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LAMININS IN STEMNESS AND GERM CELL DEVELOPMENT IN HUMAN

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Laminins in stemness and germ cell development in human
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“One never notices what has been done; one can only see what remains to be done."

- Marie Curie.

ABSTRACT

Stem cell niches regulate the fine balance between self-renewal and differentiation of various stem cells. Although crucial hallmarks of stem cell niches, such as the mechanical signalling, neural inputs communicating distant cues, the proximity to blood vessels and the structural support by the surrounding basement membrane (BM), have been evaluated in different niches, little much attention has been directed to the specific composition of the BM. As a crucial component of the BM, laminins have shown major importance for physiological development, with lack of laminin α (LAMA) 1 and 5 resulting in major developmental disruptions.

Although, the importance of laminin 521 (LN521) in the embryonic inner cell mass (ICM) from which pluripotent stem cells (PSCs) are derived and in tissues such as the pancreas, nervous and muscular system and vasculature has been established, recent mass spectrometry studies of decellularized adult testicular tissue have suggested the presence of LN521 as well as LN121 in the testis.

Hence, we aimed to investigate the role of LN521 in stemness behaviour of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) as well as the potential role of LN521 and 121 in the testicular stem cell niche.

Utilizing five human foreskin fibroblast cultured hESC lines, pluripotency after short-term culture on LN521, LN121 and Matrigel was evaluated. We found that LN521, not only supports cell adhesion and stem cell maintenance but further homogenises the pluripotency expression pattern between the cell lines.

Further, by applying LN521 as a culture substrate for derivation of Klinefelter syndrome (KS) hiPSCs, we demonstrated the beneficial effect of LN521 on fibroblast reprogramming efficiency. By utilizing LN521 as culture substrate of KS derived hiPSCs we additionally revealed a similar X chromosome inactivation (XCI) behaviour to female cultured PSCs, observed by either the maintenance of XCI with one inactive X chromosome or erosion of XCI.

Finally, testicular laminin composition was evaluated during gonadal development and its importance for spermatogonial stem cell maintenance evaluated. We found LAMA 5 to be restricted to the vasculature while LAMA 1 was the sole α laminin chain of prenatal and pre- and peripubertal seminiferous cords and tubules. LAMA 1 was further restricted to the innermost basal lamina of the adult seminiferous tubules. Interestingly, LAMA 1 loss, as a result of inadequate culture conditions, correlated with a loss of germ cells.

In conclusion we demonstrated the importance of LN521 in stem cell maintenance of hESCs and hiPSCs and revealed a correlation between germ cell maintenance and presence of LN121.

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
Stem Cells International, vol. 2018, Article ID 7127042, 9 pages, 2018

II. Sarita Panula*, **Magdalena Kurek*,** Pankaj Kumar, Halima Albalushi, Sara Padrell Sánchez, Pauliina Damdimopoulou, Jan I. Olofsson, Outi Hovatta, Fredrik Lanner, Jan-Bernd Stukenborg
Human induced pluripotent stem cells from two azoospermic patients with Klinefelter syndrome show similar X chromosome inactivation behavior to female pluripotent stem cells
Human Reproduction, accepted
*These authors contributed equally to this work.

III. **Magdalena Kurek**, Elisabet Åkesson, Masahito Yoshihara, Yanhua Cui, Elizabeth Oliver, João Pedro Alves-Lopes, Ragnar Bjarnason, Kirsi Jahnukainen, Rod T Mitchell, Jan-Bernd Stukenborg
Laminin alpha 1 is crucial for germ cell maintenance of testicular cultures applied for pre- and peripubertal fertility preservation tissue
Manuscript

ADDITIONAL PUBLICATIONS (NOT INCLUDED IN THE THESIS)

I. Jan-Bernd Stukenborg, João Pedro Alves-Lopes, **Magdalena Kurek**, Halima Albalushi, Ahmed Reda, Victoria Keros, Virpi Töhönen, Ragnar Bjarnason, Patrik Romerius, Mikael Sundin, Ulrika Norén Nyström, Cecilia Langenskiöld, Hartmut Vogt, Lars Henningsohn, Rod T Mitchell, Olle Söder, Cecilia Petersen, Kirsi Jahnukainen
Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy
Human Reproduction, vol. 33, issue 9, pages 1677-1683, 2018

II. Halima Albalushi, Lena Sahlin, Elisabet Åkesson, **Magdalena Kurek**, Kristin Rós Kjartansdóttir, Rika Lindh, Olle Söder, Emilia Rotstein, Outi Hovatta, Jan-Bernd Stukenborg
Hormone Production by Human First-Trimester Gonads in a Functional In Vitro System. Endocrinology, vol. 160, issue 1, pages 133-142, 2019

III. Iuliia Savchuk, Marie-Line Morvan, Jean-Philippe Antignac, **Magdalena Kurek**, Bruno Le Bizec, Olle Söder, Konstantin Svechnikov
Ontogenesis of human fetal testicular steroidogenesis at early gestational age
Steroids, vol. 141, pages 96-103, 2019

IV. Iuliia Savchuk, Marie-Line Morvan, Jean-Philippe Antignac, **Magdalena Kurek**, Bruno Le Bizec, Olle Söder, Konstantin Svechnikov
Steroidogenic potential of human fetal kidney at early gestational age
Steroids, vol. 149, 2019

CONTENTS

LIST OF ABBREVIATIONS

AMH	Anti-müllerian hormone
ATRX	Transcriptional regulator ATRX
BLIMP1	Beta-Interferon gene positive-regulatory domain I binding
BM	Basement membrane
BMP	Bone morphogenic protein
BOULE	Boule Homolog RNA binding protein
BSA	Bovine serum albumin
c-KIT	KIT proto-oncogene
c-MYC	v-Myc myelocytomatosis avian viral oncogene homolog
CREM	CAMP responsive element modulator
Cyp26B1	Cytochrome P450 family 26 subfamily b member 1
DAZL	Deleted in azoospermia like
DDX4	DEAD-Box Helicase 4
DMRT1	Mab-3-related transcription factor 1
ECM	Extracellular matrix
EGF	Epidermal growth factor
EpiSC	Epiblast derived stem cells
ESCs	Embryonic stem cells
f-HM	Fibroblasts healthy male
FGF	Fibroblast growth factor
FISH	Fluorescence in-situ hybridization
FPKM	Fragments per kilobase million
FSH	Follicle-stimulating hormone
GATA	GATA binding protein
GDNF	Glial cell derived neurotrophic factor
H3K27me3	Histone 3 lysin 27 trimethylation
HP-EGF	Heparin epidermal grwoth factor
ICM	Inner cell mass
iPSCs	Induced pluripotent stem cells
KLF4	Kruppel-like factor 4

KS	Klinefelter syndrome
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LIN28	Lin-28 homolog A
MAGE-A4	Melanoma-Associated Antigen A4
NANOG	Homeobox protein NANOG
NANOS3	Nanos homolog 3
NC1	C-terminal globular domain of laminins
PGC	Primordial germ cell
PLZF	Zinc finger protein PLZF
POU5F1	POU class 5 homebox 1
PSC	Pluripotent stem cells
SCID	Severe combined immunodeficiency
SF1	Steroidogenic factor 1
SOX	Sex determining region Y-box
SRY	Sex-determining region Y
SSC	Spermatogonia stem cell
SSEA	Stage-specific embryonic antigen
STRA8	Stimulated by retinoic acid 8
SYCP3	Synaptonemal complex protein 3
TFAP2C	Transcription factor AP-2 gamma
TGFb1	Transforming growth factor beta 1
TRA	Tumor-related antigen
VEGF	Vascular endothelial growth factor
WPC	Weeks post conception
WT1	Wilms Tumour 1
Xa	Active X chromosome
XACT	X active specific transcript
XCI	X chromosome inactivation
Xi	Inactive X chromosome
XIST	X inactive-specific transcript

1 INTRODUCTION

1.1 PLURIPOTENT STEM CELLS

During human development cells that give rise to and maintain the body pass through different phases of differentiation potential. Blastomeres, which form as a result of gamete fusion, originate in a totipotent state in which they can give rise to all cell types of the body in addition to extra embryonic tissue including the placenta. As cleavage continues, blastomeres begin to fuse and the blastocyst starts to form, giving rise to three cell lineages: the embryonic tissue (epiblast) and the two extra-embryonic tissues (trophectoderm and primitive endoderm or hypoblast), from which the placenta and the yolk sac develop upon implantation (see Figure 1 for post-implantation development) [1]. At this crucial step in embryonic development, the epiblast cells of the inner cell mass (ICM) possess pluripotent potential and may give rise to any cell of the human body, with the exception of extraembryonic tissue [2].

As development progresses, specialisation of the embryo continues, beginning with formation of the amniotic cavity within the epiblast leading to the formation of the amnion at the trophoblast side and the epiblast disc at the hypoblast side. By embryonic day 14 gastrulation is already initiated, leading to the formation of the three multipotent germ layers, endo-; meso- and ectoderm that may only give rise to the various cell precursors of their specific germ lineage [3]. With the progression of organogenesis and increased signalling, cell specification continues until most cells of the body specialize into a unipotent state that gives rise to only one cell type.

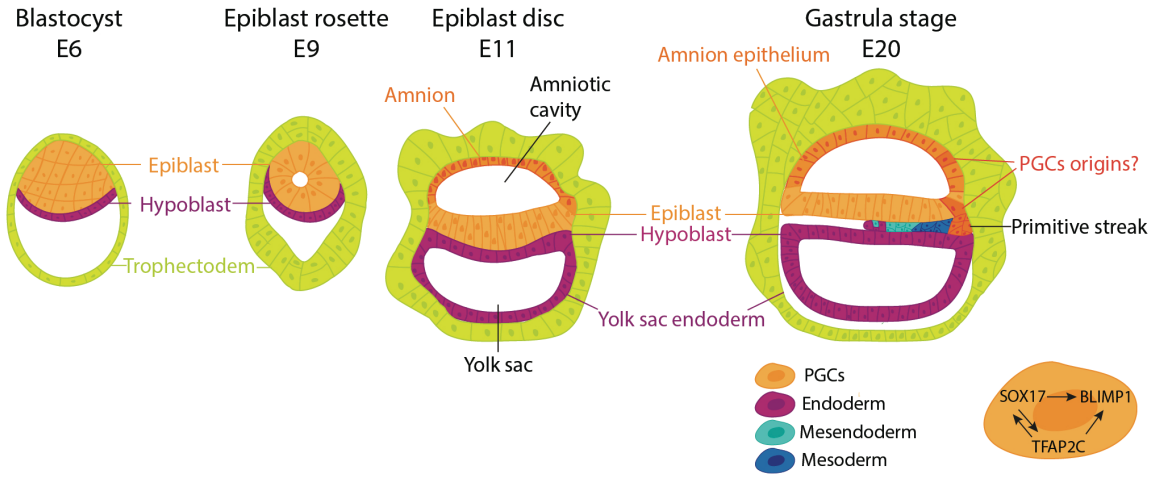

Figure 1: Human post-implantation development and primordial germ cell specification. Adapted from Shahbazi and Zernicka-Goetz 2018 with permission from Springer Nature.

Although the majority of cells in adult life lose their pluripotency, one can still find various tissues containing multipotent adult stem cells such as haematopoietic stem cells, skeletal muscle stem cells, gut stem cells, epithelial stem cells and neural stem cells [4].

1.1.1 Embryonic stem cells

In order to understand development and function of the vast variety of human cells, as well as the development or onset of disease, a closer look at human foetal development or isolation of cells from inaccessible areas of the human body would be advantageous. Thus, the majority of scientific breakthroughs until 1981 were achieved through animal studies and isolated human primary cells. However, in 1981 a major discovery by Evans and Kaufman opened-up new possibilities for the understanding of development.

Following the isolation and culture of mouse embryonic ICM, Evans and Kaufman observed that these embryonic stem cells (ESCs), were not only capable of limitless proliferation and self-renewal but, if kept under the right culture conditions, could also develop into cells of the three germ layers *in vitro* and even participate in the formation of an offspring when injected into blastocysts [5]. Following this discovery, improvement of the isolation and culture techniques led to the first isolation of human ESCs in 1998 by Thomson and co-workers leading to major breakthroughs in human developmental studies [6].

1.1.2 Human induced pluripotent stem cells

The concept of reprogramming somatic cells was first described in 1962 by John Gordon who transferred nuclei from differentiated intestinal cells into oocytes by somatic cell nuclear transfer [7].

Nevertheless, the terminology of induced pluripotent stem cells (iPSCs) and reprogramming of somatic cells as we know it today was first applied in 2006 by the group of Shinya Yamanaka who introduced variations of 24 genes, known to play a role in pluripotency, into mouse somatic cells in order to achieve a conversion into a stem cell-like state [8]. Yamanaka was able to show that as few as four transcription factors (POU class 5 homebox 1 (POU5F1), sex determining region Y-box (SOX) 2, Kruppel-like factor (KLF4) and v-Myc myelocytomatosis avian viral oncogene homolog (C-MYC)) were enough to convert a mouse somatic cell into an iPSC. In 2007 the same set of reprogramming factors were employed to generate iPSCs from human somatic cells opening the doors to a new area of research [9]. Interestingly, subsequent work by Thomson using POU5F1, SOX2, Homeobox protein NANOG (NANOG) and Lin-28 homolog A (LIN28), among others, has revealed that reprogramming of somatic cells can be achieved by various additional sets of factors and enhancers increasing the efficiency [10, 11].

The obtained hiPSCs are similar to hESCs in regard to their morphology, culture conditions, surface marker expression and their ability to differentiate to all three germ layers *in vivo* as well as *in vitro*. Nevertheless, global gene expression profiles show a subtle difference in microRNA and mRNA expression compared to hESCs as well as a persistence of epigenetic marks from their cell type of origin which continues to affect gene expression [12]. However, comparison of hiPSC and hESCs of isogenic origin, excluding human variations, has shown conflicting results. Using male lines, no significant differences in gene expression and methylation profiles could be observed in contrast to a study using female lines which could be attributed to a sex difference [13].

The main advantage of hiPSCs however, is the avoidance of ethical issues related to hESCs and their patient specificity, allowing *in vitro* disease modelling, personalized regenerative treatment and drug discovery. Nevertheless, for clinical translation to be possible, reprogramming must occur without any integration into the host genome and both derivation and maintenance must occur under xeno-free conditions.

Traditionally the derivation of hiPSCs was achieved by the use of retroviruses integrating into the host genome which could cause insertional mutagenesis and residual expression of reprogramming factors. Thus, reprogramming with viral vectors as retrovirus or lentivirus has shown to cause genetic or epigenetic as well as transcriptional abnormalities ranging from accumulation of point mutations, dysregulation of imprinted genes, chromosomal aberrations and aberrant methylation patterns etc. Therefore, the maintenance of genomic integrity by the use of episomal vectors, Sendai viral vectors, adenoviral vectors, plasmids, mRNA, small molecules and proteins has gained increasing popularity in order to avoid genetic aberrations which are strongly associated with cancer development [14].

1.1.3 The primed and naïve stem cell state

Although mESCs and hESCs both possess the ability to differentiate into any of the three germ layers they have to be distinguished based on their origin and capability.

Conventional mESCs are derived from the ICM of pre-implantation blastocysts and retain a naïve pluripotency, characteristic for the pre-implantation ICM. They are capable of differentiating into all three germ layers, form chimeras after injection into blastocysts and retain two active X chromosomes (Xa) in female lines. In contrast, mESCs derived from the epiblast of the post-implantation blastocyst (EpiSCs) retain a restricted pluripotency referred to as primed pluripotency which, while still capable of giving rise to all three germ layers, does not contribute to chimera formation and demonstrate X chromosome inactivation (XCI), as shown by one Xa and one inactive (Xi) X chromosome.

In contrast, human conventional ESCs derived from 6-7 day old blastocysts ICM, consisting of pluripotent epiblast cells, possess differing characteristics and culture requirements, having fibroblast growth factor (FGF) 2 and transforming growth factor beta 1 (TGFb1) instead of leukemia inhibitory factor (LIF) as their core signalling molecules, as compared to conventional mESCs. Referred to as primed hESCs, these cells are characterized by the expression of POU5F1, NANOG, SOX2, tumor-related antigen (TRA)- 1-81, TRA 1-60, stage-specific embryonic antigen (SSEA) 3 and SSEA4 [15].

Initially this difference between mESCs and hESCs was attributed to genetic species differences, as both cell types are derived from pre-implantation blastocysts. However, it has been shown that even ESCs derived from the mouse pre-implantation blastocyst will adapt a primed state if not provided with the correct naïve culture conditions. Hence providing hESCs with the correct naïve culture conditions allows the derivation of naïve hESCs or the conversion of existing primed hESC lines towards a naïve state [16] as demonstrated by a more diverse set

of naïve pluripotency markers as well as biallelic expression of the long non-coding RNA X inactive-specific transcript (*XIST*) in female lines [17].

1.1.4 Pluripotent stem cell culture

Initially hESCs derivation was performed with mitotically inactive mouse embryonic fibroblasts (MEF) in combination with foetal bovine serum (FBS) in order to support PSC self-renewal. However, the exposure to xenogeneic components and a batch to batch variability under such culture conditions mean the generated hPSCs are unsuitable for clinical use [6]. Given the increasing demand for clinically translated regenerative approaches, a variety of culture conditions are now available for use. Animal derived feeder cells can be substituted by human cells, such as human foreskin fibroblasts (hFF), while knockout serum replacement (KSR) and FGF2 substitute FBS and are sufficient to stimulate the self-renewal of hPSCs [18, 19]. Various matrix proteins have also been tried in order to establish feeder-free culture conditions. Matrigel, which is derived from mouse sarcoma extracts, is the most commonly used, although often presents with batch to batch variability. However, matrixes such as type IV collagen, vimentin, fibronectin and human recombinant laminin-521 (LN521) can be employed as xeno-free alternatives [20-22].

1.1.5 X chromosome inactivation

XCI is a mechanism in which one of the two X chromosomes in female somatic cells is transcriptionally silenced to equalize the dosage of X-linked genes between males and females, resulting in one Xa and one Xi chromosome in females.

Although much progress in understanding XCI was achieved in the recent years, especially by the use of fluorescence in-situ hybridization (FISH) and single cell RNA sequencing, the mechanism of human XCI is still not completely understood. Nevertheless, it has been shown that the main player in XCI is the long non-coding RNA *XIST*. Coating the entire Xi with *XIST* in cis, it contributes to the initiation and maintenance of the X chromosome silencing by nuclear reorganization and recruitment of histone-modification complexes [23, 24]. Further, studies have shown that the Xa expresses not only transcriptional regulator ATRX (*ATRX*) but also X active specific transcript (*XACT*), a long non-coding RNA produced by and covering the Xa [23].

Although XCI is initiated early on in embryonic development, during the transition from a naïve to primed pluripotency, the random inactivation of one X chromosome is not set in stone and comes in various forms [25]. Recent studies have shown that human pre-implantation embryos show biallelic expression of *XIST* without fully silencing the X chromosomes but rather dampening the net X output. In post-implantation embryos however, the XCI changes to a complete silencing of only one X chromosome resulting in a Xa/Xi state that is maintained throughout life in somatic cells.

Nevertheless, reactivation of the Xi occurs during female germ cell development to ensure that each haploid germ cell contains one Xa. While the reactivation of the, during meiosis,

transcriptionally inactivated X chromosome in sperm cells has been demonstrated directly after fertilization.

It is important to note that XCI does not silence the Xi completely. In female somatic cells around 10-15% of X-linked genes "escape" the silencing program and are still expressed, whereby expression of escape genes can vary for given genes in specific tissues explaining tissue-specific sex differences [26].

1.1.5.1 X chromosome inactivation in pluripotent stem cells

As XCI is established early in embryonic development, PSCs represent a great source to investigate the molecular mechanisms involved in XCI as they recapitulate XCI in various phases of embryonic development. Although primed female hPSC lines initially express one Xa and one Xi, the XCI maintenance during culture has shown to be unstable resulting in cells with no XCI (Xa/Xa), XCI (Xa/Xi), or XCI with erosion of silencing marks (Xa/Xi eroded) [27]. The recent optimization of naïve PSC cultures has provided new insights into XCI as such as those cells resemble not only the pre-implantation state in terms of genes expression but also XCI. Naïve PSCs derived by the 5iLA and 2i method have shown biallelic *XIST* expression without complete silencing of both X chromosomes but dampening the net X output as seen in human pre-implantation embryonic studies [2, 17].

1.1.5.2 X chromosome inactivation in Klinefelter syndrome

The Klinefelter syndrome (KS) is the most frequent sex chromosome abnormality in males with an incidence of 1:500 to 1:1000. It is an X chromosome aneuploidy characterized in 80-90% of the cases by a non-mosaic 47, XXY karyotype. However, higher-grade aneuploidies and mosaic forms of KS account for the remaining 10-20%, making KS a clinically variable condition.

The acquisition of the supernumerary X chromosome occurs due to failures in chromosome disjunction in anaphase of meiosis I, meiosis II or mitosis during either oogenesis, spermatogenesis or, occasionally, during the first cell cleavages of a fertilized oocyte [28].

As KS patients have no obvious phenotypic distinction compared to normal healthy males and possess a diverse clinical phenotype (based on the grade of aneuploidy, mosaicism, structural abnormalities and origin of the X chromosome), the diagnosis is often made once KS patients seek medical attention related to fertility issues [28].

KS patients typically present with a tall stature, mild language and speech disabilities, small testes, gynecomastia, sparse body hair, and hypogonadism, resulting in azoospermia with hyalinization and fibrosis of the seminiferous tubules [28].

As in females, the same mechanism of XCI is observed in KS patients, with incomplete silencing of the Xi (resulting in the escape of 10-15% of X-linked genes), contributing to most of the mentioned phenotypic features [28].

This is especially relevant when it comes to the reproductive life of KS patients as it is assumed that the escape genes not only affect the proper testicular somatic environment but also the meiotic progression of germ cells themselves leading to progressive germ cell depletion after the onset of puberty [28].

1.2 GERM CELL DEVELOPMENT

Germ cells are one of the most unique cell types of the human body. They not only proliferate and differentiate but are the only cell type capable of meiosis, resulting in haploid sperm and oocytes, creating, once fused with each other, a new life, passing on our genetic information to the next generation.

1.2.1 Foetal germ cell development

The first description of human germ cells dates back to 1948, when Emil Witschi observed primordial germ cells (PGC) in the extraembryonic yolk sac close to the allantois in embryos three weeks post conception (wpc) (Figure 1) [29]. Witschi was further able to observe the migration of PGCs from the yolk sac to the hindgut followed by the colonization of the gonadal ridge by PGCs from 4 until 6 wpc [30]. Many decades later however, due to the inaccessibility of early developmental stages, the process of human germ cell development and differentiation is still not fully understood.

As a response to WNT and bone morphogenic protein (BMP) signalling from the extraembryonic tissue, germ cell specification is suggested to start in the posterior epiblast or the emerging amnion, resulting in the specification of hPGC precursors [31]. This process has shown to be highly dependent on the expression of *SOX17*, as knock-out of *SOX17* prevents specification of hPGCs while over-expression induces hPGCs differentiation without the need of further external signals [31-33]. In addition to *SOX17*, transcription factor AP-2 gamma (*TFAP2C*) and specifically beta-interferon gene positive-regulatory domain I binding (*BLIMP1*), are essential for hPGCs specification (Figure 1), as well as the repression of the pluripotency gene *SOX2* and upregulation of *POU5F1, NANOG* and maintenance of the naïve pluripotency gene, *KLF4* [31, 33, 34] .

On their developmental journey to the gonadal ridges through the yolk sac, allantois and hind gut, hPGCs undergo proliferation and nuclear reprogramming as determined by the progressive loss of *XIST* and Histone 3 lysin 27 trimethylation (H3K27me3) leading to X chromosome reactivation in females [30, 35]. Once settled in the gonadal ridge around 5 wpc the hPGCs, now called gonocytes, start colonizing the gonad and initiate genome wide DNA demethylation and chromatin remodelling, enabling the erasure of imprints and the establishment of sex-specific imprints until 9 wpc [30, 32]. From 7 wpc, up until which point female and male embryos differ only in their allosomes, the bi-potential gonad starts its path towards testicular or ovarian development, initiated by signalling from the surrounding environment [30, 36].

Male sex specification in particular, is induced around 7 wpc with the expression of sex-determining region Y (*SRY*) and *SOX9* [30, 37-39] in the supporting gonadal cells [40], leading

to the specification of Sertoli cells and formation of seminiferous cords. At this point male gonocytes start populating the seminiferous cords and begin to proliferate, increasing to about 150.000 germ cells by 9 wpc [41]. Although the exact mechanisms underlying gonocyte proliferation are unknown, extrapolation from *in vivo* mouse studies and *in vitro* differentiation studies suggest that BMP4, LIF, FGF2 and epidermal growth factor (EGF) are crucial factors [42, 43].

During the first trimester, male gonocytes remain mitotically active, expressing hPGC markers such as POU5F1, NANOG, SSEA4, Nanos homolog 3 (NANOS3), KIT proto-oncogene (c-KIT), deleted in azoospermia like (DAZL) and DEAD-box helicase 4 (DDX4). In parallel to the loss of mitotic activity their expression profile changes to DDX4 and Melanoma-Associated Antigen A4 (MAGE-A4) until the onset of puberty, indicating a transition towards the spermatogonial stem cell (SSC) population. In support of this, single cell RNA sequencing of two and seven days old neonatal and adult human testicular tissue has revealed the presence of two distinct germ cell populations in the neonate, consisting of cells similar to human gonocytes and cells expressing pre-spermatogonia profiles [44].

At this point the testicular differentiation, as observed by the differentiation of the Wolffian ducts into the epididymis, seminal vesicles and vas deferens, and the virilisation of external genitalia, is independent of the pituitary axis and achieved by the androgen production of immature Leydig cells which are stimulated by chorionic gonadotrophin [45].

In contrast, oogonia specification occurs during week 9 of development and is characterised by a greater period of mitotic activity. Subsequent meiosis, which overlaps with mitosis, is initiated by retinoic acid and the expression of *STRA8*, around week 10-11 of development and results in primary oogonia which remain quiescence until the onset of puberty [41].

1.2.2 From “mini-puberty” and puberty to spermatogenesis

Although the hypothalamic-gonadotropin-axis is largely inactive until the onset of puberty at the age of 9-14 years, brief activity, referred to as mini-puberty, is observed during the first months of life, whereby gonotrophin-releasing hormone from the hypothalamus stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary [45, 46]. During this phase, LH stimulates the production of testosterone by immature Leydig cells, followed by an increase in Anti-müllerian hormone (AMH) upon FSH stimulation of Sertoli cells and subsequent germ cells proliferation. Following a peak of hormone levels at 3 months after birth, a slow decrease follows with undetectable levels by 6 months (see Figure 2 for hormonal levels and testicular growth during male development) [45].

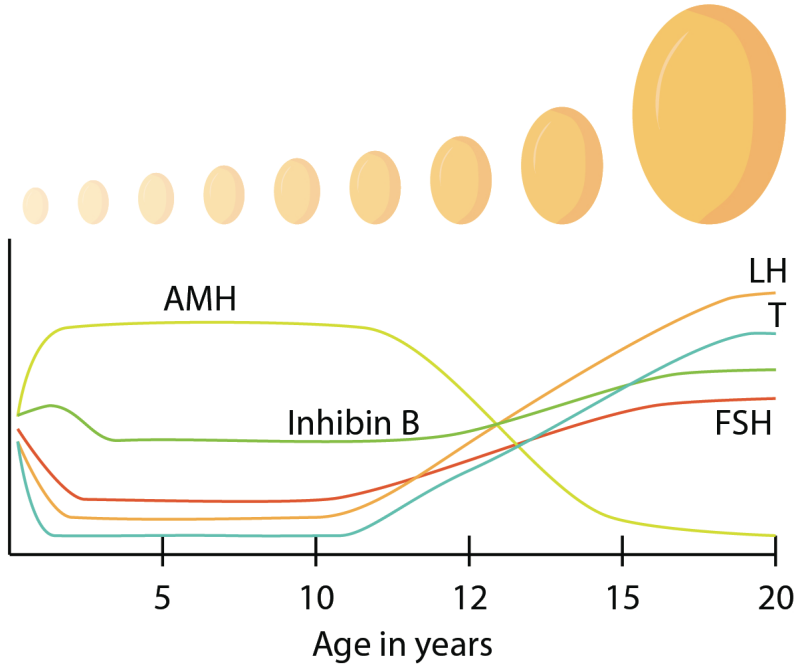

Figure 2: Hormone levels and testicular growth during male development. Adapted from Condorelli et al 2018 with permission from Springer Nature.

At the onset of puberty, before which point the testicular size doubles from 2-4 mL in size, the hypothalamic-gonadotropin-axis is revived leading to a surge in LH and FSH levels [47]. In the pubertal testis LH stimulates the maturation of Leydig cells and induces the production of testosterone [48]. Testosterone in return induces the maturation of Sertoli cells seen by the loss in proliferative capacity, establishment of the blood-testis barrier, decrease in AMH levels and increase in inhibin B levels, creating a negative-feedback loop with FSH [45, 49, 50]. Further, testosterone stimulates the production of Glial cell derived neurotrophic factor (GDNF), FGF2, BMPs and retinoic acid by Sertoli and peritubular cells via their androgen receptors and thereby influences SSC differentiation and proliferation.

Located at the basement membrane (BM) of seminiferous tubules, SSCs can be classified as either quiescent A_{dark} spermatogonia, which representing the SSCs reserve, or mitotically active A_{pale} spermatogonia, which are capable of entering meiosis to ultimately differentiate to elongated sperm. At this stage of maturation, they express markers such as Zinc finger protein PLZF (PLZF), c-KIT, MAGE-A4, DDX4 and DAZL [51].

Once A_{pale} spermatogonia receive differentiating cues they give rise to type B spermatogonia, which will continue to mitotically divide, generating two primary spermatocytes, which leave the SSC niche and cross the blood-testis barrier in order to proceed with first meiotic division [49]. At this stage, primary spermatocytes express markers such as Boule Homolog RNA binding protein (BOULE), Synaptonemal complex protein 3 (SYCP3), DAZL and DDX4. The second meiotic division leads to the formation of round spermatids, which differentiate to elongated spermatids in a morphogenic process without further proliferation. At this stage elongated spermatids express protamine, acrosin and CAMP responsive element modulator (CREM) [52]. During this phase of spermatogenesis, major structural changes such as nuclear

condensation, histone replacement, formations of cytoplasmic droplets and flagellum formation occur. Lastly, immotile spermatozoa are transported into the rete testis before entering the epididymis for final maturation. In total the process of spermatogenesis together with spermiogenesis takes around 74 days in humans giving rise to 16 spermatids from each single SSC [51] and a daily production of 150-275 million spermatozoa [46].

1.2.3 Somatic gonadal cells

Unlike PGC that migrate into the gonadal ridge, the gonadal ridge itself is proposed to arise from coelomic epithelial progenitor cells proliferating on the mesonephros surface, creating a dense cell layer [53, 54]. Fragmentation of the underlying basement membrane then [55] allows the ingression of genital ridge progenitor cells that continue to proliferate giving rise to both steroidogenic and supporting cells [56, 57] under the influence of mainly GATA binding protein 4 (GATA4), Wilms Tumour 1 (WT1), Steroidogenic factor 1 (SF1) signalling [58, 59].

In the case of male sex determination, the supporting cells, which are the first to ingress into the gonad, differentiate into Sertoli cells retaining WT1 expression, while the steroidogenic cells which appear later, differentiate into Leydig cells retaining SF1 expression [60].

1.2.3.1 Sertoli cells

The first observations of Sertoli cells date back as early as 1865, when Enrico Sertoli first described "cellule ramificate" [61], branched cells supporting the developing germ cells which were later confirmed by Von Ebner and called Sertoli cells [62].

The differentiation of Sertoli cells from the supporting cells and, with that, the path of male sex-determination, is induced by the expression of *SRY* gene [37-40, 63], located on the Y chromosome. Its sole expression in Sertoli cells begins around 4 wpc with a peak around 5 to 6 wpc and subsequent persistence at low levels even in the mature testis [59].

The expression *SRY* stimulates the up-regulation and expression of *SOX9* in supporting cells which, in turn, stimulates the expression of FGF9, creating a positive feedback loop with *SOX9* [36, 41, 65-67] leading to the specification of Sertoli cells and subsequent formation of seminiferous cords around 7 to 9 wpc. *SRY* has been shown to be the key regulator of male sex-determination as mutation leads to a female sex-reversed phenotype in humans [39] as well as male sex reversal when introduced into XX mice [68].

Nevertheless, *WT1, GATA4*, doublesex and mab-3-related transcription factor 1 (*DMRT1*), *SF1*, and *SOX9* are thought to play a key role in Sertoli cell specification, as exogeneous expression of all five factors has been shown to be sufficient for the differentiation of Sertoli-like cells from human fibroblasts [69].

In addition to male sex-determination, the Sertoli cell differentiation leads to subsequent production of AMH around 5 to 6 wpc, leading to the regression of the Müllerian ducts and prevent the development of female internal genitalia [46].

In contrasts to foetal gonad development, where global meiotic entry of germ cells is prevented by the degradation of retinoic acid by Cyp26B1, Sertoli cells form a tight barrier composed of Sertoli-to-Sertoli cell tight junctions during puberty, allowing for meiotic progression. This way Sertoli cells are able to create an immune-privileged blood-testis barrier, protecting and governing advanced meiotic germ cells in the adluminal compartment of seminiferous cords [47, 70] and preventing meiotic entry of basal SSCs. At this point Sertoli cells exhibit cytoplasmic projections showing morphological resemblance to tree branches, allowing the support and migration of different germ cell stages towards the seminiferous lumen [46].

Furthermore, they play a crucial role in SSC maturation and spermatogenesis through the expression of transferrin, acetate, lactate, androgen-binding protein, GDNF, TGFb1 and extra cellular matrix (ECM) components [46].

1.2.3.2 Peritubular myoid cells

In contrast to rodents, the seminiferous tubule wall in men is surrounded by a BM followed by acellular space and multiple layers of elongated peritubular myoid cells with contractile properties. Peritubular cells initiate proliferation after birth [71] building up a multi-layered structure surrounding the seminiferous tubules, a process which continues until the onset of puberty. Comparison of neonatal and adult peritubular cells has revealed distinct spatial separation [44] likely based on the development of smooth muscle characteristics induced by androgens [72] through their androgen receptor. Although the most important role of peritubular cells is their peristaltic action facilitating intratesticular transport of immotile spermatozoa to the rete testis, it has been demonstrated that they also fulfil important secretory functions [73].

Together with Sertoli cells they are responsible for the production and deposition of ECM proteins such as fibronectin, collagens and laminins [46, 74], establishing the seminiferous BM as well as the blood-testis barrier. Furthermore, it has been shown that peritubular myoid cells support the secretion of inhibin, transferrin and androgen-binding protein by Sertoli cells [46] as well as contributing to the production of GDNF, supporting the SSC renewal [75].

1.2.3.3 Leydig cells

Leydig cells located in the interstitium of the testes were first described in 1850 by Franz Leydig in various animal testes [76] and confirmed 1854 in humans by Albert Kölliker [77].

The differentiation of mature Leydig cells occurs in three waves [46]. In the foetus, Leydig cell differentiation from the steroidogenic cell population is induced around 6 to 16 wpc, as a downstream event of *SRY* expression, leading to the production of testosterone and secondary sexual differentiation [46, 78]. Despite LH independent initiation of Leydig cell differentiation, hCG/LH stimulation has shown to be required from 5 wpc for the maintenance of testosterone production. Following a regression of Leydig cells, the second wave occurs within the first months of birth and supports, besides testosterone, the production of insulin-like factor 3 mediating testicular descent to the scrotum [46, 79].

In contrast to foetal Leydig cell development, the differentiation of adult Leydig cells appears LH dependent, and is achieved by the LH surge which takes place at the onset of puberty, leading to the continuous production of testosterone.

In addition to their steroidogenic function, Leydig cells further produce oxytocin, stimulating the contraction of peritubular myoid cells and transportation of spermatozoa to the rete testis [46].

1.2.4 *In vitro* germ cell maturation

Infertility affects approximately 10% of the male population and can be a result of sperm-production problems, genetic mutations, infections, medications, chemicals or further unknown factors [80]. Exposure to radiation and chemotherapy as part of malignant and haematological disease treatment can additionally have severe, and often permanent, gonadotoxic effects. While adult patients with progressive spermatogenesis have the option of sperm cryopreservation prior to gonadotoxic therapy, immature testicular tissue cryopreservation is the only option for childhood cancer patients, in whom spermatogenesis has not yet started [81]. Given the destructive effect of treatments on prepubertal SSCs and increasing childhood cancer survival rates, current research is being driven towards the development of novel fertility preservation approaches such as SSC transplantation, testicular tissue transplantation or *in vitro* germ cell maturation [82]. Although two-dimensional germ and Sertoli cell co-cultures have demonstrated the potential of *in vitro* germ cell progression [83], three-dimensional cultures in scaffolds or as tissue explant have shown superior support of cell functionalization and organization [84]. Adaptation of the rat testicular explant culture in an air-liquid interphase, first developed in the 1960's, has led to the differentiation of mouse germ cells by Sato and colleagues in 2003 [85, 86], while promising results for steroidogenic functionality of human Leydig cells and maturation of human Sertoli cells have been demonstrated recently. Nevertheless, a common obstacle of this methods is the significant loss of SSCs with culture time [87-89].

1.3 STEM CELL NICHES

The first concept of a specialized stem cell environment was established in 1978, by Schofield who suggested a specific anatomical location of niches as well as differentiation of their harboured stem cells upon niche removal [90, 91].

In order to regulate the fine balance between self-renewal and differentiation, stem cells are controlled by intrinsic factors in response to extrinsic cues provided by their surrounding microenvironment, which Schofield defined as the stem cell niche [90, 91]. Although stem cell niches have a common function providing trophic support, delivering topographic information, structural support and physiological cues in order to regulate stem cell functions, they are also as versatile as the stem cells they support. Stem cell niches in the adult human include skeletal muscle stem cells found along the myofiber, gut stem cells in intestinal crypts, epithelial stem cells in the follicular bulge, neural stem cells in the hippocampus and the olfactory bulb,

haematopoietic stem cells at the endosteal surface of trabecular bone and SSCs adjacent to the BM of the testicular seminiferous tubules.

Examination of the various stem cell niches has led to the identification of certain hallmarks such as the supporting stromal cells, which interact directly with the stem cells and each other, a close proximity to blood vessels, ECM proteins, which provide structural support as well as mechanical signals, and neural inputs, allowing the communication of distant physiological cues to the environment (see Figure 3 for hallmark depiction). Given the complexity of stem cell niches, it is no surprise that vulnerable stem cells are sensitive to deregulations in the niche, which can lead to pathologies related to tissue degeneration and tumorigenesis or even stem cell depletion [4].

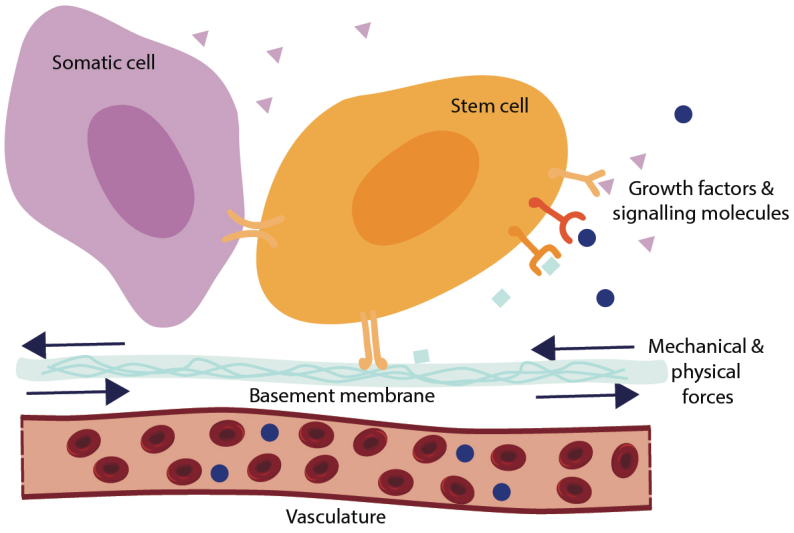

Figure 3: Hallmarks of stem cell niches.

1.3.1 Spermatogonial stem cell niche

As mentioned before, spermatogenesis is the process of SSC differentiation through mitotic and meiotic cell divisions leading to the formation of haploid spermatids. SSCs, which are situated at the basal membrane of the seminiferous tubules, are anchored to the BM and surrounded by Sertoli cells forming a blood-testis barrier [92]. Once the mitotically active SSCs (Apale SSC) differentiate and give rise to type B spermatogonia, mitotic divisions will generate spermatocytes which will cross the blood-testis barrier in order to proceed with meiotic divisions. As the initial steps of SSC differentiation happen in a two-dimensional fashion it is assumed that the SSC niche must be situated at the tubular BM and comprise discrete areas to ensure quiescence of Adark SSCs and mitotic activity of Apale SSCs [50]. Various studies could so far link the SSC niche to the close proximity of blood vessels as well as bordering to interstitial tissue, which is surrounding the seminiferous tubules, rather than bordering another tubule. Although Sertoli cells are the only cell type in direct contact with SSCs, it is possible that the sounding interstitial cells such as Leydig cells, peritubular myoid cells and macrophages, determine whether Sertoli cells produce SSC self-renewing factors, such as FGF2 or GDNF, or differentiating factors, such as activin A, BMP4 and retinoic acid.

Further, it has been shown that adhesion molecules such as integrins, expressed on SSCs and Sertoli cells, play a major role in SSC homing to the basal membrane, however extensive investigations of the role of the basal membrane in the SSC niche have yet to be carried out [93].

1.3.2 Basement membranes as part of the niche

BM are self-assembling sheets of ECM approximately 200nm in diameter [94] which coat the basal site of epithelial and endothelial cells, as well as surrounding adipocytes, nerve axons and muscles, thereby compartmentalizing different cell types [95]. Their main composition is based on a laminin and collagen polymer network in addition to other components such as nidogen, perlecan and many more [96]. Nevertheless, BMs show a tissue and organ-specific molecular composition in order to support their specific biological function [96, 97]. They provide structural support and polarity for cells via the linkage of the actin cytoskeleton through adhesion molecules, such as intergins, to the ECM facilitating biological functions such as cell adhesion, migration and signalling [98, 99].

Since 1993, when Adams and Watt demonstrated various developmental defects in response to modifications of ECM composition, the fundamental role of BM during development as well as in adult stem cell homeostasis has been recognized [100].

BMs have been shown to modify signalling not only through direct binding of cell surface receptors but also indirectly via non-canonical presentation of growth factors, such as [97] FGF2, TGFb1, heparin epidermal growth factor (HP-EGF) and vascular endothelial growth factor (VEGF) [101], through which they mediate cell signalling effects [102].

1.3.2.1 Laminin

Laminins are one of the most abundant proteins in the BM, consisting of three laminin chains, α, β, γ, which assemble through a coiled coil to a cross-shaped heterotrimer. So far 16 laminin isoforms have been found in mammals in various combinations of the five α, three β and two γ chains allowing cell type-specific functions such as adhesion, differentiation, migration and phenotype maintenance [103, 104].

The N-terminal region of the α chain consists of three globular domains which are separated by three EGF-like repeats, while the C-terminal region consists of five globules, called the G domain. In contrast, the N-terminal region of β and γ chains consist of only two globular domains separated by two EGF-repeats and no G-domains at the C-terminal region [104, 105]. The heterotrimer assembly of the α, β and γ chains to a long-coiled arm is facilitated through disulfide-bonds at the C-terminal region and the centre, leaving the N-terminal regions of α, β and γ chains free, resulting in three short arms (Figure 4) [104-106].

The assembly of higher-order laminin networks on the other hand, is facilitated by linking the N-terminal region of one α, β and γ chain of three laminin heterotrimers, while the cell-adhesion to transmembrane cell receptor, such as integrins, is facilitated through the G domain

of the C-terminal region, influencing the signalling cascade (see Figure 4 for laminin structure) [105].

1.3.2.2 Type IV collagen

Collagens are triple-helical proteins which consist of three α chains self-assembling into either homo- or heterotrimers [107]. The 28 currently recognized collagen types consist of repeated Gly-X-Y motifs, where X typically comprises proline and Y hydroxyproline [100, 107].

Type IV collagen was first discovered in 1966 by Nicholas Kefalides while studying glomerular BM protein extracts of dogs [108]. It is a non-fibrillar collagen which, in contrast to fibrillar collagens such as type I, II, III, V, XI, XXIV and XXVII collagens, has the ability to self-assemble into networks[107]. Type IV exists in three different heterotrimer forms distinguished by the assembly of six different α chains (α1-α6), while the most common variant consists of two α1 and one α2 subunit. Each of the 400nm long α chains contain a triple helical domain, important for assembly and an N-terminal 7S domain as well as a C-terminal globular domain (NCI), important for network formation [105]. For the heterotrimer assembly of type IV collagen, NCI domains of three α chains interact, followed by zippering of the triple-helical domains resulting in the formation of a promoter. Further for network-assembly, NCI domains of two promoters form dimers followed by cross-linking of 7S domains of four promoters to form a tetramer building up a network-structure which ultimately links to laminin networks through nidogen (see Figure 4 for type IV collagen structure) [95, 105].

1.3.2.3 Nidogens and perlecans

Nidogens are glycoproteins that come in two forms, N1 and N2, and consist of three globular domains which are separated by two rod-like segments. They mainly support BM assembly and stabilization by binding to the short arm of the laminin γ chain as well as the triple-helical region of type IV collagen [105].

Perlecans consist of five domains covalently-linked to three heparan sulfate proteoglycan molecules at the N-terminal region. While the heparan sulfate molecules serve as a growth factor reservoir the five core domains present binding sites for laminins and type IV collagen rendering perlecans a similar function to nidogens [104, 105].

1.3.2.4 Basement membrane assembly

In vivo BM assembly starts with laminin-cell interactions in which the laminin G domains anchor to the cell surface through intergrins, α dystroglycan and sulfate glycolipids. As soon as sufficient laminin heterotrimers are anchored to the cell surface, the short arm of the α, β, γ, chains bind to adjacent chains, forming ternary nodes. Once the laminin network has formed type IV collagen, perlecan, nidogen and argin assemble on the network allowing type IV collagen polymerization to a covalently stabilized second network. Nidogen binds type IV collagen, as well as the γ subunit of laminin, connecting both networks while agrin and perlecan

primarily bind to the coiled-coil domain of laminin and to nidogen as well as cell surface receptors (see Figure 4 for BM assembly) [95].

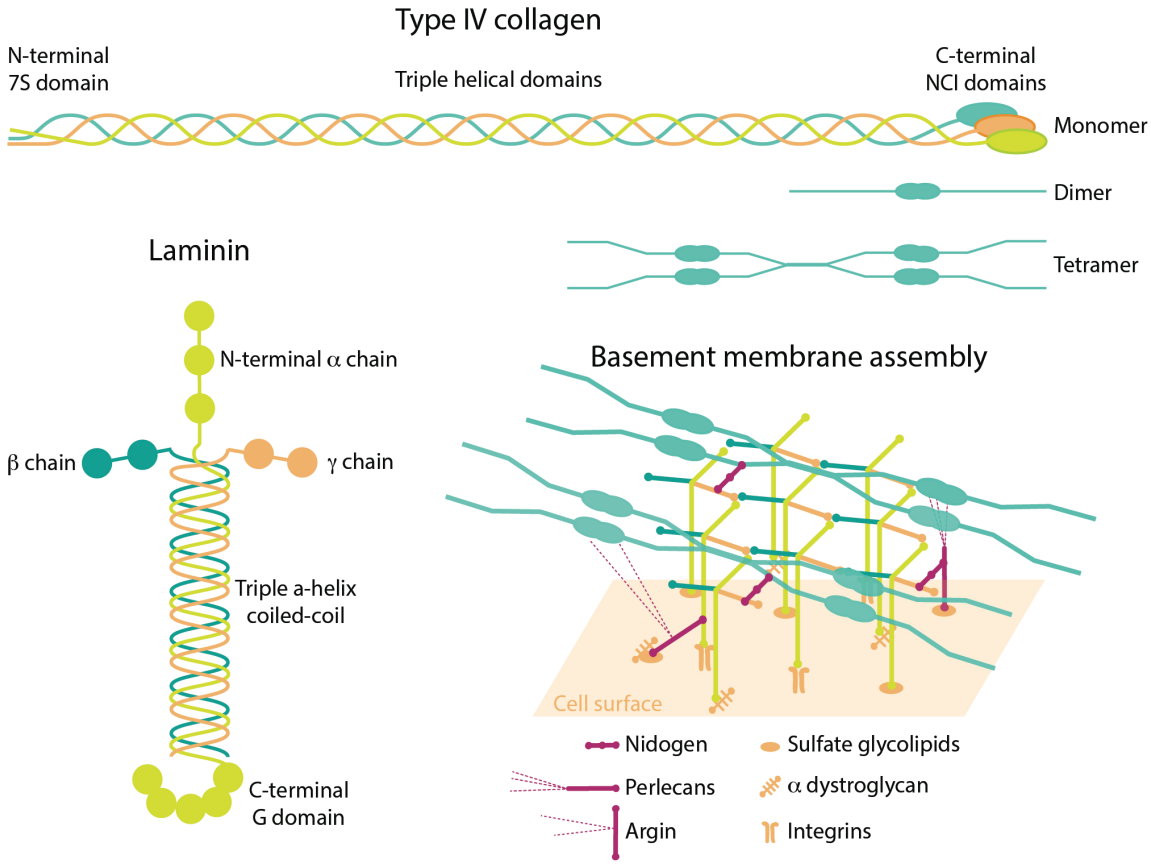

Figure 4: Laminin and type IV collagen domain structure and their basement membrane assembly. Adapted from Hohenester and Yurchenco 2012 CC 3.0 by Landes Bioscience.

1.3.2.5 Cell-extracellular matrix adhesion molecules

Cell adhesion molecules are surface proteins which mediate both cell-ECM interactions, primarily via integrins, as well as cell-cell interactions, mainly via selectins, cadherins and immunoglobulin superfamily members. Integrins, which are the biggest class of cell-ECM adhesion molecules, are heterodimeric transmembrane molecules which comprise of both α chains, participating in ECM binding, and β chains. They not only physically connect cells to the ECM but most importantly initiate cell signalling cascades, resulting in cytoskeletal reorganization, cell growth, survival and differentiation, playing an important role in stem cell fate [100]. While β1 and β3 integrins are expressed on various cell types, and mostly mediate cell-ECM adhesion, β2 integrins are exclusively expressed on white blood cells where they primarily medicate cell-cell interactions [109].

Further, integrins, as well as dystroglycans, are the primary anchorages for laminins as well as collagens and bind in a Ca^{2+} or Mg^{2+} dependent manner [100, 110].

2 AIMS

The aim of this thesis was to investigate the role of laminins in stemness of pluripotent stem cells and in the male testicular stem cell niche environment.

The specific aims were:

- To determine the role of laminin 521 in stemness stabilization between different human embryonic stem cell lines.
- To assess the clinical relevance of laminin 521 Klinefelter syndrome derived human induced pluripotent stem cells.
- To examine the basement membrane composition of the developing male gonad.
- To investigate basement membrane changes during testicular explant culture and its effect on germ cell survival.

3 MATERIAL AND METHODS

3.1 ETHICS

3.1.1 Animal work

Teratoma assays for hiPSCs were performed on severe combined immunodeficiency (SCID)/beige mice with the approval of Stockholm south ethical committee (Dnr. S14-15).

3.1.2 Human samples

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

Skin tissue biopsies from healthy male donors (HM100 31 years, HM200 34 years) and azoospermia patients diagnosed with KS (KS100 31 years, KS200 34 years) were obtained with a written consent and with the approval of the Regional Human Ethics Committee (Dnr 2013/1132-32) at the Reproduktionsmedicin at the Karolinska Hospital Huddinge.

Human male first trimester gonadal tissue ranging from 5 to 10 wpc was collected from elective surgical abortions with maternal written informed consent. Midwives were responsible for informing patients (over 18 years of age and Swedish speaking). The Regional Human Ethics Committee, Stockholm, Sweden, approved the collection (Dnr. 2007/1477-31 with complementary permissions 2011/1101-32, 2013/564-32 and Dnr. 2013/457-31/4).

Ethical approval for the use of additional human first and second trimester gonadal tissue ranging from 9 to 17 wpc was obtained from the South East Scotland Research Ethics Committee (reference number 18/NE/0290) and the NRES committee North East-Newcastle and North Tyneside 1 reference number LREC08/S1101/1). Women gave written informed consent.

Ethical approval for the use of pre- and peripubertal testicular tissue was obtained from the ethical board of Karolinska Institutet and the Reginal Ethics Board in Stockholm (Dnr. 2013-2129-31-3), the National Ethics Board of Iceland, Reykjavik (VSN 15-002) and the Ethics Board of the University of Helsinki (426/13/03/03/2015).

Ethical approval for the use of adult testicular tissue was obtained from the East of Scotland Research Ethics service committee REC Reference number – 15/ES/0094) for the use of archived human testicular tissue from the Pathology Departments at the Western General Hospital in Edinburgh.

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3.2 CELL CULTURE

3.2.1 Primary fibroblast derivation and culture

After removing the dermis of skin biopsies from healthy male donors and azoospermia patients diagnosed with KS, tissue was cut into approximately 1 mm^3 pieces and plated on FBS coated plates with human dermal fibroblast (HDF) medium consisting of Dulbeco's Modified Eagle Medium (DMEM), 10% FBS and 0.5% penicillin-streptomycin (pen-strep). The resulting fibroblast cultures (f-HM1, f-HM2, f-KS1, and f-KS2) were passaged with 0.05% Trypsin.

3.2.2 Human pluripotent stem cell culture

3.2.2.1 Human embryonic stem cell culture

For feeder derived hESC maintenance, hESCs were cultured on mitotically inactivated hFFs in knock-out DMEM containing 20% KSR, 0.5% pen-strep, 2mM L-GlutaMAX, 1% non-essential amino acids, 0.5 mM 2-mercaptoethanol and 8 ng/ml of recombinant FGF2. Cell colonies were passaged mechanically with a scalpel at a four- to six-day interval.

To enable hESC culture on LN521 or LN121-coated plates, LN521 or LN121 was slowly thawed at 4 °C and diluted to a final concentration of 10 µg/ml. Plates were then coated with either LN521 or LN121 overnight at 4 ºC followed by 37 ºC for one hour. hES cells colonies were moved from hFFs to laminin coated plates and cultured in NutriStem medium at 37 ºC in 5% CO2 with a daily media change. Upon confluency, hESCs were passaged as single cells using TrypLE Select every five to seven days.

To enable hESC culture on Matrigel, hESC-qualified Matrigel was slowly thawed overnight on ice at 4 ºC and diluted 1:84 in NutriStem. Plates were then coated with Matrigel at 37 ºC. hES cells colonies were moved from hFFs to Matrigel coated plates and cultured in NutriStem medium at 37 ºC in 5% CO2 with a daily media change. Upon confluency, hESCs were passaged as single cells using TrypLE Select every five to seven days.

3.2.2.2 Reprograming of human induced pluripotent stem cells and subsequent culture

For fibroblast reprogramming $6x10^5$ cells were electroporated with 1 µg of each plasmid; pCXLE-hOCT3/4-shp53, pCXLE-hSK, and pCXLE-hUL, using the 100 µl tip Neon System with 1650 V, 30 ms and 1 pulse settings. Transfected cells were plated onto non-coated cell culture plates with HDF medium for six days in 5% CO_2 at 37 °C. Cells were collected with 0.25% Trypsin and plated onto LN521 or hESC-qualified Matrigel coated plates with a density of $5x10^3$ cells/cm^2 and transferred to low oxygen conditions (5% O_2, 5% CO_2) at 37 °C. The following day, HDF medium was replaced by NutriStem with subsequent medium changes every two to three days. Cell colonies were manually picked and transferred onto new LN521 coated plates 23-40 days after transfection and maintained in NutriStem medium throughout culture. Upon confluency, hiPSCs were passaged as single cells using TrypLE Select. In total five hiPSC lines were derived (ips-HM1, ips-HM2, ips-KS1, ips-KS2 and ips-HF1) (see Figure 5 for hiPSC reprogramming schema).

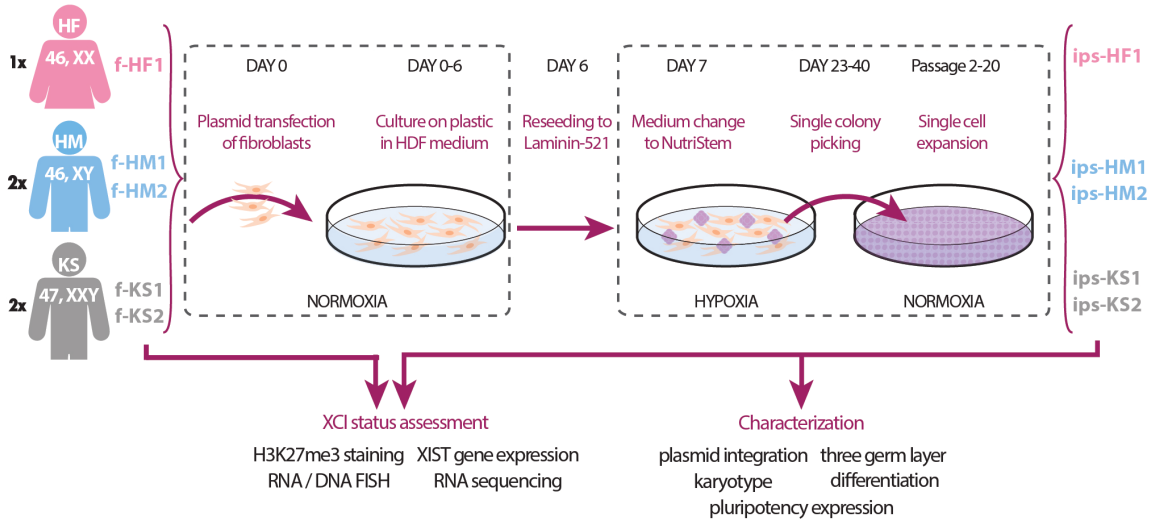

Figure 5: hiPSCs reprogramming, culture and analysis schema. Adapted from Panula et al. 2019 with permission from Oxford University Press.

3.2.3 Human pluripotent stem cell differentiation

Confluent hiPSCs were detached as small cell clumps and plated onto ultra-low adhesion plates with NutriStem medium without growth factors containing 10 µM ROCK-inhibitor Y-27632 for the first 24 h with subsequent media changes every two to three days. After two weeks the formed embryoid bodies were plated for an additional two weeks onto LN521 coated glass chamber slides and after a total culture of four weeks, the cells were fixed with 4% paraformaldehyde (PFA).

3.2.4 Prenatal tissue explant culture

First trimester male gonadal tissue samples ranging from 6-9 wpc were cultured using a testicular explant culture system established by Sato and colleagues [85]. The whole gonad was cut into three pieces and each piece cultured on top of a 0.35% agarose block in an air-liquid interface. The tissue pieces were cultured in NutriStem with 10% KSR and 1% pen-strep for 14 days with a media change at day 7 of culture. At day 14 of culture, tissues were fixed in 4% PFA and paraffin embedded.

3.2.5 Pre-and peripubertal tissue explant culture

Pre- and peripubertal testicular tissue samples from patients ranging from 1.6 to 13.4 years were cultured using above mentioned testicular explant culture system. Small tissue pieces were cultured on top of an 0.35% agarose block in an air-liquid interface. The tissue pieces were cultured in NutriStem with 10% KSR, 1% pen-strep and 10^{-7} M melatonin for 14 days with a media change at day 7 of culture. At day 7 and 14 of culture, tissues were fixed in 4% PFA and paraffin embedded.

3.3 TERATOMA ASSAY

Confluent hiPSCs were detached as small cell clumps and plated onto ultra-low adhesion plates with NutriStem medium containing 10 μM ROCK-inhibitor Y-27632 for 24 hours. Sphere suspension was then mixed with hESC-qualified Matrigel and injected subcutaneously into SCID/beige mice with approximately $1x10^6$ cells/injection. Mice were sacrificed and tumours collected three to eight weeks after injection. Tumours were fixed in 4% PFA and paraffin embedded cross-sections stained with Hematoxylin and Eosin.

3.4 PROTEIN EXPRESSION

3.4.1 Immunocytochemistry

For hiPSC characterisation, cells were fixed with 4% PFA and permeabilized with 0.03% Triton X-100 in phosphate buffered saline (PBS). Cells were blocked in PBS containing donkey serum and bovine serum albumin (BSA) for 1 h and incubated with primary antibodies in blocking buffer at 4 °C overnight. After washing, cells were incubated with secondary antibodies in blocking buffer for 1 h at room temperature and counterstained with 1 μg/ml DAPI. hESC and hiPSCs were imaged with a confocal microscope (LSM700, Zeiss).

3.4.2 Immunohistochemistry

Paraffin embedded testicular tissue samples were cut into 5 μm thick sections, de-paraffinized and rehydrated in xylene and a descending ethanol series. Sections were subjected to heat-mediated antigen retrieval at 95 ºC in either 10 mM sodium citrate or 10 mM Tris base and 1 mM EDTA solution with 0.05% Tween 20 at a pH 9. Subsequently, samples were blocked with donkey serum and BSA before overnight incubation at 4 ºC with primary antibodies. Finally, samples were incubated with fluorescence conjugated secondary antibodies at room temperature before mounting with DAPI containing anti-fade mounting medium.

3.4.3 Alkaline phosphatase

For hiPSC colony counting in reprogramming conditions, cells were fixed with 4% PFA, washed with 0.05% Tween-20 in Tris-buffered saline and stained using an Alkaline Phosphatase (AP) Detection Kit. Positive colonies were counted and imaged with an Olympus IX81.

3.5 CELL QUANTIFICATION

Quantification of POU5F1, DDX4 and MAGE-A4 positive germ cells and evaluation of seminiferous cord area as well as round seminiferous tubule was performed each on one tissue section of the whole gonad as well as one tissue section of the cultured prenatal and pre- and peripubertal tissue cultures. Results are shown as mean ± standard deviation (SD).

3.6 DNA ANALYSIS

3.6.1 Plasmid copy number

For plasmid copy number analysis, genomic DNA was collected from hiPSCs using the DNeasy Blood and Tissue kit and analysed by quantitative PCR using SYBR Green. The pCXLE-EGFP plasmid and gDNA from a hESC line (H9) were used to generate standard curves to determine the plasmid or gene copy numbers from the threshold cycle (Ct) values. Primers for Epstein-Barr virus nuclear antigen 1 (*EBNA-1*) were used to detect plasmid copies and F-box protein 15 (*FBXO15*) primers were used as genomic copy number control.

3.6.2 Karyotyping

hiPSCs were treated with 10 μg/ml colcemid, collected with TrypLE Select and centrifuged. The cell pellet was resuspended in a 0.4% potassium chloride solution and incubated with methanol:acetic acid (3:1). Chromosomal G-band analysis was performed at the Genetics Clinic at Skåne University hospital, Sweden or at Karolinska University hospital, Sweden and at least 25 metaphases were analysed.

3.6.3 FISH

For single-molecule RNA FISH, fibroblasts and their respective hiPSCs were cultured on chamber slides, fixed with 4% PFA and permeabilized with methanol at -20°C. After air-drying the cells were incubated at 70 °C in Tris-EDTA buffer at a pH 8.0 and washed with saline-sodium citrate buffer. Cells were hybridized with FISH probes for human *XIST* and human *ATRX* at 38.5 °C in hybridization buffer containing 10% deionized formamide, 10% dextran sulfate, 2 mg/ml tRNA from E. coli, 2 mM ribonucleoside vanadyl complex, 0.2 mg/ml BSA in saline-sodium citrate buffer. After washing cells with wash buffer containing 1 μg/ml Hoechst 33342 at 38.5 °C, cells were mounted with ProLong Diamond Antifade Mountant. Image stacks were acquired using Nikon Ti-E motorized inverted microscope.

DNA FISH was performed on the same chamber slides. After removing the coverslips, cells were re-fixed with 4% PFA and dehydrated in ethanol followed by brief air-drying. Cells were treated with 100 μg/ml RNaseA at 37 °C followed by incubation with 10% pepsin in 0.01M HCl at 37 °C. Cells were then re-fixed with 1% PFA and dehydrated in ethanol and air-dried. Probe mixture consisting of Vysis CEP X (DXZ1) and Vysis CEP Y (DYZ1) probes and CEP hybridization buffer was applied onto slide with a coverslip and sealed with Fastik. Samples and probes were then co-denaturated at 85 °C followed by overnight hybridization at 41 °C in a humidity chamber. Slides were washed, cells counterstained with 1 μg/ml Hoechst 33342 and mounted with ProLong Diamond Antifade Mountant. Image stacks were acquired using Nikon Ti-E motorized inverted microscope.

3.7 GENE EXPRESSION

3.7.1 qPCR

For gene expression analysis, total RNA of three biological replicates of hESCs and three consecutive passages of fibroblasts and their respective hiPSCs was extracted using the RNeasy Mini Kit with on-column DNaseI treatment. Reverse transcription (RT) of RNA to cDNA was performed using iScript cDNA synthesis kit. Gene expression was quantified using TaqMan assays and StepOnePlus Real-Time PCR System. The relative quantity was determined with comparative Ct method ($2^{-\Delta\Delta Ct}$), using *GAPDH* as an endogenous control and hESCs (HS980) or f-HF1 as reference sample for hiPSCs.

To evaluate variations in genes expression among hESCs the coefficient of variation (CV) of each gene was obtained by calculating the ratio between the SD and the mean value of all the cell lines together. Based on the CV value, variation was classified into four groups: I) up to 10%, II) 11–25%, III) 26–50%, and IV) more than 50%.

3.7.2 RNA sequencing

Total RNA was extracted using the RNeasy Mini Kit with on-column DNaseI treatment from three consecutive passages of fibroblasts and their respective hiPSCs. cDNA library preparation and sequencing were performed at the *Science for Life Laboratory, Sweden. Briefly, libraries were made using Illumina TruSeq® Stranded mRNA with poly-A selection.* A total of 2 pools were made from the 33 samples and each sample pool was sequenced on 2 lanes using Illumina HiSeq 2500 with 2x125 setup. Initial quality control of generated paired-end reads was performed using the FastQC software.

The reference index-based algorithm HISAT 2.1.0 was used to align reads with human reference genome (GRCh37). The resulting BAM files were examined by MultiQC (version 1.0) and then used to estimate transcript abundance (FPKM; **F**ragments **P**er **K**ilobase of exon per **M**illion fragments mapped) values using Cufflinks. Pearson Product-Moment Correlation coefficient was calculated to examine closeness among replicates and one replicate (f-HM100 p8) was excluded due to low correlation. Quantified expression values (FPKM) were imported into R (version 3.4.3), and principal component analysis (PCA) was conducted with top 10k highly expressing genes.

Cuffdiff v2.2.1 was used to identify differentially expressed genes. The transcripts with p value < 0.05 & fold change ≥ 1.5 were considered to be significantly expressed.

Sample specific Single Nucleotide Polymorphisms (SNPs) for chromosome X were identified using 'mpileup2snp' subcommand of VarScan2 with p value 0.5. Only uniquely mapped reads were considered (k=1). SNP locations with ≥ 30 reads were considered in downstream analysis. Informative SNPs for each donor were obtained by combining SNPs with ≥ 5 reads on minor allele in fibroblast replicates and in corresponding hiPSC replicates (f-HM1/ips-HM1: 436, f-HM2/ips-HM2: 368, f-KS1/ips-KS1: 880, f-KS2/ips-KS2: 725, f-HF1/ips-HF1: 920, HS980: 783). SNPs were considered bi-allelic/with variation when 20-

80% of reads corresponded to reference allele, and mono-allelic/no variation when <20% or >80% of reads corresponded to reference allele. Further, only transcripts with all SNPs showing either bi-allelic or mono-allelic status in all replicates were considered informative.

3.7.3 Foetal single-cell RNA sequencing data processing

The single-cell RNA-seq data of human foetal germ cells (FGCs) and their gonadal niche cells were downloaded from https://github.com/zorrodong/germcell [111]. Only male samples (1,079 of 2,210 cells) were selected, and the expression values on transcripts per million (TPM) were normalized by log2 (TPM/10+1). A total of 17,633 genes with expression levels higher than 1 that were expressed in over 10 single cell samples were analysed using the R (version 3.5.1) package Seurat (version 2.3.4) [112]. t-distributed stochastic neighbour embedding (t-SNE) dimensionality reduction was carried out following the protocol of Li L et al. [111] in which male and female samples were analysed collectively. First, 360 highly variable genes with average expression greater than 2 and dispersion more than 2 were selected using the 'FindVariableGenes' function and the scaled data were applied for PCA. Then, significant PCs were determined by the jackstraw procedure with 1,000 replicates. Here PC 1-12 were selected as significant (p < 1e-5), and t-SNE was performed using these PCs with 'RunTSNE' function. Finally, the resulting clusters were compared with Li L et al. [111], and annotated based on their classification. Even though those cells in the endothelial cell cluster were classified as outliers or into other clusters, 1,035 of 1,072 cells (96.5%) were consistently clustered. Gene expression overlays onto the t-SNE plots were performed using the 'FeaturePlot' function modified with ggplot2 (version 3.1.0).

3.8 STATISTICAL ANALYSIS

Statistical significance of reprograming efficiency was tested with unpaired t-test, n=3. Significance of pluripotency expression, *XIST* expression and total X chromosome expression for fibroblasts and their hiPSCs was assessed by one-way ANOVA using SigmaPlot 13.0 and significance accepted at $p < 0.05$.

For prenatal germ cell quantification Kruskal-Wallis one-way ANOVA on Ranks was performed to confirm the statistical significance between first and second trimester germ cells quantity. For pre- and peripubertal germ cell quantification Kruskal-Wallis one-way ANOVA on Ranks and for laminin alpha 1 (LAMA1) quantification one-way ANOVA was performed to confirm the statistical significance between control and culture conditions. Analyses were performed with SigmaPlot 13.0 software and significance accepted at $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 STABILIZING EFFECT OF LAMININ 521 ON HUMAN EMBRYONIC STEM CELLS (PAPER I)

LN521 is expressed by and supports pluripotent cells of the ICM, thus it has been established as PSC culture substrate, especially for single-cell expansion culture. Although many studies have shown the positive effect on cell proliferation, as well as long term stem cell maintenance on LN521 substrates, we aimed to evaluate the effect of LN521 on gene expression profiles of hPSC. Therefore, we investigated the short-term effect of LN521 on gene expression profiles of five hFF derived hESC lines (HS360, HS364, HS380, HS401 and HS420) in comparison to traditional hFFs, as well as LN121 and Matrigel, as alternative culture substrates.

4.1.1 Similar expression profile upon NutriStem vs. DMEM culture

As the five hFF derived hESCs lines were originally cultured in growth factor containing DMEM medium and feeder-free culture adaption was planned with NutriStem growth factor containing medium, we wanted to exclude expression profile changes due to media variation first. Therefore, gene expression profiles of 17 pluripotency, stemness and gonadal genes (*NANOG, GDF3, SOX2, POU5F1, NODAL, LEFTB, EBAF, TDGF1, KIT, UTF1, DDX4, SOX9, CYP11, SCF, SF1, StAR*) were compared. As a similar expression pattern could be observed when comparing hFF derived hESCs cultured in either DMEM or NutriStem for four passages, we concluded a lack of media influence on our following experiments.

4.1.2 Laminin 521 has superior cell support compared to laminin 121 and Matrigel

In order to investigate the effect of various feeder-free culture substrates on hESC maintenance, two hESC lines (HS360 and HS380) were transferred from hFF to either LN521, LN121 or Matrigel coated plates for up to nine passages. Although hESC qualified Matrigel is often utilized for feeder-free PSC cultures, a higher incidence of cell differentiation and changes of stemness expression profile could be observed for Matrigel as well as LN121 cultures compared to LN521. Furthermore, LN521 showed superior support of cell adhesion and proliferation rate compared to LN121 and Matrigel, which became even more distinct with increased culture period. Although hESCs express LN521, LN121, LN511 and LN111 [113], poor cell survival of hESC was shown on LN121 and Matrigel in the absence of ROCK inhibitor [20]. As hPSC express α6β1 integrin, which has its highest binding affinity to laminin α5 chains [114], this could explain the inferior cell-adhesion capacities of LN121 and Matrigel, which contain LN111, in comparison to LN521. Moreover, LN521-α6β1 integrin interactions have been shown to induce the PI3K/Akt pathway, supporting self-renewal and pluripotency expression [20, 115, 116].

4.1.3 Laminin 521 homogenizes the pluripotency expression pattern of five human embryonic stem cell lines

To analyse the effect of LN521 culture on hFF derived hESCs, all five hESC lines (HS360, HS364, HS380, HS401 and HS420) were transferred to LN521 and cultured for up to nine passages. Furthermore, variation in gene expression profiles between all five hESC lines was evaluated by comparison of the coefficient of variance, determined by the ratio of SD to mean expression value of all five cell lines.

When comparing the gene expression profile of long term hFF cultured hESCs, a gene expression variation of more than 50% in 14 out of 17 genes could be observed between all five hESC lines, compared to 11 genes in early passage four LN521 cultures. The reduced gene expression variation in LN521 cultures became even more pronounced at passage nine, demonstrated by only seven out of 17 genes with more than 50% gene variation. Although, stemness genes such as *NODAL, LEFTB, EBAF, TDGF1, UTF1*, and *LIN28* showed persistent gene expression variation of more than 50% when comparing passage four to nine, pluripotency genes such as *NANOG, GDF3, POU5F1* and *SOX2* showed a reduction in gene expression variation between all five hESC lines. Specifically, *NANOG* and *GDF3* showed reduced variation from 54% and 66% to 34% and 28% respectively, *POU5F1* and *SOX2* from 32% and 26% to 21% and 20% from passage four to passage nine, suggestive of a gene homogenizing effect of LN521 between different hESC lines.

Although comparison of 59 hESC lines, from different derivation and maintenance techniques, from 17 different laboratories has revealed largely similar expression profiles, gene variations could be observed in *POU5F1* and *SOX2* [117]. Therefore, comparison of long-term LN521 cultured hESC should be evaluated to explore further beneficial effects which could lead to less variability among different stem cell lines and more homogeneous results in *in vitro* differentiation experiments.

4.2 LAMININ 521 AND X CHROMOSOME INACTIVATION OF KLINEFELTER SYNDROME DERIVED HUMAN INDUCED PLURIPOTENT STEM CELLS (PAPER II)

Since LN521 has shown beneficial pluripotency and cell adhesion effects on hFFs derived hESCs we further wanted to evaluate LN521 culture substrate for the reprogramming of healthy male (f-HM1, f-HM1) and female (f-HF-1), as well as KS dermal fibroblasts (f-KS1, f-KS2). Therefore, fibroblast cultures from skin biopsies and one commercially available female fibroblast line were established and transfection with episomal plasmids (pCXLE-356 hOCT3/4-shp53, pCXLE-hSK and pCXLE-hUL) performed.

4.2.1 Efficient derivation of human induced pluripotent stem cells on laminin 521

To investigate the reprogramming efficiency on LN521 or Matrigel, transfected fibroblasts were plated on respective plates 6 days after transfection and AP positive staining evaluated after 15 days of culture. Reprogramming efficiency was calculated, whereby LN521 cultures showed a significantly higher reprogramming efficiency than Matrigel cultures: 0.52% vs. 0.36% ($p < 0.05$) respectively. Comparing this efficiency to the original report utilizing mouse feeder cells [118] with an efficiency of 0.39%, or laminin-511 E8 fragments with an efficiency of 0.077% [119], we could show the beneficial support of LN521 which is likely attributed to induction of pluripotency pathways of LN521-α6β1 integrin interactions [20, 115, 116].

Nevertheless, in order to achieve high reprogramming efficiency, an optimal combination of culture substrate and reprogramming factors is required, as the use of LN521 in combination with lentiviral vectors could only achieve an efficiency of 0.3% [120].

After 23 to 40 days post-transfection, reprogrammed cell colonies were manually picked and slowly adapted to single-cell enzymatic passaging on LN521 coated culture plates with loss of episomal plasmids confirmed at passage five in all five hiPSC lines (ips-HM1, ips-HM2, ips-KS1, ips-KS2 and ips-HF1). In addition, cell lines with normal karyotype were cultured up to 20 passages on LN521 coated plates and pluripotency protein and gene expression, as well as the potential for three germ layer formation *in vivo* and *in vitro,* confirmed.

In line with hFF derived hESC cultures on LN521, no significant difference in pluripotency gene expression of *POU5F1, NANOG, SOX2* and *GDF3* could be observed in all five hiPSCs lines.

4.2.2 X chromosome inactivation status of Klinefelter syndrome derived human induced pluripotent stem cells

In order to investigate the XCI status of KS derived hiPSCs, three consecutive passages of KS dermal fibroblasts from two azoospermic men and their respective hiPSCs were evaluated.

In alignment with the normal *in vivo* situation no *XIST* expression could be observed in f-HM1 and f-HM2 while f-KS1 and f-KS2 showed similar expression profiles to f-HF1, indicating normal XCI. Furthermore, RNA FISH for *XIST* showed no cloud in both HM fibroblasts while

the presence of one *XIST* in around 90% of KS (f-KS1 88% and f-KS2 94%) and HF (f-HF 95%) fibroblast could be observed, confirming normal XCI. Although a few KS and HF fibroblast showed the presence of two *XIST* clouds, DNA FISH of the same slides confirmed tetraploid cells which have shown to spontaneously occur in fibroblast cultures [121, 122].

Although *XIST* expression of KS derived hiPSCs (ips-KS1 and ips-KS2) and female hiPSCs (ips-HF1) showed no significant difference to f-HF1, H3K27me3 staining and *XIST* RNA FISH showed a variable XCI status. KS and female hiPSC lines displayed two cell populations showing either one H3K27me3 or *XIST* accumulation (*XIST*: ips-KS1 5%, ips-KS2 66%, ips-HF1 6%) or no H3K27me3 or *XIST* accumulation (*XIST*: ips-KS1 95%, ips-KS2 34%, ips-HF1 94%). As expected, male hiPSCs (ips-HM1 and ips-HM2) showed no H3K27me3 and *XIST* accumulation, while all hiPSCs displayed only a mono-allelic *ATRX* transcription site.

When analysing transcriptomic data, similar levels of total X-linked gene expression (sum of FPKM values for X-linked genes) could be observed for one KS hiPSCs line (ips-KS2) compared to HM, HF and KS fibroblasts as well as HM hiPSCs. Interestingly, ips-KS1 and ips-HF1 showed significantly ($p < 0.05$) increased expression levels compared to HM and KS fibroblasts, HM hiPSCs and ips-KS2, indicating XCI erosion.

Mono- or bi-allelic expression of X linked genes was additionally evaluated by analysing SNP variations of our RNA sequencing data against a reference genome. Based on bi-allelic observations in HM fibroblasts and hiPSCs (of those seven have a known Y homologue) 12 unique transcripts were considered false positive and excluded. The majority of X-linked transcripts of ips-KS2 showed a mono-allelic (33) expression while six bi-allelic transcripts consisted of four known XCI escapee genes and two genes of unknown status. In contrast, ips-KS1 and ips-HF1 showed a low number of mono-allelic transcripts (ips-KS1 25, ips-HF1 29) and a high number of bi-allelic transcripts (ips-KS1 50, ips-HF1 28) which included many known XCI inactive genes (ips-KS1 27, ips-HF1 14). As those results indicate an eroded XaXe XCI state for ips-KS1 and a normal XaXi XCI state for ips-KS2, DEG comparison to HM hiPSC was performed and revealed 24 X-linked genes out of 111 upregulated genes for ips-KS2, while ips-KS1 showed 123 X-linked genes out of 192 upregulated genes, supporting the XaXe status of ips-KS1 and the XaXi status of ips-KS2.

In alignment with previous studies showing various XCI states (either XaXa with both active X chromosomes, XaXi with one X chromosome inactive and covered by *XIST* or XaXe with loss of *XIST* and only partial persistence of inactivation) in long-term female hPSC cultures [123-126] our KS derived hiPSC lines display a similar behaviour to female hPSC. Although this phenomenon was mostly observed in studies applying MEF or Matrigel for hPSCs cultures, our LN521 cultures show similar behaviour, suggesting an independency of the applied culture substrate. Nevertheless, cultures under physiological oxygen conditions of 5% have shown to reduce X chromosome erosion [127] indicating a potential culture condition effect on this phenomenon.

4.3 COMPOSITION OF LAMININS IN THE DEVELOPING AND ADULT GONAD (PAPER III)

Since LN521 has shown to play a crucial role in PSC self-renewal and pluripotency expression we wanted to evaluate the laminin expression profile of the testicular stem cell niche. Therefore, prenatal male gonads ranging from 5–17 wpc, pre- and peripubertal as well as adult testicular tissues were examined in regard to their BM composition.

4.3.1 Prenatal basement membrane composition

In order to investigate the BM composition immunofluorescence staining for all five α LN chains, fibronectin, as well as the type I, IV and VI collagens was performed. Depending on the developmental state of seminiferous cords, a net-like ECM structure of fibronectin, as well as type I, IV and VI collagens could be observed in 5 and 6 wpc gonads, in the absence of seminiferous cord formation. Upon presence of seminiferous cords, fibronectin, as well as type I, IV and VI collagens, could be observed in the BM of seminiferous cords of 5 wpc to 17 wpc gonads. Furthermore, the expression of all three collagen types could be observed throughout the interstitial compartment as well as blood vessels.

Once seminiferous cords were formed, around 5-6 wpc, laminin α 1 (LAMA 1) expression could be observed as the sole LAMA chain in the seminiferous BM throughout all subsequent developmental stages examined. Interestingly, LAMA 5 could be observed from 7 wpc onwards in a few vascular BMs. In contrast LAMA 2, LAMA 3 and LAMA 4 were absent in the BM of both the seminiferous cords and the interstitial compartment with the exception of some occasional vasculature staining. Although vascular expression of LAMA 4 and 5 could be observed only in some vessels, variation of vascular laminin expression occurs based on the endothelial cell type, activation and vessel growth state, potentially explaining [128, 129] the expression of type IV collagen in all vessels compared to LAMA 4 and 5 in some vessels.

Due to limited access to fresh male gonadal samples, published single-cell transcriptomic data from male prenatal gonadal germ and somatic cells was reanalysed to investigate the BM gene expression dynamics[111]. Based on the single-cell expression profile, 10 clusters could be identified in t-SNE analysis: Leydig cell precursors, differentiated Leydig cells, Sertoli cells, macrophages and early T cells, erythrocytes, migrating, mitotic as well as mitotic arrest FGCs, FGCs contaminated by haemocytes and outliers. Looking at the expression of Sertoli cells, which are in direct contact with the seminiferous BM, we could observe high expression of type IV collagen (*COL4A1*) and *LAMA 1* and no or low expression of the remaining LAMA chains, as well as fibronectin (*FN1*) and type I (*COL1A1*) and VI collagens (*COL6A1*). Leydig cell precursors, situated in the interstitial compartment, showed expression of *COL1A1* and *COL6A1* while only low expression of *COL4A1*, *LAMA 2* and *LAMA 4* could be observed. In regards to germ cell clusters, all four clusters showed expression of *COL4A1* as well as *LAMA 1*, indicating that Sertoli and germ cells are contributing to the establishment of the seminiferous BM during prenatal life with the deposition of primarily LAMA 1 and type IV collagen.

Considering the structure of the mature adult testis, cell type specific prenatal gene expression patterns appear to be in alignment with the prospective adult BM organization, with Sertoli and germ cell expression of *COL4A1* and *LAMA 1* contributing to the deposition at the innermost lamina, and Leydig cells expression of *COL1A1* and *COL6A1*, *FN1, LAMA 2* and *LAMA 4* contributing to the deposition at the peritubular lamina as well as the vascular BM.

4.3.2 Pre- and peripubertal basement membrane composition

Since healthy pre-and peripubertal testicular tissue is difficult to obtain, analysis was performed on pre-and peripubertal tissue originating from the NORDFERTIL initiative for fertility preservation in male childhood cancer patients. For the evaluation of "normal" pre-and peripubertal BM composition samples of untreated and non-alkylating agents treated patients were evaluated. As those samples have been shown to have equivalent germ cell counts to a reported reference range and biobank controls [130], tissue samples of those pre- and peripubertal patient could be pooled, respectively.

Similar to prenatal gonads, type IV collagen and fibronectin showed protein expression in the interstitial compartment as well as the seminiferous BM. Furthermore, LAMA 1 showed sole expression in the seminiferous BM while LAMA 5 was restricted to the vascular BM.

4.3.3 Adult basement membrane composition

For the evaluation of the established BM composition in adulthood, human adult testicular tissue from three individuals was evaluated. In contrast to prenatal and pre-and peripubertal samples, the adult testicular BMs showed more organization and compartmentalization of the seminiferous BM. Although all three collagen types could be observed, type I and VI collagen were expressed in the vascular BM as well as the collagenous and peritubular layer of the seminiferous BM, while type IV collagen was expressed in the vascular BM as well as the basal lamina and peritubular layer of the seminiferous BM. Fibronectin showed a similar expression to type I and VI collagen at the collagenous and peritubular layer of the seminiferous BM, as well as the vascular BM.

Similar to prenatal, as well as pre- and peri-pubertal testicular samples, LAMA 1 showed sole expression in the seminiferous BM, with restriction to the basal lamina, while LAMA 5 persisted in the vascular BM. In agreement with previous studies [131, 132] no expression of LAMA 3 could be observed, however LAMA 2 and LAMA 4 expression was detected in the vascular BM, the interstitial compartment, as well as the peritubular layer of the seminiferous BM. In contrast to the broad seminiferous BM expression of LAMA 1, 2, 4 and 5 in mice [133], the human seminiferous BM appears more selective, with sole LAMA 1 expression throughout development and restriction to the innermost layer with direct contact to Sertoli cells and spermatogonia in adult testis, while LAMA 2 and 4 show peritubular expression, spatially separated to spermatogonia and Sertoli cell.

Although no endothelial cluster could be identified in the prenatal single-cell transcriptomic data, the peritubular type I, IV and VI collagen, fibronectin and LAMA 2 and 4 protein

expression likely indicates the contribution of peritubular cells in interstitial and seminiferous BM deposition, in line with mass spectrometry analysis of cultured adult human peritubular cells [74].

Interestingly, LAMA 2 showed further expression in early meiotic spermatocytes, while LAMA 4 could be observed as punctuate staining in spermatocytes. Disruption of Sertoli cell LAMA 2 expression has shown tight-junction perturbation in *in vitro* rodent experiments, accrediting blood-testis-barrier regulator effects to LAMA 2 [134-136]. As blood-barrier formation is initiated upon Sertoli cell maturation in puberty [47, 70] and no LAMA 2 expression could be observed in prenatal as well as pre-and peripubertal samples, LAMA 2 might play a critical blood-testis-barrier role in the adult human testis, similar to rodents. Nevertheless, further investigations are required to confirm the involvement and mechanism of LAMA 2 in the blood-testis-barrier.

4.4 EFFECT OF LAMININ BASEMENT MEMBRANE DISRUPTION ON GERM CELL SURVIVAL

In order to evaluate BM maintenance during gonadal and testicular organ culture, LAMA, type IV collagen and fibronectin protein expression was evaluated in prenatal as well as pre- and peripubertal samples after 14 days of tissue explant culture. Changes in germ cell expression patterns of POU5F1, DDX4 and MAGE-A4 were additionally evaluated in prenatal as well as pre- and peripubertal *in vivo* samples and compared to 14 days tissue explant cultures.

4.4.1 *In vivo* germ cell expression profile

In agreement with previous studies[137, 138], the presence of POU5F1, DDX4 and MAGE-A4 positive germ cells could be observed from 5 wpc. As prenatal samples have not yet fully developed seminiferous tubules and thereby do not show round tubules in cross-sections, positive germ cells were evaluated from seminiferous cords and calculated per tubular surface area. POU5F1 positive cells were present from 5 wpc (0.61 ±0.12 $POU5F1^{+ve}/mm^2$ of gonad area) with a peak at 9-10 wpc (1.12 ±0.83 $POU5F1^{+ve}/mm^2$ seminiferous cord area) and a steady decrease until 16-17 wpc (0.24 ±0.17 $POU5F1^{+ve}/mm^2$ seminiferous cord area). DDX4 positive cells were observed from 5 wpc (0.56 ±0.14 $DDX4^{+ve}/mm^2$ of gonad area) with a peak at 9-10 wpc (1.38 ±1.27 $DDX4^{+ve}/mm^2$ seminiferous cord area) and a slight decrease until 16-17 wpc (1.04 ±0.38 $DDX4^{+ve}/mm^2$ seminiferous cord area). On the contrary, only few MAGE-A4 positive cells were expressed from 5 wpc (0.01 ±0.01 MAGE $A4^{+ve}/mm^2$ of gonad area) and steadily increased until 16-17 wpc (0.28 ±0.21 MAGE-$A4^{+ve}/mm^2$ seminiferous cord area).

As no POU5F1 staining could be observed in pre- and peripubertal samples and MAGE-A4 marks (pre) spermatogonia only DDX4 evaluation was performed for pre- and peripubertal tissue to cover a broad germ cell type range. As pre- and peripubertal samples already displayed round seminiferous tubules, positive germ cell numbers were calculated per round tubules (1.55 ±1.75 $DDX4^{+ve}$/round seminiferous tubules).

4.4.2 Disruption of laminin expression during culture

Although 14 day cultured prenatal male gonads were viable and showed a similar seminiferous BM profile, expressing LAMA 1, type I, IV and VI collagen and fibronectin similar to *in vivo* age-matched controls, LAMA 5 expression was additionally observed in the seminiferous BM, indicating poor cellular support of the applied culture conditions.

Pre- and peripubertal cultures similarly showed seminiferous BM expression of type IV collagen and fibronectin with weak seminiferous LAMA 5 expression in a few samples. Surprisingly, pre- and peripubertal cultures showed a significant decrease of seminiferous LAMA 1 expression at day 7 and 14 of culture (D0 78.4% ±33.5 vs. D7 36.9% ±35.9 and D14 15.5% ±28.5 LAMA 1^{+ve} seminiferous tubules, $p < 0.001$).

4.4.3 Germ cell loss during organ culture

The presence of POU5F1, DDX4 and MAGE-A4 positive germ cells could be observed after 14 days of organ culture in prenatal gonads similar to *in vivo* age-matched controls. Although a slight decrease in DDX4 and increase in MAGE-A4 could be observed compared to age-matched *in vivo* controls further cultures are needed for proper comparison.

In contrast, pre- and peripubertal testicular cultures showed a significant decrease in DDX4 positive (D0 1.54 ±1.75 $DDX4^{+ve}$/round seminiferous tubules vs. D14 0.31 ±0.62 $DDX4^{+ve/}$round seminiferous tubules; $p < 0.001$) germ cell counts with culture time with complete germ cell loss in most samples at day 14 of culture.

Although prenatal gonadal cultures showed disruption of seminiferous BM expression with the additional presence of LAMA 5, the remaining expression of LAMA 1 as well as the lack of germ cell translocation to basal lamina at prenatal age could explain the minor effects on germ cell number. In contrast, the germ cell loss accompanied by the loss of LAMA 1 expression in pre- and peripubertal cultures, is indicative of the crucial role of LAMA 1 in germ cell signalling and maintenance and maintenance of the testicular stem cell niche homeostasis. Given collagen is capable of integrin interaction [100, 110] the significant germ cell loss upon LAMA 1 downregulation may not only be due to a loss of adhesion points but a result of disrupted LAMA 1-integrin mediated intracellular signalling as Sertoli cells still maintain within the seminiferous tubule. Furthermore, adverse effects of LAMA 1 loss on Sertoli cell signalling leading to disruption of germ cell maintenance cannot be excluded.

Therefore, further investigations of LAMA 1-gem cell and LAMA 1-Sertoli cell interaction and signalling are needed to achieve a better understanding of the key role of LAMA 1 homeostasis.

5 CONCLUSIONS

In this thesis, we investigated the effects of laminins on stemness and maintenance of α6β1 integrin expressing stem cells such as hESCs, hiPSCs and SSCs. Thus, the pluripotency of five hFF derived hESC lines was evaluated upon short-term LN521 culture. Furthermore, hiPSC reprogramming of KS fibroblasts was optimized using LN521 as culture substrate and XCI status compared to female hiPSCs. Finally, testicular laminin composition was evaluated during gonadal development and its importance for SSC maintenance evaluated.

The main conclusions are listed below.

- LN521 supports cell adhesion and hPSC maintanance of hFF derived hESCs in comparison to LN121 and Matrigel.
- LN521 homogenizes pluripotency expression profiles between hESCs lines even during short-time culture and could therefore be used to homogenise stem cell populations for more reliable differentiation protocols.
- LN521 in combination with NutriStem medium significantly improves reprogramming efficiency of hiPSCs using integration-free episomal plasmids and supports similar pluripotency behaviour.
- KS derived hiPSCs display similar XCI characteristics to female hPSCs during culture, observed by either the maintenance of XCI with a XaXi state or erosion of XCI with XaXe state. In addition, LN521 culture suggests no beneficial effect on XCI maintenanace.
- Although LN521 has shown to be a key component of the hPSC niche, LAMA 5 is only expressed in the vasculature of the developing and adult human testis.
- LAMA 1 is the sole LAMA chain expressed in the seminiferous BM of prenatal as well as pre-and peripubertal testis. Furthermore, LAMA 1 is the sole LAMA chain located at the basal lamina of the adult seminiferous tubule with direct contact to Sertoli cells and SSCs.
- Loss of testicular LAMA 1 expression, as observed following testicular explant culture, leads to germ cell loss suggesting LAMA 1 is a key component of the testicular stem cell niche.

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