age-dependent pattern, in which the patients with less than one year of age showed a constant testosterone level for up to 91 days of culture, whereas the patients with 2-11 years of age showed a decline in testosterone production after 42 days of culture.

On the other hand, evaluation of inhibin B showed that all the cultured tissues were producing inhibin B in the first three weeks of culture, with a trend for increased production after 2 weeks of culture. However, there was no specific age-dependent pattern of inhibin B production. Both results indicated the viability and functionality of the Leydig cells and Sertoli cells cultured *in vitro*.

4.4.3 Sertoli cell maturation *in vitro* using organ culture system

Morphologic evaluation of the human testicular tissues cultured *in vitro* revealed a tubular growth after 7 days of culture. Moreover, this evaluation showed morphologic changes in Sertoli cells cultured *in vitro* characteristic to Sertoli cell maturation (elongation of cell nuclei, cell migration to the basal membrane and polarization of these cells).

In addition to the morphologic evaluation, immunostaining for the Sertoli cell marker SOX9 revealed that at the beginning of the culture, the tissues had mainly nuclear SOX9 staining. While for the cultured tissues, SOX9 expression could be detected in the cytoplasm of Sertoli cells in the majority of the cultured tissues after 91 days in culture. On the other hand, the evaluation of WT-1 expression by immunostaining, another Sertoli cell marker, showed a nuclear staining in the beginning of the culture, whereas the cultured tissues showed a condensed nuclear staining after 91 days in culture.

The germ cell marker, DDX4, could be detected in beginning of culture in most of the patients. However, after 42 days of culture, it could not be detected.

4.4.4 Discussion

Anti-cancer therapies that imply high risk for gonadotoxicity can cause male infertility to the surviving patients. Since the survival rate after cancer therapy is improving dramatically in the recent years, fertility preservation is getting more attention. For adult male patients, sperm cryopreservation forms a straightforward simple clinical option. However, young cancer survivors do not have such option, as they cannot produce sperm before puberty. Fertility preservation options for such patients are all experimental till now. These options include autologous SSCs transplantation, xeno-transplantation and *in vitro* germ cell maturation. Among these techniques, *in vitro* maturation of germ cells represents one the most promising clinical options. So far, *in vitro* maturation of mouse male germ cells (489) and rat male germ cells (in paper III in this thesis) has been reported, using an organ culture system. Hence, in this study, we used testicular tissues from 12 pre-pubertal patients, before or during undergoing therapy with high risk for gonadotoxicity, for a long term culture, using an organ culture system.

Evaluation of DDX4, the germ cell marker, showed that 91% of the patient had germ cells in the beginning of the culture, indicating that the therapy did not eliminate all of the germ cells in the seminiferous tubules. However, germ cells could not be detected after 42 days in culture. This could be due to negative impact of a previous gonadotoxic treatment, as some of the patients have had already earlier treatment, or due to the negative impact of the underlying disease itself. It has been reported that some malignancies might cause abnormal germ cell maturation (358).

Meanwhile, evaluation of the Sertoli cell marker, SOX9, showed a change in the expression pattern form nuclear to cytoplasmic after culturing *in vitro*. SOX9 is expressed from the immature Sertoli cell stage. It starts as a cytoplasmic expression in the pre-Sertoli cells, and

then, SOX9 relocates to the nucleus at 6.5 weeks of gestational age. Thus, this expression pattern could indicate de-differentiation of the Sertoli cells *in vitro*, which is in contrast with the morphologic changes we have observed. De-differentiation of adult mature Sertoli cells have been reported previously (575). However, the de-differentiation of immature Sertoli cells towards pre-Sertoli cells has not been studied before, which makes it interesting to investigate this issue.

Functionality of Leydig cells and Sertoli cells was evaluated by estimating the testosterone and inhibin B production respectively. The levels of testosterone produced in the culture could not be related to the diagnoses included in the study, or even to the culture conditions used. However, a tendency to cease producing testosterone was after around 42 days of culture, which indicates the deceased viability and functionality of Leydig cells *in vitro* after 42 days. Since testosterone is crucial for spermatogenesis (307), this result suggests the importance of adding exogenous testosterone to the culture after 42 days. However, the age of the patient had an impact on the sustainability of testosterone production, as the young patients (less than one year of age) had a constant testosterone level till 91 days of culture. This is in accordance with the fact that Leydig cells in the neonatal age recruit more precursor cells and are more sensitive for hormonal stimulation (576). Inhibin B was also detected in the culture medium, which suggests the functionality of Sertoli cells as well. Both Leydig and Sertoli cells (the somatic environment) are crucial for achieving full spermatogenesis. Thus, we assume that the culture conditions were sufficient to support the viability and functionality of the somatic cell environment, which should facilitate germ cell differentiation.

Taken together, the Leydig cells and Sertoli cells showed viability and functionality after 42 days and 21 days respectively of culturing testicular tissue of pre-pubertal testicular tissue *in vitro* for 91 days using an organ culture system. However, after 91 days of culture, no germ cells could be detected.

5 CONCLUSIONS

In this thesis, we have assessed the effect of culture conditions on the differentiation potential of hES cells towards male germ cells *in vitro*. Moreover, we have established a protocol for differentiating immature rat male germ cells *in vitro*. In addition, we have also studied the effect of long term culture of human pre-pubertal testicular tissue *in vitro*. The main findings in the thesis are:

- Undifferentiated hES cell lines exhibit different gene expression profiles. In addition, the same hES cell line could have different gene expression profiles under different culture conditions.
- The suspension culture method for hES cells resulted in an improved differentiation profile, in regrads to the downregulation of the pluripotency markers *NANOG* and *POU5F1*, compared to the well-known culture method on hFFs feeders.
- BMP7 stimulation of the HS360 hES cell line during differentiation resulted in an upregulation in the germ cell markers *KIT* and *DDX4* and somatic cell markers *FSHR* and *HS3BD1*, with a downregulation in the pluripotency markers *NANOG* and *POU5F1*.
- Choice of medium and culture conditions for rat testicular cell culture *in vitro* in a 3D culture system has an effect on the Leydig cell functionality, but not on germ cell differentiation.
- Rat male germ cells are migrating towards the outside of the colonies, which contain somatic cells, in a 3D culture system *in vitro*, which makes it unfavorable for germ cell differentiation.
- Differentatition of rat undifferentiated spermatogonia (pre-meiotic) into round spermatids expressing Crem and Acrosin (post-meiotic) *in vitro* could be obtained, using an organ culture system and MEMα + 10% KSR culture medium, previously described for the mouse. Meanwhile, supplementing the culture medium with melatonin, RA or Glutamax, or co-culturing with epididymal fat did not enhace the rat male germ cell differentiation *in vitro*.
- Culturing human pre-pubertal testicular tissue *in vitro* using an organ culture system for a long term showed viability and functionality of Leydig cells and Sertoli cells for up to 42 days and 21 days respectively. However, no germ cells could be detected at 91 days of culture.

6 CRITICAL EVALUATION AND FUTURE PERSPECTIVES

Indeed, finding a clinical option for fertility preservation for young cancer survivors, as well as young survivors from other conditions necessitating gonadotoxic treatment, is an intriguing question. Although the survival rate is improving a lot in the recent years, still the fertility preservation options for those survivors did not develop too much. Hence, we tried here in this thesis to get more insights about male germ cell differentiation *in vitro*, as one of the most promising alternatives of fertility preservation.

We have investigated the differentiation potential of three hES cell lines towards male germ cells *in vitro*. Our results showed that the choice of the cell line and the culture condition could affect the differentiation potential. An upregulated germ cell markers expression, along with downregulated mesoderm, endoderm and ectoderm markers expression might predict the fate of the undifferentiated hES cells towards male germ cells. However, we should also state that these results are based on studying three hES cell lines only, which could be not sufficient to make a final judgement. Furthermore, finding the NODAL/ACTIVIN pathway relatively unchanged in the cell line suspected to differentiate towards male germ cells is a real point of interest. Further studies are needed to elucidate the role of this pathway in differentiation of hES cells *in vitro* towards germ cells. Taken together, the study is paving the way for further studies on hES cell differentiation potential towards male germ cells. However, the results should be cautiously interpreted, since we have to characterize more the differentiated cells to confirm that they are gonadal cells, and we have to investigate larger number of hES cell lines to make sure that the culture conditions, along with the gene expression profile, can be used robustly to predict the differentiation potential of the hES cells.

In addition, we have also studied the effect of the culture conditions on differentiating rat pre-pubertal testicular cell culture *in vitro* using a 3D culture system. The results showed that the culture medium *per se* can affect the functionality of Leydig cells *in vitro* in a 3D culture system, while it has no effect on germ cell differentiation. In fact, this study was initiated before we start using the MEMα culture medium in culturing testicular tissues *in vitro*. Therefore, it would be of great importance to study larger number of culture media, including the MEMα culture medium, in a further study to get a better conclusion. Moreover, the effect of glutamine on the steroidogenic enzymes, albeit proven, needs to be studied carefully to get insights about the mechanism behind this effect. The study also showed that the 3D culture system is not an optimal option for germ cell differentiation *in vitro*, due to the dis-arrangement of the germ cells in regards to the Sertoli cells in culture. This point is really interesting, and needs further studies to investigate the exact effect of germ cell positioning, in regards to Sertoli cell, on germ cell differentiation *in vitro*.

Using the organ culture system previously published for murine male germ maturation *in vitro*, we could show the differentiation of undifferentiated rat spermatogonia to post-meiotic round spermatids, expressing Crem and Acrosin on protein level. In fact, the rat model is considered a step forward between the mouse model and human, which could be seen as

unique achievement. However, the efficiency of the culture system to produce post-meiotic germ cells was extremely low, even when compared to the mouse model. This makes it important to go further in studies for optimizing the organ culture system for rat germ cell maturation *in vitro*. Moreover, the integrity of the post-meiotic cells produced by this system, along with the functionality, should be further assessed. Indeed, supplementation of the culture system with different supplements, we speculated its positive effect on the culture, could not improve the rat germ cell differentiation *in vitro*. However, more studies should follow, in order to evaluate the effect of these supplements thoroughly. Other supplements should be searched for, proposed and investigated as well, in order to optimize the organ culture system. In addition, the rat model is still not physiologically identical to humans, which makes further studies on human testicular organ culture *in vitro* inevitable.

Unequivocally, translating the achievements that we have reached into a clinical option for patients is our ultimate goal. Hence, we investigated in this thesis the possibility of differentiating human pre-pubertal immature germ cells *in vitro*, using the organ culture system. We have considered the different time periods needed by the different species in order to achieve a full spermatogenesis *in vivo* to be representative for the time needed *in vitro* to achieve the same. Knowing that human spermatogenesis *in vivo* is the longest among the three species (mouse, rat and human); we expected that that human immature testicular tissue should spend longer time in culture in order to get mature germ cells. Hence, we studied here the effect of the long term culture on the human pre-pubertal testicular tissue. However, the study has still a lot of ongoing investigation. For instance, inhibin B evaluation needs to be performed on the samples from time points up to 91 days in culture, in order to give a clearer picture about Sertoli cell functionality. In addition, characterization of Sertoli cells in the culture will give us an insight about the stage of maturation they have reached *in vitro*. This is of great importance, as having mature Sertoli cells is crucial to differentiate germ cells using the organ culture approach. Besides, the evaluation of germ cell development *in vitro* needs to be more clarified, using markers for the different stages of development of human male germ cells. Assessing the proliferation potential of the testicular cells *in vitro* is also a privilege, as it will assist in defining the maturation stage of both germ and Sertoli cells. Furthermore, we are planning to investigate the effect of adding different supplements, suspected to be beneficial, to the culture medium on the human male germ cell differentiation *in vitro*. Other methods of evaluation (e.g. qPCR and RNA sequencing for gene expression analysis and Western blotting for protein expression) should be also included, in order to confirm the results obtained so far. Therefore, this study is giving insights about the effects of long term culture on human pre-pubertal testicular tissue, which facilitates further studies towards the ultimate goal of achieving full human spermatogenesis *in vitro*.

In conclusion, this thesis provides new insights about male germ cell differentiation *in vitro*, in rat and human. However, further investigations are still needed in order to translate such insights into a clinical option.

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