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IN VITRO MODELS AIMING FOR FERTILITY PRESERVATION

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In vitro models aiming for fertility preservation

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To:

KHALID …

and my family

ABSTRACT

Fertility preservation is considered a great hope to people suffering from infertility as a consequence of developmental or genetic cause, a disease itself or its treatments sequelae. *In vitro* models are fundamental to enrich our knowledge about human germ cell development and its regulation, to generate and screen new therapeutic measures and might provide a tool that can be transferred to clinical use to treat infertility. There are, in principle, two strategies to start an *in vitro* fertility preservation protocol in male humans. First the *in vitro* generation of germ cells by differentiating human pluripotent stem cells and second the maturation and differentiation of human spermatogonia stem cells into more advanced stage of germ cells. However, both strategies are not suitable to be used in the clinic since the robust protocols for differentiation are not available. Additionally, reproducibility of the results between different laboratories is a big challenge faced by researchers working in the field.

Therefore, we focused in this thesis on these two strategies aiming to improve both models to reduce variability and improve reproducibility. In the first study, we addressed the variability occurring among hES cell lines when used for human germ cell differentiation. We aimed to find a suitable culture condition allowing a robust starting point for our cultures by using LN521 as a culture matrix for hES cells before using them in protocols of differentiation. Moreover, we investigated the only robust working system in rodents and aimed to translate this to the situation in humans by using normal fetal gonadal tissues as well as actual patient materials to investigate the potential use of these systems to mature human germ cells *in vitro*.

We found that LN521 has positive effects on hES cells growth and maintenance of their pluripotency characteristics with no influence on gonadal cells related genes expression. Furthermore, we showed that LN521 homogenized the gene expression variation among the five cell lines used in the study. In regard to *in vitro* germ cells maturation from spermatogonial stem cells, production of elongated spermatids was achieved when air liquid interphase organ culture method was used to cultivate mouse testicular tissues *in vitro*. A supplementation of 10% knockout serum replacement is shown to have positive effects on tubular maturation, germ cell proliferation and differentiation. We showed that organ culture method could be used to culture and study human first trimester gonads somatic cells *in vitro* as demonstrated by their ability to produce hormones in a manner similar to what is described for *in vivo* situation. In addition, differences in somatic cells functions in testicular tissues from different patient groups exposed to hematological and oncological

treatments could be illustrated by culturing these tissues *in vitro* by using organ culture method.

Culturing human embryonic stem cells on LN521 could be a step forward towards future applications of human embryonic stem cells in regenerative medicine by providing more predictable and controllable system to assess the behavior of human embryonic stem cells when used in different protocols for differentiation. This would be the basis for future approaches to generate more defined *in vitro* protocols for differentiation of human pluripotent stem cells towards germ cells. On the other hand, organ culture method could be a useful tool to study the process of human germ cells development and to screen the effect of different substances on human gonadal cell development and function. Moreover, it can be used as quality assessment tool of cryopreserved testicular tissues, from patients exposed to hematological and oncological treatments, before considering using them for fertility preservation purposes.

LIST OF SCIENTIFIC PAPERS

I. **Halima Albalushi**, Magdalena Kurek, Leif Karlsson, Luise Landreh, Kristín Rós Kjartansdóttir, Olle Söder, Outi Hovatta, and Jan-Bernd Stukenborg.
"Laminin 521 Stabilizes the Pluripotency Expression Pattern of Human Embryonic Stem Cells Initially Derived on Feeder Cells,"
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II. Ahmed Reda, **Halima Albalushi**, Sheyla Cisneros Montalvo, Mirja Nurmio, Zeliha Sahin, Mi Hou, Niels Geijsen, Jorma Toppari, Olle Söder,and Jan-Bernd Stukenborg.
Knock-out serum replacement and melatonin effects on germ cell differentiation in murine testicular explant cultures.
Annals of Biomedical Engineering, 2017; 45(7): 1783–1794
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III. **Halima Albalushi**, Lena Sahlin, Elisabet Åkesson, Kristín Rós Kjartansdóttir, Rika Lindh, Olle Söder, E. Rotstein, Outi Hovatta, Jan-Bernd Stukenborg.
Hormone production by a functional *in vitro* system for human first trimester gonads
Submitted to Endocrinology

IV. João Perdo Alves-Lopes, Magdalena Kurek, **Halima Albalushi**, Olle Söder, Rod T Mitchell, Cecilia Petersen, Kirsi Jahnukainen and Jan-Bernd Stukenborg
In vitro assessment of testicular function in boys subjected to treatment for haematological and oncological diseases
Manuscript

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Over expression of NANOS3 and DAZL in human embryonic stem cells.
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Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy.
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CONTENTS

LIST OF ABBREVIATION

2D	Two-dimensional
3D	Three-dimensional
3βHSD	3β-hydroxysteroid dehydrogenase
AFP	Alpha-fetoprotein
Alpha SMA	Alpha Smooth Muscle Actin
AMH	Anti-Müllerian hormone
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AR	Androgen receptor
BMP	Bone Morphogenic Protein
BTB	Blood testis barrier
CK18	Cytokeratin 18
cMyc	V-myc myelocytomatosis avian viral oncogen homolog
CREM	cAMP responsive element modulator
CRL	Crown-rump length
CYP23B1	Cytochrome P450, family 23, subfamily B, polypeptide 1
DAZ	Deleted in azoospermia
DAZL	Deleted in azoospermia-like
CT	Threshold cycle
DDX4	DEAD Box Protein 4
DMEM	Dulbecco's Modified Eagle's medium
DNMT3B	DNA Methyltransferase 3B
d*pp*	Days *postpartum*
E	Embryonic day
EBs	Embryoid bodies
ECM	Extracellular matrix
ELISA	Enzyme Linked Immunosorbent Assay
ES	Embryonic stem
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

FP	Fertility preservation
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA6	GATA binding protein 6
GDNF	Glial Cell Derived Neurotrophic Factor
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
hES	Human embryonic stem
hFF	Human foreskin fibroblast
hiPS	Human induced pluripotent stem
hPGCLCs	Human primordial germ cell-like cells
hPS	Human pluripotent stem
HSCT	Hemopoietic stem cell transplantation
HU	Hydroxyurea
IAPP	Islet Amyloid Polypeptide
ICM	Inner cell mass
IFITM1	Interferon induced transmembrane protein 1
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVS	*In vitro* spermatogenesis
KDR	Kinase Insert Domain receptor
KI67	Proliferation marker KI-67
KIT	V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene-Like protein
Klf4	Kruppel-like factor 4
KO-DMEM	Knockout Dulbecco Modified Eagl's Medium
KRT7	Keratin 7
KSR	Knockout Serum Replacement
LAMA	Laminin subunit Alpha 1
LH	Luteinizing Hormone
LIF	Leukemia Inhibitory Factor

LIN28	Lin-28 Homolog A
MAGE-4	MAGE Family Member A4
MEFs	Mouse embyonic fibroblasts
MEMα	Minimum Essential Medium Alpha
MLH1	MutL homolog 1
NANOS3	Nanos C2HC-Type Zinc Finger 3
NEUROD1	Neuronal Differentiation 1
NRL	Neck rump length
NR6A1	Nuclear Receptor Subfamily 6 Group A Member 1
PAS	Periodic Acid Schiff
PAX6	Paired Box 6
PBS	Phosphate buffered saline
Pen/strep	Penicillin/streptomycin
PGCs	Primordial germ cells
PLAP	Placental alkaline phosphatase
Plzf	Promyelocytic Leukemia zinc Finger
POU5F1	POU domain, class 5, transcription factor 1
PRDM1	Positive regulatory domain zinc finger protein
PS	Pluripotent stem cell
RA	Retinoic acid
ROCK inhibitor	Rho-associated coied-coil kinase inhibitor
SCD	Sickle cell disease
SCF	Stem cell factor
SCID	Severe combined immune-deficient
SFRP2	Secreted Frizzled Related Protein 2
SOX	SRY box
SSCs	Spermatogonial stem cells
SSEA	Stage-specific embryonic antigen
STELLAR	Developmental Pluripotency Associated Protein 3
STRA8	Stimulated by retinoic acid gene 8
SYCP3	Synaptonemal complex protein 3

TAT	Tyrosine Aminotransferase
TDGF1	Teratocarcinoma-derived growth factor 1
TESE	Testiular sperm extraction
TLD array	Taqman Low Density aaray
TRA	Tumor-related antigen
TTC	Testiculat tissue cryoprservation
TUJ	Tubulin, Beta 3
WNT3A	Wingless-type MMTV integration site family member 3 A
ZFP	Zinc Finger Protein
ZO1	Zonula occludens 1

1 INTRODUCTION

Fertility preservation for patients who are not able to produce sperm, because of genetic cause or as a consequence of treatment received, is a challenge. No clinical option available till now to preserve the fertility of such a group. *In vitro* generation and/or maturation of germ cells are considered a promising method in this regard. There are, in principle, two strategies to start an *in vitro* fertility preservation protocol in humans: the use of human pluripotent stem cells and the use of male germ cells as a starting point. To understand the work done in this thesis, the following chapters will address the basic knowledge as well as some clinical topics related to *in vitro* models for fertility preservation.

1.1 HUMAN STEM CELLS

1.1.1 Embryogenesis

Embryogenesis is the process by which human development begins. It includes all events from the union of the sperm and the oocyte to form a zygote to the establishment of the fetus[1]. The creation of a zygote takes place in the ampulla of the oviduct where one sperm penetrates through the zona pellucida of the oocyte. The formed zygote undergoes mitotic cellular division with no significant growth happening at this stage, while it is propelled towards the uterus by the cilia in the oviduct [1]. The eight-cell stage zygote is called blastomere and continues to divide further. During this process, the blastomere forms a compact sphere. The cells within the sphere interact with each other through gap junction [2]. The conceptus develops further to form a 16-cell morula. At this stage, the cells have differentiated and formed two cell layers; the inner layer (non-polarized) and the outer layer (polarized) [1, 3].

During the next step of conceptus development, a fluid filled cavity called blastocoele is formed and the conceptus is called blastocyst at this stage. The non-polarized cells give rise to the inner cell mass (ICM) which develop into all parts of the embryo and the extra-embryonic membranes including amnion, yolk sac and allantois [1, 4]. The polarized cells form a trophoblast layer which will form the chorion, the embryonic part of the placenta. The placenta consists of the chorion and the decidua (the maternal part). It provides the developing embryo with nutrition and disposes of waste. In addition, the placenta helps in blocking the immune rejection of the conceptus by the mother. Upon entry to the uterus, about 5-6 days after fertilization the blastocyst starts to produce plasmin which facilitates the process of the hatching into the uterus wall by degradation of the zona pellucida. At this stage, the ICM has formed a bilaminar disc that consists of hypoblast and epiblast. The

hypoblast develops into the extra-embryonic endoderm and the embryonic epiblast. The amniotic endoderm produces the amniotic fluid and contains and protects the embryo. The embryonic epiblast develops into three embryonic germ layers as well as germline cells by a process called gastrulation, which will be followed by the organ formation stage (organogenesis) [1, 4]. The embryonic ectoderm will form, for example, the nervous system, the dermis and lenses of the eyes. The mesoderm will develop into, for example, the skeleton, the skeletal muscles and the heart. The endoderm will give rise to, for example, the stomach, the liver and the epithelial part of the trachea [1, 4].

1.1.2 Pluripotent stem (PS) cells

As a common feature, stem cells are uncommitted cells that have two main characteristics. First is their ability for self-renewal. Second is their ability to differentiate to a diverse range of more specialized cells *in vivo* and *in vitro*. A stem cell remains uncommitted unless it gets a signal for differentiation into more specialized cell type. There are various stem cell types, shown different grades of potencies, identified in human body in embryonic, fetal and adult tissues. In addition to stem cells occurring *in vivo*, it has been possible to produce stem cells from somatic cells *in vitro*, called induced pluripotent stem cells (iPS cells), which share similarities with human embryonic stem (hES) cells. Overall, stem cells can be classified according to their differentiation potentials into: totipotent, pluripotent, multipotent and unipotent. Totipotent stem cells have full potential for differentiation and they can differentiate to embryonic three germ layers and extraembryonic tissues as well (trophectoderm). The zygote and the early stages of the embryo are the only totipotent cells [5]. Pluripotent stem cells are able to self-renew and differentiate into the three germ layers as well as the germline cells. Multipotent and unipotent stem cells lineages are restricted cells found within specialized tissues and they are also called adult stem cells. They have the ability to differentiate into different specialized cell types. In the adult body they can be found in hair, skin, bone marrow, central nervous system and the testis.

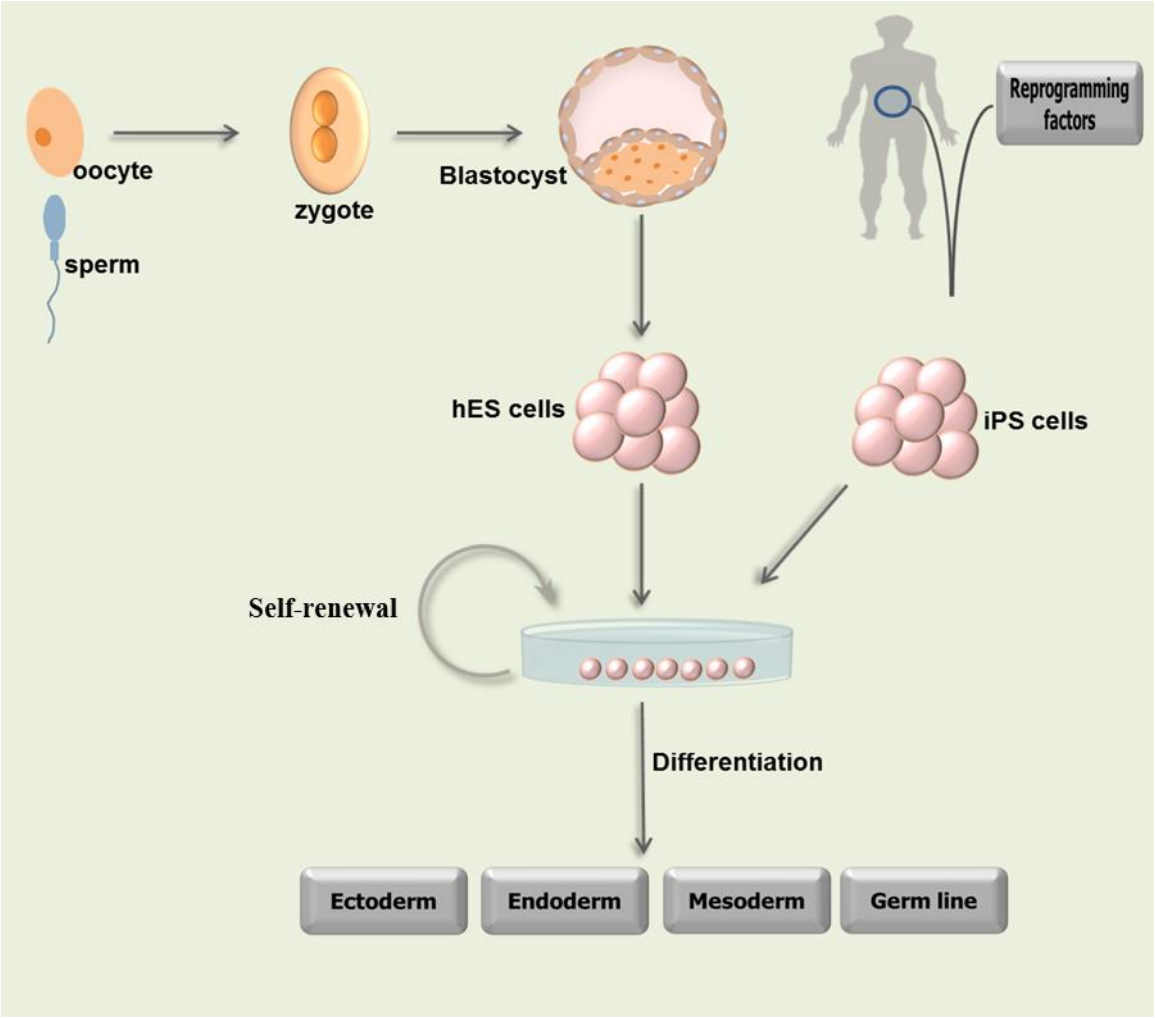

Figure 1. Human pluripotent stem cells sources. Human pluripotent stem (hPS) cells include hES cells and hiPS cells. hES cells are derived from human embryos at blastocyst stage from the ICM. hiPS cells are derived by induction of human somatic cells by specific reprogramming factors. hPS cells have the potential to renew themselves and to differentiate to ectodermal, endodermal, mesodermal and germ cells.

PS cells are able to self-renew and differentiate into the three germ layers as well as the germline cells. The cells of the ICM at blastocyst stage are characterized as pluripotent. They differ from the totipotent cells because they cannot give rise to the trophoblast and thus they are unable to differentiate to all the cells of the conceptus. The ICM cells can be derived from the blastocyst and cultured *in vitro* as pluripotent embryonic stem cells (ES cells) [6, 7]. The main characteristics of PS cells are their unlimited self-renewal capacity and their ability to differentiate to diverse cell types of the three germ layers, as well as into germline cells (Figure1). These characteristics make PS cells promising candidates for regenerative medicine in studying and treating disorders involving loss of specific cell types or tissues for example diabetes, Parkinson´s disease and infertility. In addition, these cells can be utilized for studying developmental processes and also for drug screening. PS cells include hES cells

and human iPS cells. Both types will be addressed in the following chapters with a special focus on fertility preservation.

1.1.2.1 Human embryonic stem cells (hES cells)

HES cells are pluripotent stem cells derived from supernumerary blastocysts (5-6 days after fertilization) donated from patients who underwent *in vitro* fertilization (IVF) treatment. The derivation of hES cell lines is achieved by isolating ICM of a blastocyst stage embryo and placing it into culture conditions which support undifferentiated cell proliferation [8]. There are several methods for derivation of hES cells: immunosurgical, partial or whole embryo culture and surgical isolation of the region containing the ICM with an ultra-fine glass pipette. The choice of the method depends on the quality and size of the embryo. The immunosurgical method is associated with the risk of hES cells contamination with animal pathogens[9]. Partial or whole embryo culture, in which the blastocyst is seeded directly on a STO feeder layer without its zona pellucida, helps in overcome this problem. However, it is observed that in this method the trophectodermal overgrowth hinder the expansion of the ICM. This problem of trophectodeermal overgrowth is alleviated by the surgical isolation of the region containing the ICM with an ultra-fine glass pipette. The efficiency of the surgical isolation is comparable to that of the immunosurgical isolation [10]. To avoid the destruction of the whole embryo which creates a big ethical debate, hES cells can be derived from a single blastomere of a morula (8-10 cell stage embryo) with no harm or destruction of the embryo [11, 12].

1.1.2.2 Human induced pluripotent stem cells (hiPS cells)

The principle behind the creation of induced pluripotent stem (iPS) cells is induction of somatic cells by defined factors. The first iPS cell line was produced in 2006 when Takahashi group reprogrammed mouse embryonic and adult fibroblast into iPS cells by forced expression of four transcription factors including: Core transcription factor POU class 5 homeobox 1 (*Pou5f1*), SRY (sex determining region Y)-box (*Sox2*), Krueppel-like factor 4 (*Klf4*), and v-Myc myelocytomatosis avian viral oncogene homolog (*c-Myc*) [13]. A year later, the first hiPS cells line was produced using the same transcription factors [14]. After that, many studies were published demonstrating the use of different transcription factors to produce iPS cell lines or to aim for optimization of generation of iPS cells from different types of somatic cells [15]. Initially, iPS cells were produced by using retroviral or lentiviral for induction and were derived and cultured on mouse feeders. Followed this, many approaches were reported to have improved the induction process as well as the derivation and culture methods aiming for higher efficiency integration-free and feeder-free conditions.

As a step forward to integration-free reprogramming, non-integrating viruses such as Sendai virus [16], non-integrating episomal plasmids [17, 18] as well as direct transfection with mRNA or proteins were used [19]. In addition, mouse feeders were replaced by feeder-free methods using matrices such as Matrigel and vitronectin. Despite the great efforts done by researchers in the field aiming for improvement of the reprogramming methods to make it more suitable for clinical use, it seems that an integration-free method with feeder-free and defined xeno-free condition for iPS cells production is still a challenge.

1.1.3 Characterization of human embryonic stem cells

The characteristic morphology of hES cells is a high nucleus to cytoplasmic ratio and prominent nucleoli [8]. HES cells form relatively flat compact colonies with distinct cell borders [8]. They can be cultured for extended period of time and they retain their pluripotency and normal karyotype. To assess pluripotency of hPS cells, which include hES cells and hiPS cells, there are sets of cell surface markers and transcription factors to be expressed by these cells. In addition, the capacity for differentiation *in vitro* and *in vivo* should be used also [20]. HPS cells express standard cell surface markers including: stage-specific embryonic antigen 3 (SSEA3), stage specific embryonic antigen 4 (SSEA4), tumor-related antigen (TRA)-1-60, tumor-related antigen (TRA)-1-80 and alkaline phosphatase (ALP) [8]. Core transcription factor POU class 5 homeobox 1 (POU5F1), Nanog homeobox (NANOG) and SRY (sex determining region Y)-box (SOX) 2 are required for these cells´ pluripotency and self-renewal and they are used in the characterization of hPS cells routinely [21]. The capacity of hPS cells to differentiate towards the three germ layers is assessed by *in vitro* and *in vivo* assays. By injection of hPS cells into severe combined immunodeficient (SCID) mice, *in vivo* hPS cells form teratomas which contain ectodermal, mesodermal and endodermal origin [8]. *In vitro*, the assessment is done by formation of cell aggregation called embryoid bodies (EBs) which contains spontaneously differentiated cells from all three germ layers [22]. Differentiation towards germ cells has been reported. However, the reproducibility of these protocols is rather low and new strategies to improve them by providing a robust starting point will be addressed and discussed in paper I and the following chapters.

1.1.4 Culture of human embryonic stem cells

The first hES cell line was derived in 1998 and ten years after that over a thousand new lines had been described [23]. Over the years, the derivation techniques and culture conditions have been improved. A lot of studies were done to avoid using undefined culture conditions which hinder the translation of hES cells use to the clinic. Defined culture components and

minimal exposure to animal-derived substances are required for the clinical use of hES cells [24].

Derivation of first hES cell lines were on mitotically inactivated mouse embryonic fibroblasts (MEFs) as feeder cells and the culture medium used for derivation was supplemented with fetal bovine serum (FBS) [8]. These undefined culture conditions create variability from batch to batch and also expose hES cells to pathogens and teratogenic components. Aiming for xeno-free culture conditions, initially, the animal-derived MEFs are substituted with human cells, the FBS with knockout serum replacement (KSR) as well as basic fibroblast growth factor (bFGF). However, KSR is not completely devoid of animal substances [25-27]. Few years later, it has been demonstrated that serum can be avoided and not used in the medium for culture, but bFGF should be supplemented in the medium since it is required for hES cell maintenance [28]. Feeder cells were replaced by extracellular matrices as an approach towards feeder-free and xeno-free culture condition. Matrigel is most commonly used in the coating of the hES cell culture plates. It is a mouse sarcoma tumor extracts containing a mixture of proteins, for example: laminin, collagen IV, and heparan sulfate proteoglycan. Later studies reported that recombinant vitronectin [29], a hydrogel [30], and a laminin-511(LN-511) fragment [31] can be used as alternatives to matrigel or feeder cells for culturing of hES cells. However, apoptotic inhibitors such as Rho-associated kinase inhibitor (Rock inhibitor) and small molecules inhibitor are needed for the clonal survival [32-34] and the cells need to be passaged in small clumps which can contain some differentiated cells.

In addition to the previous methods of culture, synthetic scaffolds were used and shown to support survival and colony formation of hES cells [35, 36]. However, it is shown that in this culture condition the endogenously produced laminin by the stem cells themselves was facilitating the adhesion and survival of the hES cells [37]. To overcome this challenge, researcher reported culture of hES cells as single cell suspension without a need to add Rock inhibitor to the medium by using LN521 as a culture matrix [11, 38]. The first study of this thesis focused on the effects of LN521 on hES cells when used as a culture matrix.

1.1.4.1 Laminins

Laminins are a large family of heterotrimeric basement membrane adhesion proteins. They are highly specific to the cell and tissue type [39]. Each one of these proteins consists of one α, one β and one γ chain, and according to the chain composition, these proteins being named (for example LN-521 consists of α5, β2and γ1 chains) [40]. Around 16 laminin isoforms are known to exist in mammalian tissues to date, and most of them are recombinant proteins [41,

42]. It is shown that hES cells express alpha 1, alpha 5, beta 1, beta 2 and gamma 1 laminin chains and therefore suggested to express four isoforms of laminins, LN521, LN121, LN511 and LN111 [43]. Moreover, alpha 5 chain laminins are expressed in the inner cell mass of the mouse blastocyst which makes us consider laminins containing alpha 5 chains as part of stem cell niche. Researches showed that LN521 is expressed in the embryo at early stages of development in the ICM from which all embryonic tissues developed [39]. It is also shown that it is expressed in some tissues in adult in a combination with other laminins, e.g., in the epithelium and surfaces of the oral cavity, gastrointestinal tract, urinary tract, skin, lungs and testis. It is also shown to be present in the nerve, muscle, endothelial and cardiac cells [44, 45]. Moreover, laminins with alpha 5 chains are produced *in vitro* by hPS cells in culture and have autocrine and paracrine effects in regulating hPS cells survival and self-renewal. This is proven by a study showing dramatic reduction in hPS cells self-renewal and accelerated apoptosis with disruption and knocking down LAMA (laminin subunit alpha) gene [46].

LN521 supports long term self-renewal of the ES cells when used as a culture matrix in defined and xeno-free culture conditions [11]. Studies showed that hPS cells can be expanded on LN521 efficiently without using Rock inhibitor in defined xeno-free medium [11, 38, 47]. Moreover, it is shown that LN521 with E-cadherin support the derivation of hPS cell lines without a need to destroy the embryo. Avoidance of destroying the embryo could circumvents the ethical concern related to destruction of embryos for derivation of hES cell lines [11, 38]. In addition, studies showed LN521 allows the derivation of transgene-free iPS cells and their expansion efficiently [47]. LN521 was shown also to support culturing hPS cells in agitated conditions on microcarriers coated with LN521 [44]. Furthermore, it supports the differentiation of hPS cells to dopaminergic neurons when used as a culture matrix in xeno-free conditions [47]. Using LN521 as a culture matrix provides flexible culture protocols with possible weekend-free cells feeding which reduces the demands of stem cell culture [37]. However, detailed understanding of the effect of LN521 on hPS cells gene expression which could explain its positive effects on promoting self-renewal and pluripotency of hPS cells are not fully studied. In paper 1 of this thesis we studied the impact of using LN521 as a culture matrix on short term on hES cells gene expression.

1.1.5 Differentiation of hPS cells into male germ cells

Differentiation of hPS cells into germ cells is considered an important tool to study the early events of germ cells development occurring in human embryo. Using hPS cells as a model for germ cell development and differentiation will enrich our knowledge which was otherwise extrapolated from animal models and the species difference hinder the one-to-one translation

of this knowledge to human. Moreover, differentiation of hPS cells *in vitro* provides future hopes and opportunity for treatment of infertility. Despite the great efforts done so far, we are not there yet. Different protocols were used starting from a single step embryoid body formation till multistep differentiation protocols with combination of different factors and different methods. The first attempt for *in vitro* differentiation of hPS cells towards germ cells was done by Clark and colleagues. They reported presence of Deleted in Azoospermia like (DAZL), Developmental Pluripotency Associated Protein 3 (STELLAR), DEAD-box polypeptide 4 (DDX4) and Synaptonemal complex protein 3(SYCP3) in the embryoid bodies [48]. EBs formation was used broadly as one step differentiation or as a combination of more steps on different types of cells or on matrices such as gelatin and matrigel [49-53]. Some studies used the two dimensional (2D) monolayer culture as a differentiation method in which hPS cells were cultured and differentiated on gelatin, laminin, polyornithine, feeder cells, gonadal stromal cells and matrigel [50, 54-59]. This approach leads to the generation of germ cell expressing SSEA1, DDX4, MutL Homolog 1(MLH1) and SYCP3. In 2015, a new differentiation strategy was introduced by Irie as well as Sasaki and colleagues. In their approach, they aimed to induce the cells into a mesodermal-like state before starting to direct them to differentiate towards germ cells. They used different matrices and a combination of 2D and 3D methods of culture [60, 61]. Both groups reported production of human primordial germ cell like cells (hPGCLCs) with improved efficiency compared to previous published studies. However, the reproduction of these results remains challenging.

Different factors have been tried and added to the differentiation medium. Bone morphogenic proteins (BMPs) were mostly used in the differentiation attempts and BMP4 is the most used factor [50-54, 56, 60-72]. Other factors such as leukemia inhibitory factor (LIF) and bFGF were also used as additives in the differentiation medium [55, 58, 61, 62, 70, 72-74]. Retinoic acid (RA) was reported to support inducing hPS cells differentiation into stage beyond PGCs [49, 62, 64, 70, 75-78]. In the recent years, additional factors such as, stem cell factor (SCF), Wnt family member 3A (WNT3A) and glial cell-line derived neurotropic factor (GDNF) have been employed for further experiments [60, 61, 66]. Moreover, some research groups, including our own data not included in this thesis (Ref Panula et al 2016; Plos One), used overexpression of different factors such as Deleted In Azoospermia (DAZ), DAZL, BOULE, DDX4, STELLAR and Nanos C2HC-Type Zinc Finger 3 (NANOS3), to promote hPS cells differentiation towards PGCs and male germ cells [56-58, 71, 78].

Evaluation of the germ cells resulted from the *in vitro* differentiation is challenging since early human germ cells share expression of several genes and proteins with human

pluripotent stem cells. Defining the exact appearance of early germ cells is not well characterized yet. In addition, comparing results from different groups is difficult since the definition of a common and standardized evaluation protocol is still missing for human cells. E.g. DDX4 was used broadly by many groups to evaluate the presence of human germ cells after differentiation of hPS cells. Other markers such as: POU5F1, SYCP3, DAZL, cKIT, PRDM1, SSEA1 and STELLAR were among the most commonly used protein markers for evaluation of cells resulted from hPS cells differentiation. Furthermore, many groups reported the presence of haploid cells expressing acrosin in their differentiation protocols which should be taken with caution since these cells should have specific morphological characteristics of haploid spermatids [56, 57, 71, 73, 75]. Recently, with advanced progress toward understanding human germ cell specification *in vivo*, the spectrum of markers used for evaluation of the hPS cells differentiation increased as well.

Differentiation of hPS cells into human germ cells is a challenge in the scientific field. Different approaches of analysis of the resulted cells and lack of standard criteria to define the first appearance of human germ cell hinder the comparison of the results obtained from different groups. Moreover, the overlap of the gene expression between the stem cells and the male germ cells in humans complicates the picture further. Furthermore, using different cell lines and sometimes in-house produced cell lines make it more difficult to judge the results since each hPS cells line is unique and different; furthermore, the same line behaves differently when cultured with different culture methods. This necessitates a robust culture condition, robust differentiation protocols as well as common baseline criteria to define the early specified germ cells.

1.2 THE TESTIS

The testis is a specialized organ. In early development, testes have a major impact on the regulation of normal development of male reproductive system by their hormonal secretion. Moreover, this organ is unique since it produces the gametes which transfer the genetic materials across generations. In this section, development of the human gonads, testicular cells development and spermatogenesis in mice and humans will be discussed.

In brief, the testis is divided internally into two compartments: the seminiferous tubules and the surrounding interstitial tissue. The seminiferous tubules are long looped tubules, where spermatozoa production takes place. Theses tubules contain Sertoli cells and germ cells. The differentiation of spermatogonial stem cells (SSC) to mature spermatozoa starts at the basement membrane of the seminiferous tubules towards the lumen. When the formation of

the elongated spermatids is done, it gets released into the lumen. The seminiferous tubules drain into the rete testis which unite to form the ductuli eferentes and then drain into the epididymis. In the seminiferous part of the testis, a unique arrangement of Sertoli cells forms the blood-testes barrier (BTB). BTB is unique when compared to other tissue barriers. In addition to tight junctions, gap junctions, desmosomes and ectoplasmic specializations coexist and co-function together to form the BTB[79].

BTB plays a major role in isolating the developing germ cells and thus preventing an immune reaction against their cell surface antigens [80]. The outer layer of seminiferous tubules is surrounded by peritubular myoid cells. The interstitium consists of Leydig cells, fibroblasts, macrophages, lymphocytes, blood vessels, lymph vessels, loose connective tissues and nerves. These two compartments are responsible of the testes functions of spermatogenesis and steroidogenesis [80].

1.2.1 Germ cell development

Germ cells are unique cells that secure the survival of a species by having the ability to transmit genetic information between generations. Germ cells are resulted from a complex developmental process that starts in the early stages of the embryo development and continues throughout the reproductive life of the individual. Our knowledge about the molecular and cellular mechanisms of the early germ cell development is still sparse. Inaccessibility of the embryo and the small number of germ cell population at the early stage of development lead to this limited knowledge. Most of the knowledge about human germ cell development is extrapolated from studies done in mice. Detailed information on the molecular and cellular mechanisms controlling the fate of the human germ cells is needed for better understanding of the human germ cell development.

1.2.1.1 Specification and migration

The specification events of human germ cells take place at day 9-16 of the embryo development. It is impractical to study these events since it is impossible to have human embryo at this stage for study and research purpose because of ethical and regulatory issues. As mentioned before, most of the knowledge of human germ cells development is extrapolated from mice; these early events of specification are studied in detail in mice.

Specification of mouse germ cell precursors takes place in the proximal epiblast cells on embryonic day 6.25. The specification happens as a response to BMPs produced from the extraembryonic tissue [81, 82]. At E7.2, about 40 primordial germ cells (PGCs), which are ALP positive, are generated in the posterior primitive streak from the mouse germ cell

precursors [83, 84]. Then, these PGCs reside in the mesoderm of the yolk sac close to the junction with the allantois at E8 [85]. At this point of development, the corresponding developmental age of human embryo is 3-4 weeks. The earliest human PGCs were identified in the same location as mouse PGCs, close to the junction with the allantois [86, 87]. The characteristics of human and mouse PGCs are very similar. They are large round cells with large nucleus and are ALP positive [84, 86].

The PGCs then migrate through the hindgut and its mesentery into the gonadal ridges at E8.5 for mice and 5 weeks of development for humans. In humans, the PGCs start the migration from the allantois at week 4 and start to enter the gonads at week 5[88]. At week 6, the PGCs arrive at the final destination and colonize the developing gonads which occur at E11.5 for mice [86, 87, 89].

The migration of PGCs from proximal epiblast cells towards the developing gonads is a complex process which has some active and some passive events and it is influenced by many physical and directional signals. Moreover, the PGCs must increase in number and survive throughout the migration journey [87]. The exact details about the PGCs migration is not known; however, it is believed that the morphological rearrangement of the embryo plays the major role in directing the PGCs to migrate from the yolk sac into the hindgut [90].

Later on, the PGCs may become able to migrate actively as they will have some changes in the morphology from round into irregular shape with some protrusions and pseudopodia [86]. There is a believe that the migration process is also mediated by the chemotaxic factors, KIT ligand, since it is found to be expressed in the PGCs [91, 92]. It is assumed that KIT ligand promotes the survival of the PGCs during the migration process, as it's down regulation in mouse PGCs causes apoptosis which leads to failure of migration correctly [93]. KIT ligand is believed to play a major role in PGCs migration. However, the complexity of the mechanism of PGCs migration, survival, and proliferation requires more research and study.

1.2.1.2 Colonization and sex determination

After reaching the gonads, the human PGCs proliferate. The PGCs proliferation starts during migration but happens mostly in the gonads. It results in about 150.000 germ cells in males and 450.000 in females by week 9 of development [94]. Regulation of the *in vivo* PGCs proliferation is unknown yet; however, many studies showed that *in vitro* PGCs proliferation is regulated by many factors such as BMP4, LIF, bFGF and forskolin [95, 96]. At early stages of embryo development at the gastrulation period, the PGCs are still bi-potential and can form either oocytes or sperm. The fate of PGCs is not dependent on the germ cell sex but it is

influenced by signals from the surrounding environment from the fetal gonads [97]. Germ cell sex determination process and its molecular controlling mechanism are studied in detail in mice. Results revealed that RA and fibroblast growth factor 9 (FGF9) play a major role in this process [97]. RA triggers the entry of oogonia into meiosis in the fetal ovary by induction of retinoic acid gene 8 expressions (Stra 8). However, in the fetal testes, the retinoic acid gene 8 (stra 8) level is low because retinoic acid is degraded by cytochrome P450, family 26, subfamily b, polypeptide 1 CYP26B1 (CYP26B1) which resulted in prevention of entry to meiosis. FGF9 is produced by Sertoli cells in the testis and causes inhibition of meiosis by maintaining the pluripotency markers expression [98]. In humans, it was hypothesized that RA and Stra8 have a role in the regulation of meiosis entry of the oogonia in the fetal ovary. Research results so far suggest a role of FGF9 in the regulation of prespermatogonia mitotic arrest but the triggering factors for it is not yet known, neither in humans nor in mice [98].

1.2.1.3 Bipotential gonads

The development of the human embryo of both sexes is almost identical in the early period of gestation. After this period, the process of development differs at the anatomical and physiological level and results in male and female phenotype [99]. The development of testes in males and ovaries in females is directed by the chromosomal sex established early in advance at the time of conception [99-101]. Different multistep homologous but opposite mechanisms are involved in the male and female primary gonads differentiation [102]. Interstitial cell development and secretion of hormones are important for male gonads differentiation process. Presence of germ cells is not necessary for the development of male gonads at early stages of embryo [99]. In contrast, differentiation of female gonads requires the presence of female germ cells as well as the presence of functional somatic cells [99]. The primary male and female gonads in the fetus consist of three components: the primordial germ cells, the genital ridge mesenchyme and a layer of epithelium. The female phenotype develops far in advance to the histological differentiation which is the opposite to what happens in the male. In the male, the histological differentiation occurs before the phenotype development [99]. The process of gonadal development occurs as two phases: genital ridge appearing phase which is considered as the bi-potential gonad, followed by a phase of testis or ovary development [103]. Pre-Sertoli cells aggregate around the germ cells at 6-7 weeks of gestation and thus forming the primary sex cords which is considered the earliest distinguishable remark between the testis and the ovary at the early stages of development [100, 103]. The differentiation of the somatic cells of the future gonads plays an important role in directing the fate of the arriving PGCs. In an XY gonad, the differentiation of the

somatic cells is induced by SRY which upregulates SOX9 expression [104]. This leads to cellular and morphological changes including the differentiation of the Sertoli cells and the Leydig cells, and the formation of the testis cords around 7-9 weeks of development.

1.2.1.4 Gonocytes

When PGCs reach the male gonads, they become enclosed within the testis cords and from this time on, they are called gonocytes. "Gonocytes" is a general terminology used to refer to the fetal and neonatal precursors of undifferentiated SSCs, which are the reservoir of the germline stem cells. Gonocytes are a heterogeneous cell population with different expression profiles. They can be divided into 3 subpopulations: mitotic, transitional 1 and 2 pre-spermatogonia [105]. Around the second trimester of gestation, most of the mitotic pre-spermatogonia starts to lose their pluripotency protein expression and gain the expression of germ-cells specific proteins as they differentiate to transitional spermatogonia and become quiescent mitotically [105]. Gonocytes should migrate from the center of the seminiferous cords to the basement membranes to start the differentiation process. The number of gonocytes in the human fetal testes increases from around $3*10^3$ to around $3*10^4$ between the period 6-9 weeks post-conception [106]. The number of pre-spermatogonia increases dramatically and reaches around $4*10^4$ in the third trimester. The ratio of germ cells to Sertoli cells increases from 1:10 at week 6-9 of gestatin to 1:6 at 10-41 weeks of gestation. Transitional 2 pre-spermatogonia colonizes the basement membrane and is believed to form the spermatogonial stem cell (SSC) pool which maintains spermatogenesis in adulthood [105].

1.2.1.5 SSCs

SSCs are the crucial cell type needed for the generation of sperm. These cells, like other stem cells, are defined by their ability to self-renew and to differentiate to other types of cells. SSCs result from differentiation of gonocytes. This process is characterized by changes in morphology and loss of some gonocytes markers, e.g. reduction in Placental Alkaline Phosphatase (PLAP) expression occurs first then followed by loss of expression of NANOG and POU5F1 [107]. However, the expression of some of these markers is still present and is detected in the testes in the early postnatal life. NANOG expression is found to be lost as early as 3 months and POU5F1 is still detectable till 3-4 months postnatally [108-110]. Whilst Melanoma Associated Antigen-4 (MAGE-4), Nanos C2HC-Type Zinc Finger 1(NANOS-1) and DDX4 continue to be expressed in the human SSCs. Expression of POU5F1 in the human and mouse testes is different [111]. In humans, POU5F1 is not expressed beyond the gonocytes type while in mice it is expressed in spermatogonia till

adulthood [112-114]. In each species, there are different types of spermatogonia, and different variations were observed among different species [115]. SSCs undergo self-renewal to maintain the pool of cells needed for future spermatogenesis and at the same time provide the tissue with differentiating progenitors for the ongoing spermatogenesis. This role is taken by different types of cells in different species. For example in primates, including men, it is thought that A_{dark} spermatogoina are the SSC which is considered as the regenerative reserve, and A_{pale} are the functional reserve[116]. On the other hand, in mice, the A-single spermatogonia is considered as the stem cell which acts as the reserve and the progenitor cell as well [117].

The process of self-renewal of SSCs is believed to be controlled by a unique microenvironment surrounding the SSCs: SSC-niche. This niche consists of several compartments such as: Sertoli cells, vasculature, interstitial compartment and the basement membrane [118]. It is believed that SSCs reside on their niche and to start differentiation a movement away from the self-renewal promoting niche is needed. Research done on murine SSC transplantation into a recipient mouse testes provided a support evidence of such phenomenon to occur in the testes when SSCs migrate towards their niche on the basement membrane after transplantation [119, 120].

1.2.2 Testicular somatic cell development

1.2.2.1 Sertoli cell

Differentiation and appearance of pre-Sertoli cells under the influence of *SRY* gene expression in the human embryo at 6 weeks post conception considered a crucial step in the development of human testes. In the human male fetus, somatic cells which express SRY, start to express sry-related high motility group (HMG)-box DNA binding protein (SOX9) as well [121]. Human female fetus somatic cells express SOX9 at early stages of development but it is lost dramatically since its expression lacks the enhancement from *SRY* gene expression as in male fetus [122]. Sertoli cells have a critical role during fetal development since they are the first cells differentiating from the precursor supporting cells. Their appearance is considered as the first indication that the bipotential gonads pass toward testis development. They surround the germ cells and form the testicular cord. During postnatal period, it is found that Sertoli cells dominate the cell population in the testes with limited number of undifferentiated germ cells [123, 124]. During puberty, the mean nuclear volume of Sertoli cells increases significantly, which is an indicator that Sertoli cells undergo maturation at puberty [123]. Sertoli cells have several functions to ensure normal maturation

of germ cells. They provide germ cells with physical scaffold toward the lumen of the seminiferous tubules during the process of differentiation. They also form the blood-testis barrier.Moreover; they create a suitable microenvironment for germ cells and their maturation and survival. Sertoli cells produce steroid and peptide hormones as they are equipped with rough and smooth endoplasmic reticulum and Golgi apparatus.

Sertoli cells secrete peptide hormones such as anti-Mullarian hormone (AMH) and inhibin B. These hormones belong to a superfamily of glycoproteins similar in their structure to transforming growth factor-beta [125]. AMH, also known as Mullarian inhibiting substance, is produced by immature Sertoli cells after their initial differentiation around 8.5 weeks of gestation in humans. Expression of AMH by Sertoli cells is triggered by SOX9 as soon as testicular cord starts to assemble and Sertoli cells start to differentiate [126-128]. This hormone causes the Mullarian ducts regression during early periods of gonadal development which is considered an essential step in male sexual differentiation [129, 130]. Moreover, AMH is one of the earliest proteins, specific for Sertoli cells, expressed by the fetal gonads [126]. AMH was detectable in the male serum at the fetal life as well as perinatal period and is found to decline towards puberty which suggests its potential use to serve as a Sertoli cell maturation marker [131].

In female fetus, AMH is produced later and then the production continues throughout the female productive life by granulosa cells of small antral and primary follicles in the ovaries [129, 130, 132]. It has been shown that AMH was not detectable in the female fetus serum in human and other mammalian species [133]. The first time AMH expression was demonstrated in the developing ovaries was at the end of fetal life around 36-38 weeks of gestation [132]. In mature ovary, AMH is expressed by granulosa cells and is correlated with follicular development and ovarian cycle [132, 134]. These findings are in agreement with results shown in our *in vitro* study of human fetal gonadal tissue included in the thesis (paper III).

Sertoli cells produce inhibins which play a role in FSH secretion control by negative feedback as shown by evidence from experiments on different species [135]. In addition, inhibins act as paracrine regulators in human testes and ovaries and they are produced by Sertoli cells in the testis and by granulosa and theca cells in the ovary. Active inhibins are found in two active molecular forms in the circulation, inhibin A and inhibin B, which consist of one a subunit and either bA or bB subunits for inhibin A and inhibin B respectively [136]. Both subunits are expressed in the testes from fetal life. Inhibin B is principally produced by Sertoli cells and its secretion and expression are correlated positively to their function [135].

It is shown that inhibin B concentration in the circulation is correlated with sperm concentration, sperm count and testicular volume [137, 138]. Moreover, circulating levels of inhibin B reflect the normal and impaired process of spermatogenesis [137, 139-141]. Inhibin B is considered as a marker for spermatogenesis [135].

Studies done on inhibin B concentration in the male and female fetuses showed that there is a clear cut difference in which higher inhibin B concentration was detected in the cord blood of male babies compared to female babies and it was suggested to use this difference as a marker for fetal sex at the second trimester of pregnancy [142]. In females, inhibin B is considered the principal inhibin form during the follicular phase of maturation while inhibin A presents in the luteal phase of female menstrual cycle. Inhibin A can be used in the prognostic assessment in the prediction of resumption of ovarian function, while inhibin B is considered as an ovarian function marker and thus can be used to identify the potential response of the ovaries to ovulation induction [135].

1.2.2.2 Leydig cells

Leydig cells are located in the interstitium of the testes and their main function is production of testosterone from cholesterol. There are two known types of Leydig cells, fetal and adult Leydig cells. Fetal Leydig cells arise from the mesenchymal precursors of the gonadal ridge at the beginning of the 8^{th} week of gestation [143-146]. During the period 9-14 weeks of gestation, Leydig cells number and size increase and they occupy more than half of the parenchyma of the testes [143]. At about week 17-18 of gestation, the number of Leydig cells start to reduce gradually [143]. Production of testosterone at 7-8 weeks of gestation is found to be LH/hCG independent. However, the fetal Leydig cell main function in masculinization of the developing urogenital system is found to be LH/hCG dependent in the late gestational life [147]. The adult mature Leydig cells is found to be packed with smooth endoplasmic reticulum, which explains the main function of this type of Leydig cells in initiation and maintenance of spermatogenesis [143, 144, 146]. The Leydig cell function is regulated by luteinizing hormone (LH) and some autoregulatory factors acting autonomously on Leydig cells. Moreover, some paracrine factors secreted from cells in the seminiferous tubules regulate the function of Leydig cells. Leydig cells produce testosterone which is a fundamental player in initiation and regulation of spermatogenesis. During fetal life, higher concentration of testosterone was detected in male fetal serum compared to female fetal serum and this difference tends to decrease with advancement of fetal age [142]. During fetal life, testosterone plays a major role in the stabilization of the Wolfian duct derivatives, formation of external genitalia and accessory glands, and regulates the testes descend [99].

During early neonatal life, at approximately 6 months after birth, the concentration of testosterone has been found to increase transiently with increase in LH and FSH [148]. During male reproductive life, testosterone acts with FSH hormone at different cellular sites and in a manner specific for each stage of sperm formation to ensure normal process of spermatogenesis [149].

1.3 SPERMATOGENESIS

The process of male germ cell proliferation and differentiation is called spermatogenesis. It starts when differentiating diploid SSCs start their cell division followed by sequential cell division of spermatogonia cells and finally the meiosis of spermatocytes which results in round spermatids formation [150-152]. Further differentiation of round spermatids into the spermatozoa is called spermiogenesis [150, 153]. Spermatogenesis takes 35 days in mice and 64 days in humans [154]. In the mammalian testes, one SSC can produce two stem cells by undergoing self-renewing division which can be asymmetric division resulting in one stem cell and one differentiating cell, or symmetric division which results in two differentiated cells [154].

1.3.1 Mouse spermatogenesis

In mice, at embryonic day 7.25–7.5, PGCs appear in the distal portion of the primitive streak and at the base of allantoic buds within the extraembryonic mesoderm. They are normally identified by high alkaline phosphatase (ALP) activity [84, 155].

Spermatogenesis in mice starts two days after birth when gonocytes located in the center of the seminiferous tubule [156] start to proliferate and migrate toward the basement membrane. By day six after birth all gonocytes would have migrated and resided at the basement membrane and became spermatogonia [155].

A primitive set of spermatogonia, known as undifferentiated spermatogonia, include: A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia has stem cell properties [157-160]. In addition to the self-renewing characteristics, undifferentiated spermatogonia generate differentiating spermatogonia which include A1, A2, A3, A4, intermediate, and B spermatogonia. B spermatogonia differentiate further into meiotic spermatocytes, haploid spermatids and spermatozoa. Spermatogonia cell types (A_{single} to type B) are located on the peripheral basement membrane and the other cell types are arranged sequentially toward the lumen of the seminiferous tubule [161].

1.3.2 Human spermatogenesis

During this process, it is found that from one SSC 16 spermatids are formed in humans. This process is highly well controlled resulting in production of an average of 60 million sperm per ml of ejaculate [162, 163]. The process of sperm production is continuous all through the man's life but it declines after 40 years of age [162].

Undifferentiated spermatogonia are located in the basement membrane of the seminiferous tubules and are semicircular in shape. Since the 1960s, human undifferentiated spermatogonia are classified into two groups: A_{dark} and $A_{pale,}$ according to the intensity of their staining with hematoxylin [164]. Nevertheless, the identity of human SSCs is not described thoroughly. It is believed that A_{dark} spermatogonia are quiescent and represent the reserve SSCs, while A_{pale} are active mitotically and represent the active SSCs. A_{pale} differentiate to type B spermatogonia, which itself is divided by mitosis and forms two cells that then enter meiosis and are named primary spermatocytes [165]. Before entering meiosis 1, the primary spermatocyte undergoes one round of DNA replication in preleptotene phase. After that, it enters the meiosis 1 which consists of four steps: leptotene, zygotene, pachytene and diplotene. During meiosis I, primary spermatocytes become bigger in size and move towards the lumen of the seminiferous tubules. After completion of meiosis I, secondary spermatocytes are formed and they are smaller in size and undergo meiosis II immediately to form haploid round spermatids [165]. In the early stages of spermatogenesis during the mitotic division, the resulting cells stay connected together by intercellular cytoplasmic bridges which makes the maturation events happen in a synchronized way [162]. Some weeks following the production of round spermatid, the process of spermiogenesis starts and it results in the production of spermatozoa. It is divided into different phases: the Golgi phase, the cap phase, tail formation phase and the maturation stage. This process is controlled and guided by Sertoli cells [162]. During the conversion from spermatid to spermatozoa, dramatic changes happen to the spermatid, including condensation of the chromatin and the nucleus, reduction in the cytoplasm volume, and morphology changes, such as appearance of acrosome cap and a tail [165]. The mature spermatozoa are released to the lumen and then they mature further in the epididymis to become a functional sperm which is able to fertilize an oocyte [166].

1.4 FERTILITY PRESERVATION IN MALES

Advanced achievements in the early detection and treatment of cancer resulted in increasing the number of cancer survivors [167, 168]. Thus, consequences of cancer treatment and

quality of life post cancer treatment became a hot topic and gained a lot of attention in the medical field recently. Most of cancer survivors desire having kids and families in the future and they feel that there was insufficient counselling about fertility preservation from their physicians before the initiation of the treatment [169, 170]. This brings the issue of fertility preservation (FP) into the focus of health care professionals, researchers, patients, patient families and the whole society.

1.4.1 Effects of radiotherapy and chemotherapy on the gonads

Improved chemotherapy and radiotherapy treatment protocols fortunately increased the survival rates over the years and more improvement is suspected in the coming years. However, these treatments are accompanied with late effects and may result in infertility or subfertility in those survivors. In addition, increased survival rates for childhood cancer lead to increased numbers of survivors reaching adulthood and here is when the issue of fertility preservation comes into the picture. Gonadotoxic agents are used to treat cancer and other non-cancer conditions such as autoimmune diseases [171]. Moreover, fertility preservation is an option for those who are receiving this gonadotoxic agents as well as patients with some chromosomal abnormalities leading to infertility in adulthood such as Klinefilter syndrome [171]. The radiotherapy and chemotherapy often have negative effects on the gonads and the reproductive capacity. This effect depends on factors, such as the duration of the treatment, the intensity of the treatment regimen, the dose of the drugs used and the type of the anticancer treatment used [171]. Alkylating agents-based chemotherapy and high doses cranial radiotherapy are considered the most harmful regimens to both ovaries and testes [172]. The risk of gonadotoxicity can be classified as low, in which only less than 20% of receivers experience infertility, intermediate, in which 21-80% experience infertility and high, in which more than 80% experience infertility [173].

1.4.1.1 Effects of radiotherapy on the testis

Radiation is used as a treatment for the infiltration of the cancer itself or as a conditioning treatment before bone marrow transplantation. It exerts macroscopic and microscopic effects on adult testes resulting in reduction in the testicular weight and depletion in the seminiferous tubules and in the number of spermatozoa [174-176]. The main consequences for radiation in testes are reduced fertility and impaired DNA integrity [177-179]. The effect of radiation on the testes is found to be dose-dependent [171]; radiation can cause impairment of spermatogenesis at very low dose as low as 0.1-1.2 Gy. Higher doses of radiation can cause permanent azoospermia [178]. It is found that male germ cells are more sensitive to radiation compared to somatic cells which are found to be more resistant [180]. In prepubertal boys, it

is shown that dysfunction of Leydig cells appear at 20 Gy dose and in mature male at 30 Gy dose [180]. However, previous research showed that a dose of 9-10 Gy has produced gonadal dysfunction [181].

1.4.1.2 Effects of chemotherapy on the testis

Chemotherapy is considered the main stay in the treatment of cancer. Increased cytotoxicity of germinal epithelium when compared to Leydig cells, as is the case with radiotherapy, causes more pronounced and first adverse effects, appearing on spermatogenesis than in testosterone production. The effects of chemotherapy in causing azoospermia is agent and dose dependent and it is not dependent on the prepubertal status of the patient at the time of receiving the chemotherapy [182]. Leydig cells get affected after exposure to higher cumulative doses of gonadotoxic chemotherapy [183]. Nonetheless, recent data showed that there are more men with impaired testosterone production after chemotherapy at chemical and even at clinical level could be observed than what was described previously, and it manifests as metabolic syndrome, osteoporosis and erectile dysfunction as examples and they recommended initiation of testosterone replacement with monitoring [184, 185].

Based on their mechanism of action, chemotherapy agents can be classified into different classes; some of them are cell cycle specific while some are not. Alkylating agents, such as cyclophosphamide, are not cell cycle specific, but they modify the cell DNA chemically, and therefore affect the cell function. Carboplatin and cisplatin are considered alkylating-like agents since they work on DNA by cross linking the DNA. Antimetabolites, alkaloids and topoisomerase are cell cycle specific chemotherapeutics. Antimetabolites, such as methotrexate, are cell cycle specific and they act during the S phase of the cell cycle by interfering with the synthesis of DNA and RNA. Alkaloids interfere with the cell division at M phase by inhibiting the function of microtubules. Topoisomerase inhibitors act by inhibiting the proper coiling of DNA, and thus interrupting the normal process of DNA transcription and replication [178].

Since type B spermatogonia are proliferating rapidly, it is the type affected primarily by the chemotherapeutic agents as well as type A pale spermatogonia. On the other hand, the quiescent type A dark spermatogonia can restore the spermatogonial population (type A pale) if it is not affected by the chemotherapeutic agents which can explain the reversibility of azoospermia in some patients after receiving chemotherapy. However, if type A spermatogonia get affected partially or completely by chemotherapeutic agents, then irreversible loss of the stem cell population, and therefore of the whole process of

spermatogenesis can occur. There were initial thoughts that the prepubertal testes are less susceptible to insults from cytotoxic agents since the testes are less mature at prepubertal stage [186, 187]. However, later studies demonstrate that there is no difference between prepubertal and postpubertal testes in their susceptibility to the effect of chemotherapeutic agents [188, 189].

Alkylating agents are found to be more gonadotoxic when used for cancer treatment compared to non-alkylating agents. Use of cancer treatment regimen containing alkylating agents is shown to cause prolonged azoospermia in 90-100% of the patients [190]. On the other hand, regimen containing non-alkylating agents caused transient azoospermia in about one third of the patients and most of those patients will have full recovery later on in life [191]. When chemotherapeutic agents with harmful effects to stem spermatogonia or to the process of their differentiation is avoided in the cancer treatment regimen, normospermia is found to be restored within three months after receiving the treatment [192]. However, when gonadotoxic agents are used, the stem spermatogonia cells get depleted and those surviving can remain in the testes, but their differentiation properties is affected for years after stopping the cancer treatment [193]. Some researchers demonstrated the negative impact of chemotherapeutic agents on Sertoli cells which could contribute to the impairment of spermatogenesis in patients receiving chemotherapy as treatment for cancer [194].

1.4.2 Options for FP for patients receiving gonadotoxic treatments

There are several options for fertility preservation for children and adolescents receiving chemotherapy and radiotherapy as treatment which include: sperm cryopreservation and testicular tissue cryopreservation (TTC). Finding cancer treatment with no or minimal effects on gonads will be a great progress in the field of cancer treatment. However, improving malignant diseases control and treatment strategies with minimal side effects without affecting their efficacy is less likely. Prepubertal boys have no definite option for fertility preservation yet since they cannot produce sperm. The experimental option available for them is the testicular tissue cryopreservation which will be discussed in details here.

1.4.2.1 Gonadal protection

Shielding the testes or removing them from the irradiation field is well known as a protective measure [195]. Moreover, hormonal suppression of the teste by GnRH analogs is also known to be used as a protection measure, based on rendering the gonads less sensitive to the effect of the cytotoxic drugs by disruption of the gametogenesis [192]. Attempts towards development of hormonal gonadal protection strategy were not successful in both humans

and primates [192, 196, 197]. It is shown that treatment with GnRH analogs failed to preserve male fertility post sterilizing chemotherapy or to affect the spermatogenesis recovery speed after radiotherapy [198, 199].

1.4.2.2 Sperm Cryopreservation

This method is the established fertility preservation method for those who are postpubertal at least Tanner stage 3 in the pubertal development [200-202]. It is recommended to be offered to all postpubertal patients with a recent cancer diagnosis as per the updated American Society of Clinical Oncology guidelines [200]. The best time for sperm cryopreservation is before the start of the therapy to insure having normal sperm for the collection since the DNA integrity and the semen quality may be affected by the chemotherapy even after only a single round of chemotherapy [200]. There are no guidelines for neither the quality of the semen before considering cryopreservation nor the duration of storage of sperm for fertility preservation [173]. The sperm samples can be obtained by masturbation or testicular sperm extraction (TESE), which is a direct sperm retrieval procedure from the testis for assisted reproduction technology in patients having azoospermia. In this procedure, identification of spermatogenesis foci in the seminiferous tubules is done under a microscope with microsurgical extraction of testicular tissues. This technique is used recently in patients with cancer to retrieve sperms and it is called onco-TESE [203].

1.4.2.3 Testicular tissue cryopreservation

Prior to puberty onset, the fertility preservation options are limited to testicular tissue cryopreservation (TTC) because of the absence of mature sperm in the testes. In this approach, germ cells can be stored and can be used later on in different techniques for the purpose of fertility preservation. TTC is still experimental; however, it has great potential when it comes to fertility preservation in prepubertal children. It is performed by transscrotal excision through which testicular biopsy is harvested and then cryopreserved by slow freezing techniques either as cell suspension [204] or as tissue pieces [205]. This procedure is found to be well tolerated with minimal complications post operatively and with no delay in the cancer treatment [206-208]. Very low temperatures are used to preserve the intact cells or tissue. Cell survival after cryopreservation is affected by many factors including the effect of ice during the freezing and thawing process, type of cryoprotectant used and the rate of cooling. It is still a challenge to find optimal parameters for cryopreservation that are suitable for all types of cells needed to be cryopreserved [209]. This option of fertility preservation relies mainly on the future experimental techniques to produce mature sperm from SSCs [173].

1.4.3 Options for FP for prepubertal boys receiving gonadotoxic treatments

Prepubertal boys are not able to produce sperm yet. Hence, the only option to preserve their future fertility is to cryopreserve their testicular tissue for possible use in the future. All available methods are still experimental, and the ongoing research is promising but faces many challenges. There are various techniques to use the cryopreserved prepubertal tissues as an option for fertility preservation: autologous transplantation, xeno-grafting and *in vitro* maturation of germ cells. In addition, use of hPS cells and differentiating them *in vitro* into germ cells might also be an option, which needs to be taken in consideration especially for patients who do not have any germ cells left at the time of tissue collection and cryopreservation, as we showed in our group publication in Human Reproduction journal which is not included in this thesis [210].

Since the *in vitro* maturation of gonadal cells is the focus of this thesis, we will discuss it more in detail.

1.4.3.1 Male germ cells/ tissue autologous transplantation

Transplantation of the cryopreserved testicular tissue or cells is considered a future option for fertility preservation for prepubertal boys. Transplantation of SSCs was successful in animal models mice, rat and rhesus monkey, and functional sperm production was reported [211-214]. Moreover, it was also investigated in humans and reported that a percentage of 55% of the recipient testicular tissues contained transplanted cells [215, 216]. It is reported that quality and quantity of the transplanted SSCs play a major role in the success of such method [211]. Transplantation of testicular tissue in mice proved to be more successful when compared to transplantation of SSCs suspension [217]. Testicular tissue grafting was shown to be successful in mice, rabbits, pigs and sheep and production of functional sperm from those grafting experiments was reported [218]. On the other hand, no report showed a successful grafting of human testicular tissue grafting experiments [218]. Transplanting testicular tissue or SSC in patients with cancer has a higher risk to reintroduce the cancer because of high possibilities of cancerous cells contamination [219]. However, transplantation of SSCs is considered a better option, but we are not there yet since decontamination protocols are still not achieved [220, 221]. The main difficulty here is that there are no definite specific markers to isolate SSCs purely [222]. Moreover, it is essential to increase the number of SSCs before the transplantation to ensure good success rate. This necessitates purification, isolation and *in vitro* expansion protocols for SSC before transplanting them. A lot of work has been done but such protocols are not available yet [223,

224]. All these challenges need to be addressed carefully to consider autologous transplantation as a clinical option for fertility preservation.

1.4.3.2 Male germ cells/tissue xenotransplantation

Transplantation of testicular cells across species is considered an option for fertility preservation. This technique was used in different species, such as mice, rats, hamsters, dogs and rabbits [225-228]. However, results showed success in some species while the approach in less closely related species failed. In these less closely related species, colonization of the donor germ cells into the recipient testes is observed, but the maturation to functional sperm was not achieved. And when it is achieved, the sperms were not normal [226, 227]. In humans, observation of meiotic activity in xeno-grafted human testicular tissue was shown, but no report yet of complete human spermatogenesis from this method [229-232]. Better results were demonstrated when immature tissues were used compared to mature tissues which indicates that this method could work for prepubertal human testicular tissues if used in the future as fertility preservation method [218, 232, 233]. However, this method is less favorable because of the risk of zoonosis and is suggested to be used as a method for investigating human tissue contamination with cancer cells before transplantation [234]. Nevertheless, in the absence of any other methods of *in vitro* maturation of human testicular tissue, this method is considered an option for future fertility preservation even though it is still experimental nowadays.

1.4.3.3 In vitro maturation of germ cells

There are two ways of generation *in vitro* germ cells: the first is by differentiation of hPS cells *in vitro* into mature germ cells, and the second is by maturation of SSCs *in vitro*. IVM of germ cells is considered a future option for fertility preservation by producing haploid germ cells *in vitro,* which could be used in principle for assisted reproduction therapy (ART) and embryo generation, and thus restoration of men fertility and ability to father kids. The drawback of using other methods such as germ cells transplantation with high risk of cancer re-introduction and the germ cells xenotransplantation with a risk of zoonosis can be overcome by *in vitro* maturation (IVM) of germ cells [235]. Remarkable effort was put in and good progress was achieved since this method was explored by researchers. Male sperms were produced from *in vitro* maturation of mouse testicular tissue and were functional [236]. However, the efficiency was low, and more modification and research are needed to optimize the results. On the other hand, no full spermatogenesis was reported in other species and in most experiments a stage of meiotic arrest was achieved which necessitates more research to understand the differences between the species and to explore the exact cause of failure of

mature germ cell production. *In vitro* maturation of germ cells in mice and humans was studied in paper II, III and IV in this thesis.

As mentioned previously, the use of hPS cells can be an option for germ cells generation *in vitro*. The production of human pluripotent stem cells creates a lot of hope to the scientific field to differentiate and mature these cells into male germ cells *in vitro* and use the resulted cells in ART. The generation of hiPS cells makes it more attractive to potential clinical protocols to use those cells to generate the patient's own germ cells *in vitro* using different differentiation protocols. Thus far, there is no report of functional mature human sperm generated from stem cell, neither hES cells nor hiPS cells. However, some forward steps were reported (see stem cells section 1.1.5). Use of stem cells in this aspect may provide new information towards establishing robust *in vitro* methods for germ cells maturation. Therefore, *in vitro* maturation of human germ cells is a promising option for fertility preservation for prepubertal patients receiving gonadotoxic treatment. Different methods have been used, including two-dimensional culture systems, three-dimensional culture systems and organ culture. In the following chapter these methods will be discussed with more focus on organ culture methodologies, as this is the technique which has been mainly used in the studies included in this thesis.

Two-dimensional systems

Culture of testicular cells as monolayer, alone or with other types of cells, was used broadly as attempts for functional spermatogenesis *in vitro*. This method was applied to different animals testicular tissues to study various aspects in regard to *in vitro* spermatogenesis (IVS), including: cell to cell and cell to extracellular matrix (ECM) interaction [237-241], properties of different testicular cell types when cultured together [242], and the effect of different growth factors and signaling molecules on the IVS [243-245]. As animal testicular tissues, human testicular tissues were cultured in 2D methods to explore the effect of different types of feeder cells when co-cultured with human germ cells and different types of supplements on the progress toward IVS [246-250]. Great effort has been done using this method of culture, although, no full IVS was reported. This can be explained by the lack of spatial arrangement of the testicular cells when cultured in 2D systems.

Three-dimensional systems

3D culture systems, either by culturing testicular tissue fragments or a combination of dissociated testicular cell in a supportive scaffold, were used as an attempt to allow the spatial arrangement of the testicular tissues. There are many publications showing some

advancement towards IVS using 3D to culture testicular cells combination in a scaffold. Different types of matrices were used as scaffolds (soft agar, methycellulose, collagen, calcium aliginate, poly-D,L-co-glcolic acid and matrigel) in combination with testicular cells from different species (mice, rats, calves, rhesus monkeys and humans) [251]. Furthermore, other researchers used carbon nanotubes and collagen sponges to explore the rat testicular organogenesis [252, 253]. Some groups reported that testicular cells aggregates could be used as scaffolds themselves when they are cultured *in vitro* and they could support germ cell maturation.[254]. Recently, testicular organoids, testis organ-like structure, were described by different groups and presented as a model applicable for establishment and study of IVS [255-257]. Despite the tremendous work scientists have carried out so far, they showed progression towards maturation of immature male germ cells until the stage of elongated spermatids in mice with low efficiency so far. More obstacles are needed to be overcome in order to establish a functional full IVS in human to consider this method as a clinical option for fertility preservation.

Organ culture

The organ culture methodology conserves the microenvironment arrangement and the spatial cellular arrangement of the tissue when cultured *in vitro*. This methodology was introduced in the early 1900s, when *in vitro* differentiation of germ cells was reported for the first time using an organ culture method [258]. Organ culture methodology includes hanging drop culture and air-liquid interphase culture. In hanging drop culture, the piece of the tissue is cultured within a small volume of medium and placed inversely in the lid of a culture dish. This method was used to study human and murine gonadal development including the testes as well as to study the effect of different factors on the development and maturation of germ cells [259-261]. In air-liquid interphase culture, the tissue fragments are cultured on a stand, allowing it to reach the medium to get the nutrients and the air to get the oxygen. This method was described in the early fifties by Trowell [262], and later on adapted by Steinberger when he used it to culture immature and adult rat testicular tissue [263, 264]. Since then, many research groups used the same method to promote IVS. Some groups used agarose as a stand to culture animal testicular tissue, e.g. murine [236, 265-274], rat [275, 276] and calf [277] testicular tissue. On the other hand, other research groups used a membrane as a stand for the testicular tissue fragments during air-liquid interphase culture and they cultured human foetal, prepubertal and adult testicular tissues [88, 278, 279]. Moreover, this method of culture was used to study and differentiate female gonads from different species, as well as to study male

and female gonadal development by culturing fetal gonadal tissues from mice, rats and humans [280-283].

Despite the great effort and considerable knowledge provided by these studies, functional full IVS was achieved only in mice by Sato and his group and the efficiency was low. More research is needed to explore the early germ cell development and factors controlling the process of human gametes formation and differentiation. Ongoing projects will enrich the field with more clues towards establishing a proper method for IVM of human testicular cells. This great step towards generating live fertile pups from mice *in vitro* cultured testicular tissues demonstrates that it could be possible in the future to have *in vitro* differentiated human sperm as an option for fertility preservation for prepubertal boys receiving gonadotoxic treatment.

2 AIMS

The main aim of this work was to study the process of human male gonadal cell development and maturation using different *in vitro* models. Moreover, we focused in the study of *in vitro* models for human gonadal cell maturation and differentiation, aiming to assist in finding fertility preservation clinical options.

The specific aims for each paper included in this thesis were:

- To investigate the short term effects of LN521, when used as a matrix to culture hES cells, on the expression of stemness, pluripotency, and human gonadal cells related genes as an approach for future robust *in vitro* hES differentiation protocols towards germ cells.
- To investigate the effect of supplementation of KSR, melatonin, and glutamax on murine testiculr tissue maturation *in vitro*.
- To use air-liquid interphase explant culture method to culture human fetal gonadal tissues *in vitro* and evaluate their endocrine functions..
- To assess the *in vitro* functions of Leydig cells and Sertoli cells of human testicular tissues, from prepubertal boys subjected to treatment for hematological and oncological diseases, cultured *in vitro* using air-liquid interphase explant culture method.

Paper	*In vitro* Model	Cells/ Tissue	Species	Age	Study focus
I	Culture of hES cells on LN521	cells	Human	Pre-natal	Short term effects of LN521 on hES cells gene expression
II	Organ culture	tissues	Mouse	Post-natal	Establishment of organ culture method and investigation of the role of KSR on germ cells maturation
III	Organ culture	tissues	Human	Pre-natal	Assessment of gonadal somatic cells function in vitro
IV	Organ culture	tissues	Human	prepubertal	Assessment of immature prepubertal testicular tissues function in vitro

3 MATERIALS AND METHODS

3.1 ETHICS

3.1.1 Animal tissues

The approval for animals use and handling was obtained from the ethics committee for experimental laboratory animals at Karolinska Institutet (N489/11 and N280/14).

3.1.2 Human tissues

3.1.2.1 First trimester gonads

The Regional Human Ethics Committee, Stockholm, Sweden, approved the tissues collection (Dnr 2007/1437-31 with complementary permissions 2011/1101-32 and 2013/564-32). The ethical approval to perform the gonadal studies: Dnr 2013/457-31/4.

3.1.2.2 Human prepubertal boy testicular tissue

The approval for testicular tissues collection from the patients was obtained from the Ethics Board in Stockholm (Dnr 2013-2129-31-3, Dnr 2014/267-31/4, the National Ethics Board of Iceland, Reykjavik (VSN 15-002) and the Ethics Board of the University of Helsinki (426/13/03/03/2015).

3.1.3 Stem cells

Ethics approval for the derivation and differentiation of the hES cell lines was obtained from the Regional Ethics Committee in Stockholm (Dnr. 454/02).

3.2 CELL LINES AND TISSUES

3.2.1 hES cell lines (Study I)

Human embryonic stem-cell lines (HS360, HS364, HS380, HS401 and HS420), all karyotype 46 XY, were derived by mechanical isolation of the inner cell masses of six-day-old embryos.

3.2.2 Mouse tissue (Study II)

Wild-type C57/BL6 pregnant mice were purchased from Charles River (Sulzfeld, Germany). Newborn animals were transported with their mothers to Karolinska Institutet (Stockholm, Sweden).

3.2.3 Human tissue

3.2.3.1 First trimester gonads (Study III)

Human first trimester embryonic/foetal gonadal tissues (4.5-10.5 weeks post conception) were collected after elective surgical abortions and maternal written informed consent. All cases were tested and negative for HTLV, HIV, HBV, HCV and Syphilis infection. A total of 27 ovaries and 28 testes, from 55 individual embryos, were collected and included in the study. The tissues were dissected in physiological sodium chloride solution under sterile conditions within 1 hour after surgery. Directly thereafter, the tissue was transferred to Knock-out Dulbecco's Modified Eagle Medium (KO-DMEM, 10829-018, Invitrogen).

3.2.3.2 Human prepubertal testicular tissue (Study IV)

Eighteen prepubertal boys from Sweden, Finland and Iceland, undergoing treatments implying a very high risk of infertility, were enrolled in an experimental procedure for testicular cryopreservation, within the NORDFERTIL research programme. Inclusion criteria were planned allogeneic or autologous HSCT, or testicular radiotherapy. Testicular volumes >10ml (measured by orchidometer), a high bleeding or infection risk were considered exclusion criteria. Written and verbal information about the research project was given to the parents, and when appropriate to the patient, before they agreed to give their written informed consent. After unilateral open testicular biopsy, where less than 20% of the testicular volume of one testis was collected, two thirds of the tissue was cryopreserved for clinical fertility preservation [284] and the other third was transported to the NORDFERTIL research laboratory at Karolinska Institutet.

3.3 CELL AND TISSUE CULTURE

3.3.1 Stem cell culture

3.3.1.1 On feeders

hES cells were cultured on mitotically inactivated hFFs (CRL-2429, ATCC, Manassas, VA, USA) in knock-out (KO) DMEM medium (KO-DMEM, 10829018, Invitrogen, Life Technologies, Paisley, Scotland) containing 20% KO Serum Replacement (KO-SR, 10828028, Invitrogen), 0.5% penicillin-streptomycin (15140122, Invitrogen), 2mM L-GlutaMAX (35050038, Invitrogen), 1% non-essential amino acids (11140035, Invitrogen), 0.5mM 2-mercaptoethanol (31350010, Invitrogen) and recombinant human fibroblast growth factor 2 at 8 ng/ml (FGF2, 234-FSE/CF, R&D Systems, Minneapolis, USA).

3.3.1.2 On matrices

To enable hES cell culture on LN521- and LN121-coated plates, LN521 and LN121 were slowly thawed at 4°C for one to two hours and diluted at a 1:10 ratio with Dulbecco's phosphate-buffered saline containing CaCl2 and MgCl2 (DPBS++) to a final concentration of 10 µg/ml. The plates were then coated with LN521 or LN121 at 5µg/cm² overnight at 4℃ followed by one hour at 37℃ or for two hours at 37℃ according to the manufacturer's instructions. The hES cells were cultured in NutriStem medium (NutriStem hESC XF, 05-100-1A, Biological Industries Israel Beit Haemek 120 Ltd., Israel) at 37℃ and with 5% CO2, with daily medium change. To perform hES cell culture on Matrigel-coated plates, Matrigel was thawed on ice at 4°C overnight. Plates were incubated with Matrigel diluted 1:84 in NutriStem at 4°C overnight or at room temperature for 1 hour. The cells were passaged enzymatically using TrypLE select (12563011, Invitrogen) every five to seven days. After three to four passages, the cells were expanded to collect material for analysis.

3.3.2 Stem cell differentiation

After reaching confluence on LN521-coated plates at passage nine, hES cells were allowed to form embryoid bodies (EBs) in 24-well ultra-low attachment plates (#3473; Corning) for 14 days in NutriStem without growth factors (#06-5100-01-1A; Biological Industries). The EBs were then either plated on LN521 -coated culture plates or chamber slides (#354104, Corning) for an additional 14 days. After a total of 28 days, differentiated cells were either used for gene expression analysis or fixed with 4% paraformaldehyde for immunocytofluorescence analysis.

3.3.3 Mouse testicular tissue culture

To culture mouse testicular tissue, we used the testicular explant culture method described originally by Sato et al [236] (see figure 2). To prepare the agarose pillar, pre-autoclaved 0.7% SeaKem_ LE agarose (50004, Lonza, Basel, Switzerland) was mixed with the relevant culture medium 1:1 v/v to give a final concentration of 0.35% agarose. The different culture conditions used were; (1) minimum essential medium alpha (MEMa; 22561-021, Gibco) + melatonin (M5250, Sigma Aldrich, Munich, Germany, final concentration 1027 M) (2) MEMa + Glutamax (32561-029, Gibco, 1.87 mM Glutamax), (3) MEMa + 10% Knockout Serum Replacement (KSR, 10828-028, Gibco), (4) MEMa + Glutamax + melatonin, (5) MEMa + Glutamax + 10% KSR, (6) MEMa + Glutamax + 10% KSR + melatonin, (7) MEMa + melatonin + 0.1% KSR, (8) MEMa + melatonin + 1% KSR, (9) MEMa + melatonin + 10% KSR, and (10) MEMa + melatonin + 20% KSR. The tissue was cultured at 34.5℃, and 5% CO2.

3.3.4 Human first trimester gonads culture

The first trimester gonadal tissue samples were cultured using a culture method adapted from testicular explant culture method published by Sato et al [236]. The first trimester gonadal tissues were cultured on agar blocks in air-liqiud interphase (as described in section 3.3.3.). The cell culture medium was Knock-out Dulbecco's Modified Eagle Medium (KO-DMEM, 10829-018, Invitrogen) supplemented with 10% (v/v) knock-out serum replacement (KO-SR, 10828-028, Invitrogen) and 1% (v/v) penicillin/streptomycin (Pen/Strep, 15140-122, Invitrogen). The gonadal tissue was received as both gonads with mesonephros from the same fetus. One gonad was used as control to the experiments. The remaining gonad was cut into three small pieces; each approximately three mm3 in size and placed on top of the agarose pillars, one piece per pillar being soaked, but not covered, by the culture medium. The gonadal tissue was cultured at 37°C and 5% CO2 for 14 days. The culture medium was collected every seven days, stored in -80°C until evaluation, and replaced by fresh medium. At the day of culture termination, both media and tissue were collected. Media was stored in -80°C until evaluation.

3.3.5 Human prepubertal testicular tissue culture

Testicular samples were cultured in a modified air-liquid interface system as described previously. Upon arrival to the lab, samples were washed with NutriStem® (05-100-1A, Biological Industries, CT, USA), 1% (v/v) pen/strep (1% pen/strep; 15070-063, Gibco, Thermo Fisher Scientific, MA, USA) and their size and weight were registered. Samples were dissected into small fragments (approx. 1mm3) and cultured on top of soft-agarose (50004, Lonza, Basel, Switzerland) stands in an air-liquid interface up to 21 days. NutriStem® supplemented with 1% pen/strep, 10−7 M melatonin (M5250, Sigma Aldrich, Munich, Germany) and 10% (v/v) KSR XenoFree (KSR; A1099201, Gibco, Thermo Fisher Scientific) was used as culture medium for all patient groups. Culture medium was added to the culture wells (2 mL) up to the edge of the agarose-stands without covering the testicular fragments and changed weekly.

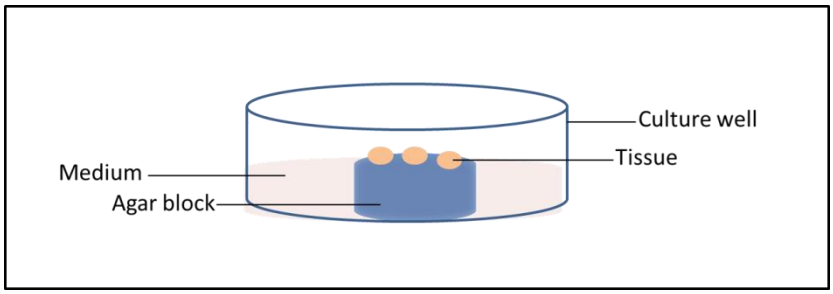

Figure 2. Schematic representation of the air-liquid interphase organ culture used in the thesis

3.3.6 First trimester tissues age determination

The postconceptional age of tissue was determined by examination of anatomical landmarks such as limb, nervous system, gonadal and eye development according to the atlas of life before birth [285]. The tissue collected was not intact, why crown rump length (CRL) or neck rump length (NRL) often was not possible to measure. The exactness of staging may differ a maximum of ± 0.5 week.

3.4 STAINING

3.4.1 Embedding and sectioning

Tissues collected at the end of the culture were fixed either in Bouin's solution (HT10132, Sigma-Aldrich) or in PFA (02176, Histolab, Gothenburg, Sweden) at 4°C overnight. After that dehydration was done in ascending ethanol concentrations (30%, 50% and 70% ethanol) for 24 hours each and (80%, 96% and 99.6% ethanol) at least for 6 hours each, at RT. Then the samples were transferred to butyl acetate (45860, Sigma-Aldrich) overnight at RT. Samples were embedded in melted paraffin (ParaplastX-TRA®, P3808, Sigma-Aldrich) at 61°C overnight, left to cool and solidify and then cut into 5µm sections by a Biocut sectioning machine (Reichert-Jung, NY, USA). The sections were placed on microscope slides (10143352, Superfrost Plus, Thermo Scientific, MA, USA) and dried in an oven overnight at 37°C.

3.4.2 Immunohistochemistry

After de-paraffinization for the sections of formaldehyde 4% fixed samples, antigen retrieval was performed in 50 mM glycine (pH 3.5, ≥90 °C maintained for 10 min). The primary antibody was applied for overnight incubation at 4 °C in 0.1% BSA/TBS. Negative control sections were incubated with 0.1% BSA/TBS without primary antibodies. Subsequent steps were performed at RT, with TBS washes between incubations. Primary antibody binding was detected using Vectastain ABC kit Universal according to the manufacturer's instructions (PK-7200, Vector Laboratories).

3.4.3 Immunofluorescence

For immunocytofluorescence analysis of cells, cells were fixed with 4% paraformaldehyde for 15 minutes, washed three times with 1× phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton X-100 (#108643, MERCK, Germany) in PBS for ten minutes. After blocking for one hour with 5% donkey serum (125558, Jackson Immune research), 1% bovine serum albumin (BSA; 001-000-162, Jackson-immunoresearch) and

0.1% Tween20 (#655205, MERCK) in 1× PBS at room temperature, the cells were incubated with primary antibodies in antibody solution (1% donkey serum, 0.1% BSA and 0.1% Tween20 in PBS) at 4◦ C overnight (Supplementary Table 2). Next day, the cells were washed three times with 1× PBS and incubated with secondary antibodies (donkey anti-mouse Alexa Fluor 488 conjugated (715-546-150, Jackson-immune research) or donkey anti-rabbit Cys3 conjugated (11488299, Fischer Sci.)) in antibody solution for one hour at room temperature in the dark and afterwards counterstained with DAPI at 1 µg/ml (PureBlu DAPI, 135-1303, BioRad) for 15 minutes.

For paraffin embedded sections, the sections were de-paraffinized a dehydrated using xylene followed by a descending ethanol series. After that, samples were antigen retrieved in 10mM sodium citrate buffer at PH of 6 at 95◦C. Subsequently, tissues were blocked with serum from the same host where the secondary antibody was produced. Incubation with the primary antibodies of interest overnight at 4◦C was followed by washing steps. In the last step, the tissue sections incubated with fluorescent-dye conjugated secondary antibodies and mounted with mounting medium containing DAPI.

3.4.4 Periodic acid Schiff staining

Sections of the samples were de-paraffinized in xylene and rehydrated in descending ethanol concentrations; 99.6%, 96% and 70%, for ten minutes each at RT. The PAS kit (101646, Merck, Darmstadt, Germany) was applied to stain the sections in accordance with the manufacturer's protocol. The sections were then washed in distilled water twice for five minutes each. Afterwards, the sections were incubated with periodic acid for five minutes and washed under tap water for three minutes, followed by washing in distilled water twice for five minutes each. The sections were then incubated with Schiff's reagent for 15 minutes at RT and washed again under tap water for three minutes, followed by washing in distilled water twice for five minutes each. Afterwards, some tissue sections were counter-stained with haematoxylin (Mayer's Hemalaun solution, 1092491000, Merck) for two minutes, followed by washing under tap water. The sections were then de-hydrated in ascending ethanol concentrations; 70%, 96%, and 99.6%, followed by xylene, each for ten minutes at RT. To mount the sections, Entellan® new (1079610100, Merck) was applied on the sections.

3.5 HORMONE MEASUREMENT

3.5.1 Enzyme-Linked Immunosorbent Assays (ELISA)

Conditioned medium from organ cultures was collected in order to evaluate the production of testosterone, inhibin B and AMH during the culture period. Hormone concentrations were

determined by specific enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Two technical replicates were performed for each sample. The microplate reader Fluostar Omega (BMG LabTech, Ortenberg, Germany) was used for reading the absorbance at 450nm. The standards provided with the kits were used for generating a standard curve.

3.5.2 Mass spectrometry

The concentration of total testosterone in the media was determined by Liquid Chromatography-tandem Mass Spectrometry LC-MS/MS at the Karolinska University Hospital Laboratory, Stockholm, Sweden.

3.6 GENE EXPRESSION ANALYSIS

3.6.1 RNA isolation and cDNA amplification

HES cells were harvested using enzymatic digestion with TrypLE select. RNA isolation was performed using a RNeasy kit (#74104, Qiagen, Germany) according to the manufacturer´s protocol and treated with DNase (RNase-free DNase SET, 79254, Qiagen). CDNA was synthesized using an iScript cDNA synthesis kit (#170-8891, Bio-Rad), following the manufacturer´s protocol.

3.6.2 Taqman Low Density array

To compare the five hES cell lines, the expression of 96 genes was analysed using Taqman low density (TLD) array cards (4385344, Applied Biosystems, Carlsbad, CA 92008, USA), designed by the International Stem Cell Initiative, according to the manufacturer's protocol. Briefly, the TLD array cards were preloaded with 96 TaqMan probes which were run at the same time. Included in the 96 genes were markers of undifferentiated stem cells, pluripotency maintenance, stemness and differentiation, and six internal controls. The data was analysed using RQ-Manager 1.2 Software. All the hES cell lines were run in three biological replicates.

3.6.3 Quantitative polymerase chain reactions

Quantitative PCR was performed using TaqMan Universal PCR Master Mix (#4369016, Applied Biosystem) with TaqMan probes (Applied Biosystem) according to the manufacturer's protocol. GAPDH was used as an internal control.

3.7 FLOW CYTOMETRY

Samples for flow cytometric analysis were prepared as previously described by Rotgers and colleagues [286]. Briefly, cultured testis pieces were cut using McPherson- Vannas scissors and enzymatically digested by using 1 mg/mL collagenase/dispase (10269638001, Roche, Basel, Switzerland), 1 mg/mL hyaluronidase (H3506, Sigma–Aldrich), 1 mg/mL DNAse1 (DN-25, Sigma–Aldrich). Resulted Cell suspensions were filtered and subsequently fixed and permeabilized using 4% paraformaldehyde and 90% methanol. Immunolabeling with mouse anti- γH2AX-Ser139 antibody (05-636, EMD Millipore Billerica, MA, USA), which is a marker for meiosis, was performed to assess the different germ cell population in the testicular pieces.

3.8 STATISTICAL ANALYSIS

To perform the statistical analysis, t test, One-way ANOVA, ANOVA on ranks and Spearman rank correlation were specifically applied for the different experimental contexts as described for each study, using the Sigma Plot software ver.12.0 (Systat Software Inc.,IL, USA). The means and standard deviations were used in the figures as indicated and each experimental condition was repeated at least 3 times. A p value <0.05 was considered to indicate a significant difference.

For TLD array analysis, TLD array data was normalized using GAPDH as a housekeeping gene control and undetected genes were removed. To separate and compare gene expression profiles of cell lines, the dCT means of replicates were plotted in a heatmap, separating cell lines using hierarchical clustering. Moreover, common patterns of expression were investigated via intersection of genes with high expression, arbitrarily defined as exhibiting $dCT < 5$, as well as low expression ($dCT > 15$).

4 RESULTS AND DISCUSSION

4.1 GENE EXPRESSION ANALYSIS OF HUMAN ES CELLS CULTURED ON LN521

The derivation of clinically safe hES cell lines has proven to be challenging, and this limits the use of PS cells in personalized medicine. In addition, more defined culture conditions will help in interpretation of results obtained from hES cell experiments and improve our understanding of the mechanisms controlling hES properties and characteristics. Moreover, establishing robust and reproducible culture conditions will make PS cells more suitable to be used for future challenging differentiation protocols, such as differentiation towards germ cells. To obtain this, the use of the synthetic matrices to culture hES cells would result in better-controlled conditions. The introduction of LN521 as a matrix for hES cell culture was a step forward for clinically safe hES culture condition. In this paper, we cultured five male hES cell lines initially derived on hFFs using LN521 as a matrix to examine the behavior of these cells in terms of pluripotency characteristics as well as important genes for male gonadal development when cultured for short period on LN521. Furthermore, we compared the gene expression of these hES cell line cultured on LN 521 with the gene expression when they were cultured on LN121, hFFs and matrigel, to investigate the differences of using these substances as matrices for hES culture. Moreover, we explored the effect of LN521 on the expression of genes related to human gonadal cells.

4.1.1 Pluripotency state of hES cells cultured on LN521

After being cultured on LN521 for four passages, the hES cells from five hES cell lines (HS360, HS364, HS380, HS401 and HS420) analysed by TLD arrays. Results of this analysis showed higher expression of genes related to stemness (ZFP42, TDGF1, SFRP2, PODXL, NR6A1, CD9, DNMT3B, IFITM1 and LIN28) and lower expression of genes related to differentiation (TAT, PAX4, IAPP and DDX4). The karyotypes of all five cell lines were 46, XY after four passages on LN521.

The five hES cell lines then cultured on LN521 for nine passages and after the ninth passage their pluripotency characteristics were examined by gene expression, protein expression and by differentiating them to three different germ lines cells. Immunofluorescent staining revealed positive staining of five known pluripotency markers NANOG, POU5F1, SOX2, TRA-1-60 and SSEA4. Quantitative analysis of the expression of NANOG, POU5F1 and SOX2 revealed no significant differences between the five hES cell lines.

The ability of the cells from all five hES cell lines to differentiate spontaneously into the three germ layers was evaluated by differentiating the cells *in vitro* by formation of embryoid bodies. After 28 of *in vitro* spontaneous culture as embryoid bodies, all the hES cell lines expressed ectoderm (TUJ1), mesoderm (alpha SMA) and endoderm (AFP) markers. At the gene level cells from all hES cell lines showed expression of ectodermal (NEUROD 1, PAX6), mesodermal (KDR, ACTC1), endodermal (GATA6, AFP, DDX4) and trophoblast (KRT7) markers.

4.1.2 Homogenized gene expression profile for hES cells cultured on LN521

To investigate the effect of utilizing LN521 as a matrix for hES cell culture, we analyzed the expression of pluripotency, stemness and male gonadal cells related genes by qPCR. First of all, we compare the expression of the genes when hES cells cultured on feeders using two different media, DMEM and NUTRISTEM. The analysis of this part revealed a similar expression pattern and by this, we can confirm that the change of the culture medium to NUTRISTEM when culturing the cells on matrices has no effect.

When gene expression of hES cells cultured on hFFs and on LN521 at p4 analyzed, we observed more gene expression variation of cells cultured on hFF compared to cells cultured on LN521 at p4.

Comparing the pluripotency gene expression of the hES cells cultured on LN521 for four and nine passages showed less variation with prolonged culture. The variation of a specific gene was analyzed by comparing the coefficient of variance which is determined by calculating the ratio between the SD and the mean value of all cell lines together. NANOG and GDF3 showed a variation of less than 50% at p9 from more than 50% at p4. POU5F1 and SOX2 showed a reduction in the variation from less than 50% at p4 to less than 25% at p9.

4.1.3 Gene expression profile of hES cultured on LN521, LN121 and hFF

To study the difference between LN521 and other matrices used for hES cells culture, two hES cell lines (HS360 and HS380) were cultured on LN521, LN121 and Matrigel. Cells cultured on LN121 and Matrigel had a higher incidence of differentiation compared to those cultured on LN521. Moreover, hES cells cultured on LN121 were slower in attachment and growth compared to hES cells cultured on LN521. This difference between LN521, LN121 and Matrigel became more pronounced with prolonged culture.

4.1.4 Effect of culturing hES cells on LN521 on the expression of gandal cell genes.

LN521 has been shown by other researchers to be present in the decellularized matrix of human adult testis [45]. To investigate the effects of LN521, when used as a matrix for hES cells culture on the expression of genes related to male gonadal cells, we included male gonadal cell related genes in the gene analysis of the hES cells after p4 and p9 of culture. The results showed different patterns of variation of such genes between p4 and p9.

4.1.5 Discussion

Incomplete defined culture conditions and the use of xenogenic materials for hES cell culture and expansion are main challenges towards establishment of well-defined culture condition for clinically safe hES cell lines. The use of synthetic matrices would result in better controlled conditions for hES culture. HES cells express alpha1, alpha5, beta 1, beta 2 and gamma 1 laminin chains. Therefore, LN521 was suggested to be expressed in the hES cells with other forms of laminin, LN121, LN511 and LN111 [43]. Moreover, alpha 5 containing laminin isoforms thought to be part of stem cell niches since the alpha chain laminins are found in the inner cell mass of the mouse blastocyst [287]. Therefore, in this work, we aimed to investigate if LN521 could be an optimal matrix for hES cells previously derived and cultured on feeders as a step for well-characterized hES lines to be used in the defined differentiation protocols for regenerative medicine applications purposes.

Our results showed stable hES cell lines after being transferred from hFF to LN521 and cultured for a short period on LN521. Cells were growing as monolayer and reaching confluency within five to seven days without any signs of differentiation. Our results agree with previous studies showing feasibility of using LN521 as a matrix for hES cell culture in xeno-free and chemically defined culture systems. However, our results showed the short term culture effect of LN521. A future study of the longterm effects of using LN521 as a culture matrix for hES cells is needed to confirm our results.

In our study, we observed variations in the expression of pluripotency genes and differentiation genes among the five cell lines which indicate the cell line specific expression pattern. However, investigating the expression of pluripotency genes of the hES cells cultured on LN521, we observed a homogenized expression profile compared to those cultured on feeders and more pronounced when the culture on LN521 was prolonged. This is an indication for more predictable and robust culture conditions of hES cells which is of great help toward establishment of directed and cell line independent differentiation protocols.

Our results showed a similar picture of variation in the gene expression for cells cultured on feeder using two different media (DMEM and NUTRISTEM) which indicates that the change to NUTRISTEM when culturing the cells on LN521 had no obvious effect. We concluded that the homogenization effect we observed is a result of using LN521 as a matrix for hES cell culture.

When LN521, LN121, and Matrigel were compared when used as a matrix for hES cell culture, we showed that LN521 provides a suitable matrix for hES cell attachment, growth, and self-renewal. The difference in the alpha chain between two types of laminins; LN521 and LN121 could be an explanation of the differences we observed. However, future studies are needed to investigate the effect of this differences and its impact in the long term culture.

Since LN521 was shown to be expressed in the human testis decellularized matrix, we investigated the potential effects of LN521 on gene expression levels of early gonadal germ and somatic cells. Genes related to the specification and appearance of early stage of primordial germ cells in humans, genes related to gonadal somatic cells and NODAL pathway related genes which play a great role in early cell fate including germ-cell specification were included in the analysis. We showed here that LN521 had no effects on the expression levels of genes related to early gonadal cells. This indicates the possibility of using LN521 to culture hPS cells tend to be used for differentiation toward human germ cells.

However, more obstacles in the differentiation of hPS cells towards germ cells are related to our lack of understanding about the exact mechanisms and process of human germ cells differentiation which necessitates studying normal and pathological human gonadal tissues as well as mature and immature gonadal tissues. Moreover, finding a robust *in vitro* system that can mimic *in vivo* system will provide great help in differentiating PS cells towards germ cells *in vitro*. The aspect of finding robust *in vitro* system to culture and mature human germ cells as a basis for fertility preservation as well as for better understanding of human gonadal cells development and maturation is addressed in papers II, III and IV.

4.2 EFFECTS OF KSR AND MELATONIN ON GERM CELL DIFFERENTIATION IN MURINE TESTICULAR EXPLANT CULTURE.

In vitro maturation of male germ cells is a complex process and it is still a challenge for scientists to mimic the *in vivo* situation. A promising step was achieved when a testis explant culture method using KSR was introduced. On the other hand, the efficiency of this system is not optimal yet. In this paper, we aimed to establish this culture condition in two different laboratories, to improve the reliability if the culture systems to generate mature germ cells *in*

vitro and to investigate the effects of different concentrations of KSR, melatonin and glutamax on germ cells maturation *in vitro*.

4.2.1 The impact of the duration of murine testicular tissue explant culture

We showed here that the duration of murine testicular tissue explant culture had an impact on germ cell proliferation and on the efficiency of germ cell maturation up to late/post meiotic stage.

To assess the proliferation of germ cells in culture, the cultured tissues were stained for DDX4 as germ cells markers and for KI-67 as a proliferation marker. Thirty five days culture samples showed the highest percentage of seminiferous tubules containing cells positively stained for DDX4/KI-67 which stained proliferating germ cells, compared to 18 or 56 days culture samples. When we compared day 18 and day 56 culture samples, cultured tissues at day 18 showed significantly higher proliferation index for germ cells.

Aiming to assess the degree of germ cells maturation *in vitro*, the cultured tissues were stained for DDX4 as germ cell marker and CREM as post meiotic stage germ cell marker. When comparing the number of cells stained positively for DDX4 or DDX4/CREM in the seminiferous tubules, tissue cultured for 18 days showed significantly more cells expressing DDX4 or DDX4/CREM compared to tissue cultured for 35 days and 56 days. We observed round spermatids in all tissue pieces cultured for 18, 35 and 56 days *in vitro*. Elongated spermatid could be observed in 67% or 60% of all analyzed fragments cultured for 35 and 56 days, respectively.

We could show presence of viable spermatogonia population in the whole culture periods by positive staining for DAZL, which is a marker for differentiating spermatogonia and early spermatocytes.

4.2.2 The effect of supplementation with melatonin and glutamax

We demonstrated that supplementation with melatonin and glutamax showed a positive effect on germ cell differentiation efficiency.

To test the effect of glutamax and melatonin on germ cell maturation, testicular tissues were cultured for 35 days and the basic medium consisting of DMEM and 10% KSR was supplemented with glutamax, melatonin or both. For evaluation, expression of DDX4/KI67, DDX4/CREM, and morphologic feature of germ cell subtypes were carried out. Evaluation of the number of cells stained positively for DDX4 and DDX4/CREM in the seminiferous tubules showed significantly higher number of those cells in culture conditions containing

10% KSR and melatonin in contrast to other conditions. Moreover, culture condition with 10% KSR and glutamax has more DDX4/CREM positive cells when compared with condition with 10% KSR only as supplements.

Cultured germ cells proliferation activity was investigated by evaluating the percentage of seminiferous tubules with double positive expression of DDX4 and KI-67. The results revealed no significant difference between different groups of culture conditions. Morphological evaluation of tissue fragments stained with PAS showed presence of spermatocytes and round spermatids in all fragments cultured with 10% KSR and melatonin, glutamax, and combination of both. Elongated spermatid could be recognized in 33% of tissue fragments when 10% KSR and glutamax added to the culture medium, in 83% of fragments when 10% KSR, and melatonin added to the culture medium and in 67% of the fragments when10% KSR with both melatonin and glutamax added to the culture medium.

4.2.3 The effect of supplementation with different concentrations of KSR.

Our results revealed that KSR supplementation has a prominent effect on tubule maturation, germ cell maturation, and testosterone production with a concentration of at least 10%.

The results showed that KSR actually has a prominent effect on spermatogenesis *in vitro*, in a dose dependent manner, which proves that KSR is essential for IVM of murine germ cells. Testicular tissue fragments were cultured with different concentration of KSR (0%, 0.1%, 1%, 10%, and 20%) and evaluated by the expression of CREM which indicates germ cell maturation beyond the late meiotic/post-meiotic stage. Expression of DDX4 as germ cell marker with and without KSR was also investigated. The evaluation revealed significantly more CREM-positive cells per tubules in fragments cultured in medium containing 10% KSR. No supplementation of KSR or supplementation with 0.1% KSR resulted in absence of any detection of CREM-positive cells in any tubules of all the cultured fragments. This indicates that there was no germ cell maturation beyond the late meiotic/post-meiotic stage. On the other hand, supplementation with 1% KSR resulted in a significant increase in the CREM-positive cells per tubules (34 ± 16%) compared to supplementation with 0% or 0.1%.

The supplementation with 10% and 20% KSR resulted in a significant increase in the CREM cells number per tubules compared to supplementation with 0.1% and 1% KSR. However, comparing the supplementation with 10% and 20% of KSR revealed no significant differences between the two conditions. Evaluation of DDX4 and DDX4/CREM cells per tubules revealed the same results of supplementation with 10% and 20% KSR resulted in a significant increase compared to supplementation with 0%, 0.1% and 1% of KSR.

Morphological evaluation of PAS stained section from different condition of culture showed no pachytene spermatocytes could be observed when culture medium supplemented with 0% or 0.1% KSR. On the other hand, with supplementation with 10% and 20% of KSR round and elongated spermatids could be observed in 83% and 67% of analyzed tissue fragments, respectively.

The initiation of germ cell differentiation and meiosis could be observed when tissue fragments were cultured for 3 weeks in the absence of KSR followed by 2 weeks with supplementation of 10% KSR, and few round spermatids could be observed in 33% of the tissue fragments from this culture setup. In regard to supplementation with melatonin, a significant higher number of DDX4/CREM-positive cells could be observed when melatonin was added to the culture medium.

Testosterone production per week by the tissue fragments cultured in media with different concentration of KSR was evaluated. The results of testosterone production were in line with the CREM expression and morphologic evaluation results. Testosterone levels increased with increasing KSR concentration supplemented to the medium of culture. Supplementation of 10 % and 20% of KSR to the culture media resulted in significantly higher testosterone level after 5 weeks of culture (7 ± 2 and 8 ± 1 ng/mL respectively), compared to supplementation with 0%, 0.1% and 1% of KSR (1 ± 0.9, 0.5 ± 0.2 and 1 ± 0.3 ng/mL respectively).

However, supplementation with either 10% or 20% KSR has no significant difference in testosterone production. Testosterone production pattern was persistent in all the media collected weekly, from the first week till the fifth week of culture.

4.2.4 Discussion

In this study, we used the organ explant culture method which has a basic principle of culturing the tissue fragments in air-liquid interphase allowing access to oxygen and nutrient without disturbing the tissue architecture. To ensure that undifferentiated spermatogonia were the most advanced germ cells present in the tissue used for culture, we used 3 d*pp* mice.

The reduction in the proliferation around day 56 of culture, as shown in DDX4/KI67 analysis and decrease in the number of DDX4 and DDX4/CREM positive cells observed in day 35 and 56 of culture, indicates that organ culture system might not be stable for the differentiation of germ cells beyond 35 days. Our results are in line with what has been published previously [271].

In this study, we investigated the effect of glutamax, melatonin and KSR on germ cells proliferation and maturation. We previously showed a positive effect of glutamax on rat germ cell viability *in vitro* in our lab [288] and wanted to test if it has the same effect on cultured murine germ cells. On the other hand, melatonin was used because it has anti-cytotoxic effect by scavenging the reactive oxygen species [289, 290] and previous results showed its positive effects on testicular tissues of rats[275] and CD-1 mice[271]. We aimed here to look for melatonin effects on cultured murine germ cells. We used a concentration of 10-7 M which is shown by other researchers to produce the antioxidant effect [271, 291-293].

Sato and his group substituted FBS, which resulted in meiotic arrest when used previously [263, 294], with KSR. KSR was introduced to keep stem cells in a pluripotent state and preventing differentiation and thus helping in propagation of stem cells *in vitro* [295]. Surprisingly, complete meiosis and production of mature haploid germ cells was achieved when 10% KSR was used. When we started this work, the optimal concentration of KSR was not reported yet.

When the number of positive cells for DDX4 and DDX4/CREM per tubules was evaluated, we were able to observe a significantly higher number of cells per tubules expressing DDX4 and DDX4/CREM. When we evaluate the morphology of the cultured sections we could see some elongated spermatids when the culture medium supplemented with melatonin. These results suggested a supportive effect of melatonin on germ cell differentiation. However, the effect of different doses of melatonin should be evaluated by additional experiments.

Adding KSR to the culture medium with different concentrations and evaluating the cultured sections by looking for CREM staining and evaluating the morphology showed dose response pattern on germ cell differentiation. The same effect could be seen with tubular maturation in terms of formation of the lumen and increase in total cell number per tubule. Germ cell differentiation was improved with increasing KSR concentration. However, no added benefit on germ cell differentiation could be observed when comparing 20% KSR supplementation with 10% KSR supplementation. From these results we could conclude that a concentration of 10% KSR is the optimal concentration to use in this setting.

Evaluation of testosterone produced by the cultured tissue fragments showed the same results as germ cell differentiation with different KSR concentration. Testosterone production increased with increased KSR concentration supplemented to the culture medium and no difference was found between supplementation with 10%KSR and 20% KSR. Since KSR

contains lipid rich albumin, we speculate that KSR is responsible for the increase in the testosterone production.

The progression of differentiation of murine germ cells in our culture condition with at least 10% KSR supplementation was similar to the situation *in vivo*. However, detailed studies on stage specific expression pattern are needed to compare the aspects of spermatogenesis *in vitro* and *in vivo*.

4.3 HORMONE PRODUCTION BY HUMAN FIRST TRIMESTER GONADS TISSUES CULUTRED *IN VITRO*

Study of human gonadal development will help in advancing our knowledge in understanding and treating conditions related to fertility. Limited access to human embryo makes it challenging to study the early period of gonadal development *in vivo*. *In vitro,* study of embryological gonads may provide us with clues and information needed for *in vitro* maturation of human germ cells. Organ culture methodologies, as hanging drop and air liquid interphase, were used to study human gonadal development; however, the results demonstrated a limited progress in germ cell differentiation and mainly focused on testicular development and germ cell maturation. Since information about first trimester gonadal tissues functionality *in vitro* cultures is not well studied, we evaluate in this study the functionality of human first trimester gonads when cultured *in vitro* in terms of the ability to produce hormones known to have a great impact on human gonadal development. We could demonstrate in this study that human first trimester gonads have the potential to produce hormones and were functional when cultured *in vitro* using explant culture conditions. Production of hormones from *in vitro* cultured male and female gonads showing resemblance to *in vivo* normal production of these hormones from gonads at equivalent age.

4.3.1 Production of testosterone

In this study, we cultured 27 female and 28 male gonadal tissues at postconceptional age 4.5-10.5 weeks using air-liquid interphase culture method for 14 days. Media collected at seven and 14 days of culture were analyzed for testosterone. The samples were grouped into four different groups based on post-conceptional age (4.5-5.5 weeks, 6-7.5 weeks, 8-9.5 weeks and 10-10.5 weeks).

No testosterone was detected above the detection limit (0.03ng/mL) in the media collected from female gonads cultures. In contrast, male gonads culture media had more testosterone at day 7 and day 14 of culture. A significant difference was observed when testosterone

production from male gonads culture of 4.5-5.5 weeks group compared to 10-10.5 weeks group at day 7 of culture.

4.3.2 Production of AMH and inhibin B

Female cultured gonads did not produce AMH above the detection limit. On the hand, AMH was detected in all media samples from cultured male gonads. Analysis of the media collected from male gonads culture revealed a significant amount of AMH produced by male gonads at age of 8-9.5 weeks compared to male gonads at age of 4.5-5.5 weeks at day seven and day 14 of culture.

Inhibin B produced by cultured male gonads was higher compared to cultured female gonads. Inhibin B detected in the media of female gonads culture ranged between 13.5-132 pg/mL and there were no significant differences between the age groups. Significantly higher inhibin B concentration was detected in the media samples from male gonads cultures in the age group 8-9.5 weeks and 10-10.5 weeks compared to those in the age group 4.5-5.5 weeks, at day seven and day 14 of culture.

4.3.3 Discussion

We did not detect any testosterone in the media collected from female gonads cultures, which is shown before that no testosterone is detected in the first trimester ovaries [296]. Higher concentrations of testosterone were detected in the media samples from male gonads cultures. These results reflect what has been shown before in regard to *in vivo* situation when higher concentration of testosterone was detected in the plasma of male fetuses compared to female fetuses and the speculation drawn from this findings that plasma testosterone level is predictive of fetal sex [142]. Moreover, previous findings showed that at six weeks post conception the testosterone production starts in the human fetus at the same time when Leydig cells start to differentiate [143, 296, 297].

We did not detect any AMH in the media samples from female gonads cultures. In contrast to that, AMH was detected in the media from male gonads cultures as early as four and half weeks. Previous studies showed no detection of AMH in the female fetal serum. Moreover, in situ hybridization, results showed the absence of AMH transcripts in fetal ovaries [298]. In regard to male gonad, it is shown that it is produced by Sertoli cell very early in the fetal life from the onset of testicular differentiation [298]. Moreover, AMH detection in the cytoplasm of the Sertoli cells was demonstrated as early as six weeks post conception. All these findings are in agreement with our *in vitro* culture results.

In our cultures we detected inhibin B in the media from both gonad cultures, male and female. However, higher concentration of inhibin was detected from male gonad cultures. Previous studies showed that Inhibin B was measured in human chorionic villous extracts from male embryos in the first trimester of pregnancy [142]. Moreover, the differences in the AMH levels in the male and female sera were demonstrated before and the authors suggested to use inhibin B serum concentration as a biochemical marker for fetal sex in the early second trimester [142]. These *in vivo* findings about the first trimester gonads are in line with what we have shown in our *in vitro* culture study. These findings demonstrate that air-liquid interphase explant culture method could be a robust method to study human gonadal cells development and to provide us with knowledge needed to establish a method for fertility preservation by *in vitro* maturation of human germ cells.

4.4 ASSESSMENT OF SERTOLI CELLS AND LEYDIG CELLS FUNCTIONS IN HUMAN PREPUBERTAL TESTICULAR TISSUES CULTURED *IN VITRO*

Increased survival rate of patients treated for malignant and hematological diseases is accompanied with increased burden caused by side effects of the treatment regimens used to treat such diseases. Prepubertal boys receiving conditioning therapies for hematopoietic stem cell transplantation (HSCT), irradiation exposure of the gonads (>=6 Gy), and high doses of alkylating agents (>=4000 mg/M2 CED) are at high risk of future infertility. While post pubertal boys and men can cryopreserve their sperm for future use to father kids, prepubertal boys have some experimental methods with hope to success in the future to restore their fertility. These experimental options include cryopreservation of testicular tissue followed by transplantation of spermatogonial stem cells, tissue grafting, or *in vitro* maturation of primary SSCs or PS cells. Cryopreservation of testicular tissues is considered as a future option in many clinical units worldwide; however, differentiating spermatogonia into functional spermatozoa is still a challenge, and proper methodologies for this differentiation are still not available.

Nowadays, *in vitro* spermatogenesis using testicular explant culture conditions is considered a promising option especially after the success reported in maturing murine germ cells *in vitro* as reported by many groups including our group as mentioned in paper II. This achievement raised the hope to establish a functional *in vitro* method for human fertility preservation. However, we are not there yet to have a reliable human fertility preservation method despite the great achievements in animals. This aspect was addressed in papers III and IV, as they aimed to translate using the air-liquid interphase explant culture method to culture and mature human germ cells *in vitro*.

In this study, we included patients exposed to high gonadotoxic treatment, divided into four groups according to their treatment (Alkylating group, non-alkylating group, sickle cell disease patients treated with hydroxyurea, and no-chemotherapy group) were included. Testicular tissues were obtained before or during the treatment. For all the groups, testicular tissues were cultured for 21 days using explant tissue culture method. Testicular tissues were analyzed by germ cell number and markers for Sertoli cell functionality at the day of biopsy. Functionality of Sertoli cell and Leydig cell during the course of the *in vitro* culture was analyzed by measurement of Inhibin B, AMH, and testosterone in the conditioned medium.

4.4.1 Reduction of germ cell number in testicular explant tissue cultures

Number of germ cells per total seminiferous cord was counted for all four groups at four different time points of culture (day 0, 7, 14 and 21 days). At day 0, SCD patients treated with HU group and the group receiving alkylating agents showed lower germ cell numbers per seminiferous cord. However, this difference in germ cells number at day 0 among the groups did not show statistically significant difference due to high variation in the group of patients receiving no chemotherapy and the group of patients treated with no alkylating agents. On the other hand, we observed that number of germ cells per seminiferous cord decreased in all groups to values close to zero at day 21 of culture.

4.4.2 Similar protein expression of Sertoli cells *in vitro* cultured testicular tissues from four different patient groups

Aiming to assess Sertoli cell functionality *in vitro*, we analyzed the protein expression of ZO1, AMH, and CK18 for all the groups at day 0, 7, 14, and 21.

Expression of ZO1 was constant throughout the culture period as evaluated by the percentage of positive seminiferous cord for ZO1. Although a decreasing trend in ZO1 expression in the group of patients of SCD treated with HU was observed, no statistically significant difference was observed among the four groups and in each group at different time points of culture.

As regards to AMH expression over the period of culture, a decreasing trend was observed when the percentage of positive seminiferous cord for AMH expression was evaluated. A statistically significant difference in AMH expression reduction was only observed for testicular samples from the group of patients not exposed to chemotherapy at day 7, day 14, and day 21 when compared to day 0.

Concerning the expression of CK18, analysis of the percentage of positive seminiferous cords showed an increasing trend over the period of culture. Although an increasing trend in CK18 expression was observed in all the groups over the time of culture, it was only statistically

significantly different in tissues from patients not treated with chemotherapy between day 14 and day 0 as well as day 21 and day 0.

4.4.3 Impaired somatic cell functions in testicular tissues from SCD patients treated with HU

Testosterone, inhibin B, and AMH concentrations were measured in conditioned medium obtained from testicular tissues from different patient groups at day 7, 14 and 21 of culture.

Analysis of testosterone and inhibin B concentrations showed a trend of increase of both from day 7 to day 14 which followed by a decreasing trend from day14 to day 21 in all four groups.

In regards to AMH concentration quantification, when comparing other groups to the group of patients not exposed to chemotherapy at different time points of culture, lower concentration of AMH was detected in the medium collected from cultured testicular tissue of SCD patients treated with HU at day 14 and day 21.

4.4.4 Discussion:

Low number of germ cells observed in the testicular tissue from the group of SCD patients treated with HU and the group of patients receiving alkylating agents indicates the negative impact of the SCD itself as a disease or its treatment using HU and the alkylating agents on germ cells *in vivo*. On the other hand, the reduction of germ cells in all the groups over the course of culture to reach a value of zero at day 21 indicate that the current condition of *in vitro* explant culture used to culture the testicular tissues is not optimal.

We used three markers to assess the maturation of Sertoli cells, ZO-1, AMH and CK-18.

Immature Sertoli cells have higher expression of CK-18 and AMH compared to mature ones[299]. Moreover, ZO1 expression increases with blood testes barrier formation in maturing testis and considered as a Sertoli cell maturation marker[300].

Analysis of ZO-1 expression in all the four groups showed constant expression over the course of culture. AMH expression evaluation revealed a decreasing trend over the culture period. While the expression of ZO-1 and AMH go hand in hand with what is expected as markers of Sertoli cell maturation, expression of CK-18 does not. We observed an increasing trend in CK-18 expression over the period of culture which could indicate de-differentiation or malfunction of Sertoli cells in this set up of culture. This de-differentiation or malfunction of Sertoli cells could explain the loss of germ cells over the period of culture which demonstrate that explant culture conditions did not support testicular tissues *in vitro*.

Our results suggested that the SCD itself and/or the treatment with HU impair the normal hormone production of Sertoli and Leydig cells, which most likely occurred *in vivo* (before the testicular biopsy) and was reflected *in vitro*. More studies will be necessary in order to explain whether the cause of this reduced somatic functionality is due to the SCD itself or to the treatment with HU. However, the existence of this compromised somatic environment, together with the fact that few germ cells were present at the time of biopsy, highlights the need of alternative fertility preservation strategies for this group of patients that might not rely on the utilization of their biopsied testicular tissue.

Low levels of testosterone, inhibin B, and AMH at all time points of culture in testicular tissues from SCD patients treated with HU propose the negative effects of SCD as a disease and/or its treatment on Sertoli cells and Leydig cells functions. Further studies are needed to verify the exact cause of the resulted effects on Sertoli cells and Leydig cells; the SCD itself or its treatment using HU. Furthermore, more considerations should be taken concerning fertility preservation strategies for patients with SCD. Low counts of germ cells at day 0 and impaired somatic environment in testicular tissues from patients with SCD might indicate the need to look for alternatives as fertility preservation options for such patients other than using testicular tissue biopsies. Nevertheless, more studies are needed for this purpose.

5 LIMITATIONS AND FUTURE PERSPECTIVES

The introduction of human PS cells was a great step in science. Human hES are an important tool to study early germ cell development in human. Moreover, human ES cells and hiPS cells are a promising future option for infertility treatment. The use of human pluripotent stem cell in the reproductive medicine as a therapeutic tool necessitates a culture condition that is feeder-free and xeno-free, robust enough to keep these cells pluripotent and to minimize the handling effects. Moreover, difficulties in repetition of differentiation methods between different laboratories due to lack of such culture condition is considered to be a challenge. Remarkable effort has been done and advanced steps were made towards achieving such robust culture condition and minimizing the differences in the gene composition of these cells resulting from differences in handling and changes in laboratories in which these cells being cultured or derived.
The use of human recombinant laminin LN521 in human embryonic stem cell culture is considered a step forward towards generating clinically safe conditions. We studied the effect of using LN521, compared to other matrices, as a culture matrix on the characteristics of human embryonic stem cells at the gene expression level. We demonstrated the positive effect of LN521 in supporting hES cells and maintaining them in a normal balanced pluripotency state which could be considered a step towards a robust culture condition for hES cells. Homogenization of the gene expression profiles of five male hES cell lines initially derived on feeders was observed when these lines were cultured on LN521. Having a similar expression profiles might result in more controllable and predictable behavior of these different cell lines when used in various differentiation protocols which is needed for the use of these cell lines in reproductive medicine. Using five hES cell lines in our study strengthens our conclusion and makes it more reliable. However, our study spanned for nine passages and it would be of interest to look for the effect of LN521 in long term culture. The difference effect we observed between LN521 and LN121 makes it interesting to study the effect of the difference in alpha chain composition of these laminins in producing such differences between the two laminin isoforms. More molecular and mechanistic study of impact of this difference is needed in the future.

After the positive results shown by Sato and his group in using explant culture method and supplementing the medium with KSR resulted in complete spermatogenesis in mice, we planned this work as an establishment of the method in our laboratory and our collaborators

laboratory. At the beginning of this project, the impact of different concentrations of KSR added to the culture medium was not known. We used wild type mice of the same transgenic strain that Sato used. We demonstrated the impact of different concentration of KSR on germ cell maturation and found that at least 10% of KSR needed to be supplemented to the culture medium to get the predominant effect of tubular maturation, germ cell maturation, and testosterone production. We showed that supplementation the culture medium with glutamax and melatonin resulted in more germ cells achieving post-meiotic stage of development. However, melatonin seemed to have better effect compared to glutamax. More studies are needed to investigate the optimal concentrations needed to increase the efficiency of the culture system and to look for the impact of different concentrations. This information will help in translating the method and its additive to other species to optimize the results obtained using the same culture method. We did not test the functionality of the elongated spermatids resulted from the culture because the efficiency of the culture and the yield of haploid cells isolation by flow cytometry were very low.

Our understanding of human gonadal cell development, specification and differentiation is poor and necessitates more efforts and studies. Since the access to early human embryo is out of reach and the number of existing PGCs in the gonads at these stages is small, using *in vitro* model will be a great advancement in our knowledge and understanding. A lot of previous and ongoing researches still face a great challenge in achieving a robust *in vitro* method to mature human gonadal cells and tissues *in vitro*. Our lack of knowledge about the exact mechanisms of human gonadal cells specification, characteristic, and main players hinder our advancement in achieving robust *in vitro* methodology and protocols for human gonadal development. Moreover, the impact of different molecules in the normal development and function of gonads was studied in animal models which made the translation to human impractical. Culturing human fetal gonads *in vitro* will be a great advancement in studying the impact of different substances in human gonadal development and will help in understanding cells functions in normal development and diseases. We demonstrated that cultured fetal gonads *in vitro* using explant culture method produce hormones in resemblance to *in vivo* situation. This could propose the use of such method to elucidate the effect of different substances on fetal gonadal tissue, specifically somatic cells. However, our study was limited to somatic cells function analysis. Analysis of germ cells maturation will be of great help to understand the development and maturation of germ cells. Moreover, more molecular studies are needed to understand the mechanism of fetal gonadal somatic cell development, function, and factors controlling them.

Explant culture conditions showed success in culturing mouse and rat testicular tissues as shown before [236, 275]. However, it is not the case when the same methodology used to culture human testicular tissues. Our studies here and other researchers as well showed the limitation of explant culture conditions to support testicular somatic environment *in vitro*, and thus its failure to maintain germ cells as well. The differences in the timeline maturation of somatic cells and germ cells in human and rodents could explain these different outcomes of *in vitro* explant culture of testicular tissues. However, in this study, we demonstrate that the explant culture conditions could be used to elucidate the somatic environment health and condition in different groups of patients which could be used as a tool to assess the testicular tissue biopsies prior to consider the proper fertility preservation modality for each patient exposed to hematological and oncological treatments. Moreover, the impairment of somatic environment and low germ cells counts in testicular tissues obtained from SCD patients treated with HU indicate the negative effect of the SCD itself and/or the treatment with HU. However, more studies are needed to elucidate the exact cause of such pathology in the testis of this group of patients, as well as to search for the proper modality and timing for fertility preservation for such group of patients.

6 CONCLUSION

- LN521 has a positive effect on maintaining hES initially derived on feeders in a normal balanced pluripotent state by homogenizing their gene expression profile. This could be a step towards more predictable and controllable behavior of these cells when used in various differentiation protocols for possible use of pluripotent stem cells in the future applications in regenerative medicine, e.g. their use in fertility preservation.
- LN521 had no effects on expression of genes related to human gonadal cells development in hES cells cultured on LN521. This could help in establishing robust and reproducible hPS cells culture conditions to enable use of these cells for future challenging differentiation protocols such as differentiation towards germ cells.
- Differentiation of mouse undifferentiated spermatogonia to elongated spermatid stage could be achieved *in vitro* using air-liquid interphase organ culture methods.
- Supplementation of at least 10% KSR to the mouse testicular tissues *in vitro* organ culture had a prominent effect on tubular maturation, germ cell maturation, and testosterone production.
- Supplementation of glutamax and melatonin to the mouse testicular tissue organ culture showed a positive effect on germ cell differentiation efficiency. However, more elongated spermatids could be observed when melatonin with 10% KSR was used as supplements.
- First trimester human gonadal cells have the potential to produce hormones known to have a significant impact in normal human gonadal development when cultured *in vitro*.
- Human first trimester gonads cultured *in vitro* in air-liquid interphase explant culture conditions showed the functionality and the ability to produce steroid and protein hormones in resemblance to *in vivo* normal hormone production at equivalent developmental stages.
- Air-liquid interphase explant tissue-culture condition is robust for culturing human first trimester gonadal somatic cells *in vitro*. It can be used to study human gonadal development process *in vitro*.
- Culturing human first trimester gonads in air-liquid interphase explant tissue-culture condition can be used to study the effect of substances suspected to cause hormone disturbances at the early period of human gonadal development.
- Explant culture conditions used to culture human prepubertal testicular tissues *in vitro* are not yet optimal to support germ cell differentiation.
- Explant culture of human prepubertal testicular tissues *in vitro* could be used as a tool to assess the functionality of the somatic environment of the testicular tissue of patients treated for hematological and oncological diseases, ahead of further steps towards fertility preservation modalities, such as testicular tissue auto-transplantation.

- Low germ cells count and impaired somatic environment in testicular tissues obtained from patients with SCD treated with HU indicate a need for fertility preservation strategies which might not rely on the use of patient's testicular tissue biopsy. More studies are needed in this regard to understand the cause of testicular tissues pathology in SCD patients treated with HU.

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