MODELING GONADAL DEVELOPMENT AND FUNCTION WITH HUMAN PLURIPOTENT STEM CELLS

Kirsi Sepponen

Department of Obstetrics and Gynecology

Doctoral Programme in Biomedicine

Faculty of Medicine

University of Helsinki

Helsinki, Finland

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Supervised by

Professor Juha Tapanainen
Department of Obstetrics and Gynecology,
University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Docent Timo Tuuri
Department of Obstetrics and Gynecology,
University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Doctor Karolina Lundin
Department of Obstetrics and Gynecology,
University of Helsinki and Helsinki University Hospital, Helsinki, Finland

Reviewed by

Docent Juho-Antti Mäkelä
Institute of Biomedicine, University of Turku, Finland

Assistant Professor Renata Prunskaite-Hyyryläinen
Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland

Opponent

Professor Noora Kotaja
Institute of Biomedicine, University of Turku, Finland

Cover: Immunofluorescence of SF1-induced gonadal-like cells: AMH, magenta; HSD3B2, green/grey

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For my family

TABLE OF CONTENTS

ABSTRACT

Human gonads (testes and ovaries) develop from mesoderm as paired organs and are initially identical, or bipotent, in males and females. Upon sex determination, the gonads initiate differential transcription, molecular signaling, and hormonal patterns and begin to display their sex-specific characteristics. Occasionally, these complex processes become disrupted, causing impaired gonadal development at embryonic and early fetal stages and altered gonadal functions, which may lead to infertility or even abnormal sex development. The molecular mechanisms delineating early gonadal development and sex determination are not thoroughly understood and have been challenging to investigate in humans.

Human pluripotent stem cells (hPSCs), comprising human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are a versatile tool for studying regulatory mechanisms of human organ development and related disease processes. hPSCs can form all three germ layers of an early embryo and have an endless capacity to self-renew, providing an infinite source of cells equivalent to the undifferentiated cells of an embryo.

Follicle-stimulating hormone (FSH) and its receptor (FSHR) are necessary for normal development and function of the reproductive system. Any disruptions in FSH-FSHR signaling may cause severe fertility issues. For example, inactivating FSHR mutation resulting in an alanine to valine (A189V) amino acid substitution leads in women to amenorrhea and infertility due to blocked follicle development and in men to markedly reduced spermatogenic capacity but partially preserved fertility. The disease mechanisms for the observed phenotypes remain obscure.

In this study, the first aim was to reveal regulatory pathways and signaling mechanisms involved in early gonadal development by developing an hPSC-based *in vitro* model. The second aim was to evaluate the regulatory role of nuclear receptor subfamily 5 group A member 1 (*NR5A1*), a gene encoding steroidogenic factor 1 (SF1), in early gonadal cell transcriptomics. The third aim was to examine the function of human wild-type (WT) FSHR and FSHR with an inactivating A189V mutation.

To model gonadal development, we differentiated hPSCs into bipotential gonadal-like cells via primitive streak- and intermediate mesoderm-like stages by stimulating activin, bone morphogenetic protein, and Wingless-related integration site-dependent signaling, demonstrating the importance of the proper balance between these signaling pathways in early gonadal differentiation. hiPSCs of male origin, differentiated to the bipotential gonadal-like stage, were further directed towards more mature gonadal-like anti-Müllerian hormone-producing and steroidogenic cells by activating endogenous *NR5A1* with CRISPR-Cas9 technology. Cells differentiated in the absence of *NR5A1* activation did not exhibit these characteristics, as assayed by gene expression and immunodetection-based methods and mass spectrometry. Moreover, by performing whole sample RNA sequencing analysis for the cells in the presence and absence of

NR5A1 activation, we identified many novel targets of *NR5A1* and altered gene expression patterns of many gonad-related genes and genes not previously associated with gonads in response to *NR5A1*.

We also established a protocol for directed differentiation of hPSCs into cells endogenously expressing *FSHR*. By differentiating hESCs expressing WT *FSHR* and patient-specific hiPSCs expressing mutated A189V *FSHR* and by performing immunosorbent-based functional assays in the differentiated cells, we demonstrated distinct functional response to FSH between the mutated and WT receptors. In addition, we identified several novel FSHR-protein interactions by using affinity proximity mass spectrometry in HEK293 cells stably expressing WT or A189V FSHRs in stimulated and unstimulated conditions.

This research project provided novel insights into signaling and gene regulatory mechanisms during early gonadal development. The established differentiation protocols can serve as promising tools also in future studies. For example, the gonadal models can be used to examine gonadal dysgenesis or causes of infertility with patient-derived hiPSCs. This project also yielded a considerable amount of publicly available transcriptomics and proteomics data, which can be utilized in evaluating SF1 and FSHR biology.

TIIVISTELMÄ

Ihmisen sukurauhaset (kivekset, munasarjat) muodostuvat mesodermista ja ovat alun perin identtiset eli bipotentit molemmilla sukupuolilla. Sukupuolenmääräytymisestä lähtien miehen ja naisen sukurauhasten geenienkspressio, signalointi- ja hormonitoiminta poikkeavat toisistaan ja sukurauhaset alkavat ilmentää kunkin sukupuolen piirteitä. Toisinaan nämä monimutkaiset toiminnot häiriintyvät johtaen sukurauhasten varhaiskehityksen häiriöihin ja normaalista poikkeavaan toimintaan, mikä puolestaan saattaa johtaa hedelmättömyyteen tai häiriöihin sukupuolenmääräytymisessä. Ihmisen sukurauhasten varhaiskehitykseen ja sukupuolenmääräytymiseen liittyviä mekanismeja tunnetaan edelleen huonosti ja niiden selvittäminen on haastavaa.

Ihmisen monikykyiset kantasolut (hPS-solut), joihin luetaan sikiöaikaiset kantasolut (hES-solut) ja indusoidut monikykyiset kantasolut (hiPS-solut), ovat hyödyllisiä ihmisen elinten kehitykseen liittyvien säätelytekijöiden vaikutusten ja tautimekanismien mallinnuksessa. hPS-solut muodostavat kaikki kolme alkiokerrosta ja jakautuvat määrättömästi, tarjoten loputtoman soluvarannon alkionkehityksen mallintamiseen.

Follikkeleita stimuloiva hormoni (FSH) ja sen reseptori (FSHR) ovat välttämättömiä lisääntymiselinten normaalin kehityksen ja toiminnan kannalta ja siten FSH-reseptori-signaloinnin häiriöt voivat aiheuttaa vakavia hedelmällisyysongelmia. Esimerkiksi eräs FSH-reseptorin inaktivoiva mutaatio (A189V), joka johtaa alaniinin korvautumiseen valiini-aminohapolla, johtaa naisilla follikkeleiden kehityksen pysähtymiseen ja edelleen sen seurauksena amenorreaan ja hedelmättömyyteen. Sen sijaan vastaava mutaatio miehillä heikentää siittiötuotantoa, vaikka hedelmällisyys mutaation seurauksena osittain säilyy. Näihin fenotyyppeihin johtavat tautimekanismit eivät ole vielä kovin hyvin selvillä.

Tämän tutkimuksen ensimmäisenä tavoitteena oli selvittää varhaisten sukurauhasten kehityksen säätely-ja signalointimekanismeja ja kehittää sitä varten kantasoluihin perustuva *in vitro*-malli. Toisena tavoitteena oli tutkia tumareseptori 5 A 1 (NR5A1)-geenin säätelyvaikutusta kehittyvien sukurauhasten geeniekpressioon. Kyseinen geeni koodittaa steroidogeenistä tekijä 1:tä (SF1). Kolmantena tavoitteena oli tutkia villityypin ja A189V mutantti-FSHR:n toimintaa.

Tässä tutkimuksessa mallinsimme sukurauhasten kehitystä erilaistamalla hPS-soluja alku-uurre- ja välimesodermi-vaiheiden kautta bipotentin sukurauhasen kaltaisiksi soluiksi stimuloimalla aktiviini-, luun morfogeneettinen proteiini- ja Wnt-signalointireittejä. Samalla osoitimme, että tasapaino kyseisten signalointimekanismien välillä on tärkeää sukurauhasten erilaistumiseksi. Lisäksi erilaistimme bipotentin sukurauhasen kaltaisista soluista anti-müllerian hormonia ja sukupuolihormoneja tuottavia sukurauhasen kaltaisia soluja. Tämä toteutettiin aktivoimalla NR5A1-geeniä CRISPR-Cas9-teknologian avulla. Sen sijaan geenienkspressio-, vasta-aine- ja massaspektrometriaan perustuvat määritykset osoittivat, että ne solut, joissa NR5A1-geeniä ei aktivoitu, eivät ilmentäneet samoja piirteitä. Tämän lisäksi RNA sekvensointi-

analyysi indusoiduista ja indusoimattomista soluista osoitti useita uusia *NR5A1*:n säätelykohteita, muutoksia sukurauhaseen liittyvien geenien ekspressiossa ja geenejä, joiden yhteyttä *NR5A1*:n ja sukurauhasten kanssa ei ole aiemmin osoitettu.

Lisäksi kehitimme menetelmän hPS-solujen erilaistamiseksi soluiksi, jotka ekspressoivat FSHR-geeniä. Erilaistimme villityypin ja mutantti FSH-reseptoria ilmentäviä soluja, joille teimme ELISA vasta-ainemäärityksiä. Erilaistuneiden ja stimuloitujen solujen vaste FSH:lle oli erilainen villityypin ja mutantti FSH-reseptoria ilmentävissä soluissa. Tämän lisäksi identifioimme affiniteettimassaspektrometrialla useita uusia vuorovaikutussuhteita FSH-reseptorin ja muiden proteiinien välillä FSH:lla stimuloiduissa ja kontrolli HEK293 soluissa, jotka ilmensivät joko villityypin tai mutantti FSH-reseptoria.

Tämän työn ansiosta saatiin tietoa sukurauhasten varhaiskehityksen säätelystä geeni-ja signalointitasoilla. Kehitettyjä menetelmiä voidaan hyödyntää työkaluina selvitettäessä sukurauhasten kehityshäiriöiden ja hedelmättömyyden syitä. Projektista saatiin runsaasti julkista transkriptomiikka-ja proteomiikka-aineistoa, jota voidaan hyödyntää jatkotutkimuksissa.

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications (I-III)

I. Sepponen, K., Lundin, K., Knuus, K., Väyrynen, P., Raivio, T., Tapanainen, J. S., and Tuuri, T. The role of sequential BMP signaling in directing human embryonic stem cells to bipotential gonadal cells. J. Clin. Endocrinol. Metab. 102, 4303–4314 (2017). doi: 10.1210/jc.2017-01469.

II. Lundin, K.*, Sepponen, K.*, Väyrynen, P., Liu, X., Yohannes, D. A., Survila, M., Ghimire, B., Känsäkoski, J., Katayama, S., Partanen, J., Vuoristo, S., Paloviita, P., Rahman, N., Raivio, T., Luiro, K., Huhtaniemi, I., Varjosalo, M., Tuuri, T., and Tapanainen, J. S. Human pluripotent stem cell-derived cells endogenously expressing follicle-stimulating hormone receptors: modeling the function of an inactivating receptor mutation. Mol. Hum. Reprod. 28 (2022). doi: 10.1093/MOLEHR/GAAC012.

*Equal contribution as first authors

III. Sepponen, K., Lundin, K., Yohannes, D. A., Vuoristo, S., Balboa, D., Poutanen, M., Ohlsson, C., Hustad, S., Bifulco, E., Paloviita, P., Otonkoski, T., Ritvos, O., Sainio, K., Tapanainen, J. S., and Tuuri, T. Steroidogenic factor 1 (NR5A1) induces multiple transcriptional changes during differentiation of human gonadal-like cells. Differentiation (2022). doi: 10.1016/J.DIFF.2022.08.001.

In addition, some unpublished data are presented.

ABBREVATIONS

AAVS1	adeno-associated virus integration site 1
AC	adenylyl cyclase
αGSU	glycoprotein hormone α subunit
AMH	anti-Müllerian hormone
AP-MS	affinity purification mass spectrometry
APPL	adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper
BMP	bone morphogenetic protein
cAMP	3',5'-cyclic adenosine monophosphate
Cas	CRISPR associated
CRISPR	clustered regularly-interspaced short palindromic repeats
crRNA	CRISPR RNA
DAG	diacylglycerol
dd	destabilizing domain
DE	differential (gene) expression
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DOX	doxycycline (hyclate)
dpc	day(s) post coitum
DSD	differences in sex development
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMX2	empty spiracles homeobox 2
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated protein degradation
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cell
FBS	fetal bovine serum
FGF	fibroblast growth factor
FOXL2	forkhead box L2
FOXO1	forkhead box protein O1
FSH	follicle-stimulating hormone
FSHβ	follicle-stimulating hormone β subunit
FSHR	follicle-stimulating hormone receptor

FST	follistatin
Gαs	Gs alpha subunit
GATA4	GATA binding protein 4
GC	granulosa cell
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
GTP	guanosine-5'-triphosphate
hESC	human embryonic stem cell
hiPSC	human induced pluripotent stem cell
hPSC	human pluripotent stem cell
HSD3B2	3β-hydroxysteroid dehydrogenase type II
IGF	insulin-like growth factor
IGF1R	insulin-like growth factor 1 receptor
IM	intermediate mesoderm
INSL3	insulin-like 3
INSR	insulin receptor
IP3	inositol 1,4,5-trisphosphate
iPSC	induced pluripotent stem cell
kb	kilobase
KO	knockout
LC	Leydig cell
LH	luteinizing hormone
LHβ	luteinizing hormone β subunit
LHCGR	luteinizing hormone/choriogonadotropin receptor
LHX1	LIM homeobox 1
LHX9	LIM Homeobox 9
LPM	lateral plate mesoderm
MAC	multiple approaches combined
MAPK	mitogen-activated protein kinase
NR5A1	nuclear receptor subfamily 5 group A member 1
OCT4	octamer-binding transcription factor 4
-OH	-hydroxyl
OSR1	odd-skipped related transcription factor 1
P450SCC	P450 side-chain cleavage
PAX2	paired box 2
PB	PiggyBac

PBS	phosphate-buffered saline
PGC	primordial germ cell
PGD	prostaglandin
PI3K	phosphoinositide 3-kinase
PIP3	phosphatidylinositol 3,4,5-triphosphate
PKA	protein kinase A
PKB/Akt	Akt serine/threonine kinase
PLC	phospholipase C
PMC	peritubular myoid cell
PS	primitive streak
PSC	pluripotent stem cell
qRT-PCR	quantitative reverse transcription polymerase chain reaction
ROCKi	Rho-associated coiled coil forming protein serine/threonine kinase inhibitor
rtTA	reverse tetracycline-controlled transactivator
SC	Sertoli cell
SCNT	somatic cell nuclear transfer
scRNA	single-cell RNA
SF1	steroidogenic factor 1
sgRNA	single guide RNA
SRY	sex-determining region Y
SSC	spermatogonial stem cell
STAR	steroidogenic acute regulatory protein
TC	theca cell
TGFβ	transforming growth factor β
TMP	trimethoprim
VMH	ventromedial hypothalamus
Wnt	Wingless type mouse mammary tumor virus integration site family
WT	wild-type
WT1	Wilms' tumor suppressor 1

INTRODUCTION

The utilization of rodents as models to study developmental disorders of the reproductive system in humans relies on the assumption that the development of human gonads closely follows that of rodents. The sequence of events and signaling mechanisms activated during the development of mammalian gonads are well-known in rodents, but the molecular signals driving gonadal development in humans during fetal stages are not fully understood and may display substantial species-specific differences (Warr et al., 2011; McClelland et al., 2012; Larney et al., 2014). Human gonadal development has been challenging to investigate due to limited access to suitable human material, difficulties in culturing primary cells and tissues for extended periods, and ethical issues associated with use of human fetal material in research.

Human pluripotent stem cells (hPSCs), which constitute human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have been widely used to delineate developmental processes and disease mechanisms in the past two decades. Since the derivation of the first human hiPSC lines (Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Park et al., 2008), these cells have become a workhorse for many research laboratories, substituting or complementing more conventional *in vitro* models.

Stem cell-based models to investigate human gonadal development and to identify causes of infertility have primarily been developed to direct germ cell development, and models for the differentiation of somatic gonadal cells were not available until recently. In this study, we describe derivation of early gonadal cells using hPSCs and elucidate signaling pathways contributing to their differentiation. We also describe how activation of gonadal marker gene NR5A1 affects the differentiation of male gonadal-like cells.

REVIEW OF THE LITERATURE

1. Determining pluripotent stem cells (PSCs)

1.1. Potency within an embryo

During development, cells specialize to fulfill various functions of somatic tissues. As the cell differentiates to become more specialized, it gradually loses its potential to retain a broad range of distinct cellular fates (Zakrzewski et al., 2019). Zygotes and blastomeres in cleavage stage embryos up to a 4- to 8-cell stage have been historically regarded as the most potent mammalian cell types with the ability to give rise to all embryonic and extraembryonic tissues and are hence coined 'totipotent'. The blastocyst, which is generated by cleavage and differentiation of blastomeres, consists of two parts: an inner cell mass and an outer trophoblast. Cells allocated to the inner cell mass give rise to all tissue types within an embryo and are therefore called 'pluripotent', whereas cells destined to become trophoblasts give rise to extraembryonic tissues such as the umbilical cord, placenta, and chorion (Hardy et al., 1989). This dogma is gradually changing as increasing evidence demonstrates that the cells of the inner cell mass under certain *in vitro* conditions may also give rise to cell types resembling trophectodermal tissue, although these cells lack some of the characteristics of their *in vivo* counterparts (Gamage et al., 2016).

1.2. Pluripotent cells can be derived from various sources

Stem cells are able to self-renew, *i.e*. to continuously produce a new progeny of stem cells, and to differentiate into other cell types. Adult stem cells, such as testicular spermatogonial stem cells (SSCs), persist in adult tissues, possess limited differentiation capabilities, and typically act in maintaining tissue homeostasis and renewal. In contrast, PSCs exist only transiently during embryogenesis. They can be derived by isolation of the inner cell mass of an early blastocyst and cultured *in vitro* (Evans and Kaufman, 1981; Martin, 1981) (Fig. 1). In addition to these so-called embryonic stem cells (ESCs), PSCs can be derived from postnatal germ cells under specific culturing conditions (Kanatsu-Shinohara et al., 2004; Golestaneh et al., 2009). Pluripotency in somatic cells can also be artificially induced in several ways: by reprogramming the differentiated cell or its nucleus into pluripotent state by somatic cell nuclear transfer (SCNT) technology (Gurdon, 1962; Wakayama et al., 2001), by cell fusion (Tada et al., 2001; Cowan et al., 2005), or by direct *in vitro* reprogramming (Takahashi and Yamanaka, 2006) (Fig. 1). All of these strategies generate pluripotent cells that are suitable for different purposes.

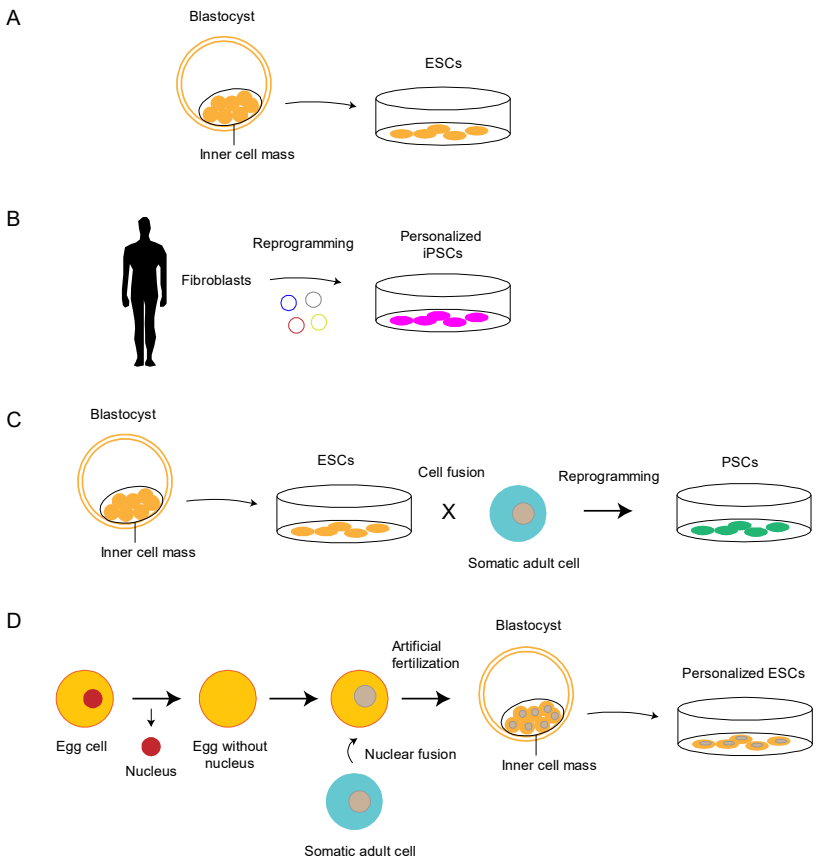

Figure 1. Different ways to generate PSCs. A. ESCs can be generated from the inner cell mass of the blastocyst stage embryo. B-C. Pluripotency in somatic cells can be induced by reprogramming factors either directly or by generating a somatic cell-ESC hybrid. D. Alternatively, the somatic cell nucleus can be fused with an enucleated egg to generate personalized ESCs. Abbreviations: ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; PSC, pluripotent stem cell.

ESCs are an ideal tool for investigating self-renewal, development, and disease mechanisms (Hochedlinger and Jaenisch, 2015). However, as their derivation leads to the destruction of an embryo, the utilization of ESCs raises ethical and in some countries also legal questions (Volarevic et al., 2018).

Technically even more challenging, cost-inefficient, and ethically and legally troublesome is the generation of nuclear transfer ESCs. In this technique, a differentiated cell nucleus from a somatic cell is transferred into an enucleated oocyte (Munsie et al., 2000). Similar methodology was used to clone the first mammal, sheep Dolly (Wilmut et al., 1997). Despite its disadvantages, SCNT provides an unbiased model for epigenetic reprogramming studies (Hochedlinger and Jaenisch, 2015).

Different cell types can also be fused together to generate a hybrid, a method that has been utilized in reprogramming somatic cells by hybridizing them with ESCs (Tada et al., 2001; Cowan et al., 2005). The induced pluripotency due to fusion of ESCs and

differentiated somatic cells originally proved that ESCs contain all factors required for pluripotency, therefore greatly advancing the field of stem cell reprogramming. However, the resulting cells retain genetic material of both somatic and ESC origin, limiting their application. In addition, cell aneuploidy might cause problems in developmental and genomic studies (Hochedlinger and Jaenisch, 2015).

Besides reprogramming methods that require ESCs or oocytes, reprogramming with direct conversion of terminally differentiated postnatal cells back to pluripotent state generates induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). These can be generated with various viral or non-viral reprogramming methods, which aim at inducing the expression of specific reprogramming factors within their target cells (Malik and Rao, 2013). iPSC technology is a simple, versatile tool with less ethical and legal challenges than ESCs and SCNT. It has shown great promise regarding disease modeling, drug screening, and toxicological and cell transplantation approaches (Karagiannis et al., 2019).

1.3. PSCs of human origin

hESCs and hiPSCs refer to the human equivalents of PSCs. The first hESC line was generated in the late 1990s by American scientist James Thomson (Thomson, 1998), whereas the first hiPSC lines emerged in the first decade of the 2000s (Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Park et al., 2008). Since then, both have been widely applied in basic and translational research and in several ongoing clinical trials, although their inherent tumorigenic and heterogenic properties and possible immunological risks stemming especially from transplantation of heterologous hESC-derived cells still raise concerns and may restrict their application (Yamanaka, 2020). The derivation of the first hESC and hiPSC lines was followed by studies that questioned their similarity regarding, for instance, their gene expression patterns (Chin et al., 2009; Ghosh et al., 2010). However, later studies have revealed that hESCs and hiPSCs are transcriptionally and epigenetically highly similar (Gupta et al., 2010; Mallon et al., 2014; Choi et al., 2015), and that the genetic background plays a major role in the observed transcriptional changes (Rouhani et al., 2014; Choi et al., 2015). More importantly, the established hPSC lines tend not to be similar in their differentiation capabilities towards distinct cellular fates (Mikkola et al., 2006; Osafune et al., 2008; Hu et al., 2010), thus individual lines favor differentiation into certain lineages at the expense of others.

2. The gonads – structure and function

A gonad or sex gland is the primary reproductive organ, producing gametes and hormones for sexual reproduction. In vertebrates, the gonads are usually paired glands.

2.1. Testicular architecture

The mammalian male reproductive tract comprises testes, ductus deferentes, epididymides, accessory sex glands, and the penis (Older and Watson, 1996). The testes

are located on each side of the scrotum covered with the tunica albuginea, a protective capsule of fibrous tissue, which also regulates blood flow, intertesticular pressure, and sperm movement (Older and Watson, 1996; Aganovic and Cassidy, 2012) (Fig. 2A). Within the testes, thin cords called seminiferous tubules make up most of the testicular volume (Orth, 1984) (Fig. 2A).

Seminiferous tubules consist of germ cells, somatic Sertoli cells (SCs) and peritubular cells (Fig. 2A). SCs are the first somatic counterpart to emerge during testicular development and they have a key role in orchestrating testicular development and triggering differentiation and maintaining survival of developing germ cells (Svingen and Koopman, 2013). They are polarized epithelial cells that extend from the basal compartment of the seminiferous tubule to the luminal side, from which mature spermatids are released to the lumen (Hess, 1990) (Fig. 2A). The size of the SC population determines the number of germ cells and other testicular somatic cells (Rebourcet et al., 2017). SCs exert their actions on germ cells and other testicular somatic cell types both by providing physical structural support and via paracrine signaling (Svingen and Koopman, 2013; Griswold, 2018). An important SC secretory factor is anti-Müllerian hormone (AMH), previously known as Müllerian inhibiting substance (Tran et al., 1977; Münsterberg and Lovell-Badge, 1991). It is involved in male sex differentiation by inducing regression of the Müllerian ducts, which give rise to structures of the female reproductive tract.

The backbone of seminiferous tubules is surrounded by peritubular myoid cells (PMCs, Fig. 2A), which are smooth muscle cells that physically support the tubule and participate in regulation of spermatogenesis and SC secretory functions (Skinner and Fritz, 1985; Zhou et al., 2019). They interact with SCs in providing structural integrity for the seminiferous tubules by producing components of the lamina propria (Skinner et al., 1985), which separates seminiferous tubules from the interstitial compartment (Fig. 2A). In humans, lamina propria comprises a continuous basal lamina, a thin sheet of extracellular matrix glycoproteins and proteoglycans, in the immediate vicinity of seminiferous tubules and multiple layers of myofibroblasts and fibrocyte-like cells separated by connective tissue (Davidoff et al., 1990; Holstein et al., 1996). The interstitial compartment consists of mature and immature steroidogenic Leydig cells (LCs), interstitial macrophages, lymphatic and vascular endothelial cells, pericytes, and lymphatic space (Mendis‐Handagama et al., 1987) (Fig. 2A). The exchange of gas, nutrients, and waste products within the testes is conducted by blood vessels, which run within the interstitial space (Coveney et al., 2008) (Fig. 2A).

Within seminiferous tubules, a special immunoprivileged microenvironment is created to ensure undisturbed spermatogenesis. Close to the basement membrane of seminiferous epithelium, adjacent SCs form a blood-testis barrier, which comprises tight junctions, gap junctions, desmosomes, and ectoplasmic specializations that divide the seminiferous tubule into basal and adluminal compartments (Yan Cheng and Mruk, 2012) (Fig. 2A). The function of the blood-testis barrier is to block large molecules, such as antibodies, and immune cells originating from circulatory or lymphatic systems

from entering the lumen of the seminiferous tubules. The blood-testis barrier locally prevents autoimmune attacks against the developing sperm, which express antigens that are foreign to the immune cells (Yan Cheng and Mruk, 2012). In addition to the blood-testis barrier, the immunoprivileged environment within the testis is maintained by various immunomodulatory factors secreted by testicular cells (Qu et al., 2020).

2.2. Ovarian architecture

Ovaries are almond-shaped organs situated on both sides of the uterus, adjacent to the uterine horns. They are held in place by utero-ovarian and infundibulopelvic ligaments and surrounded by tunica albuginea. The ovary is separated into three distinct compartments: an outer cortex containing the follicles and the germinal epithelium (*i.e.* ovarian surface epithelium), an inner medulla containing dense fibrous connective tissue, blood vessels and myoid-like contractile cells, and the periovarian hilum that serves as a point of entry for blood vessels, lymphatic ducts, and nerves and physically connects the ovary to the rest of the reproductive tract (Hoffman et al., 2020) (Fig. 2B).

The ovarian follicles manifest as functional anatomical structures of the ovary that consist of developing germ cells and supporting somatic cells (Fig. 2B). Oocytes residing within follicles derive from oogonia, i.e. mitotically active female germ cells, and eventually give rise to fertilizable eggs (Stephenson et al., 2012) (Fig. 2B). Within each ovarian follicle, an oocyte is surrounded by one or more layers of somatic granulosa cells (GCs) (Oktem and Oktay, 2008; Stephenson et al., 2012) (Fig. 2B). They act as structural components of the follicle and support follicle development by secreting cytokines such as leukemia inhibitory factor and kit ligand (Manova et al., 1993; Parrott and Skinner, 1999). Conversely, GC differentiation and proliferation is supported by the oocytes (Nilsson et al., 2001; Belli and Shimasaki, 2018; Barros et al., 2019), which develop during folliculogenesis. It is a dynamic and cyclic process in which the immature oocyte develops into a mature oocyte within an ovarian follicle and involves coincidental proliferation and differentiation of the pre-GCs into GCs (Rimon-Dahari et al., 2016).

The basal lamina separates the oocyte and surrounding GCs from the interstitial (stromal) tissue (Weakley, 1966; Oktem and Oktay, 2008) (Fig. 2B). Theca cells (TCs) laying within the interstitial compartment are endocrine cells that provide structural integrity for the follicle, synthesize androgens, and mediate signals between GCs, thereby having an essential role in the development of ovarian follicles from primordial follicles into preovulatory/Graafian follicles during folliculogenesis (Sasano et al., 1989; Young and McNeilly, 2010) (Fig. 2B). TCs differentiate from the ovarian stromal cells and eventually undergo hormonally-dependent terminal differentiation to form an estrogen and progesterone-producing corpus luteum (Young and McNeilly, 2010) (Fig. 2B).

As described above in both males and females, the germ cells and the somatic cells surrounding them reciprocally interact both physically and via paracrine signaling factors. In addition, advancements in methodology, especially the evolvement of single-cell RNA (scRNA) sequencing-based transcriptomic approaches in recent years, have

enabled the discovery of novel interactions between somatic and germ line cells of the testes and the ovaries, and shed light on the transcriptomic changes underlying specification of distinct gonadal cell types (Li et al., 2017; Stévant et al., 2018, 2019).

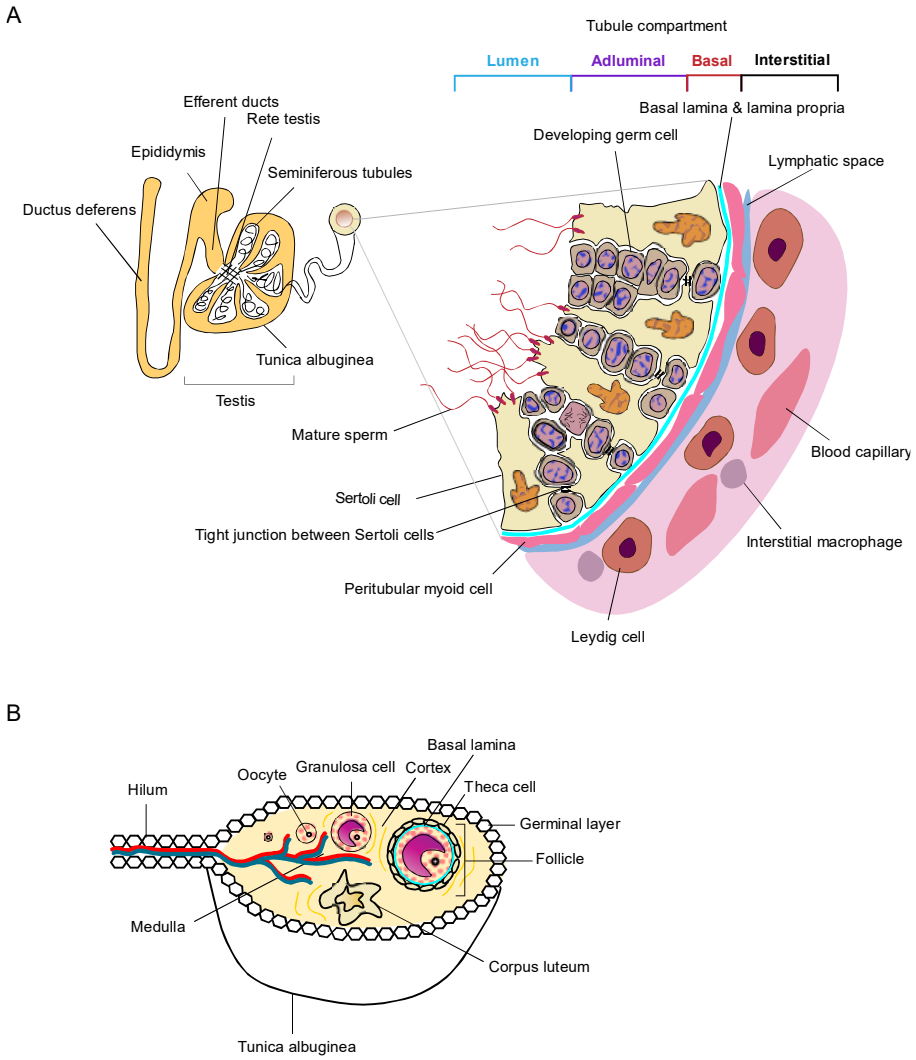

Figure 2. A. Adult testis, the associated ducts and the structure of seminiferous tubules within testis. The tubule is divided into dissimilar compartments that host different cell types. SCs guide sperm development at the basal and adluminal compartments of the seminiferous tubules until sperm is released to the lumen. From there, spermatozoa travel first to the rete testis, an anastomosing tubule network located in the hilum of the testicle, and then into efferent ducts and epididymis for their final maturation until they are released via ductus deferens during spermiation. B. Structure of an adult ovary. During their maturation, oocytes become surrounded by several layers of granulosa cells and form follicles that are located in the ovarian cortex. The ovary contains follicles at various developmental stages. The ovarian medulla is a highly vascular stroma and the hilum connects the ovary to the reproductive tract.

3. Development of the gonads

3.1. Origin of the gonads

The gonads derive from an embryonic germ layer referred to as an intermediate mesoderm (IM) (Dressler, 2006; Sasaki et al., 2021) arising as a consequence of mesodermal segregation into paraxial, lateral plate mesoderm (LPM) and IM subtypes during gastrulation. The fate of cells forming distinct mesodermal subtypes is determined by their location along the primitive streak (PS); the cells defining the IM lay between the anterior and posterior sites. The anterior parts of the IM form transient fetal kidneys, pronephros and mesonephros, and the Wolffian (mesonephric) ducts, which later regress in females but develop into internal genitalia (epididymis, vas deferens, and seminal vesicles) in males (Smith and Mackay, 1991; Joseph et al., 2009). In addition, the IM-derived mesonephric coelomic epithelium gives rise to the Müllerian (paramesonephric) ducts that regress in males and persist in females (Santana Gonzalez et al., 2021). According to a recent lineage-tracing study conducted in mice and monkeys, gonadal development initiates in mammals at posterior IM (Sasaki et al., 2021).

3.2. Active signaling pathways in mesoderm specification

3.2.1. Wingless-type mouse mammary tumor virus integration site family (Wnt)/β-catenin

Canonical Wnt/β-catenin signaling drives several events of morphogenetic and cellular differentiation during embryogenesis (Wang and Wynshaw-Boris, 2004). Briefly, in the absence of Wnt, β-catenin is bound by a destruction complex and becomes degraded in proteosomes (Fig. 3). In the presence of Wnt, the destruction complex is disrupted, allowing β-catenin to accumulate in the nucleus and activate gene expression. Canonical Wnt/β-catenin signaling contributes to the mesodermal fate decision. Paraxial mesoderm is induced with high Wnt signaling, whereas low Wnt signaling induces LPM, as demonstrated by single-cell transplantation studies performed in transgenic zebrafish (Martin and Kimelman, 2012). The duration of Wnt signaling can also affect the mesodermal fate. Upon differentiation of hiPSCs, stimulation of the Wnt signaling pathway for different time periods has been reported to affect the fate decision between anterior and posterior IM (Takasato et al., 2015).

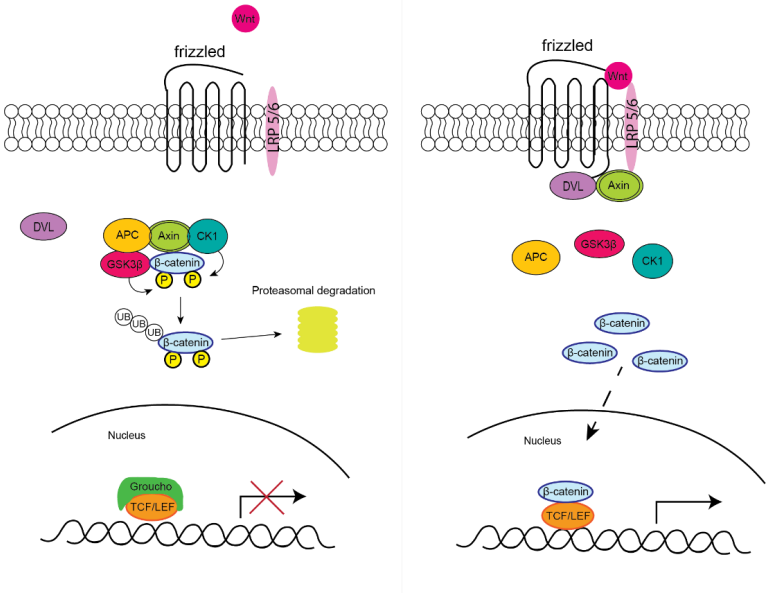

Figure 3. Wnt/β-catenin signaling pathway. In the absence of Wnt ligand, constitutively expressed β-catenin becomes ubiquitinated by the destruction complex and is targeted to proteasomes for degradation. In the presence of Wnt ligand, the protein forms a complex with frizzled/LRP6 or LRP5 receptors and recruits DVL to the plasma membrane. LRP5/6 becomes phosphorylated and suppresses GSK-3β activity, which subsequently leads to translocation of the β-catenin destruction complex to the membrane, away from cytosolic β-catenin. The accumulating β-catenin enters the nucleus and activates target gene expression. Abbreviations: APC, adenomatous polyposis coli; CK1, casein kinase 1; GSK-3β; glycogen synthase kinase 3β; DVL, dishevelled; LRP, low-density lipoprotein receptor-related protein; UB, ubiquitin; TCF/LEF, T cell factor/lymphoid enhancer factor.

3.2.2. The transforming growth factor β (TGFβ) superfamily

The TGFβ superfamily consists of over 30 structurally related secreted regulatory proteins, including nodals, activins, inhibins, bone morphogenetic proteins (BMPs), AMH, and growth and differentiation factors (Hinck et al., 2016). The members of the superfamily are evolutionally conserved (Hinck et al., 2016) and widely but spatiotemporarily expressed during the lifetime of an organism. Proteins of the TGFβ superfamily signal via Smad transcription factor-dependent or -independent pathways (Wrana, 2013; Zhang, 2017), and Smads can also cross-talk with other signalling pathways depending on the developmental stage or cell type (Luo, 2017).

Activin/Nodal

Upon early gastrulation, a TGFβ family member Nodal initiates gastrulation and specifies the mesodermal cell fate via graded signals (Thisse et al., 2000; Vincent et al., 2003). In *in vitro* assays, Nodal is commonly replaced by Activin A, which shares a common membrane receptor with Nodal and interacts with the same intracellular Smads (Kumar et al., 2001; D'Amour et al., 2005). Activin/Nodal binds to serine/threonine TGFβ type II receptors, which complex with and phosphorylate type I receptor (Fig. 4A). This catalyzes phosphorylation of Smad2 and Smad3, which together interact with the common co-mediator Smad (co-Smad), Smad4, and the complex translocates to the nucleus to act as a transcriptional regulator.

BMPs

A separate group of the TGFβ superfamily, BMPs, induce and specify mesoderm subtypes at the dorsal-ventral and mediolateral axes (Dosch et al., 1997; Tonegawa et al., 1997). BMP subgroup involves 15 distinct proteins that play a role in the development of a wide array of organs (Katagiri and Watabe, 2016). BMP signals derived from the overlying ectoderm and the lateral plate are suppressed by the opposing signals from the paraxial mesoderm (somites), forming a morphogen gradient along the mesoderm (Fig. 4B). The BMP gradient is crucial for mesodermal induction; low levels of BMP drive differentiation of the IM, whereas high levels induce the LPM (James and Schultheiss, 2005).

BMP4 is one of the key players in mesoderm specification, as demonstrated by embryonic lethality and lack of mesoderm in *Bmp4* knockout (KO) mice (Winnier et al., 1995). Additionally, studies conducted in *Xenopus* embryos report that BMP4 is responsible for the ventralization of the gastrulating embryo (Jones et al., 1996) and the patterning of the distinct mesodermal subtypes in a post-blastula embryo (Dosch et al., 1997).

Similarly to the absence of functional BMP4, *Bmp2* KO mice die upon embryogenesis, exhibit defects in amnion and chorion, and suffer from compromised cardiac development (Zhang and Bradley, 1996). Furthermore, mice lacking BMP7 die after birth, suffer from kidney, eye, and skeletal defects, and during kidney development BMP7 has been reported to maintain proliferation of nephron progenitors and prevent their precocious differentiation (Wang et al., 2014). All BMPs preferentially signal via type II and type I serine–threonine kinase receptor complexes and involve R-Smads1, -5, or -8 (Smad 1/5/8) for canonical signal transduction (Fig. 4A). However, BMPs display dissimilar affinities to different type II BMP receptors (Nickel et al., 2009). BMP signaling is tightly controlled, for instance, by extracellular agonists or antagonists, such as natural antagonists Noggin or Chordin *in vivo* (Piccolo et al., 1996; McMahon et al., 1998), or by a synthetic small molecule BMP inhibitor Dorsomorphin *in vitro* (Yu et al., 2008). TGFβ superfamily signaling can also be regulated by co-receptors and intracellular regulators, as presented for BMP signaling in Figure 4A. In BMP signaling, regulation may be implemented *e.g.* by a co-receptor membrane glycoprotein endoglin, inhibitory Smads, and microRNAs (Wang et al., 2014).

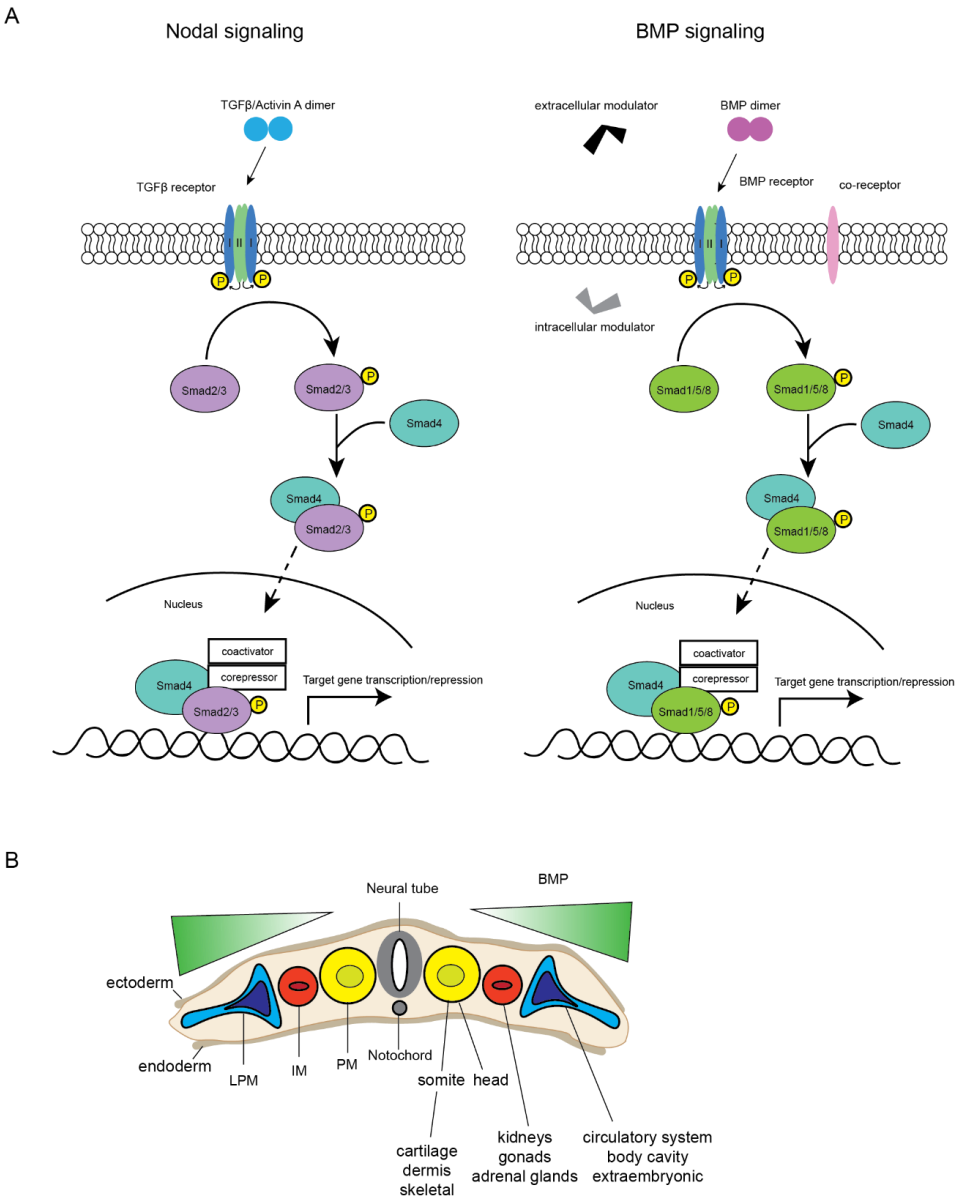

Figure 4. A. Nodal and BMP signaling pathways. Ligand binding as a dimer to the type I or type II receptor triggers formation of a heterotetrameric receptor complex. Constitutively active type II receptor transphosphorylates type I receptor, which leads to receptor internalization and intracellular recruitment of Smad proteins. In Nodal signaling, Smad2 or -3, and in BMP signaling, Smad1, -5, or -8 become phoshorylated. Active Smad proteins complex with Smad4 and translocate to the nucleus, where they regulate target gene expression. Intracellular and extracellular factors may modulate BMP signaling. B. BMP morphogen gradient (green) in a gastrulating embryo (horizontal plane) induces mesodermal subtypes, which develop into distinct mesodermal derivatives. Abbreviations: BMP, bone morphogenetic protein; IM, intermediate mesoderm, LPM, lateral plate mesoderm, P, phosphorylation; PM, paraxial mesoderm, TGFβ, transforming growth factor β.

3.3. Key IM markers and role of BMP7 in inducing the IM *in vitro*

The earliest markers identifying IM formation during gastrulation are LIM homeobox 1 (LHX1) (Tsang et al., 2000), odd-skipped related transcription factor 1 (OSR1) (James and Schultheiss, 2005; James et al., 2006) and paired box 2 (PAX2) (Bouchard et al., 2002). The expression of these markers initiates in the mouse IM between 8.5 to 9.5 days post-coitum (dpc) (Fujii et al., 1994; Tsang et al., 2000; Bouchard et al., 2002; James et al., 2006) and mutations or deletions of each of these genes in mice severely affect the reproductive organs (Table 1).

Table 1. IM markers and associated mutation defects in mice

IM marker	**Mutation phenotypes and biological consequences**
PAX2	Mutant *Pax2* mice suffer from complete renal and reproductive tract agenesis (Torres et al., 1995).
LHX1	Homozygous *Lhx1* KO mice lack both the kidneys and the gonads, in addition to displaying missing anterior head structures (Shawlot and Behringer, 1995). A null mutation also leads to disorganized IM and the mice display reduced expression of Pax2 and homeodomain transgene *Hoxb6-lacZ* (Tsang et al., 2000).
OSR1	Loss of *Osr1* in mice leads to severe heart defects and complete agenesis of the adrenal glands, the metanephric kidneys and the gonads (Wang et al., 2005).

Lhx1 and *Osr1* expression can be first detected at the prospective LPM and at the IM, but later *Lhx1* expression narrows down to the IM and the future nephric duct (Fujii et al., 1994; Tsang et al., 2000), whereas *Osr1* remains expressed in the more posterior structures and in the mesenchyme surrounding the nephric duct (James et al., 2006). *Pax2* expression is restricted to the IM from the beginning (Bouchard et al., 2002).

OSR1 can be induced in BMP7-expressing mesoderm-like cells following their *in vitro* differentiation from mouse ESCs and retinoic acid administration (Mae et al., 2010). In subsequent studies, *OSR1*-expressing IM derivatives have been generated from hPSCs by using BMP7 (Mae et al., 2013; Sepponen et al., 2017), suggesting that BMP7 is involved in IM induction, likely by promoting *OSR1* expression. BMP7 also regulates proliferation of PAX2-positive progenitors via Janus kinase-mediated signaling (Blank et al., 2009) and PAX2 contributes to specification of the IM via epigenetic mechanisms and by targeting genes that repress formation of the paraxial mesoderm (Ranghini and Dressler, 2015).

3.4. Morphological events during development of the genital ridge

The gonads and the adrenal glands are thought to separate from a common adrenogonadal primordium (Hatano et al., 1994, 1996; Ikeda et al., 1994; Bandiera et al., 2013). The site of gonad development is commonly termed a genital ridge, which refers to a ridge of embryonic mesoblast originating from the mesonephros. The gonadal anlage develops on the ventromedial surface of the mesonephroi between 4.5 to 5 weeks of fetal development in humans (Francavilla et al., 1990; Satoh, 1991) and between 9.0 to 10.5 dpc in mice (Koopman et al., 1990; Hacker et al., 1995; Karl and Capel, 1995; Tanaka and Nishinakamura, 2014). The proliferation of the coelomic epithelial cells overlaying the mesonephros initiates gonad development and is followed by disintegration of the underlying basement layer and migration of the epithelial cells into the mesenchyme, where they undergo epithelial to mesenchymal transition (DeFalco and Capel, 2009). In mammals, the primordial germ cells (PGCs), from which the gametes are later derived, are specified from post-implantation epiblast cells by extraembryonic tissue-derived inductive signals. PGC specification takes place near the base of allantois and from there PGCs migrate to the gonads via the hindgut and dorsal mesentery, reaching the genital ridges between the 4th and the 6th week of fetal development in humans and at around 10-12.5 dpc in mice (Molyneaux et al., 2001; Mamsen et al., 2012) (Fig. 5). In rodents, the embryonic development of gonads is preserved in the absence of germ cells (Merchant, 1975). Nevertheless, during embryogenesis the somatic cells and germ cells reciprocally interact (Motta et al., 1997), and germ cells can contribute to differentiation of male and female somatic cells of the gonads (Adams and McLaren, 2002; Yao et al., 2003).

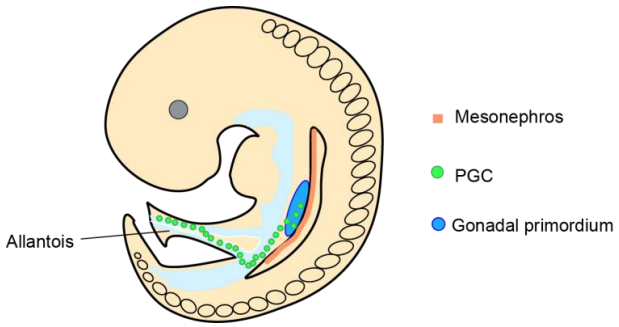

Figure 5. PGCs are specified near the base of allantois and migrate to the gonadal primordia via hindgut epithelium. The gonadal primordia develop as thickenings of mesonephros that arises from the IM. Abbreviation: PGC, primordial germ cell.

3.4.1. Signaling pathways driving formation of the genital ridge

The proliferation of somatic cells of the coelomic epithelium initiates formation of the genital ridge and is controlled by Wnt/β-catenin signaling (Chassot et al., 2012) (Fig. 3). Another pathway suggested to mediate signaling events during the early stages of gonadal development is the insulin/insulin-like growth factor (IGF) signaling pathway (Fig. 6). Mice possessing homozygous deletions of insulin receptor (*Insr*) and insulin-like growth factor 1 receptor (*Igf1r*) display compromised proliferation of the XY and XX genital ridges and subsequent downregulation of ovarian and testicular transcription factors that coordinate sex determination (Pitetti et al., 2013). Canonical Wnt signaling and insulin/IGF signaling pathways are thought to interact during gonadal development (Windley and Wilhelm, 2015).

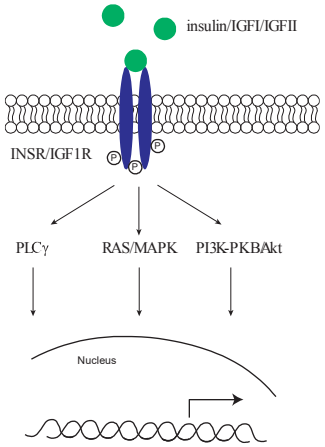

Figure 6. Insulin/IGF signaling. The binding of insulin, IGFI, or IGFII ligand to the insulin or IGF1 receptors triggers activation of downstream signaling cascades, and target gene expression. Abbreviations: IGFI, insulin-like growth factor I; IGFII, insulin-like growth factor II; IGF1R, insulin-like growth factor 1 receptor, INSR, insulin receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PKB/Akt, Akt serine/threonine kinase; PLCγ, phospholipase C gamma.

3.5. Transcription factors identifying the bipotential gonad

At the early stage of their development, gonadal primordia are sexually bipotent, *i.e.* they can form either the testes or the ovaries. In mice, the transcriptomes of XX and XY gonads are nearly identical until 11.2 dpc (Munger et al., 2013) and the bipotential gonads express several characteristic biomarkers such as Wilms' tumor suppressor 1 (WT1), GATA binding protein 4 (GATA4), LIM Homeobox 9 (LHX9), empty spiracles homeobox 2 (EMX2), and steroidogenic factor 1 (SF1, encoded by nuclear receptor subfamily 5 group A member 1, *Nr5a1*) (Armstrong et al., 1993; Luo et al., 1994; Miyamoto et al., 1997; Birk et al., 2000; Hammes et al., 2001; Kusaka et al., 2010; Hu et al., 2013).

3.5.1. WT1

WT1 is one of the first markers detected in the gonadal primordium and it is expressed within the entire urogenital ridge, from which the future gonads and kidneys

differentiate (Armstrong et al., 1993; Sasaki et al., 2021). WT1 is a zinc-finger transcription factor that has at least 36 potent isoforms in mammals (Hastie, 2017). Two of these isoforms differing only in composition of three amino acids (KTS) are crucial for gonadal development. In the absence of functional WT1(-KTS), the development of genital ridges in mice is perturbed and the gonads regress due to extensive cell death (Hammes et al., 2001). WT1(+KTS) has a role later in sex determination of the gonads, as demonstrated by XY sex reversal in the absence of WT1(+KTS) (Hammes et al., 2001).

3.5.2. GATA4

GATA4 is the only marker identified in mice that if absent is reported to be solely responsible for the abolishment of the entire gonadal primordium. This event was reported using *Gata4* ablation in a tamoxifen-inducible gene deletion, which prevented thickening of the coelomic epithelium and disintegration of the basement membrane. GATA4 is a zinc-finger transcription factor, the expression of which in mice begins at 10.0 dpc in the anterior half of the gonadal primordium, gradually extending posteriorly along the coelomic epithelium. In addition to the genital ridges, GATA4 expression has been reported in the adjacent mesenchyme, mesentery, and gut endoderm (Hu et al., 2013).

3.5.3. LHX9, EMX2, and SF1/*Nr5a1*

GATA4 expression is closely followed by the expression of LHX9, EMX2 and SF1. The absence of any of these transcription factors leads to regression of the initial thickening of the coelomic epithelium by either increased cell death (*Emx2*, SF1/*Nr5a1*) or reduced cell proliferation (*Lhx9*) (Luo et al., 1994; Miyamoto et al., 1997; Birk et al., 2000; Kusaka et al., 2010). In mice, LHX9 is expressed in the genital ridges and some *Lhx9* expression has also been detected in the mesentery (Kusaka et al., 2010; Hu et al., 2013), whereas EMX2 expression can be detected in the coelomic epithelium at the site of the nascent genital ridge, in the adjacent mesenchyme, mesonephric tubules, and nephric duct (Kusaka et al., 2010). However, the expression of these markers is not restricted solely to the genital ridges.

SF1/*Nr5a1*, by contrast, is one of the earliest markers and a specific biomarker of the adrenal glands and the genital ridges (Hatano et al., 1996). After segregation of the adrenogonadal primordium (at 11.0 dpc in mice, at approximately the 5th developmental week in humans) into adrenal and gonadal primordia, SF1/*Nr5a1* expression is considerably higher in the developing steroidogenic cortex of the adrenal gland than in the bipotential gonad (Hanley et al., 1999). In the gonads, SF1/*Nr5a1* is accompanied by higher GATA4 and WT1 expression levels than in the adrenal glands (Hanley et al., 1999; Bandiera et al., 2013). In mouse gonads, SF1/*Nr5a1* expression is initiated at 9.5 to 10.2 dpc in the anterior half of the nascent genital ridge (Ikeda et al., 2001; Hu et al., 2013), whereas in humans it first appears in a cell population underlying the genital ridge between the 4th and the 5th week of fetal development (Hanley et al., 1999).

3.6. Sex determination

Previously, the female reproductive structures were considered to develop in the absence of inductive signals, following a default pathway. However, nowadays, the common understanding is that delicate alterations in the balance of the molecular machineries promoting male and female fates define the sex of an organism (Kim et al., 2006). Upon sex determination, the balance in the gonadal transcriptional networks tilts towards the male- or female-specific fate (Fig. 7). The key gene initiating the male fate is a 35 kilobase (kb) Y-chromosome-linked gene sex-determining region Y (*SRY)*, a transiently expressed gene and evolutionally conserved high-mobility group box transcription factor (Sinclair et al., 1990; Hacker et al., 1995). SRY is both essential and sufficient for inducing testis development, as demonstrated by introduction of *Sry* alone to XX gonads and the consequential formation of the testis (Koopman et al., 1991).

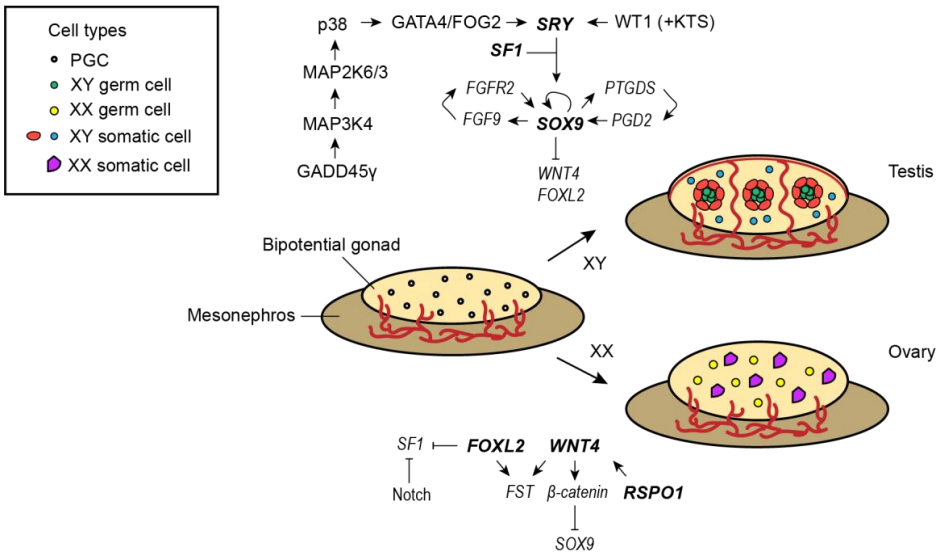

Figure 7. Development of the bipotential gonads towards the testes or the ovaries. Transcription factors/secretory factors/kinases mediating activation and/or inhibition of the development of male or female gonads. Abbreviations: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FST, follistatin; MAPK, mitogen-activated protein kinase; PGC, primordial germ cell; PGD, prostaglandin; PTGDS, prostaglandin D2 synthase.

3.6.1. Activation of *SRY*

Mitogen-activated protein kinases (MAPKs) and GADD45γ have been suggested to activate *SRY* in a cascade involving a MAPK kinase kinase (MAP3K4), a MAPK kinase (MAP2K6, MAP2K3), and a MAPK (p38) (Miyake et al., 2007; Bogani et al., 2009; Warr et al., 2016). Ultimately, p38 MAPK activates GATA4, which binds to the *Sry* promoter together with its co-factor FOG2, and this activates transient *Sry* expression and initiates testis differentiation (Gierl et al., 2012; Warr et al., 2012). In addition to MAPKs and GATA4/FOG2, WT1(+KTS) participates in cell-autonomous regulation of SRY expression, as demonstrated in *Wt1*(+KTS) KO mice (Bradford et al., 2009).

3.6.2. Male pathway is triggered after *SRY* activation

In mouse, SRY directly upregulates the expression of testicular marker SOX9 by binding to *Sox9* enhancer together with SF1 (Sekido and Lovell-Badge, 2008). *Sox9* is expressed at a low level in the bipotential gonads, but the expression rapidly increases in pre-SCs of the male gonads at 11.5 dpc and persists later in SCs from 12.5 dpc onwards in mouse (Sekido et al., 2004). In the absence of *SOX9*, the male gonads of human and mouse encounter sex reversal (Foster et al., 1994; Chaboissier et al., 2004; Barrionuevo et al., 2006). SOX9 as a downstream transcription factor of *SRY* is sufficient for determining testicular fate, as demonstrated by testicular development induced by ectopic expression (in mouse) or duplication of *SOX9* (in human) in the absence of *SRY* (Huang et al., 1999; Vidal et al., 2001).

The expression of SOX9 is followed by upregulation of fibroblast growth factor (FGF) 9 (Colvin et al., 2001; Schmahl et al., 2004; Kim et al., 2006, 2007; Bagheri-Fam et al., 2008), which signals via FGF receptor 2 in the gonads (Kim et al., 2007; Bagheri-Fam et al., 2008). FGF9 is a secreted protein similarly expressed in XX and XY gonads prior to sex determination but thereafter localizing to SCs (Kim et al., 2006). Loss of *Fgf9* in XY mouse leads to male-to-female sex reversal (Colvin et al., 2001; Kim et al., 2006) because the expression of SOX9 is not maintained in XY gonads without FGF9, and therefore, testis-specific morphological events (described in Section 3.6.4) are not induced (Colvin et al., 2001). Another factor affecting SOX9 expression is prostaglandin (PGD) 2, which is produced by PGD synthase expressed by the gonads and acts independently of FGF9 (Wilhelm et al., 2005; Moniot et al., 2009).

3.6.3. Female pathway

Similarly to *Sox9*, *Wnt4* is expressed equally in the mesenchyme of the bipotential gonads in both XY and XX embryos in mouse (Vainio et al., 1999). Its expression is maintained in the female gonads, whereas upregulation of *Sox9* and *Fgf9* in the male gonads suppresses *Wnt4*, thereby resulting in the sex-specific expression patterns of *Wnt4* (Vainio et al., 1999; Kim et al., 2006). Due to a homozygous mutation of *Wnt4*, female mice become masculinized with persistent Wolffian but absent Müllerian ducts and increased steroidogenesis (Vainio et al., 1999). Overexpression of human *WNT4* in male mice disrupts testis vasculature and interferes with testosterone biosynthesis, however, the resulting sex reversal is not complete (Jordan et al., 2003). WNT4 upregulates follistatin (*Fst*) and downregulates inhibin bb expression in directing gonadal specification towards the female fate (Yao et al., 2004, 2006). WNT4 itself is regulated by R-spondin 1, a Wnt signaling modulator acting via LGR4/5 receptors, and a homozygous R-spondin 1 gene deletion in female mice leads to formation of ovotestes that contain both testicular and ovarian tissue (Chassot et al., 2008; Tomizuka et al., 2008; Carmon et al., 2011). Both WNT4 and R-spondin 1 maintain pre-GCs in an unproliferative and undifferentiated state and prevent transdifferentiation of pre-GCs into SCs during fetal development (Maatouk et al., 2013).

Furthermore, ablation of an ovarian transcription factor forkhead box L2 (*Foxl2*) in female mice leads to abnormal ovarian development (Uda et al., 2004). FOXL2 is required to maintain ovarian identity within the developing gonads (Schmidt et al., 2004; Ottolenghi et al., 2005) *e.g.* via FOXL2-mediated repression of SF1/*Nr5a1* (Takasawa et al., 2014) and upregulation of *Fst* expression together with Smad3 (Blount et al., 2009).

3.6.4. Morphological events during sex determination

The earliest morphological difference between the male and female gonads is characterized by massive growth of the testes exclusively, which is mainly undertaken by proliferation of pre-SCs (Schmahl et al., 2000). Proliferation of the pre-SCs involves FGF9, PGD2, and Activin A (Karl and Capel, 1998; Brennan et al., 2003; Schmahl et al., 2004; Archambeault and Yao, 2010).

As SCs differentiate, they encircle germ cells by forming protrusions around them. These clusters of SCs and germ cells are segregated into testicular cords by mesonephros-derived endothelial cells, which start to migrate into the testes at 11.5 dpc in mice (Martineau et al., 1997). This event is subsequently accompanied by migration of myoepithelial cells and PMCs from the mesonephros (Coveney et al., 2008). In humans, testicular cords appear between the 6th and the 7th week of fetal development (Hanley et al., 1999) and later elongate to give rise to seminiferous tubules. The migration of endothelial cells leads to the appearance of the major testicular artery, the coelomic vessel, on the surface of the male gonads, whereas a similar structure does not form in females (Coveney et al., 2008) (Fig. 7). Some of the PMCs and fetal LCs differentiate directly from interstitial mesenchymal cells as a response to signals derived from SCs, and PMCs and SCs together secrete extracellular matrix proteins to form basal lamina at their interface (Skinner et al., 1985; DeFalco et al., 2011).

3.7. Gametogenesis

In both males and females, gametogenesis refers to a complex process in which diploid precursor cells (oogonia and spermatogonia) undergo cell division and differentiation to eventually generate mature haploid gametes.

3.7.1. Spermatogenesis

In males during spermatogenesis, SSCs transit into differentiating spermatogonia, which divide mitotically and develop into spermatocytes that undergo meiotic divisions to generate haploid spermatids, and finally mature into spermatozoa (Griswold, 2016). Within seminiferous tubules, the entire process takes place within SCs, whereby differentiating spermatogonia initiate spermatogenesis at the basal compartment, enter the adluminal compartment as spermatocytes, and mature spermatids are released from the seminiferous epithelium to the lumen (Fig. 2A).

Spermatogenesis begins in puberty and continues throughout a male's life. It is empowered by the endless capacity of SSCs to self-renew and give rise to differentiating progeny (DeFalco and Capel, 2009; Kanatsu-Shinohara and Shinohara, 2013). In human, spermatogenesis takes typically about 74 days to complete, although temporal variation exists between individuals (Misell et al., 2006; Amann, 2008), and typical daily sperm production capacity ranges from 150 to 275 million (Amann, 2008).

3.7.2. Oogenesis and folliculogenesis

In females, gametogenesis initiates already during fetal life. Within ovaries, oogonia divide several times mitotically and proliferate clonally before they enter meiosis (Oktem and Oktay, 2008). At the final stage of the first meiotic division (Prophase I), meiosis halts (Borum, 1961) until it becomes reactivated in the postnatal ovaries at the onset of puberty (Hsueh et al., 2015). At the end of the first meiotic division, the oocyte becomes enclosed by GCs, forming a primordial follicle (Oktem and Oktay, 2008).

In contrast to males, in whom spermatogenesis generates sperm throughout adulthood, the number of oocytes produced during the woman's lifetime is considered to be predetermined already in fetal life. Merely a small fraction of oocytes undergo the full process of folliculogenesis and ovulation, whereas the majority are apoptotically cleared from the ovaries both perinatally and postnatally (Pepling and Spradling, 2001; Matsuda et al., 2012). The number of oocytes within an ovary dramatically decreases from 6-7 million in an 18-week-old female fetus to about a million oocytes at birth (Oktem and Oktay, 2008). Apoptosis is activated in the oocytes to be eliminated, but the definite reasons behind massive loss of oocytes during fetal life are not yet known (Grive and Kathryn Grive, 2020). At the beginning of puberty, a woman has about 400 000 oocytes left in her ovaries, of which approximately 400 undergo ovulation and form mature gametes during her lifetime (Grive and Kathryn Grive, 2020). When almost the entire follicular reserve is consumed, a woman undergoes menopause and loses her reproductive capability.

3.8. Sex hormone steroidogenesis and signaling

3.8.1. Steroid hormone nuclear receptors mediate acquisition of sex characteristics

In addition to providing gametes for sexual reproduction, the gonads hormonally support reproductive and sex-dependent development and function in males and females (Alonso and Rosenfield, 2002; Dohle et al., 2003). Sex hormones (androgens, estrogens, and progestogens) are the final products in a process of steroid biosynthesis or steroidogenesis that occurs within the testes and the ovaries. Generally, androgens (such as testosterone and dihydrotestosterone, DHT) control the development of internal and external male reproductive structures and male characteristics, and support spermatogenesis, while estrogens (estrone, estradiol, and estriol) are responsible for the development and maintenance of female characteristics and regulation of folliculogenesis and reproduction. Gonadal steroids act in their target tissues via sex

steroid receptors, which include androgen receptors, three receptors belonging to the estrogen receptor subfamily, including classical estrogen receptors α and β, and the more recently identified membrane-associated G protein-coupled estrogen receptor (Revankar et al., 2005; Thomas et al., 2005), and progesterone receptors. Sex hormones and their receptors are also involved in several physiological functions and diseases in both males and females (Jia et al., 2015; O'Hara and Smith, 2015; Liu et al., 2019).

3.8.2. Steroidogenesis

In steroidogenesis, cholesterol is enzymatically converted into several precursor hormones, which are subsequently converted into mature biologically active hormones (Miller and Auchus, 2011). The enzymatic reactions are catalyzed by cytochrome P450 steroid hydroxylases and hydroxysteroid dehydrogenases, which are regulated by NR5A family orphan nuclear receptors (Lala et al., 1992; Morohashi et al., 1992; Saxena et al., 2007). The main steroidogenic organs are the gonads and the adrenal glands, and their enzymatic pathways for steroid biosynthesis partially overlap (Fig. 8A). Typically, steroid biosynthesis is initiated by the steroidogenic cells of the main steroidogenic tissues, but final enzymatic conversions in the pathway can also take place at peripheral sites.

Steroidogenic acute regulatory protein (StAR) shuttles cholesterol across the mitochondrial membrane. When this is impaired, affected individuals are unable to produce sufficient amounts of adrenal and gonadal steroids (Lin et al., 1995). Pituitary-derived hormones or analogs of an intracellular mediator, 3',5'-cyclic adenosine monophosphate (cAMP), typically induce *StAR* expression by enhancing its transcription or by stabilizing the *StAR* mRNA (Manna et al., 2003; Duan and Jefcoate, 2007), thereby boosting cholesterol transport.

The first enzymatic step within mitochondria involves the P450 side-chain cleavage (P450SCC/*CYP11A1*) enzyme, which converts the internalized cholesterol into cytosolic pregnenolone in three steps involving hydroxylation and scission of the cholesterol side chain (Miller and Auchus, 2011). This is the rate-limiting step in the production of steroids, as pregnenolone serves as a precursor for sex hormones (Fig. 8A). The following enzymatic processing of the hormones occurs within the smooth endoplasmic reticulum (ER). Within the ER, 3β-hydroxysteroid dehydrogenase type II (encoded by *HSD3B2*) catalyzes formation of progesterone from pregnenolone and progesterone is further converted into hydroxyl (OH)-progesterone and subsequently into androstenedione in two enzymatic reactions involving 17α-hydroxylation and 17,20-lyase activities of cytochrome P450 17A1 (encoded by *CYP17A1*) (Miller and Auchus, 2011). The enzymatic conversion from androstenedione into testosterone, mainly taking place in the testes, involves 17β-HSD type III (encoded by *HSD17B3*) (Ge and Hardy, 1998). DHT, the most potent form of androgens, can be converted from testosterone by two isoforms of 5α-reductase type I or II, encoded by *SRD5A1* and *SRD5A2*, respectively (Ge and Hardy, 1998). *SRD5A1* also participates in conversion of 17-OH-progesterone into DHT via an alternative "backdoor" pathway, which was originally identified in tammar wallaby testes (Wilson et al., 2003), but has since been

revealed to be active also in humans under specific circumstances such as in patients with 21-Hydroxylase Deficiency (Kamrath et al., 2012). 17β-HSD type III together with aromatase (encoded by *CYP19A1*) can convert androstenedione and testosterone into estrogens in the gonads and in peripheral tissues (Alonso and Rosenfield, 2002).

Within the gonads and the adrenal glands, the same enzymatic reactions of *CYP17A1* also convert pregnenolone into dehydroepiandrosterone (DHEA) (Alonso and Rosenfield, 2002), which is progressively converted to dehydroepiandrosterone sulfate (DHEA-S). DHEA and DHEA-S can be further processed into potent sex steroids. DHEA-S is converted by dehydroepiandrosterone sulfotransferase, which is encoded by *SULT2A1*. Immunohistochemical staining of human fetal tissues has revealed that the protein is abundantly expressed in fetal adrenal glands and to a lesser extent in testicular interstitial cells (Parker et al., 1994). Furthermore, active DHEA-S conversion in the adrenal glands has been reported during fetal development (Parker, 1999). Adrenal glands also produce mineralocorticoids corticosterone and aldosterone and glucocorticoid cortisol (Turcu and Auchus, 2015). Their biosynthesis from intermediate steroid metabolites involves cytochrome P450 enzymes encoded by *CYP21A2*, *CYP11B1,* and *CYP11B2* (Fig. 8A).

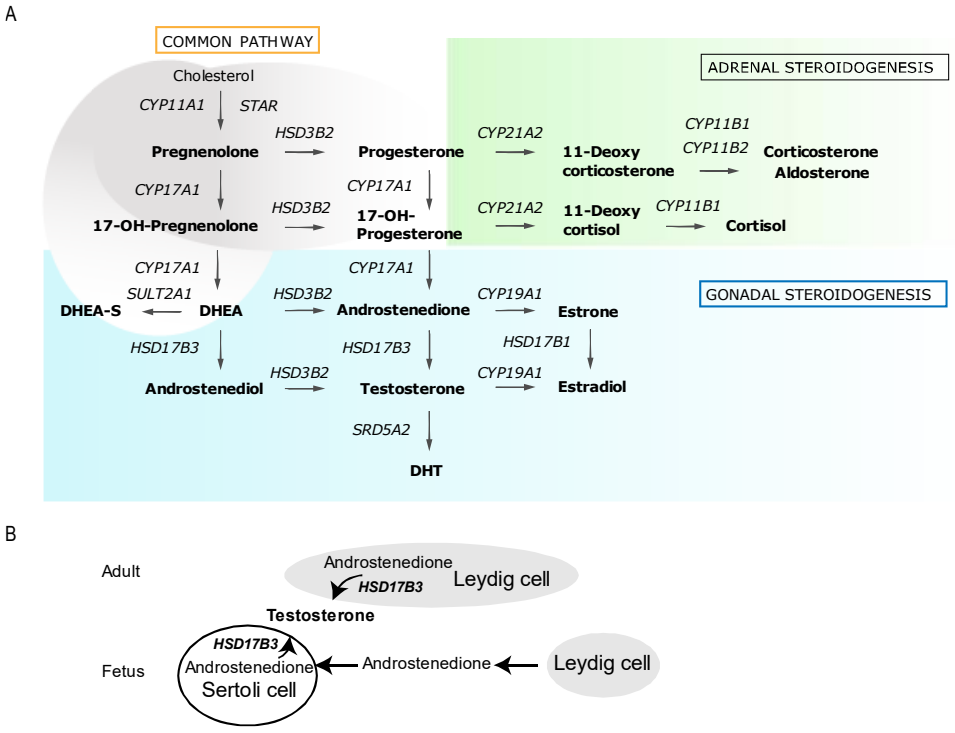

Figure 8. A. Human gonadal and adrenal steroidogenesis and their shared steroidogenic pathways. Genes encoding enzymes of the steroid biosynthesis pathways are presented in *italics,* and steroids are indicated in **bold**. Abbreviations: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone. B. An illustration of testosterone secretion by LCs and SCs of adult and fetal gonads (mainly supported by studies in mice). HSD17B3, which is required to convert androstenedione to testosterone is not expressed by fetal LCs but can be found in fetal SCs.

3.8.3. Fetal gonadal steroidogenesis

Fetal testicular steroidogenesis is crucial for the sex-specific masculinization of the male fetus, whereby the internal (epididymis, vas deferens, seminal vesicles, and prostate) and external (penis, scrotum, and perineum) genitalia develop, testis descent begins, and the fetal brain differentiates (McCarthy et al., 2018; Mäkelä et al., 2019). Mutations in genes encoding the enzymes of steroidogenic pathways may cause undervirilization, which impairs development of the male genitalia and can lead to development of female genitalia despite a 46,XY karyotype (Al Kandari et al., 2006; Rubtsov et al., 2009). In contrast to males, steroidogenic activity within fetal ovaries can be considered minimal (Reyes et al., 1973, 1974; Voutilainen and Miller, 1986), and the absence of testosterone in the female embryo is crucial for the degeneration of the Wolffian ducts.

The steroids produced by the fetal testes differs from those detected in adults, and also the developmental state of the fetus has an impact on steroid production (Ge and Hardy, 1998; O'Shaughnessy et al., 2000; Jarow et al., 2001; Savchuk et al., 2019). For example, the fetal human testes at developmental weeks 6 and 7 produce mainly progesterone and 17-OH progesterone, whereas during later stages of fetal development androstenedione, testosterone, and DHEA become the major steroids produced (Savchuk et al., 2019). The change in the identity of secreted hormones is due to the initiation of testosterone production at the 6^{th} week of development in humans, peaking at around the 9^{th} to 12^{th} week of development (Reyes et al., 1974; Tapanainen et al., 1981). In adults, only LCs are able to synthethize testosterone, but in the fetal testes SCs expressing *Hsd17b3* catalyze the conversion of androstenedione to testosterone, as demonstrated in mice (O'Shaughnessy et al., 2000; Shima et al., 2013) (Fig. 8B). In contrast, fetal LCs do not express *Hsd17b3* in mice (O'Shaughnessy et al., 2000; Shima et al., 2013), an enzyme required for testosterone biosynthesis within the mammalian testes (Andersson et al., 1996; Ge and Hardy, 1998), and therefore are not able to contribute to all enzymatic steps of steroidogenesis (Fig. 8A). In support of this, *HSD17B3* expression has recently been found in fetal SCs but is lacking in early fetal LCs in human (Guo et al., 2021).

Testicular steroidogenesis is also hormonally controlled with a unique temporal pattern at fetal stages compared with adulthood. In human, the evidence received from fetuses lacking a hypothalamus and pituitary indicate that the pituitary hormones start to regulate testicular function and androgen production at the earliest at midgestation (O'Shaughnessy and Fowler, 2011). Prior to this, production of gonadal androgens is regulated by placental chorionic gonadotropin (O'Shaughnessy and Fowler, 2014).

4. Pituitary gonadotropins and their receptors regulate gonadal functions

Hormone secretion and gametogenesis within the gonads are regulated by neuroendocrine control of the hypothalamic–pituitary–gonadal axis, whereby gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus stimulates the anterior pituitary gonadotrope cells to release pituitary gonadotropins, which stimulate gonadal functions (Fig. 9A). The sex hormones and peptide hormones released from the testes and the ovaries in response to pituitary gonadotropin stimulation act on the hypothalamus and anterior pituitary by a negative-feedback mechanism (Fig. 9A).

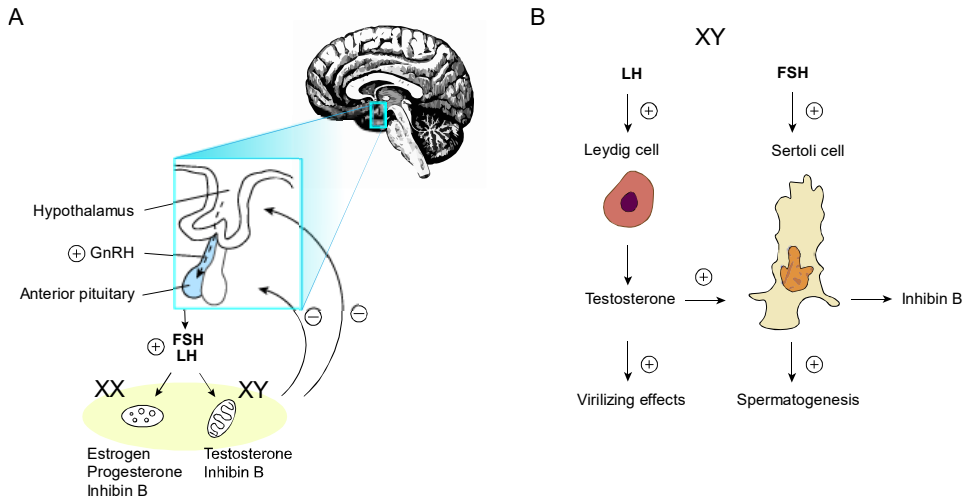

Figure 9. A. Schematic representation of the human hypothalamic-pituitary-gonadal axis. A subset of neurons within the hypothalamus secretes GnRH, which triggers the release of gonadotropins FSH and LH from the anterior pituitary. Gonadotropins stimulate the gonads to secrete steroids and glycoproteins, which inhibit hypothalamic neurons and the anterior pituitary via a negative feedback loop. B. Illustration of somatic gonadal cell types targeted by pituitary gonadotropins in males, the main hormones secreted by gonadal cells in response to stimulation, and their sex-specific reproductive functions. + indicates stimulation, - indicates inhibition. Abbreviations: FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

Pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are heterodimeric proteins consisting of two subunits: a common α-subunit (αGSU, encoded by *CGA*) and a distinct β-subunit (FSHβ or LHβ, encoded by *FSHB* and *LHB*, respectively), which confers specificity for the receptor (Papkoff and Anantha Samy, 1967; Papkoff and Ekblad, 1970; Closset et al., 1975; Boime and Ben-Menahem, 1999). The same αGSU subunit is also present in the structure of LH-related hormone human chorionic gonadotropin, secreted by the placenta, and in a pituitary-secreted thyroid-stimulating hormone thyrotropin. FSH and LH influence gonadal functions by signaling via their cognate receptors, follicle-stimulating hormone receptor (FSHR) and luteinizing hormone/choriogonadotropin receptor (LHCGR), respectively. The receptors are members of the largest class of G-protein-coupled receptors (GPCRs), the evolutionally conserved class A GPCRs (Kakarala and Jamil, 2014) exhibiting a leucine-rich repeat-containing extracellular ectodomain, a large NH_2-terminal domain

specialized for ligand binding, and an intracellular C-terminal end consisting of motifs for signaling (Puett et al., 2005; Ulloa-Aguirre et al., 2018b). Like other GPCRs, they contain a transmembrane domain with seven hydrophobic stretches connected by alternating extracellular and intracellular loops (Puett et al., 2005; Ulloa-Aguirre et al., 2018b), and thereby, are located in the plasma membrane (Means and Vaitukaitis, 1972; Gospodarowicz, 1973).

4.1. Tissue distribution of FSHR and LHR

Within the gonads, FSHR is expressed in testicular SCs (Orth and Christensen, 1977) and in ovarian GCs (Camp et al., 1991; Tisdall et al., 1995; Zheng et al., 1996). LHR is expressed in steroidogenic LCs of the testis and in TCs, luteal cells, and GCs of the ovary (Mamluk et al., 1998; Yung et al., 2014). Both receptors are expressed in fetal urogenital tissues (Rannikko et al., 1995; Apaja et al., 2005). Additionally, several reports have suggested a role for FSHR or LHR in extragonadal contexts based on their detected expression in normal/abnormal tissues such as neoplasms or tumor vasculature (Hudelist et al., 2009; Radu et al., 2010; Siraj et al., 2013), osteoclasts, monocytes, and blood vessel smooth muscle cells (Reshef et al., 1990; Robinson et al., 2010), placenta (Reshef et al., 1990; Stilley et al., 2014b), umbilical cord (Rao et al., 1993; Stilley et al., 2014a), or female reproductive tissues beyond the ovaries (reviewed in Pakarainen et al., 2007; Chrusciel et al., 2019), although controversies exist about the accuracy and significance of these findings (Pakarainen et al., 2007; Chrusciel et al., 2019; Pawlikowski, 2019).

4.2. Main functional roles of FSH and LH in reproduction

4.2.1. FSH and LH regulate spermatogenesis and steroidogenesis in males

In males, FSH and LH contribute to spermatogenesis and testosterone production via SCs and LCs (Levalle et al., 1998; Oduwole et al., 2018) (Fig. 9B). Studies in rodents have revealed that FSH stimulates proliferation of SCs during different stages of their development (Orth, 1984; Sasaki et al., 2000; Sharpe et al., 2003), promotes formation of SC-SC interactions (Sluka et al., 2006) and induces SCs to secrete inhibin B, a glycoprotein hormone that negatively regulates FSH release from the pituitary in several mammals, including primates (Ling et al., 1985; Ultee-Van Gessel and De Jong, 1987; Handelsman et al., 1990).

In addition to its proliferative and self-regulatory actions, FSH regulates completion of meiosis and initiation of the final stage of spermatogenesis together with testosterone (Abel et al., 2008). Testosterone and FSH also support germ cell survival, which has been well-documented in rodents (Tapanainen et al., 1993; Henriksen et al., 1995). These hormones stimulate SCs to produce antiapoptotic factors and regulate SC-dependent control of cell adhesion complexes for germ cell survival and release (Ruwanpura et al., 2010). Although prepubertal SCs do not markedly contribute to androgen signaling (O'Shaughnessy et al., 2012), FSH has been reported to stimulate

production of steroids in immature SCs (Welsh and Wiebe, 1976; McDonald et al., 2006). In contrast, LH controls spermatogenesis in males by stimulating production of androgens in LCs (Fig. 9B), and disruption of androgen-mediated events leads to spermatogenic defects in rodents (O'Shaughnessy et al., 2012).

4.2.2. Ovarian function is maintained by FSH and LH in females

In females, FSH and LH coordinate folliculogenesis by acting via GCs and TCs (Richards and Pangas, 2010). FSH directs the differentiation of GCs, regulates estrogen production, and is essential for follicular growth and maturation (Aittomäki et al., 1995; Kumar et al., 1997; Richards and Pangas, 2010). LH initiates the ovulatory processes and triggers luteinization, a process resulting in development of the corpus luteum (Richards and Pangas, 2010).

4.3. Gonadotropin-stimulated intracellular signaling mechanisms

Gonadotropins activate signaling pathways associated with multiple cellular processes such as proliferation, differentiation, differential gene expression (DE) and functional selectivity (McLean et al., 2002; Sharpe et al., 2003; Wayne et al., 2007; Son et al., 2011). Binding of the ligand to its corresponding receptor activates a complex network of signalling events, which may change according to, for instance, cell identity, developmental stage of the cell, and number of available receptors and their ligands (Dierich et al., 1998; Crépieux et al., 2001; Donadeu and Ascoli, 2005; Wayne et al., 2007).

4.3.1. Activation and inactivation of canonical GPCR signaling

In canonical GPCR signaling, heterotrimeric guanine (G) nucleotide-binding regulatory proteins consisting of α, β, and γ subunits serve as primary upstream activators of enzyme effectors, which trigger production of second messengers and transduction of intracellular signals (Oldham and Hamm, 2008). Upon receptor-mediated activation, the G protein subunits separate (Kahn and Gilman, 1984). The α subunit bound by guanosine-5'-triphosphate (GTP) and the βγ dimer modulate various intracellular signaling cascades (Koch et al., 1994; Ulloa-Aguirre et al., 2018a; Casarini and Crépieux, 2019) prior to the functional deactivation of the α subunit by GTP hydrolysis and re-association of the G protein complex (Oldham and Hamm, 2008). Additionally, gonadotropin-mediated signal can be abolished by clathrin-dependent internalization of the receptor by a family of scaffolding proteins known as arrestins (Nakamura et al., 1998; Troispoux et al., 1999; Kara et al., 2006) (Fig. 10). Typically internalized GPCRs traffic to early endosomes for postendocytic sorting, however, recently, FSHR trafficking has been reported to progress instead via very early endosomes (Jean-Alphonse et al., 2014; Sayers and Hanyaloglu, 2018) (Fig. 10).

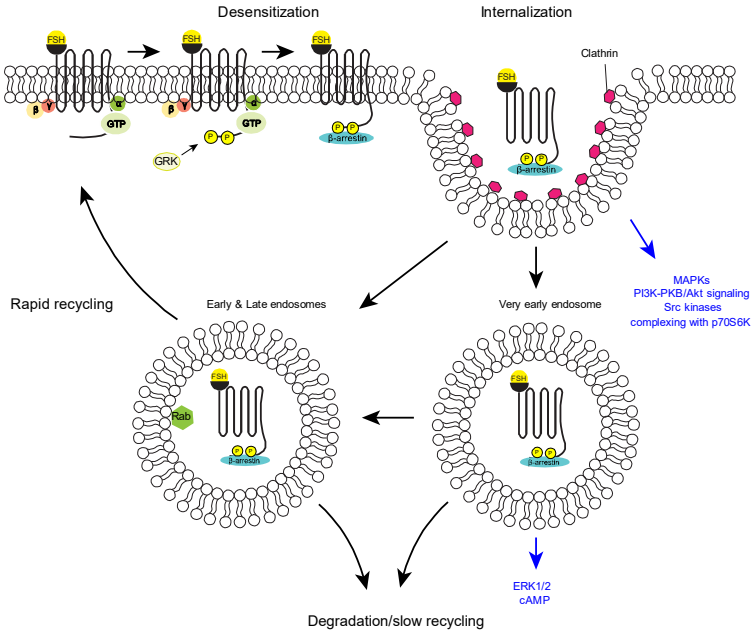

Figure 10. β-arrestin-mediated FSHR desensitization, internalization, and recycling. During receptor desensitization and internalization the responsiveness to the agonist under chronic or repetitive exposure is decreased and the receptor is uncoupled from its signaling cascade. GRKs phosphorylate FSHR C-terminal tail at serine/threonine residues. β-arrestin binds to the phosphorylated tail, thereby preventing binding of the G-protein subunits. The receptor becomes internalized into clathrin-coated pits and is recycled to the plasma membrane (rapid recycling) or targeted to early endosomes (followed by late endosomes) or very early endosomes for receptor downregulation (degradation/slow recycling). The clathrin-coated pit and the very early endosome can serve as platforms for β-arrestin-bound FSHR signaling (blue). Abbreviations: ERK, extracellular signal-regulated kinase; GRK, G-protein-coupled receptor kinase; GTP, guanosine-5'-triphosphate; MAPK, mitogen-activated protein kinase; P, phosphorylation; p70S6K, p70 S6 kinase; PI3K, phosphoinositide 3-kinase; PKB/Akt, Akt serine/threonine kinase; Rab, Rab GTPase.

4.3.2. G-protein-dependent signal transduction of FSHR

Stimulation of FSHR can trigger complex G-protein-dependent or -independent signaling cascades (Fig. 11). These signaling pathways have typically been studied in cultured SCs and GCs, which were originally thought to be the only cell types expressing the receptor.

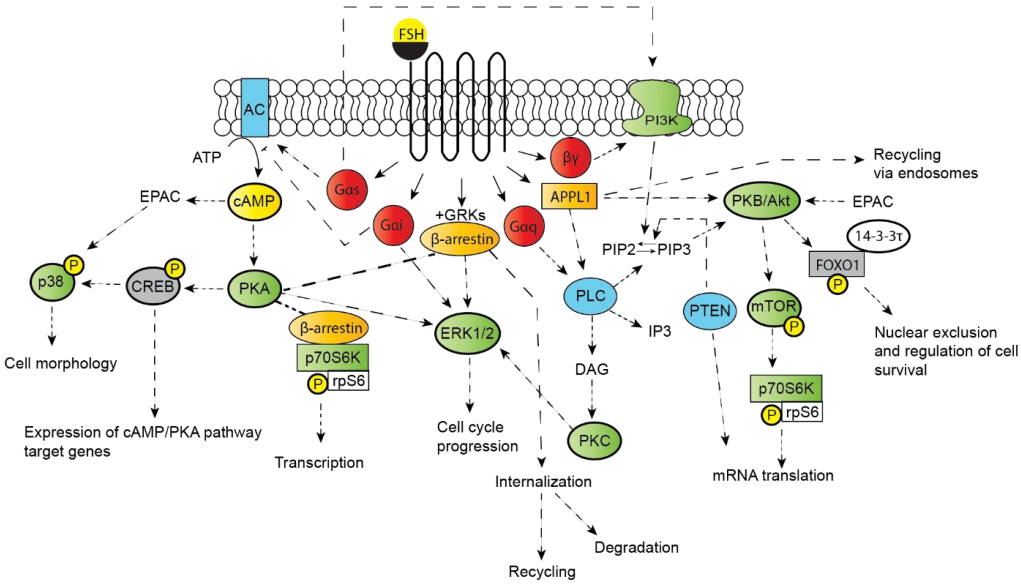

Figure 11. Illustration of the multiple intracellular signaling pathways triggered by FSHR activation. Typically, G-protein subunits act as primary activators of small effector molecules that activate/inhibit enzymatic cascades, eliciting various biological functions. Additionally, other proteins such as β-arrestins, may transduce signals exerted by activated FSHR. G-protein subunits appear in red, kinases in light green, other enzymes in blue, multifunctional intracellular proteins in gold, and transcription factors in grey. Gαs stimulates canonical AC/cAMP/PKA signalling, which activates cAMP reponse element-binding protein. In addition, increased cAMP levels can activate EPAC, which stimulates PKB/Akt signaling. PKA-mediated signaling can indirectly promote phosphorylation and activation of ERK1/2. Gαs can also stimulate the PI3K/PIP3–Akt/mTOR signaling. Gαi inhibits AC and thereby prevents cAMP production by Gαs. Upon FSHR interaction with Gαq, PLC-mediated PKC signaling is activated, leading to release of secondary messenger molecules IP3 and DAG, which activate PKC and induce release of Ca^{2+}. PLC can also activate the PI3K/PIP3 pathway, which induces phosphorylation and activation of the PKB/Akt. FSHR interaction with β-arrestin activates ERK1/2 signaling and interaction with APPL1 activates PLC and PKB/Akt. Abbreviations: AC, adenylyl cyclase; APPL, adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper; ATP, adenosine triphosphate; cAMP, 3',5'-cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; DAG, diacylglycerol; EPAC, the exchange protein directly activated by cAMP; ERK, extracellular signal-regulated kinase; FOXO1, forkhead box protein O1; G, G-protein; Gαs, Gs alpha subunit; GRK, G-protein–coupled receptor kinase; IP3, inositol 1,4,5-trisphosphate; mTOR, mammalian target of rapamycin; P, phosphorylation; p38, p38 mitogen-activated protein kinase; p70S6K, p70 S6 kinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-biphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; PKA, protein kinase A, PKC, protein kinase C; PKB/Akt, Akt serine/threonine kinase; PLC, phospholipase C; rpS6, ribosomal protein S6.

In the most well-characterized G-protein-dependent pathway, primary transduction effector Gs alpha subunit (Gαs) stimulates canonical adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) signaling, which activates cAMP response element-binding protein, a gene transcriptional modulator. PKA-mediated signaling or alternatively destabilization of a PKA-dependent phosphatase can also indirectly promote phosphorylation and activation of the extracellular signal-regulated kinase (ERK)1/2 MAPK involved in cell cycle progression. Instead of activating AC, FSH-induced signaling through Gαs can also stimulate the phosphoinositide 3-kinase (PI3K)/phosphatidylinositol 3,4,5-triphosphate (PIP3)–Akt/mammalian target of rapamycin pathway, which interacts with the PKA pathway in regulation of mRNA translation (Ulloa-Aguirre et al., 2018a).

FSHR can also interact with other G-protein subunits, such as Gαi, which inhibits AC and thereby prevents cAMP production by Gαs (Fig. 11). This has been originally demonstrated in cultured hamster SCs pre-treated with pertussis toxin, which inhibits Gαi activity (Davenport and Heindel, 1987). Another G-protein subunit typically interacting with FSHR is Gαq (Fig. 11). The association of Gαq with phosphoinositide-specific phospholipase C (PLC) leads to release of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which are important secondary messenger molecules in intracellular signaling (Berridge, 2009; Eichmann and Lass, 2015). DAG and IP3 are involved in the activation of protein kinase C and Ca^{2+} mediated signaling, which can support GC and SC functions (Jayes et al., 2000; Seals et al., 2004; Lin et al., 2006) and affect cytoskeletal structure in SCs (Spruill et al., 1983; Franchi and Camatini, 1985).

PLC can also activate the PI3K/PIP3 pathway, which induces phosphorylation and activation of the Akt serine/threonine kinase (PKB/Akt) (Gonzalez-Robayna et al., 2000; Nechamen et al., 2004; McDonald et al., 2006), contributing to biological processes such as cell proliferation, growth, metabolism, migration, differentiation, or survival in the ovaries and the testes (Meroni et al., 2002; Cecconi et al., 2012; Riera et al., 2012; Nascimento et al., 2016).

4.3.3. G-protein-independent signal transduction of FSHR

Beyond G-proteins, FSH induces signaling via other mediators, such as β-arrestins, which are scaffolding proteins involved in FSHR desensitization, internalization, and recycling (Nakamura et al., 1998; Troispoux et al., 1999; Kara et al., 2006; Reiter and Lefkowitz, 2006), but which also have been implicated in FSHR- activated and cAMP-independent ERK1/2 signaling (Crépieux et al., 2001; Kara et al., 2006) (Fig. 11), and interaction of FSHR with adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper (APPL) 1, a forkhead family transcription factor forkhead box protein O1 (FOXO1) a, or a cofactor protein 14-3-3τ (Cohen et al., 2004; Nechamen et al., 2004, 2007; Dias et al., 2010). Despite independent signaling transduction mechanisms, β-arrestins and G-proteins can trigger spatially diverse synergistic or opposing signals, depending on the cellular context (DeFea, 2008).

Of the proteins interacting with FSHR, APPL1 is expressed in several tissues, including the testis and the ovary (Zhou et al., 2017; Zakrzewski et al., 2020), and regulates receptor intracellular signaling and trafficking events (Thomas et al., 2011; Diggins and Webb, 2017) by interacting with *e.g.* PKB/Akt (Mitsuuchi et al., 1999) and the membrane-fusion mediator small GTPase Rab5 (Miaczynska et al., 2004). FOXO1 is involved in many cellular processes, including regulation of FSH-responsive autophagy-related genes in GCs (Herndon et al., 2016), thus regulating follicular atresia. The 14-3-3τ proteins serve as cofactors in serine/threonine phosphorylation, thereby contributing to signaling cascades (Tzivion and Avruch, 2002). The interplay between FSHR, APPL1, and the 14-3-3τ proteins is thought to lead to the abrogation of autophagocytosis in follicles (Ulloa-Aguirre et al., 2018a).

4.4. FSHR and FSHB mutations impair human fertility

4.4.1. Sex- and species-specific effects on mutation phenotype

Originally, GPCR signaling was thought to be mediated by secondary messengers only, and thereby, mutations in GPCRs affecting production of *e.g.* cAMP were referred to as activating or inactivating mutations. Both of these mutation types have been reported in FSHR, although the natural prevalence of the mutations has been considered to be low compared with the natural mutations occurring in LHR (Desai et al., 2013). Some of the mutations identified in FSHR have been suggested to alter the balance between distinct signaling pathways (Aittomäki et al., 1995; Tranchant et al., 2011; Uchida et al., 2013).

The impact of mutations in FSHR or its ligand depend on sex and species. In female mice, KO of *Fshr* or *Fshb* impairs reproduction (Kumar et al., 1997; Dierich et al., 1998). Parallelly in women, inactivating mutations of *FSHR* or *FSHB* can lead to primary amenorrhea and infertility (Matthews et al., 1993; Aittomäki et al., 1995; Layman et al., 1997, 2002). In male mice, a substantially milder effect on fertility follows deletion of either *Fshr* or *Fshb* (Kumar et al., 1997; Dierich et al., 1998; Krishnamurthy et al., 2000). Also in men, reproduction is partially preserved in the presence of mutated *FSHR* despite observed variable degrees of oligozoospermia (<15 million sperm/ml of semen) (Tapanainen et al., 1997). In contrast to *FSHR* mutations, inactivating mutations of *FSHB* in men lead to complete infertility as a result of azoospermia (Lindstedt et al., 1998; Phillip et al., 1998; Layman et al., 2002).

4.4.2. Inactivating A189V mutation in FSHR

The first inactivating mutation in *FSHR* associated with reproduction was a C566T transition predicted for A189V substitution at the extracellular region (exon 7) of the receptor (Aittomäki et al., 1995) (Fig. 12). This mutation is positioned at a stretch of amino acids common to FSHR, LHR, and thyroid stimulating hormone receptor (Nagayama et al., 1989; Minegishi et al., 1990, 1991) and at a region of DNA that is highly conserved across mammals (Sprengel et al., 1990; Gromoll et al., 1993). The sequence and characteristics of molecular events causing the A189V mutation phenotypes, such as primary amenorrhea and infertility in women and oligozoospermia in men (Aittomäki et al., 1995; Tapanainen et al., 1997), are still unknown. However, the current dogma is that the considerably reduced number of A189V receptors at the cell membrane compared with the wild-type (WT) receptors is causing the observed phenotypes (Rannikko et al., 2002; Tranchant et al., 2011). In support of this, despite severely impaired receptor binding both WT and mutated receptors maintain receptor affinity for their ligands in FSH-stimulated immortalized mouse GCs, human embryonic kidney cells, or mouse SCs either stably or transiently transfected with WT or A189V FSHR (Aittomäki et al., 1995; Rannikko et al., 2002; Tranchant et al., 2011). In addition, to further support the capability of the mutated FSHR to maintain receptor affinity for its hormonal ligand despite of the base transition, C566T transition introduced into *LHR* induces a similar effect on the identical transition in *FSHR* (Rannikko et al., 2002).

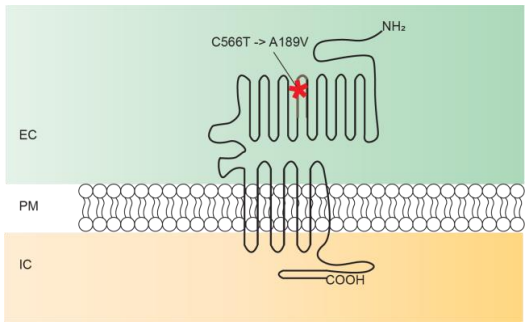

Figure 12. Inactivating mutation at the extracellular region of FSHR gene (exon 7, gray line) encoding a corresponding amino acid change in the protein. Abbreviations: EC, extracellular; IC, intracellular; PM, plasma membrane.

4.4.3. Altered FSHR density may lead to biased signal transduction

The regulatory and functional interplay between FSH and FSHR has been traditionally studied using *in vivo* animal models or different cell lines such as immortalized cell lines overexpressing FSHR, tumor cell lines, or primary cells (Maguire et al., 1997; Rannikko et al., 2002; Tranchant et al., 2011; Casarini et al., 2016). However, considerable limitations exist when using cell lines ectopically expressing FSHR, or tumor cells, due to acquired non-physiological receptor densities or abnormal cellular changes in long-term culture (Rannikko et al., 2002; Tranchant et al., 2011; Casarini et al., 2016). Current evidence indicates that the receptor density at the plasma membrane affects signal transduction downstream of FSHR (Tranchant et al., 2011; Stilley et al., 2016; Sayers and Hanyaloglu, 2018). Furthermore, at least signaling via the conventional cAMP/PKA pathway has been reported to be greatly suppressed in the cells transiently/stably expressing the A189V FSHR, as demonstrated by reduced cAMP production and PKA activity in response to FSH (Aittomäki et al., 1995; Tranchant et al., 2011). Also PKC signaling is disturbed, demonstrated by abolished production of another secondary messenger, IP3 (Rannikko et al., 2002). However, A189V mutant receptor is capable of signaling via ERK1/2 MAPKs, although the protein kinases remain more weakly and transiently phosphorylated than those activated by WT FSHR (Tranchant et al., 2011). Furthermore, ERK activation in cells expressing the mutant receptor has been shown to be β-arrestin-mediated, as signaling was completely abolished in the presence of small interfering RNAs targeting β-arrestins 1 and 2 (Tranchant et al., 2011). When WT FSHR was expressed at reduced levels similar to those of A189V FSHR, β-arrestin mediated signaling became the preferred signal transduction pathway, further underlining the importance of adequate receptor density at the cell membrane (Tranchant et al., 2011).

5. SF1/*NR5A1* in reproduction

In the early 1990s two research groups independently discovered a nuclear protein that acts as a common regulator of nearly all steroidogenic enzymes. It was designated Ad4BP for its ability to bind to adrenal site 4 in the bovine adrenal cortex (Morohashi et al., 1992) and SF1 for its ability to interact with related promoter elements of several steroidogenic enzymes (Lala et al., 1992). Ad4BP/SF1 is encoded by *NR5A1*, a highly

conserved 30 kb gene located on chromosome 9 in humans (Oba et al., 1996; Brandt et al., 2013). Soon after these first discoveries, SF1 was identified in mice as a mammalian homolog of the gene encoding the cofactor fushi-tarazu factor 1 (Lala et al., 1992), which activates *fushi tarazu* and is involved in embryo segmentation in *Drosophila* (Lavorgna et al., 1991; Ohno and Petkovich, 1993). The amino acid sequence of SF1 is well-conserved across vertebrate and invertebrate species, and SF1 ortholog inter-species substitutions have been reported to be able to rescue the phenotype caused by *Nr5a1* deletion (Yussa et al., 2001; Karpova et al., 2005).

5.1. Regulation of SF1/*NR5A1* transcriptional activity

The human SF1 has a size of 461 amino acids, and the main structure of the protein is similar to other nuclear receptors, including a DNA-binding domain containing two zinc fingers and a FTZ-F1 box, an accessory hinge domain, a ligand-binding domain, and two activation domains (Oba et al., 1996; Mullican et al., 2013; Meinsohn et al., 2019). Originally, SF1 was considered to be an orphan nuclear receptor due to its constitutive activation and ligand-independent stable conformation upon activation (Desclozeaux et al., 2002). However, later structural and functional studies suggest that its interactions with different lipids, such as phospholipids or sphingolipids, might regulate the ability of SF1 to transcriptionally activate its target genes (Krylova et al., 2005; Li et al., 2005; Urs et al., 2006; Sablin et al., 2009). Controversies exist about the physiological significance of these lipid-protein interactions *in vivo*, as demonstrated by equal capability of mouse and *Drosophila melanogaster Nr5a1* orthologs, which are thought to have distinct requirements for the presence of ligand, to rescue mutant phenotypes in *Drosophila* (Lu et al., 2013). These studies indicate that no consensus exists about whether SF1 requires an endogenous ligand to become activated.

In addition to interactions with putative ligands, SF1 can interact with a number of co-activator and co-repressor molecules, which either activate or repress, respectively, the transcriptional activity of SF1 in a tissue- or cell context-specific manner (Meinsohn et al., 2019). Other processes modulating SF1 transcriptional activity involve posttranslational and microRNA-mediated regulatory mechanisms (Meinsohn et al., 2019) and SF1 dosage (Achermann et al., 2002; Fatchiyah et al., 2006; Doghman et al., 2013). For example, overexpression of SF1 due to gene amplification in adrenocortical tumors increases the transcriptional activity of SF1 (Doghman et al., 2013). Instead, mutations that impair SF1 binding to target promoters cause altering phenotypes depending on how severely SF1 transcriptional activity is compromised (Achermann et al., 2002).

5.2. Tissue distribution of SF1/*NR5A1*

Nr5a1 is primarily expressed in steroidogenic organs such as the gonads and the adrenal cortex (Ikeda et al., 1994, 1996). In the adult testes, SF1/*Nr5a1* is expressed in SCs and LCs (Ikeda et al., 1993; Buaas et al., 2012; Anamthathmakula et al., 2019), whereas in the adult ovaries, SF1/*Nr5a1* is expressed in GCs, luteal cells and TCs (Ikeda et al., 1993; Buaas et al., 2012). During fetal development SF1/*Nr5a1* is present in the gonadal ridge,

and exhibits a sexually dimorphic expression pattern in rodents and pigs both during and after sex determination (Hatano et al., 1994; Ikeda et al., 1994; Pilon et al., 1998). According to immunohistochemical staining of the human fetal testes and ovaries, *NR5A1* expression persists in the gonads of both sexes (Hanley et al., 1999). In mouse, the developing testis maintains *Nr5a1* expression in pre-SCs and fetal LCs of the testes (Ikeda et al., 1996), however, *Nr5a1* expression disappears from the ovaries between 13.3 dpc and 16.5 dpc, reappearing at 18.5 dpc, presumably marking the beginning of folliculogenesis (Ikeda et al., 1994).

SF1/*Nr5a1* expression is not only limited to steroidogenic tissues but has also been detected in several nonsteroidogenic tissues, such as the ventromedial hypothalamus (VMH) and pituitary gonadotroph cells (Ingraham et al., 1994; Shinoda et al., 1995; Asa et al., 1996; Ikeda et al., 1996), in the skin (Patel et al., 2001), in the placenta (Sadovsky et al., 1995), and in the endothelial cells of venous sinuses and pulp vein of the spleen (Morohashi et al., 1999). The functional significance of SF1/*Nr5a1* expression in these tissues is not clear, but it seems to be closely associated with gene regulation during tissue development (Shinoda et al., 1995; Tran et al., 2003; Zangen et al., 2014). In the VMH and pituitary, SF1 is additionally involved in regulation of energy metabolism and behavior (Zhao et al., 2008; Fosch et al., 2021) and in coordinating endocrine functions (Zhao et al., 2001).

A study performed already in the late 1990s revealed that overexpression of SF1 in non-steroidogenic cells converts these cells into steroidogenic cells. In this study, nonsteroidogenic HeLa cervival cancer cells co-transfected with a reporter construct and an expression vector for SF1 induced transcriptional regulation of gonadal steroidogenesis pathway gene HSD3B2 (Leers-Sucheta et al., 1997), highlighting the fundamental role of SF1 in promoting steroidogenesis.

5.3. Targets of SF1/*NR5A1*

SF1 binds to its targets as a monomer (Wilson et al., 1993), and therefore, its binding differs from most of the other members of the nuclear hormone receptor family, which bind to their targets via receptor dimerization (Helsen et al., 2012). In steroidogenic cells, SF1 is the major regulator of cholesterol metabolism and essential for regulating cytochrome P450 steroid hydroxylases, which convert cholesterol into various steroids (Lala et al., 1992; Ikeda et al., 1993; Morohashi et al., 1993). SF1 triggers the expression of steroidogenic enzymes, such as cytochrome P450 steroid hydroxylases P450SCC (Morohashi et al., 1993), P450c17 (Zhang and Mellon, 1996) and aromatase (Lynch et al., 1993), and upregulates HSD3B2 expression (Leers-Sucheta et al., 1997). In addition to these, SF1 induces expression of a testicular-development associated gene, vanin-1, encoding glycosylphosphatidylinositol-linked membrane-associated pantetheinase, which has a role in cholesterol biosynthesis (Wilson et al., 2005). SF1 also regulates the dosage-sensitive sex reversal and adrenal hypoplasia congenita critical region on the X chromosome, gene 1; *NR0B1* (DAX1) (Yu et al., 1998; Kawabe et al., 1999).

In addition to having a fundamental role in steroid hormone biosynthesis *per se*, SF1 also regulates transcription of steroidogenesis-related genes involved in cholesterol mobilization, including *STAR* (Sugawara et al., 1996; Sandhoff et al., 1998) and electron transporters such as cytochrome b5 (encoded by *CYB5A*) (Huang et al., 2005) and ferredoxin reductase (Imamichi et al., 2014). SF1 is also involved in cholesterol storage and its liberation (Ferraz-de-Souza et al., 2011a; Hołlysz et al., 2011) and regulates the genes of the cholesterogenic pathway (Baba et al., 2018). In addition, SF1 has been reported to directly regulate almost all genes in the glycolytic pathway (Baba et al., 2014). As the glycolysis product pyruvate is converted to cholesterol biosynthesis precursor acetyl-coenzyme A, and steroidogenesis requires cholesterol as a starting material, SF1 has been suggested to link cholesterogenesis, glycolysis, and steroidogenesis pathways, possibly to enable efficient steroidogenesis (Baba et al., 2018).

SF1 is involved in regulation at all levels of the hypothalamus-pituitary-gonadal axis. It regulates the neuronal nitric oxide synthase gene (Wei et al., 2002), which modulates the secretion of GnRH from the hypothalamus, and FSH and LH from the pituitary gonadotropes. In pituitary cells, it stimulates transcription of the genes encoding GnRH receptor (Duval et al., 1997), αGSU (Barnhart and Mellon, 1994), FSHβ (Ingraham et al., 1994; Zhao et al., 2001), and LHβ (Halvorson et al., 1996). In addition, SF1 is crucial in promoting male phenotype, as it stimulates AMH expression (together with GATA4 and SOX9) (Shen et al., 1994; Giuili et al., 1997; De Santa Barbara et al., 1998), thereby inducing regression of Müllerian ducts and development of female genitalia. SF1 is implicated in sex determination since it activates transcription of *SRY* (De Santa Barbara et al., 2001), and interacts directly with SRY at *Sox9* enhancer to induce SOX9 expression (Sekido and Lovell-Badge, 2008). Later during testicular development, SF1 induces the expression of insulin-like 3 (*INSL3*) and thereby enhances testicular descent (Zimmermann et al., 1998).

In addition to its multiple roles in steroidogenesis and endocrine organ development, SF1 is also likely to contribute to cell proliferation and regulation of the cell cycle. This is suggested based on various genetic studies in mice, mechanistic studies in chicken gonads, and various cell lines reviewed by Morohashi et al. (2020). Furthermore, SF1 has also been implicated in stem cell pluripotency and differentiation. SF1 is able to bind to and transactivate proximal octamer-binding transcription factor 4 (*OCT4/POU5F1*) promoter in undifferentiated human embryonal carcinoma cells (Yang et al., 2007) and to maintain and induce specific states of pluripotency in hPSCs (Yamauchi et al., 2020). As stem cells differentiate, the expression of both SF1 and OCT4 decreases (Yang et al., 2007). SF1 has been reported to drive the differentiation of murine ES cells into steroidogenic cells, adipose tissue mesenchymal stem cells into corticosterone-producing steroidogenic cells, and human bone marrow-derived mesenchymal stem cells into steroidogenic cells producing gonadal and adrenal steroids (reviewed in Morohashi et al., 2020). However, differentiation of steroidogenic cells into specific gonadal cell types requires additional factors to SF1, which has been demonstrated in several gene overexpression studies (Buganim et al., 2012; Yang et al., 2017; Liang et al., 2019).

5.4. SF1/*NR5A1 in vivo* studies

5.4.1. First homozygous *Nr5a1* KO mouse models

Mouse embryos lacking both alleles of *Nr5a1* exhibit gonadal and adrenal gland degeneration, XY sex reversal with persistent Müllerian structures, incomplete hypogonadotropic hypogonadism, and VMH anomalies (Ingraham et al., 1994; Luo et al., 1994; Ikeda et al., 1995; Sadovsky et al., 1995; Shinoda et al., 1995; Morohashi and Omura, 1996). These mice are born alive, but most pups die postnatally within a few hours of birth (Luo et al., 1994), likely due to the lack of adrenocortical steroids (Luo et al., 1994; Majdic et al., 2002).

The *Nr5a1*-/- KO mouse models created in the 1990s presented universal phenotypes, but to reveal the tissue- or cell-specific effects of SF1, animal models with targeted ablation of *Nr5a1* specifically in the gonads (Jeyasuria et al., 2004; Pelusi et al., 2008) have been valuable. Targeted deletion of *Nr5a1* in testicular LCs resulted in hypoplastic and cryptorchid testes in male mice (Jeyasuria et al., 2004). Deletion of *Nr5a1* in ovarian GCs led to missing corpora lutea and decreased total number of ovarian follicles. Both male and female mice were sterile. A later study utilized postnatal GC-specific deletion of *Nr5a1* in female mice and reported impaired development of follicles and reduced fertility (Pelusi et al., 2008).

5.4.2. Phenotype of a heterozygous *Nr5a1* deletion in mouse

The gonads and the adrenal glands respond differently to disrupted SF1 expression. Although the ablation of both *Nr5a1* alleles results in complete absence of the adrenal glands and the gonads, the influence of a heterozygous deletion of *Nr5a1* in mice is limited to adrenal development, while gonadal development remains unchanged (Luo et al., 1994; Bland et al., 2000, 2004). However, in adult animals the size of the testes and the ovaries in *Nr5a1*+/− mice is reduced relative to WT mice (Bland et al., 2000), and also the developing testes display lower expression of LC and SC markers (Park et al., 2005). Nevertheless, external genitalia of *Nr5a1*+/− mice are similar to the WT, indicating rather minor consequences of heterozygous *Nr5a1* deletion on gonadal development (Luo et al., 1994). Therefore, in mice, the gonads seem to be less sensitive to SF1 dosage than the adrenal glands.

5.4.3. *Nr5a1* mutations and varying phenotypes in humans

In humans, a single heterozygous mutation in *NR5A1* may lead to gonadal dysgenesis or 46,XY sex reversal (Achermann et al., 1999; Correa et al., 2004; Mallet et al., 2004), but the phenotype can also be milder. Since the identification of the first disease variant of *NR5A1* in a phenotypic female suffering from primary adrenal insufficiency and 46,XY differences in sex development (DSD) with complete sex reversal due to a heterozygous p.Gly35Glu mutation (Achermann et al., 1999), close to 200 *NR5A1* variants have been identified (Fabbri-Scallet et al., 2020). In humans, most *NR5A1* variants present heterozygous mutations, and variants causing adrenal defects are rare (Fabbri-Scallet et al., 2020). Furthermore, a wide spectrum of disease phenotypes exists

due to the different *NR5A1* variants (Ferraz-de-Souza et al., 2011b; Domenice et al., 2016; Fabbri-Scallet et al., 2020), and these are often associated with DSD and infertility. For example, in 46,XY males the phenotype may include hypospadia, partial or complete sex reversal, or infertility (Bashamboo et al., 2010; Domenice et al., 2016; Fabbri-Scallet et al., 2020). In 46,XX females, heterozygous *NR5A1* mutations have been reported to cause ovarian failure (Lourenço et al., 2009; Camats et al., 2012). Moreover, the same *NR5A1* variant may present varying phenotypes (Ferraz-de-Souza et al., 2011b; Fabbri-Scallet et al., 2020), suggesting that, for example, coexisting mutations in other gene(s) encoding protein(s) that interact with SF1/*NR5A1,* or that are associated with sex development, or that are known as epigenetic modifiers at distinct developmental stages may explain the different phenotypes (Ferraz-de-Souza et al., 2011b; Camats et al., 2018).

Similarly, overexpression of *Nr5a1* can have pathological consequences, as demonstrated by induced adrenocortical tumorigenesis in mice and in humans in response to increased SF1 expression (Pianovski et al., 2006; Doghman et al., 2007), highlighting the importance of correct *NR5A1*/SF1 dosage for the development and function of endocrine glands.

5.5. Genes regulating SF1 during the development of gonads

During fetal gonadal development SF1 associates with several factors that regulate its expression (Fig. 13). WT1(-KTS) and LHX9 cooperatively regulate *Nr5a1*, by binding to its promoter region (Wilhelm and Englert, 2002). WT1 binding has been demonstrated to be required for the early development of bipotential gonadal cells, however, SF1 becomes downregulated by WT1 after sex determination (Chen et al., 2017). Also insulin/Igf signaling influences SF1 expression and loss of *Insr/Igf1r* has been reported to reduce SF1 expression and the proliferation of somatic gonadal progenitors in mouse, which can lead to XY sex reversal and a delay in ovarian differentiation (Pitetti et al., 2013). Interestingly, SF1 expression is abolished in *Emx2* mutant mouse (Kusaka et al., 2010), which suggests that Emx2 has a role in regulating SF1.

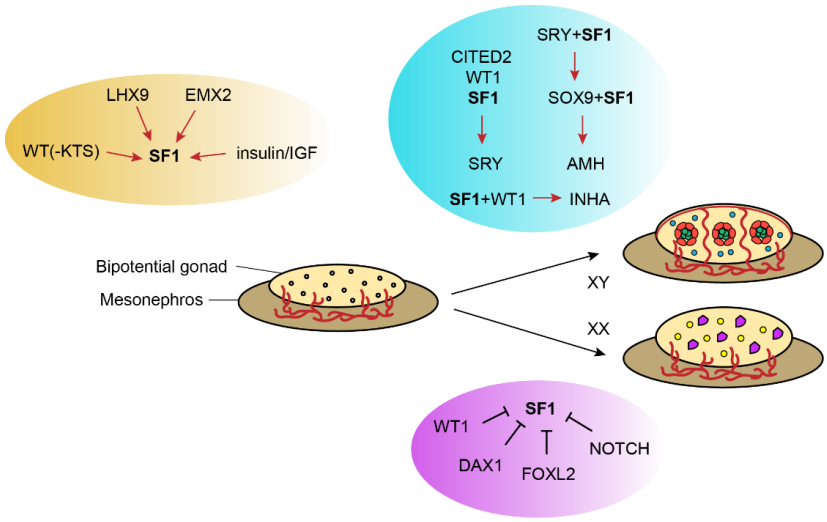

Figure 13. An overview of the factors that regulate SF1 and its downstream targets during early gonadal development. Factors activating or inhibiting SF1 prior to (yellow) and following sex determination (blue in males, pink in females) are shown.

In males, SF1 contributes to the male pathway by enhancing the expression of SRY, AMH, SOX9, and INHA (Giuili et al., 1997; De Santa Barbara et al., 2001; Sekido and Lovell-Badge, 2008; Buaas et al., 2009; Ji et al., 2013), but in females enforced SF1 signaling alone is insufficient to induce testicular differentiation, indicating presence of inhibitory factors or lack of coregulatory factors (Nomura et al., 2019). FOXL2 has been identified to be involved in the transcriptional repression of SF1 in ovarian development by binding to *Nr5a1* proximal promoter, preventing binding of WT1 (Takasawa et al., 2014). A recent study proposed that SF1 suppression in ovaries also involves Notch signaling (Nomura et al., 2019), which mediates interactions between somatic pre-GCs and germ cells during formation of primordial follicles and follicular growth (Vanorny et al., 2014; Terauchi et al., 2016). Furthermore, nuclear receptor protein DAX1, which is expressed in the bipotential gonad but the expression is maintained exclusively in females following sex determination, inhibits SF1-mediated transactivation of SF1 targets (Ito et al., 1997).

6. Current models for human gonadal supporting cell development

Embryonic development of the gonads has been mostly studied in animal models due to ethical reasons, shortage of human material, and difficulties in isolating the genital ridges in very early fetuses (< developmental week 5). In addition, the immortalized and tumor-derived cell lines generated from adult tissues do not typically display characteristics of fetal cells, and therefore, are not sufficient for modeling development of fetal gonads. For these reasons, most of the studies have been originally performed in rodents, especially in mice, although sex determination and development differ between mouse and human (Warr et al., 2011; McClelland et al., 2012; Larney et al., 2014). For example, GATA4 has been shown to directly transactivate porcine and

murine *Sry* promoters in HeLa cells, but not the human *SRY* promoter (Miyamoto et al., 2008), indicating a species-specific effect on *Sry* activation.

Over the years, alternative approaches have been taken to model human gonadal development and function *in vitro* by generating somatic cells of the testes and the ovaries with different methods, and varying cell types of human origin (Table 2).

Table 2. Published methods for gonadal cell modeling *in vitro* using cells of human origin.

Starting cell type	**Method**	**Gonadal somatic cell lineages generated**	**References**
hESCs	Spontaneous differentiation in a 2D culture	Germ-like cells (primarily) and SC-like cells	Bucay et al., 2009
Human umbilical cord blood-derived mesenchymal stem cells	Retrovirus-mediated forced expression of SF1 in a 2D culture	GC-luteal-like cells	Yazawa et al., 2010
hESCs	Directed differentiation as embryoid bodies and in a 2D culture	GC-like cells	Lan et al., 2013
hESCs	Directed differentiation in a 2D or 3D culture	Germ-like cells, SC- and LC-like cells	Kjartansdóttir et al., 2015
Human bone marrow-derived stem cells	Directed differentiation in a 2D culture	LC-like cells	Hou et al., 2016
hiPSCs	Directed differentiation in a 2D culture	GC-like cells	Liu et al., 2016
hESCs	Directed differentiation and overexpression	oocytes and GC cells	Jung et al., 2017
Human umbilical cord-derived perivascular cells	Directed differentiation in a 2D culture	Germ-like cells (primarily) and SC-like cells	Shlush et al., 2017
hESCs	Directed differentiation in a 2D culture	bipotential gonadal-like cells	Sepponen et al., 2017

Human amniocyte-derived iPSCs	Spontaneous differentiation in 2D and 3D cultures	GC-like cells	Lipskind et al., 2018
hiPSCs	Directed differentiation in a 2D culture	SC-like cells	Rodríguez Gutiérrez et al., 2018
hiPSCs	Directed differentiation in a 2D culture	LC-like cells	Chen et al., 2019
hiPSCs	Directed differentiation and overexpression in a 2D culture	LC-like cells	Li et al., 2019
Primary human fibroblasts and hESC-derived fibroblasts	Overexpression in 2D with NR5A1, GATA4, SOX9, WT1 and DMRT1, or with NR5A1 and GATA4	SC-like cells	Liang et al., 2019
hiPSCs	Directed differentiation in a 2D or 3D culture	SC-like cells (primarily) and LC-like cells	Knarston et al., 2020
hESCs	Directed differentiation in a 2D culture and as embryoid bodies	GC-like cells	Yao et al., 2021
hESCs	Directed differentiation in a 2D culture	LC-like cells	Shin et al., 2021
hiPSCs	Overexpression of NR5A1 in a 3D culture	LC-like cells	Ishida et al., 2021

Common methods have relied on direct conversion with transcriptional induction, spontaneous (Bucay et al., 2009; Lipskind et al., 2018) or growth factor- and small molecule-mediated directed differentiation from hPSCs (Lan et al., 2013; Kjartansdóttir et al., 2015; Liu et al., 2016; Sepponen et al., 2017; Rodríguez Gutiérrez et al., 2018; Chen et al., 2019; Knarston et al., 2020; Shin et al., 2021; Yao et al., 2021) or multipotent stem cells (Yazawa et al., 2010; Hou et al., 2016; Shlush et al., 2017), or transcriptional induction of hPSCs combined with exogenous growth factors (Jung et al., 2017; Li et al., 2019; Ishida et al., 2021). However, the differentiation of gonadal-like cells by following their presumed embryonic development stage-by-stage has been only rarely reported (Lan et al., 2013; Sepponen et al., 2017; Knarston et al., 2020; Shin et al., 2021), and adequate monitoring of the induction of the early intermediate developmental stages at and prior to the emergence of the gonadal primordium seems to be neglected in the majority of these studies.

6.1. Induction of SF1/*NR5A1 in vitro*

Although several genes induce SF1 expression during gonadal differentiation (Fig. 13), growth factors or cytokines contributing to this are not known. Yet, knowledge about the triggering cues would be crucial in understanding and mimicking the early steps of gonadal development. Some growth factors, such as IGF and epidermal growth factor (EGF), enhance SF1 phosphorylation or expression in tumor cells (Sweeney et al., 2001; Sirianni et al., 2007), but whether they would increase SF1 expression also in the developing gonads has not been reported. Furthermore, a slight increase in SF1 expression has been recently reported by Wnt signaling agonist administration for several days, or alternatively, by absent or low concentration of BMP4 in differentiated hiPSCs (Knarston et al., 2020). In another study, SC-like cells expressing moderate levels of *Nr5a1* mRNA were generated from mouse ESCs by adding multiple growth factors (FGF2, FGF9, PGD2, FSH, and glia cell line-derived neurotrophic factor) together, without specifying the effect of each individually (Seol et al., 2018). Thus, growth factors inducing SF1 expression in cells mimicking gonadal identity have remained elusive in the literature, and instead of triggering SF1 expression with growth factors or small molecules, SF1 has been overexpressed together with other gonad-associated transcription factors to directly reprogram rodent or human fibroblasts or ESCs into somatic testicular cell types (Yazawa et al., 2006; Jadhav and Jameson, 2011; Buganim et al., 2012; Yang et al., 2015, 2017; Xu et al., 2019; Rore et al., 2021).

7. CRISPR/Cas system and gene activation

7.1. Discovery of CRISPR/Cas

The history of clustered regularly interspaced short palindromic repeats (CRISPR) dates back to the late 1980s, when Ishino et al. (1987) reported their findings in *Escherichia coli*, where they had identified a nearly palindromic pattern of unusual repeat sequences separated by non-repetitive sequences. Later, these repeat sequences were reported to contain associated protein-coding sequences, called CRISPR-associated protein (Cas) (Jansen et al., 2002) and both CRISPR and Cas were demonstrated to act in bacteria as a defense mechanism to overcome phage infections (Barrangou et al., 2007) (Fig. 14). The CRISPR/Cas system has been recognized in nearly half of the identified bacterial and archaeal genomes (Makarova et al., 2011), and it is classified into subgroups, involving different effector proteins such as *Streptococcus pyogenes* Cas9 (Ishino et al., 2018).

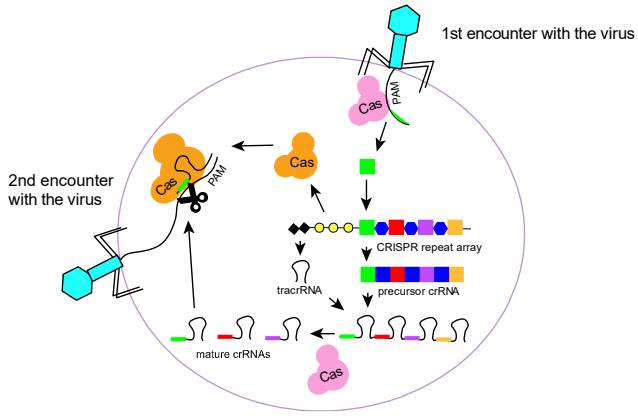

Figure 14. In CRISPR/Cas-mediated adaptive immunity foreign viral DNA is cut by Cas endonucleases and the resulting DNA fragments are incorporated into CRISPR repeat arrays. These consist of short repeat sequences separated by unique spacer sequences of viral or plasmid DNA. Upon second encounter with the virus, the incorporated fragments, transcribed now as precursor crRNAs and small non-coding tracrRNAs, are processed into mature crRNAs with Cas endonucleases. The resulting guide RNAs and Cas endonucleases form a complex to cleave the cognate virus genome at a specific sequence both complementary and non-complementary to the guide RNA sequence and adjacent to a short DNA motif termed PAM (Jinek et al., 2012). PAM sequence is only present in the invading viral genome, allowing the host prokaryote to discriminate self from non-self DNA. Abbreviations: crRNA, CRISPR RNA; PAM, protospacer-adjacent motif; tracrRNA, trans-activating crRNA.

7.2. CRISPR/Cas in genome engineering

In a relatively short time, CRISPR/Cas has revolutionized the field of targeted genome engineering and has proven to be superior in scalability, cost-effectiveness, and ease to engineer. However, the realization of the full potential of early findings and the development of natural CRISPR/Cas systems in prokaryotes into versatile genome and epigenome editing tools, typically derived from type II CRISPR/Cas systems, have required multiple steps in the past decades (Ishino et al., 2018). For example, to facilitate targeted gene editing the prokaryotic CRISPR RNA (crRNA) duplex originally made up of crRNA and trans-activating crRNA components has been modified to a single guide RNA (sgRNA) molecule (Jinek et al., 2012).

7.2.1. CRISPR/Cas9

CRISPR/Cas9 mechanism derived from *Streptococcus pyogenes* has been intensively used in mammalian genome editing. Originally, CRISPR/Cas9 system was recognized as an RNA-guided endonuclease (Gasiunas et al., 2012; Jinek et al., 2012). The first engineered version of CRISPR/Cas9 genome editing tool *in vitro* that was performed in mammals (human- and mouse-derived cell lines) was reported to be able to induce a precise cleavage at endogenous genomic loci and to facilitate homology-directed repair with minimal side effects (Cong et al., 2013). Since then, an array of CRISPR-based applications to modify the genomes or epigenomes of mammals has been published, and the technology is constantly and rapidly evolving (Hendriks et al., 2020). The common applications used in mammals include a gene KO for disrupting gene function, gene editing to create a specific change in the target gene sequence, and introduction of a mutated Cas9 (tagged with *e.g.* a fluorescent dye, a repressor, or an activator domain) to enable long-term cell imaging or modification of endogenous gene expression levels (Cong et al., 2013; Qi et al., 2013; Tanenbaum et al., 2014; Balboa et al., 2015). These mutation(s) in Cas9 can yield a catalytically inactive enzyme that binds to a DNA target sequence without altering it (Qi et al., 2013). In Cas9 protein, the two nuclease domains responsible for creating nicks in target DNA, namely HNH and RuvC, are typically engineered to contain inactivating mutations H840A and D10A, respectively (Jinek et al., 2012). The resulting mutated Cas9 is called dead Cas9 or dCas9.

7.3. Triggering transcriptional activity by introducing transcriptional activator domains

To induce transcriptional activity, dCas9 is fused with transcriptional activator domains (Fig. 15A). One of these transcriptional activator domains is herpes simplex virus-derived VP16 protein, which recruits basal transcription factors (such as TFIIA) (Kobayashi et al., 1995) and transcriptional co-factors, *i.e.* proteins that regulate the activity of transcription factors (such as PC4) (Kretzschmar et al., 1994) to the site of transcription (Uhlmann et al., 2007). In addition, VP16 recruits histone acetyltransferases, *i.e.* p300 (Kundu et al., 2000), modifying the chromatin structure to enable transcription. By fusing dCas9 with an increasing number of VP16 domains (Cheng et al., 2013; Balboa et al., 2015), or by using a combination of different activator domains (Chavez et al., 2015), the transcriptional activation of dCas9 can be further increased. In addition, dCas9 activation can be markedly enhanced, probably by triggering nonlinear cumulative effects, by using multiple non-overlapping gRNAs for targeting dCas9 to the promoter region (Cheng et al., 2013; Balboa et al., 2015).

7.4. Enhancing control of transcriptional activation

The transcriptional control of CRISPR-mediated editing can be enhanced by, for example, placing Cas9 under doxycycline (DOX, tetracycline derivative) -inducible promoter, whereby a conditionally inducible production of Cas9 protein is triggered upon DOX administration. The system requires additional components, such as viral reverse tetracycline-controlled transactivator (rtTA) domain, which binds to a

tetracycline responsive promoter element in DNA to activate gene expression (Fig. 15B). However, DOX-inducible gene expression may be leaky, thus, gene expression may be unintentionally triggered in the absence of DOX. To improve stability of Cas9 protein, a small molecule drug trimethoprim (TMP), which regulates fusion protein stability reversibly and dose-dependently (Iwamoto et al., 2010), has been used (Balboa et al., 2015). It reversibly shields the protein fused with a destabilizing domain (dd) derived from *Escherichia coli* dihydrofolate reductase from degradation (Iwamoto et al., 2010). In the absence of TMP, the fusion protein carrying the dd domain is targeted to proteasomes for degradation (Iwamoto et al., 2010).

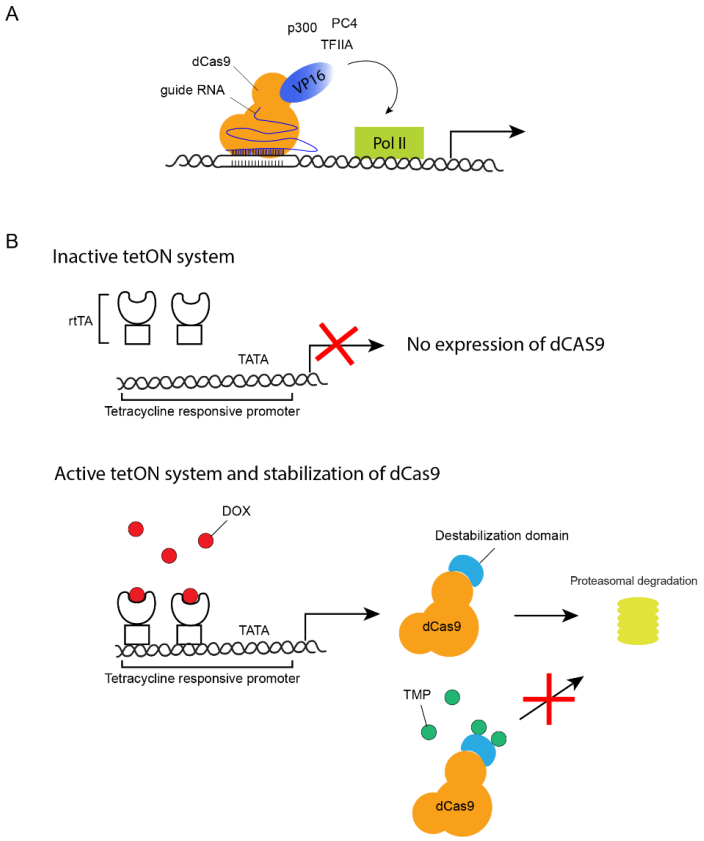

Figure 15. A. CRISPR-Cas9 activation utilizing inactivated Cas9 (dCas9) and guide RNAs, which target Cas9 to the promoter region of the gene of interest. The transactivating domain (*e.g.* VP16) recruits transcription initiation factors and Pol II to activate transcription. B. Dual mechanism for controlling gene expression via TetON system and destabilization of dCas9 protein. In the absence of DOX, rtTA does not bind to the tetracycline responsive promoter and, as a result, dCAS9 is not expressed. In the presence of DOX, rtTA binds to the promoter region and dCas9 is expressed. When fused with a destabilization domain, dCas9 is targeted to proteasomes for degradation. In the presence of TMP, dCas9 is stabilized and escapes degradation. Abbreviations: dCas9, dead Cas9; DOX, doxycycline hyclate; Pol II, RNA polymerase II; rtTA, reverse tetracycline-controlled transcriptional activator; TMP, trimethoprim.

AIMS OF THE STUDY

The general aim was to investigate regulatory and signaling mechanisms in the early human gonads and to generate hPSC-based models for differentiation of SCs and GCs.

Specific aims were as follows:

To establish an *in vitro* model for mimicking development of the bipotential gonads and gonadal cells in humans (Study I)

To evaluate the effects of *NR5A1*/SF1 activation on early gonadal development (Study III)

To establish a differentiation model for examining the function of WT FSHR and FSHR with an inactivating A189V mutation (Study II)

MATERIALS AND METHODS

1. Ethical considerations

The generation and use of the hiPSC lines described in this thesis at the Department of Obstetrics and Gynecology, University of Helsinki was approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District (HEL127.6 and HEL128.5 cell lines: No. HUS/2064/2019, 333/13/03/03/2013) with informed consent from the donors.

2. Cell lines

All cell differentiation studies were performed with hPSC lines. Human ESC line H9 (WA09, 46,XX) (Thomson, 1998) was obtained from WiCell Research Institute (Madison, WI, USA). HEL127.6 and HEL128.5 hiPSC lines were generated at the Biomedicum Stem Cell Center (University of Helsinki, Helsinki, Finland) from fibroblasts obtained from two individuals (both 46,XX) with FSH-resistant ovaries (FSHRO) caused by an inactivating A189V mutation in the *FSHR*. HEL46.11 (46,XY) and HEL11.4 (46,XY) hiPSCs and HEK293 cells were kindly provided by the Biomedicum Stem Cell Center.

2.1. Cell maintenance (Studies I-III)

All hPSCs were maintained at 37˚C in humid atmosphere containing 5% CO_2. Cells occasionally underwent mycoplasma testing and were negative for mycoplasma. hPSCs pluripotency was maintained by culturing them on gelatinous matrices derived from mouse sarcomas, Growth Factor Reduced Matrigel® (Corning) or Geltrex® Lactose Dehydrogenase Elevating Virus-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), in either StemPro™ medium or in Essential 8™ medium (both from Thermo Fisher Scientific). The medium was changed every 1-2 days depending on cell confluency. To enhance the post-thaw survival rate of single hPSCs, Rho-associated coiled coil forming protein serine/threonine kinase inhibitor (ROCKi) Y-27632 2HCl (Selleckchem, #S1049) was used for 24 h after the thawing of cells.

hPSCs were passaged with 0.5 mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen™, Thermo Fisher Scientific, Grand Island, NY, USA) in Dulbecco's phosphate-buffered saline solution (PBS; all from Thermo Fisher Scientific, Waltham, MA, USA) approximately twice a week. HEK293 cells were passaged every 2-4 days with Trypsin-EDTA (Thermo Fisher Scientific) and cultured on Tissue-Culture Treated Dishes (Corning) in high-glucose DMEM supplemented with GlutaMAX™ (Gibco™) and 10% fetal bovine serum (FBS).

2.2. Generation of hiPSCs expressing endogenous *EMX2* or *NR5A1* (Study III and unpublished results)

DDdCas9-VP192 was introduced to the HEL46.11 cell line (kindly provided by the Biomedicum Stem Cell Center, University of Helsinki, Helsinki, Finland) for CRISPR-mediated gene activation. DDdCas9-VP192 contains a dCas9 with D10A and H840A mutations, a DD domain attached to dCAS9 for protein destabilization, and a transactivation domain, which is a 12x multiplication of VP16 domains. A tight promoter-DDdCas9VP192-T2A-GFP-IRES-Neo cassette was cloned to a destination plasmid with PCR cloning the corresponding sequence in the transposon system plasmid PiggyBac (PB)-tight-DDdCas9VP192-T2A-GFP-IRES-Neo (Addgene_102889). The destination plasmid contained homology arms for a human genome safe harbor locus adeno-associated virus integration site 1 (AAVS1), which ensures integration of a single copy of the cassette. Similarly, CAG-rtTA DOX-responsive transactivator was PCR-cloned to a destination vector containing the AAVS1 homology arms. Both destination vectors were electroporated to HEL46.11 parental cell line together with eSpCas9(1.1)_No_FLAG_AAVS1_T2 (Addgene_79888) expressing high specificity *S. pyogenes* Cas9. eSpCas9(1.1) was introduced to generate breaks in DNA strands at the AAVS1 locus and to allow homologous recombination of the donor plasmids.

Subsequently, cells were expanded and clonally selected with an aminoglycoside antibiotic geneticin (G418, Thermo Fisher Scientific) for 2 weeks. The capability of the viable clones exhibiting DDdCas9-VP192-GFP construct to induce its expression was tested by treating the clones with DOX, and subsequently, single-cell clones were generated. Clone 3H/G4 was used for all SF1 induction experiments.

RNA guides were designed to target the sequence upstream of the gene transcription initiation site, just prior to the Cas9 binding site. For inducing *EMX2* or *NR5A1* expression, the proximal promoter region (approximately -350 to -50 bp) was selected and guides were designed with online CRISPR designing tools (http://crispr.mit.edu/ or https://benchling.com) to target this location. The guides to be tested in HEK293 for their efficiency to induce gene expression were selected based on their target efficiency scores and location on the promoter sequence.

Each guide was incorporated into guide RNA transcriptional units with PCR following a previously published protocol (Balboa et al., 2015). The guides were equipped with U6 RNA polymerase III promoters to drive expression of small transcripts and terminator products amplified from the pX335 plasmid. To test the ability of the guide RNAs to induce target gene expression, the purified transcriptional units alone and in various combinations, were transfected to HEK293 cells. For this, 10^5 cells/well were seeded on gelatin-coated 24-well plates and cultured for one day until the cells were transfected with 200 ng of each guide-transcriptional unit or their combination and 500 ng of CAG-dCAS9-VP192-T2A-EGFP-ires-puro transactivating domain encoding plasmid. Samples were collected after 72 h. Transfections were performed with FUGENE® HD Transfection Reagent (Promega Corporation, Madison, WI, USA),

which was added in high-glucose DMEM supplemented with GlutaMAX™ + 10% FBS (both from Gibco™). Transfections were performed in at least four wells/construct, and from three of the wells, mRNA was isolated for real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). One well/construct was used for immunocytochemistry. Additionally, non-tranfected wells served as controls.

A combination of guide-transcriptional units inducing the highest *NR5A1/*SF1 expression in qRT-PCR and immunocytochemistry was selected and concatenated into a GG-dest vector (Addgene_69538) (Balboa et al., 2015) with Golden Gate Assembly (Cermak et al., 2011). The assembly reactions were transfected into DH5α chemically competent bacteria (New England Biolabs, Inc.). Clones positive for GG-dest plasmid were screened on Luria-Bertani agar plates containing ampicillin (Gibco, Cat. #11593-027). In addition, the plates were coated with isopropyl thiogalactoside (IPTG, Sigma, Cat. #16758) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (Xgal, dissolved in DMSO, Sigma, Cat. #B4252) to enable screening of the clones containing the guide insert. Five positive clones were processed into plasmid minipreps as described previously (Balboa et al., 2015). Correct guide concatenation was verified by restriction analysis with EcoRI and NotI and by Sanger sequencing analysis at GATC Biotech, Eurofins Genomics, Germany. The guide cassette was subsequently subcloned to an EBNA episome plasmid (EBNA plasmid backbone Addgene_102898, Addgene, Weltner et al., 2018, both kindly provided by the Biomedicum Stem Cell Center), due to the suitable cloning sites available, prior to its cloning into a PB plasmid (PB plasmid backbone Addgene_102893, Addgene, Weltner et al., 2018).

The guides selected for concatenation to activate *NR5A1* expression (U6 promoter sequence; G nucleotide for transcription in human cells; **sgRNA sequence**; trans-activating crRNA):

NR5A1_1:GTGGAAAGGACGAAACACCG**GAGGCCTGCAGAGTCACGTG**GT
TTTAGAGCTAGAAATAG

NR5A1_2:GTGGAAAGGACGAAACACCG**AGGCCTGCAGAGTCACGTGG**GT
TTTAGAGCTAGAAATAG

NR5A1_3:GTGGAAAGGACGAAACACCG**CACCCGGTTTCTAACAAGCG**GT
TTTAGAGCTAGAAATAG

NR5A1_4:GTGGAAAGGACGAAACACCG**CAGGGAGGTAGCCATTCACA**GT
TTTAGAGCTAGAAATAG

For *EMX2* activation in HEL46.11 cells, a sgRNA (sequence 5'-CCGGGGTGCCCCGGAGGGAG-3') demonstrating the highest *EMX2* mRNA expression was selected and cloned into a PB plasmid (plasmid backbone Addgene_ 102908, Addgene, Weltner et al., 2018) via 3-piece cloning using EcoRI+NotI on the gRNA, and EcoRI+XbaI and XbaI+NotI restriction sites in the plasmid. Upon

establishing HEL46.11-EMX2-VP192 and HEL11.4-EMX2-VP192 lines, the DDdCas9VP192 activation domain was introduced to HEL46.11 hiPSCs within a safety harbor locus, while in HEL11.4 hiPSCs the activation domain was introduced within a PB plasmid (PB-tight-DDdCas9VP192-T2A-GFP-IRES-Neo, Addgene_102889), thus control of the integrating dCas9 copy number was better maintained in the former.

Clonal hiPSC lines were generated as described in Study III. The clones containing RNA guides were selected with 2.5-5 µg/ml puromycin (Gibco, Grand Island, NY, USA). HEL46.11-EMX2 clone 2 and HEL46.11-NR5A1 clone 14 were used for inducing *EMX2* and *NR5A1* expression, respectively, in subsequent studies. Studies to induce *EMX2* expression in HEL11.4 background were performed with a pool of clones.

3. Differentiation of gonadal-like cells and cells expressing *FSHR* (Studies I-III)

hESCs in Study I were differentiated according to 17 distinct protocols, two of which (D and M) were further modified and followed in Studies II and III, respectively. In all differentiation protocols, cells at about 80% confluency were dissociated with 0.5 mM EDTA, resuspended in medium containing DMEM/F12 + Glutamax supplemented with 2% B27-supplement (both from Thermo Fisher Scientific) and counted manually with a hemocytometer. Subsequently, cells were centrifuged at 70 g (Studies I and III) and resuspended in medium containing 100 ng/ml Nodal signaling substitute Activin A (Q-kine, University of Cambridge, UK), 5 µM glycogen synthase kinase inhibitor CHIR-99021 (Selleckchem), and 10 µM Rho kinase inhibitor Y-27632. Cells differentiated in all but one protocol (Study I, Fig. 1A) were additionally exposed to a small molecule BMP signaling inhibitor Dorsomorphin (Selleckchem), which was added to the cells at 2 µM concentration.

A total of $1.5x10^5$ cell/cm^2 were plated on tissue-culture-treated 12-well plates (Corning) coated with 0.5 µg/cm^2 human Collagen type 1 (Corning, NY, USA). However, due to uncertain access of the product, in a subset of experiments in Study III, CellAdhere™ Type I Collagen (StemCell Technologies) replaced the Corning product. For immunocytochemistry, 10-day gonadal differentiations were performed on Collagen type 1-coated glass coverslips (Study I) or μ-slide 4 wells (polymer-coated, ibidi GmbH, Gräfelding, Germany) (Study III).

Media were changed every 1-2 days according to the overview of protocols presented in Study I (Fig. 1A), and between changes of different growth factors/cytokine inhibitors, cells were washed at least once with 1x Dulbecco's PBS (containing $CaCl_2$ and $MgCl_2$, Sigma, Cat. #D1283). For example, cells differentiated according to protocol M were washed once with PBS 24 h after seeding and the medium was changed to a basal medium containing 10 ng/ml BMP7 (Peprotech, Cranbury, NJ, USA) and 3 µM CHIR-99021. After another 24 h, cells were carefully washed with PBS and basal medium containing 2 µM Dorsomorphin and 3 µM CHIR-99021 was added to the cells. Cells were incubated for 48 h, washed with PBS, and cultured in basal medium with (Study

III) or without DOX and TMP (Studies I and III) until completion of differentiation. In Studies I and III, cells were differentiated for 8 and 10 days, respectively.

For combined 2D and 3D culturing studies, cells differentiated to day 8 were washed twice with 1x Dulbecco's PBS (Thermo Fisher Scientific, Cat. #14200-067). They were then dissociated into single cells with StemPro®Accutase®Gibco® (Life Technologies™) for 5 min at 37°C until DMEM/F12 (including Glutamax) + 2% B27 was added to inhibit enzymatic activation. Cells were subsequently counted with an automated cell counter (Countess II, Thermo Scientific) using Trypan Blue (Invitrogen, Cat. #T10282) to stain dead cells and an estimation of 1000 cells/microwell (each microwell 400 µm in size, $1.2x10^6$ cells/culture well) of live cells were seeded to pre-rinsed (Aggrewell™ Rinsing Solution; Cat. #0701) 24-well cell culture plates (Aggrewell™ 400, Stem Cell Technologies, Cat. #34411) according to the manufacturer's instructions. ROCKi Y-27632 was used at 10 µM concentration in DMEM/F12 + 2% B27 in the presence and absence of DOX and TMP for 2 days, with daily replacement of the majority of the media.

Differentiation into FSHR-expressing cells (Study II) was similar to that described above for protocol D, except for minor modifications. Cells were dissociated with Accutase as described above, which was followed by manual counting of cells and harvesting at 107 g before resuspension into differentiation medium containing Activin A, CHIR-99021 and Y-27632 at concentrations described above. After 24 h, medium containing 50 ng/ml BMP7 and 3 µM CHIR-99021 was changed on the cells. In the following days, cells were differentiated as described above.

4. Analysis methods

4.1. RNA isolation and reverse transcription (Studies I-III)

To examine changes in gene expression upon differentiation, cell samples were washed with PBS (Thermo Fisher Scientific), harvested, and lyzed into RA1 Lysis buffer (Macherey Nagel), and RNA was extracted by Nucleospin RNA kit (Macherey Nagel). Manufacturer's instructions were followed, except for steps regarding DNAse treatment, which was performed separately from the kit to ensure efficient removal of residual genomic DNA. Isolated RNA was treated for 30 min at 37˚C with RQ1 RNase-Free DNase in RQ1 DNase Reaction Buffer (both from Promega). RNA integrity was ensured by adding RiboLock RNAse Inhibitor (Thermo Fisher Scientific) into each reaction. Subsequently, samples were purified with RNA Clean-up kit (Macherey Nagel), and RNA concentration and purity were measured with Denovix spectrophotometer. To evaluate gene expression changes in undifferentiated cells upon 3-day *NR5A1* induction, cells were washed with PBS, harvested, and lyzed into LBP buffer (Macherey Nagel), and RNA was extracted with Nucleospin RNA Plus kit (Macherey Nagel). The on-column removal of genomic DNA and subsequent sample purification were performed as instructed by the manufacturer.

For each reverse-transcription reaction (20 µl), 2 µg of total purified RNA was mixed with nuclease-free H_2O and incubated at 65˚C for 1 min to break double-stranded RNA structures. After this, samples were combined with a mix of all deoxynucleotide triphosphates, Oligo(dt) 15 primers, Random Hexamer Primers, RiboLock RNAse Inhibitor (all from Thermo Fisher Scientific), and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega), and the reactions were incubated at 37˚C for 90 min, followed by enzyme inactivation at 95˚C for 5 min. For qRT-PCR, the transcribed cDNA was diluted with nuclease-free H_2O in such a way that each reaction contained cDNA converted from 0.05 µg/µl RNA.

4.2. qRT-PCR (Studies I-III)

Forward and reverse primers (Metabion, Planegg/Steinkirchen, Germany) at a final concentration of 0.5 µM each, HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia) diluted to 1x and cDNA was combined in a 20 µl reaction volume that was filled with nuclease-free H_2O. Relative mRNA expression levels were analyzed with LightCycler96 System (Roche Diagnostics, Mannheim, Germany). Relative quantification of gene expression was performed by following the ΔΔCt method (Livak and Schmittgen, 2001). Expression levels were normalized using *CYCLOPHILIN G* (also known as peptidylprolyl isomerase, *PPIG*) as an endogenous control and presented as relative to expression levels in undifferentiated cells. Primer sequences are listed in Table 3.

Table 3. qRT-PCR primer sequences

Gene symbol	**Sequence**
HAND1	Forward: 5'-AATCCTCTTCTCGACTGGGC-3' Reverse: 5'-CCTTCAAGGCTGAACTCAAGA-3'
GATA6	Forward: 5'-CGCTGTTTGTTTAGGGCTCGG-3' Reverse: 5'-CCTCTTACTGCTCTGCCGGA-3'
EMX2	Forward: 5'-GTCATCGCTTCCAAGGGAAC-3' Reverse: 5'-GGCGTGTTCCAGCCTTAGAA-3'
LHX9	Forward: 5'-GCGAACCTCTTTCAAGCATC-3' Reverse: 5'-TCCTTCTGAATTTGGCTCGT-3'
CYP11A1	Forward: 5'-TGGTGACAATGGCTGGCTAAA-3' Reverse: 5'-ATAAACCGACTCCACGTTGC-3'
CYP17A1	Forward: 5'-TCAGCCGCACACCAACTATC-3' Reverse: 5'-GCAAACTCACCGATGCTGGA-3'
CYP19A1	Forward: 5'-GTGGCCCATGGCATTTTATA-3' Reverse: 5'-GAGCTCTACTGGGGAACCAG-3'
STAR	Forward: 5'-GAGTCAGCAGGACAATGGGG-3' Reverse: 5'-CGCTCCACGAGCTCTTCATA-3'
HSD17B3	Forward: 5'-CCACAGAGATCGAGCGGAC-3' Reverse: 5'-GGTTTGGAAGCATTCCGACAT-3'
HSD3B2	Forward: 5'-GAGGCAGTAAGGACTTGGACT-3' Reverse: 5'-TGACCCAGAAGAGGGCGTAA-3'
CYP21A2	Forward: 5'-CTTGGGCTGCAAGATGTGGT-3' Reverse: 5'-TCAGGTCTGCCAGCAAAGTC-3'
LHCGR	Forward: 5'-CCGGTCTCACTCGACTATCAC-3' Reverse: 5'-TGAGGAGGTTGTCAAAGGCAT-3'
SCARA5	Forward: 5'-GAGATCTGAGGGGACAAGGC-3' Reverse: 5'-TCGCTGACGGTGTGTAGGTA-3'
MAGEB1	Forward: 5'-CTCAGAAAACAGGACCTTGATGT-3' Reverse: 5'-AGGAGATGTGGCACCTTGAC-3'
ADAMTS14	Forward: 5'-ACTTTGGGCCCTCAGGGTAT-3' Reverse: 5'-CTCATGGTTGAGGGCACAGC-3'
FSHR	Forward: 5'-ATCTGTCACTGCTCTAACAGGGT-3' Reverse: 5'-TCTCCAGGTCCCCAAATCCT-3'
CYCLOPHILIN G	Forward: 5'-TCTTGTCAATGGCCAACAGAG-3' Reverse: 5'-GCCCATCTAAATGAGGAGTTG-3'

GATA4	Forward: 5'-CAGGCGTTGCACAGATAGTG-3' Reverse: 5'-CCCGACACCCCAATCTC-3'
NR5A1	Forward: 5'-CAGGAGTTTGTCTGCCTCAA-3' Reverse: 5'-GCACAGGGTGTAGTCAAGCA-3'
WT1	Forward: 5'-GGCAGCACAGTGTGTGAACT-3' Reverse: 5'-CCAGGCACACCTGGTAGTTT-3'
AMH	Forward: 5'-CGCCTGGTGGTCCTACAC-3' Reverse: 5'-GAACCTCAGCGAGGGTGTT-3'
INHA	Forward: 5'-CTCGGATGGAGGTTACTCTTTCAA-3' Reverse: 5'-GAAGACCCCCCACCCTTAGA-3'
LHX1	Forward: 5'-TCATGCAGGTGAAGCAGTTC-3' Reverse: 5'-TCCAGGGAAGGCAAACTCTA-3'
PAX2	Forward: 5'-GGAAAGGCTGCTGAACTTTG-3' Reverse: 5'-TATGTTCGCCTGGGAGATTC-3'
OSR1	Forward: 5'-GCTGTCCACAAGACGCTACA-3' Reverse: 5'-CCAGAGTCAGGCTTCTGGTC-3'
BRACHYURY/T	Forward: 5'-GCATGATCACCAGCCACTG-3' Reverse: 5'-TTAAGAGCTGTGATCTCCTC-3'
OCT4	Forward: 5'-TTGGGCTCGAGAAGGATGTG-3' Reverse: 5'-TCCTCTCGTTGTGCATAGTCG-3'
PAX6	Forward: 5'-TTTGCCCGAGAAAGACTAGC-3' Reverse: 5'-CATTTGGCCCTTCGATTAGA-3'
SOX1	Forward: 5'-CCCATGCACCGCTACGACAT-3' Reverse: 5'-AGGGCGACGCGCTCATGTA-3'
DLK1	Forward: 5'-GGACGGGGAGCTCTGTGATA-3' Reverse: 5'-CGTCCTTTTTCTGGCAGTCC-3'

4.3. Flow cytometric analysis (Study I)

Undifferentiated and differentiated cells at day 8 were dissociated with TrypLE (Gibco), and after cell counting, 1 million cells were resuspended in 5% FBS in Dulbecco's PBS. Cells were pelleted by centrifuging, fixed, and permeabilized on ice for 20 min with Cytofix/Cytoperm, ready-to-use (BD Biosciences). Following this, cells were washed twice with BD Perm/Wash buffer (BD Biosciences). Antigen blocking was performed on ice for 20 min using Perm/Wash supplemented with 10% FBS. For antibody labeling, the primary antibodies were diluted in Perm/Wash buffer and incubated on cells for 1 h on ice. Subsequently, cells were pelleted and washed twice prior to the addition of secondary antibodies diluted in Perm/Wash buffer, which had been supplemented with

4% FBS. Following a 40-min incubation on ice in the dark, cells were washed twice and resuspended in 0.5 ml of Dulbecco's PBS containing 5% FBS. The flow cytometric analysis was performed with BD Accuri™ C6 equipment (BD Biosciences) with 30 000 cells and the data was analyzed with BD Accuri™ C6 Software (BD Biosciences).

4.4. Immunocytochemistry (Studies I and III)

Differentiated cells, either cultured on collagen-coated round glass coverslips (diameter 18 mm, #1.5, Menzel-Gläzer, Study I) or on polymer-coated µ-slide wells (Study III) were fixed with 4% PFA in PBS for 15-20 min at room temperature and washed at least three times with PBS. Permeabilization was performed using 0.5% Triton® X-100 (Fisher Scientific) in PBS, which was incubated on cells for 8 min (Study III) or 15 min (Study I). Subsequently cells were washed and incubated with antigen blocking solution (Ultra Vision Protein Block, Thermo Scientific) for 10 min. Primary antibodies were diluted in 0.1% Tween (Fisher Scientific) in PBS and incubated on cells overnight at 4˚C. The next day, cells were washed with PBS as described above and incubated with secondary antibodies, diluted in 0.1% Tween in PBS for 30 min at room temperature, in the dark. The antibodies used in this study are presented in Table 4.

Table 4. List of antibodies

Primary antibodies			
Antigen	**Host species**	**Dilution**	**Manufacturer, catalog #, clone, lot**
AMH	Rabbit	1:200	Abcam, Cat# ab84415, polyclonal, lot GR136308-6(N/A)
GATA4	Goat	1:250	Santa Cruz Biotechnology, Cat# sc-1237 (C-20), polyclonal, lot G2414
HSD3B2	Mouse	1:100	Sigma-Aldrich, SAB1402232, clone 1E8, lot 08274-1E8
Inhibin α (N-terminal region)	Mouse	26 µg/ml	Ansh labs, kindly provided by Dr. Ajay Kumar, 20/32, monoclonal
MAGEB1	Rabbit	1:100	Bio-Techne, Novus, Cat# NBP1-85405, lot R04032
P450SCC	Rabbit	1:200	Sigma-Aldrich, Cat# HPA016436, polyclonal, lot B106749
PAX6 (N-terminal region)	Mouse	1:50	Developmental Studies Hybridoma Bank, AB_528427
SCARA5	Rabbit	1:100	Sigma-Aldrich Cat# HPA024661, lot A106036
SF1	Mouse	1:250	R&D Systems, Cat# PP-N1665-00, clone N1665, lot A-3
StAR	Rabbit	1:100	Cell Signaling Technology, Cat# 8449, clone D10H12, lot 1
WT1	Mouse	1:350	Sigma-Aldrich, Cat# SAB1409796 (clone 1E9), lot 11179-1E9

Secondary antibodies			
Species, antigen, conjugate	**Host species**	**Dilution**	**Manufacturer, catalog #, lot**
Mouse, IgGs H+L chains, Alexa Fluor® 488	Donkey	1:1000	Molecular Probes Cat# A-21202, lot 1890861
Goat, IgGs H+L chains, Alexa Fluor® 594	Donkey	1:1000	Thermo Fisher Scientific, Cat# A-11058, lot 1180089
Rabbit, IgGs H+L chains, Alexa Fluor® 594	Donkey	1:1000	Molecular Probes Cat# A-21207, lot 1938375

Differentiated cells cultured on collagen-coated glass coverslips were mounted on objective slides in VECTASHIELD® Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, #H-1200) and analyzed using an Axioimager upright epifluorescence microscope (Zeiss). Nuclei in cells differentiated on polymer-coated μ-slide wells were labeled with DAPI Dilactate (Invitrogen, #D3571) at a 1:1000 ratio in PBS for 8 to 10 min in the dark. Subsequently, cells were washed two to three times with PBS. Image acquisition was performed with a TCS SP8 laser scanning confocal microscope using a 1024 x 1024 scan format and a HC PL APO CS2 40x/1.10NA water objective (Leica Microsystems, Mannheim, Germany).

Images in Study III were processed using Fiji ImageJ (version 1.53, http://fiji.sc) for image smoothing and channel recoloring. A Gaussian filter with one pixel kernel radius was used. An adequate image size for publishing was set with Adobe Photoshop (version 23.0.0, Adobe, San Jose, CA, USA).

4.5. Next-generation RNA sequencing (Study III)

Differentiated cells at days 4, 6, 8, and 10 were lyzed, and total RNA was extracted and purified according to instructions of miRNeasy Mini Kit (Qiagen, Hilden, Germany) using QlAzol Lysis Reagent (Qiagen, MD, USA). An additional On-Column DNase Digestion with the RNase-Free DNase Set was applied to ensure adequate RNA quality for RNA sequencing. The quality of purified RNA was determined by Bioanalyzer RNA Quality Control Assay, and the RNA libraries were prepared using TruSeq Stranded Total with Ribosomal RNA depletion. The RNA quality control, library construction, and sequencing were undertaken at the Sequencing Unit of the Institute for Molecular Medicine Finland FIMM Technology Centre, University of Helsinki, Finland. All sequencing runs were performed with an Illumina NovaSeq system using an S4 flow cell (Illumina, San Diego, CA, USA). Sequencing was performed with at least 25 million read pair depth with read length 151+8+8+151 bp for the paired-end run.

Data processing and analysis have been explained in detail in the Study III. In brief, raw sequencing data quality was monitored and the reads were trimmed and filtered before they were aligned with the human genome (GRCh38, release 82). After confirming the quality of the RNA sequencing data, the reads were counted using Ensembl gene-ids and were aligned to genetic features containing both protein-coding genes and non-coding genes using featureCounts software (Subread-1.4.5-p1). For analyzing the aligned data, a gene expression matrix was generated and used for all downstream analyses. To compare expression changes between the induced and non-induced samples at each time point (d6, d8, d10), and at different time points between samples that were derived from the same condition (either induced or non-induced), pairwise DE analyses were performed using DESeq2 (version 1.24) (Love et al., 2014). The method enables differential analysis of read count data in high-throughput RNA-sequencing. To analyze dynamic expression changes of DE genes, ordered time-series analyses were performed with EBSeq-HMM (version 1.18) (Leng et al., 2015). In all analyses with DESeq2 and EBSeqHMM, default normalization and statistical methods were applied. To generate PCA plots for expression data visualization, variance-stabilized data derived from DESeq2 analyses were used. In addition, heatmaps were constructed by combining expression changes from all comparisons in the pairwise DE seq analyses and from EBSeq-HMM analyses that presented dynamic changes soon after the onset of induction. The categories of EBSeq-HMM used for the heatmap were constant upregulation (U-U-U), upregulation followed by stable expression (U-S-S), downregulation followed by stable expression (D-S-S), or constant downregulation (D-D-D).

Cell type predictions were conducted with R package scCATCH (version 3.0; https://github.com/ZJUFanLab/scCATCH) (Shao et al., 2020) using reference tissues related to the gonad. Samples at each time point were compared with samples at all other time points, and markers specific for sample-clusters with logFC≥1 were detected with the findmarkergene function in scCATCH. Finally, a score of similarity was calculated.

To identify biological processes that were enriched in induced or non-induced cells at differentiation day 6, genes upregulated or downregulated at least two-fold (log2FC≥1 or log2FC≥-1, respectively) were selected and analyzed using DAVID Functional Annotation Tool (Huang et al., 2009, https://david.ncifcrf.gov/). In addition, the tool was used for annotating processes enriched among all genes, which were exhibiting dynamic expression patterns (in the 4 preselected categories: U-U-U, U-S-S, D-S-S and D-D-D) in the induced cells. The annotated GO biological processes were sorted according to their statistical significance (false discovery rate) and power (fold enrichment values).

4.6. Hormonal stimulations and cAMP production (Studies II and III)

Intracellular cAMP was assayed in cells differentiated until day 8 by following the gonadal protocol (M, Study III) or the protocol optimized for FSHR expression (Study

II). Cells were washed with Dulbecco's PBS (Thermo Fisher Scientific) and incubated for 1 or 8 h with vehicle [0.1% bovine serum albumin (BSA, Thermo Fisher Scientific) in H_2O], 1, 10, or 100 ng/ml FSH or 10 or 100 ng/ml LH, or with forskolin (10 μM, Sigma-Aldrich) in corresponding differentiation media. Forskolin was used to activate AC receptor independently. Lysates collected after 1 h stimulation and lyzed cells after 8 h stimulation were frozen at -80°C for later use. Levels of cAMP were measured from lysates with Direct cAMP ELISA Kit (EnzoLife Sciences (ELS) AG, Lausen, Switzerland, Cat. # ADI-900-066) in non-acetylated format (presumably typical cAMP levels) according to the manufacturer's instructions. Sample absorbances were read using a Multiscan EX, version 1.1 (type 355; Labsystems, Vantaa, Finland) microplate photometer. Standard curves were prepared using a Point-to-Point method. Relative expression of mRNA in lyzed cells was measured with qRT-PCR.

4.7. AMH and steroid assays (Study III)

To analyze AMH production in non-induced and induced cells (Study III), cells were differentiated to day 10 and supernatants were collected and stored until AMH levels were measured with picoAMH ELISA (Ansh Labs, Webster, TX, USA, Cat. #: AL-124-i) assay. Moreover, to analyze steroid production in non-induced and induced cells (Study III), cells were washed with Dulbecco's PBS (Thermo Fisher Scientific) and stimulated for 24 h with vehicle (0.1% BSA in H_2O), 10 or 100 ng/ml FSH or LH, or forskolin (10 μM, Sigma-Aldrich) in differentiation medium. Steroid secretion was assayed with liquid chromatography tandem-mass spectrometry (LC-MS/MS) at the Department of Internal Medicine and Clinical Nutrition, University of Gothenburg, Gothenburg, Sweden. Results were verified with a similar method at the Department of Clinical Science, University of Bergen, Bergen, Norway. Steroids measured in spent culture media included androstenedione (lower limit of quantification, LLOQ = 0.018 nmol/l), progesterone (LLOQ = 0.016 nmol/l), DHEA (LLOQ = 0.87 nmol/l), DHEA-S (LLOQ = 0.021 μmol/l and 10.4%), DHT (LLOQ = 44.8 pmol/l), estradiol (LLOQ = 1.84 pmol/l), estrone (LLOQ = 1.85 pmol/l), 17-OH progesterone (LLOQ = 0.060 nmol/l), testosterone (LLOQ = 0.017 nmol/l), cortisol (0.59 nmol/l and 4.0%), cortisone (0.17 nmol/l and 4.2%), and aldosterone (13 pmol/l and 7.5%). Human serum was used as the analysis matrix and LLOQ was defined with an accuracy of 80%-120%.

4.8. ScRNA sequencing of *FSHR*-expressing cells (Study II)

WT H9 cells differentiated until day 8 were evaluated by Chromium Single-Cell Gene Expression analysis. Cells were washed twice with Dulbecco's PBS (Thermo Fisher Scientific) and dissociated into single cells using a 1:1 mixture of Trypsin-EDTA and TrypLE™ Express (all from Thermo Fisher Scientific), that was incubated on cells for 10 min at 37˚C. Cells were resuspended into DMEM/F12 + 2% B27 and mixed, before subsequent removal of cell clumps by passing the cell suspension through a 40 μm cell strainer (Falcon, Thermo Fisher Scientific). The removal of excess floating RNA was ensured by pelleting cells at 300 g for 5 min, which was followed by resuspending of cells into Dulbecco's PBS, including 0.04% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA). Following this, cells were gently mixed and cell pelleting

was repeated until the cells were finally resuspended in cold 0.04% BSA in PBS by gentle repetitive pipetting. The number of doublets possibly interfering with the analysis was minimized by passing the cells through a cell strainer. Cells to be analyzed with Chromium were kept on ice until analysis, and their viability (>90%) and the number of doublets (<8%) was determined using a LUNA-STEM Automated Fluorescence Cell Counter (Logos Biosystems, South Korea). The library preparation and sequencing using 10x Genomics Chromium Single Cell 3'RNASeq platform were conducted at the Institute for Molecular Medicine Finland (FIMM, Helsinki, Finland). The library preparations and the sequencing were performed using the Chromium Next GEM Single Cell 3'Gene Expression, version 3.1 Dual Index chemistry. Sequencing of the sample libraries was conducted with Illumina NovaSeq 6000 Sequencing system (Illumina, San Diego, CA, USA) using read lengths 28 bp (Read 1), 10 bp (i7 Index), 10 bp (i5 Index), and 90 bp (Read 2). The resulting reads were processed and data analysis was performed as described in Study II. Cell type predictions were performed with scCATCH using the gonad, the kidney, the embryo and the fetus as reference tissues.

4.9. Protein interaction analysis using multiple approaches combined (MAC)-tagged FSHR (Study II)

First, cell lines were generated that stably expressed MAC-tagged A189V or WT FSHR baits for combined affinity purification mass spectrometry (AP-MS) and complementary proximity-based labeling with BioID. A Gateway entry clone was established by introducing flanking attB sites to both ends of the full-length FSHR cDNA, which was derived from HEL127.6 cells differentiated until day 8 according to the modified protocol D, and by performing a Gateway BP reaction. To avoid interference from possible single-nucleotide polymorphisms, the WT FSHR was generated by correcting the C566T mutation with the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The success of the correction was monitored by Sanger sequencing. The WT and A189V FSHR entry clones were recombined with the MAC-C destination vector, which enabled generation of clones exhibiting C-terminal MAC-tags as described in Liu et al. (2018). In the protein interaction studies, a green fluorescent protein (GFP) expression vector attached to a MAC-tag served as a control.

Next, tetracycline-inducible Flp-In™ 293 T-REx cells (Thermo Fisher Scientific, Cat. # R78007) were co-transfected with the MAC-tagged WT FSHR, A189V mutant FSHR, or GFP expression vector and a pOG44 Flp-recombinase expression vector (Thermo Fisher Scientific) using the DreamFect™ reagent (Oz Biosciences, San Diego, CA, USA), and clonal cell lines were derived as previously described (Varjosalo et al., 2013).

Tetracycline at 1 µg/ml concentration was added to the cells for 24 h to induce FSHR expression. For biotinylation in BioID, 50 µM biotin was added with tetracycline. To analyze receptor interactions in the presence of a FSH ligand, cells were incubated at 37°C with 100 ng/ml human recombinant FSH (Prospec) for 2 h, which was considered to be sufficient for the establishment of protein interactions. Approximately 5 x 10^7 cells

from 5 x 150 mm dishes were collected for a biological sample replicate separately in AP-MS and BioID. The final results are derived from altogether three biological replicates. Before protein purification with Strep-Tactin column, harvested cells to be used for AP-MS were lysed in mild lysis buffer, whereas cells to be used for BioID were treated with harsh lysis buffer, sonication, and benzonase treatment. Purified proteins were processed and AP-MS and BioID analyses were performed according to the previously published protocol (Liu et al., 2020).

5. Statistical methods

IBM SPSS Statistics software (version 25 or 28) or bioinformatics tool R were used for statistical analyses. qRT-PCR and AMH immunoassay data were analyzed according to the sample set by conducting pairwise comparisons using Mann-Whitney *U*-test with mean ranks or independent samples *t*-test (2-sided), or multiple comparisons by using one-way analysis of variance (ANOVA) with Bonferroni/Dunnett *t*-test for multiple sample adjustment, or independent samples Kruskal-Wallis one-way ANOVA. cAMP data were analyzed with two-way ANOVA. Normal distribution of the data was assessed using Shapiro-Wilk test and the homogeneity of variances was tested with Levene's test. The α error in multiple comparisons of qPCR and RNA-sequenced samples was controlled with the Benjamini and Hochberg method. $P<0.05$ was considered statistically significant, and the statistical analyses were performed at a confidence level of 95%.

RESULTS AND DISCUSSION

1. Differentiation of bipotential gonadal-like cells from hPSCs

The bipotential gonads develop as thickenings of the coelomic epithelium overlying the IM (Karl and Capel, 1998; Schmahl et al., 2000; Sasaki et al., 2021) and subsequently differentiate into male and female counterparts. For differentiation of the gonadal-like cells, we utilized a protocol originally developed for generation of the IM that gives rise to kidney progenitors (Mae et al., 2013). After the first protocol that described derivation of IM-like cells using hPSCs was published in 2013, protocols introduced by other research laboratories to induce the IM followed (Lam et al., 2014; Takasato et al., 2014; Morizane et al., 2015).

In the induction method of Mae et al. (2013), a Nodal substitute Activin A, a Wnt signaling agonist CHIR99021, and BMP7 directed cell differentiation towards the mesendoderm and the IM. Activin A and Wnt3a had been reported to induce mesendodermal differentiation in the previous studies involving human and mouse PSCs (D'Amour et al., 2006; Gadue et al., 2006), and after discovering CHIR99021 as a more potent mesendoderm inducer, CHIR99021 replaced Wnt3a in the IM induction protocol of Mae et al. (2013). In addition, BMP7 was identified among multiple growth factors tested to markedly induce expression of *OSR1* (Mae et al., 2013), a gene corresponding to the IM, thereby contributing to cells of gonadal, adrenal, and renal identity (Mugford et al., 2008).

BMPs are potent mesoderm inductors *in vivo* and *in vitro* (Dosch et al., 1997; Zhang et al., 2008; Drukker et al., 2012), and the current evidence suggests that they also contribute to reproductive system development and function widely across the Animalia (Lochab and Extavour, 2017). We examined the effects of Activin A, CHIR99021, three BMP ligands, and BMP signaling inhibitor Dorsomorphin (Yu et al., 2008) in directing hESCs towards bipotential gonadal-like cells. Our first approach was to test sequential BMP signaling activation and inhibition at various time points and to monitor the relative expression of several commonly known marker genes of the bipotential gonad (*GATA4*, *WT1*, *EMX2*, *LHX9*) and genes related to neural differentiation (*SOX1*, *PAX6*), LPM and extraembryonic development (*HAND1*, *GATA6*) (Study I, Fig. 1A-B). Of the 17 conditions differing in the activation and inhibition of BMP signaling, two (L and M) were selected for more detailed studies, as in these the expression of bipotential gonadal markers was induced with minimal expression of extraembryonic genes. In both protocols, BMP signaling was sequentially activated and inhibited, and the only difference between L and M was the duration of BMP signaling activation, which was applied for one (protocol M) or two days (protocol L) (Fig. 16).

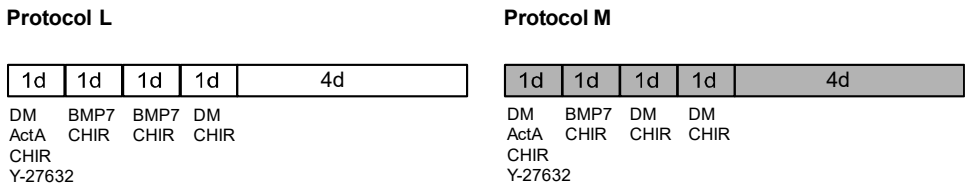

Figure 16. Growth factors and kinase inhibitors used to guide differentiation in protocols L and M. Abbreviations: Act, Activin A; BMP, bone morphogenetic protein; CHIR, CHIR99021; d, day of differentiation; DM, dorsomorphin.

Differentiation according to protocols L and M induced markers indicative of the developmental stages, through which the mammalian fetal gonads differentiate (Study I, Fig. 1D and E) (Sasaki et al., 2021). Thus, cells expressed *BRA*, a marker of PS, during the first two days of differentiation (Study I, Fig. 1D). This was closely followed by a marked increase in the expression of IM markers (*LHX1, PAX2* and *OSR1*) at days 2-4 and markers of the bipotential gonad (*WT1, EMX2, GATA4* and *LHX9*) at day 8 (Study I, Fig. 1D and E). The protocols L and M induced slightly different gene expression patterns: *GATA4* and *LHX9* expression at higher levels and *EMX2* expression at lower levels were detected in cells differentiated according to protocol M when compared with protocol L.

Differentiation was further optimized by testing the influence of different types of BMP ligands (BMP2, -4 and -7), different concentrations of BMP7 or Activin A, or the duration of Wnt signaling on derivation of gonadal-like cells. Expression of all bipotential gonadal markers was induced with each BMP subtype tested in differentiation following protocol M (Study I, Fig. 2B). However, while in protocol L BMP7 and -2 were able to induce gonadal markers (Study I, Fig. 2A), BMP4 induced the expression of LPM/extraembryonic markers and the expression levels of neural markers *PAX6* and *SOX1* was reduced. BMP7 and BMP2 specified the gonadal versus non-gonadal cell fates similarly and BMP7 was selected for all downstream studies. As BMPs exhibit different affinities to serine-threonine receptors (Nickel et al., 2009), the observed differences in the capabilities of BMP2, -4, or -7 to induce gonadal fate could be explained by dissimilar receptor binding. However, this was not delineated in our study. In addition, the different bioactivities of the tested BMPs may have affected the biological responses of each BMP.

Interestingly, studies performed to reveal the optimal BMP7 concentration for gonadal differentiation indicated that 2-day BMP exposure (protocol L) with the lowest concentration (10 ng/ml) of BMP7 most markedly upregulated gonadal markers, although it did not sufficiently suppress neural differentiation (Study I, Fig. 3A). By contrast, intermediate to high BMP7 concentration (50 or 100 ng/ml) suppressed neural differentiation and instead promoted extraembryonic/LPM differentiation (Study I, Fig. 3A). Therefore, we conclude that both the duration of BMP signaling and the concentration of BMP ligand define the differentiation outcome.

1.1. Modulating Wnt and Nodal signaling for gonadal-like cell differentiation

The duration of Wnt signaling has been shown to affect the differentiation of mesodermal subtypes from hESCs (Takasato et al., 2015). Therefore, the optimal length of Wnt signaling in gonadal-like cell differentiation was tested. Following differentiation protocol L, the expression of gonadal markers was markedly induced with 2-day CHIR-99021 supplementation, whereas in cells differentiated according to protocol M, 4 days of Wnt signaling activation was required to sufficiently induce expression of bipotential gonadal markers (Study I, Fig. 4A-B). Instead, the expression of the neural marker PAX6 was induced with 1-day Wnt signaling activation (Study I, Fig. 4A-C). This indicates that the duration of Wnt signaling has a considerable impact on germ layer specification and gonadal differentiation.

Nodal induces mesendoderm development *in vivo* (Agius et al., 2000; Thisse et al., 2000; Vincent et al., 2003), and Nodal signaling agonist Activin A drives *in vitro* differentiation of hPSCs towards PS-derived cell fates (D'Amour et al., 2005). We tested increasing concentrations (1, 10, 100 ng/ml) of Activin A in cells differentiated according to protocol L or M. Cells differentiated with 10 or 100 ng/ml Activin A presented similar morphology, whereas 1 ng/ml Activin A generated spheres that detached from the wells (Study I, Fig. 5A). The highest concentration of Activin A (100 ng/ml) induced the highest expression levels of bipotential gonadal markers (*WT1, GATA4,* and *LHX9*) and the lowest level of neural markers *SOX1* and *PAX6* (Study I, Fig. 5B and C). In contrast, the lowest concentration of Activin A (1 ng/ml) induced expression of neural markers and suppressed gonadal differentiation. The percentage of GATA4 and PAX6 positive differentiated cells after treating the cells with 1 or 100 ng/ml Activin was in concordance with the levels of corresponding mRNAs detected (Study I, Fig. 5B and C). Based on these data, it can be concluded that adequate Nodal signaling is required in order to trigger bipotential gonadal cell fate.

2. Induction of more mature gonadal-like cells by inducing SF1 expression

Despite the increasing demand for models that could recapitulate fetal testicular development and the development of key testicular cell types in humans, only a handful of studies exist in which SC-like cells of human origin have been generated *in vitro* (Bucay et al., 2009; Kjartansdóttir et al., 2015; Shlush et al., 2017; Rodríguez Gutiérrez et al., 2018; Liang et al., 2019; Knarston et al., 2020). In the majority of these, differentiation was performed spontaneously or with growth factors (Bucay et al., 2009; Kjartansdóttir et al., 2015; Shlush et al., 2017; Rodríguez Gutiérrez et al., 2018; Knarston et al., 2020), and in only one of them *NR5A1,* a crucial gene for gonadal development (Luo et al., 1994; Sadovsky et al., 1995), was reported to be slightly upregulated (Knarston et al., 2020). In another study, overexpression of *NR5A1* and *GATA4* induced differentiation of SC-like cells (Liang et al., 2019), confirming the importance of *NR5A1*/SF1 in inducing SC-like fate *in vitro*, in addition to its essential

and well-established role in gonadal development *in vivo* (Luo et al., 1994; Sadovsky et al., 1995).

We did not detect the expression of *NR5A1* in differentiated gonadal-like cells when compared with undifferentiated hPSCs (Sepponen, unpublished results, Fig. 17). FGF2, FGF9, and FSH have previously been reported to induce moderate *Nr5a1* expression and SC-like fate in differentiated mouse ESCs (Seol et al., 2018), and EGF has been shown to induce *NR5A1* expression in cultured breast cancer cells (Sweeney et al., 2001). We tested these factors during the differentiation of HEL46.11 male hiPSCs individually and in a combination involving FGF2, FGF9, and FSH and observed no induction in *NR5A1* expression levels compared with undifferentiated cells (Sepponen, unpublished results, Fig. 17).

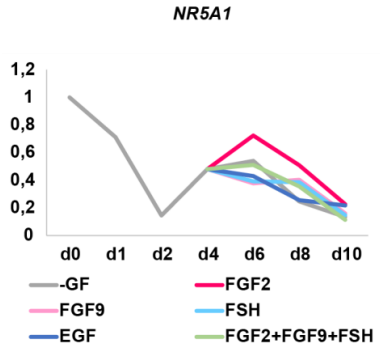

Figure 17. *NR5A1* expression in HEL46.11 hiPSCs differentiated with extended gonadal protocol M in the absence or presence of 100 ng/ml FGF9, 30 ng/ml EGF, 100 ng/ml FGF2, 10 ng/ml FSH, or a combination of FGF2, FGF9 and FSH at days 4-10. Expression levels are derived from two technical replicate wells/sample and are relative to undifferentiated hiPSCs. Abbreviations: d, day of differentiation; EGF, epidermal growth factor; FGF, fibroblast growth factor; FSH, follicle-stimulating hormone; GF, growth factor (Sepponen, unpublished results).

EMX2 may regulate *NR5A1*/SF1 since *Emx2* mice KOs have been reported to display strongly underdeveloped transient gonads and exhibit transient expression of gonadal SF1 (Kusaka et al., 2010) (Fig. 13). We utilized CRISPR gene activation technology to establish a clonally-derived HEL46.11-DDdCas9VP192-EMX2 cell line, in which endogenous EMX2 expression can be activated in a conditionally-inducible manner, to examine whether EMX2 induces *NR5A1* expression. In these cells, *EMX2* was upregulated with DOX and TMP, as demonstrated in induced cells on days 6-10 of differentiation (Sepponen, unpublished results, Fig. 18). In contrast, *EMX2* expression was not upregulated in non-induced cells. However, *NR5A1* was not upregulated and the expression of its targets (*AMH* and *SOX9*) remained unaltered despite *EMX2* induction (Sepponen, unpublished results, Fig. 18). Furthermore, in another hiPSC background *EMX2* activation induced *PAX6* expression, implying induction of neural differentiation (Sepponen, unpublished results, Fig. 18). Our results indicate that in the cellular context of our study *EMX2* activation was not sufficient to induce *NR5A1* expression and the data suggest that *NR5A1* expression might not be regulated by EMX2 *in vitro*.

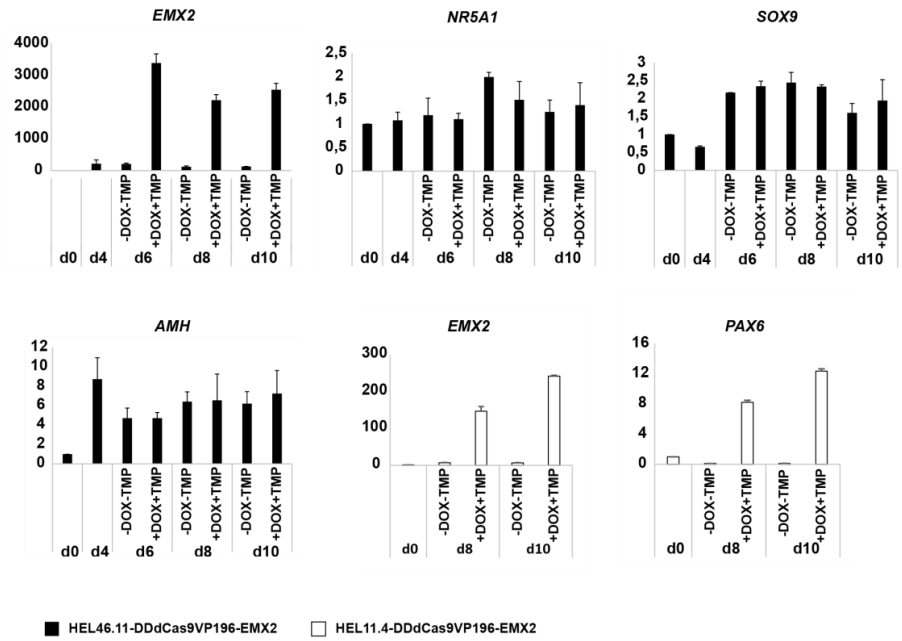

Figure 18. Expression of selected gonadal markers or neural marker *PAX6* in the presence or absence of 10 µg/ml DOX and 1 µM TMP in hiPSCs differentiated with extended gonadal protocol M. Expression levels are relative to undifferentiated hiPSCs. Bars ± SEMs represent an average of two technical replicates. Abbreviations: d, day of differentiation; DOX, doxycycline hyclate; TMP, trimethoprim (Sepponen, unpublished results).

Next, we directly activated endogenous *NR5A1*, which encodes SF1, in a conditionally inducible manner at the appropriate stage of gonadal cell differentiation. Studies by others have described protocols for derivation of somatic SCs or LCs by overexpressing SF1 and in most cases using additional transcription factors (Yazawa et al., 2006; Jadhav and Jameson, 2011; Buganim et al., 2012; Yang et al., 2015, 2017; Rore et al., 2021). However, the approach chosen in the majority of these studies to induce SF1 and possible other gonadal genes may target molecules in an unspecific manner due to the high levels of triggered gene expression. By activating endogenous expression of *NR5A1,* we expected the risk for non-physiologically high levels of SF1 and possible SF1 off-target effects to be reduced.

Targeted *NR5A1* activation using DOX and TMP markedly upregulated the expression levels of *NR5A1* mRNA in undifferentiated and differentiated HEL46.11-DDdCas9VP192-NR5A1 cells (Sepponen, unpublished results, Fig. 19; Study III, Fig. 1B). In the absence of DOX and TMP and in the undifferentiated HEL46.11 hiPSCs, *NR5A1* was not expressed. Induced cells expressed SF1 at varying levels, as demonstrated by heterogeneous staining intensity (Study III, Fig. 1D). The variable level of SF1 expression between individual cells may have affected the differentiation outcome and increased the heterogeneity within the SF1-induced cell population.

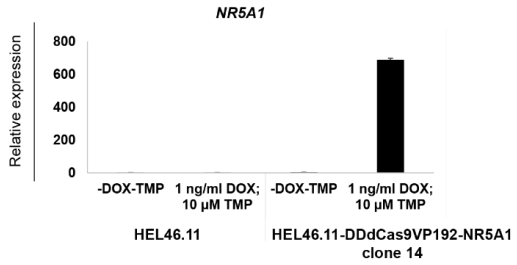

Figure 19. *NR5A1* expression in undifferentiated HEL46.11 and HEL46.11-DDdCas9VP192-NR5A1 clone 14 hiPSCs in the absence or presence of DOX and TMP for 3 days. Expression levels are relative to *NR5A1* expression in undifferentiated HEL46.11 cells in the absence of 1 ng/ml DOX and 10 µM TMP, and the bar values represent an average of the expression in 4 culture wells ± SEM. Abbreviations: DOX, doxycycline hyclate; TMP, trimethoprim (Sepponen, unpublished results).

SF1 regulates SOX9, AMH and inhibin α subunit expression in SCs (Shen et al., 1994; Giuili et al., 1997; Watanabe et al., 2000; Sekido and Lovell-Badge, 2008; Ji et al., 2013). In our study, SF1 induction in bipotential gonadal-like cells triggered pre-SC-like characteristics, such as the expression of early SC markers *SOX9*, AMH, and inhibin α subunit (Study III, Fig. 1B and D). SF1 induction also stimulated secretion of AMH (Study III, Fig. 1E), which is expressed by fetal SCs (Tran et al., 1977; Rajpert-De Meyts et al., 1999). Surprisingly, vanin-1, a testicular development-associated protein expressed in SCs and regulated by *Nr5a1* in mice (Bowles et al., 2000; Grimmond et al., 2000; Wilson et al., 2005), was not induced. Moreover, *FSHR*, which is considered a later marker of SCs and GCs, was expressed in the differentiated cells both in the presence and absence of induction (Study III, Supplementary Fig. S2B). Also, high *GATA4* and *WT1* mRNA expression and the expression of GATA4 protein was maintained in the presence of SF1 induction (Study III, Fig. 1B and D), suggesting that the cells had a gonadal identity.

DAX1 (encoded by *NR0B1*) is known to repress SF1 activity (Ito et al., 1997), and although DAX1 is expressed in the fetal bipotential gonads, it becomes downregulated in males and upregulated in females during sex determination (Ikeda et al., 2001). Interestingly, the expression of *NR0B1* was upregulated in induced cells at days 6, 8, and 10 (Study III, Supplementary Data 1). The induction of *NR0B1* in the differentiated cells suggests the presence of a negative feedback mechanism against upregulated SF1. In fetal transgenic male mice, DAX1 overexpression reduces expression of *Sox9* by reducing binding of SF1 to the testis enhancer of *Sox9* (Ludbrook et al., 2012), presumably explaining the relatively weak induction of *SOX9* expression in our male SF1-induced gonadal-like cells, in which *NR0B1* is upregulated (Study III, Fig. 1B).

INSL3 is regulated by SF1 in cultured LCs (Zimmermann et al., 1998). *INSL3* encodes a peptide hormone secreted by fetal and postnatal LCs that is involved in testis descent. In our study, *NR5A1* activation was followed by upregulation of *INSL3* expression at day 10 of differentiation (Study III, Supplementary Data 1), and the delayed response suggests that *INSL3* was likely not directly targeted by SF1. The induction of *INSL3* expression may indicate generation of a subpopulation of LC-like cells. However,

PTCH1 is another LC gene that was not upregulated in SF1-induced cells (Study III, Supplementary Data 1). *PTCH1*, which encodes a receptor for the hedgehog signaling pathway, is expressed in early fetal LCs and progenitors of adult LCs (Yao et al., 2002; Park et al., 2007; Karpova et al., 2015) and has previously been upregulated in artificially generated LCs (Jadhav and Jameson, 2011; Li et al., 2019).

The differentiated SF1-induced cells at days 6-8 were predicted to be mainly SCs, LCs, or GCs by a cluster-based annotation tool single-cell Cluster-based Automatic Annotation Toolkit for Cellular Heterogeneity (scCATCH) (Shao et al., 2020), while at day 4 the differentiated cells were annotated to be more heterogeneous (Study III, Fig. 1C). Without induction, gonadal differentiation was more elusive with a multitude of predicted gonad-associated cell types, suggesting that SF1 promotes targeted gonadal somatic cell differentiation. Overall, the cell identities at different time points remained ambiguous.

2.1. Gonadal-like cells were steroidogenic and responded to hormonal stimulation

SF1 regulates steroidogenesis in both the developing and adult gonads, which has been demonstrated by SF1 deletion specifically in steroidogenic cells of transgenic mice (Buaas et al., 2012). Steroidogenesis in our study was activated in SF1-induced cells, which exhibited upregulation of *CYP11A1*, *CYP17A1*, *CYP19A1,* and *HSD3B2*, and displayed enhanced steroid secretion following *NR5A1* activation (Study III, Fig. 2A-C). In contrast, expression of these markers was not induced in the differentiated non-induced cells. The steroidogenic markers induced in this study can also be found in fetal SCs and the majority of them also in fetal LCs of human origin (Pollack et al., 1997; Li et al., 2017; Savchuk et al., 2019). In addition to steroidogenic genes, also genes involved in cholesterol mobilization, such as *STAR*, ferredoxin reductase*,* and *CYB5A,* were upregulated in SF1-induced cells (Study III, Fig. 2A, Supplementary Data 1).

The sex steroids and their metabolic intermediates secreted by the differentiated and SF1-induced cells in this study included progesterone, androstenedione, 17-OH progesterone, estrone, and estradiol and their levels increased after FSH stimulation (Study III, Fig. 2C), in line with earlier studies demonstrating that FSH can stimulate steroidogenesis in immature SCs (Welsh and Wiebe, 1976; McDonald et al., 2006). In contrast, the differentiated non-induced cells secreted merely progesterone, and at a markedly lower level than the induced cells (Study III, Fig. 2C). The fetal human testes secrete mainly progesterone and 17-OH progesterone at the early stages of development, and at later stages their secretion is outweighed by secretion of mainly testosterone (Savchuk et al., 2019). In this study, testosterone and DHT, which are crucial for fetal virilization in males, were virtually undetected in spent medium — an indication of the premature nature of the differentiated male gonadal-like cells.

Studies performed in mice have demonstrated that testosterone in the fetal gonads is converted from androstenedione by fetal SCs, and that this conversion is catalyzed by

hydroxysteroid 17-β dehydrogenase 3 encoded by *HSD17B3* (O'Shaughnessy et al., 2000; Shima et al., 2013). *HSD17B3*, which was upregulated by SF1 induction in this study (Study III, Fig. 2A), is not expressed in fetal LCs but can be found in fetal SCs in human and in mouse (O'Shaughnessy et al., 2000; Shima et al., 2013; Guo et al., 2021). This evidence further supports the pre-SC-like identity of the differentiated SF1-induced cells. The presence of steroids previously identified in the human gonads during early fetal development indicates that, although male gonadal-like cells generated in this study were not capable of producing testosterone and were therefore functionally immature, SF1 induced their steroidogenic activity with characteristics similar to the human fetal testes.

In addition, SF1 enhanced the expression of a few enzymes expressed in the fetal adrenal glands, such as *SULT2A1* encoding a sulfotransferase, which typically converts DHEA into DHEA-S (Parker et al., 1994; Parker, 1999) and *CYP21A2* encoding a cytochrome family enzyme involved in adrenal mineralo- and glucocorticoid production (Voutilainen and Miller, 1986; Turcu and Auchus, 2015; del Valle et al., 2017; Melau et al., 2019). These enzymes were upregulated on days 6-10 or 8-10, respectively, in the induced cells when compared with the non-induced cells (Study III, Supplementary Data 1). However, no corticosterone, aldosterone, cortisol, DHEA, or its derivative DHEA-S were detected in stimulated or unstimulated differentiated cells, suggesting that if some contaminating adrenal-like cells were present they were not demonstrated to be functionally active in this study.

Several gonadal functions are implemented via gonadotropins and their receptors. In this study, both non-induced and induced cells responded to FSH stimulation by secreting cAMP (Study III, Supplementary Fig. S2C). In contrast, cAMP was not secreted after stimulation with LH (Study III, Supplementary Fig. S2C). The differentiated cells are therefore responsive to hormonal stimulation by FSH, a characteristic that has been previously associated with fetal SCs (Orth, 1984). Similarly to the findings in the literature (Klaij et al., 1990; Themmen et al., 1991; Johnson and Bridgham, 2001), FSH stimulation of the differentiated induced and non-induced cells suppressed the expression of *FSHR* and induced the expression of *STAR* and *INHA* (Study III, Supplementary Fig. S2D). The observed changes in gene expression levels were significant for *FSHR* in both induced and non-induced conditions [Study III, Supplementary Fig. S2D, one-way ANOVA, Dunnett *t*-test (2-sided), *P*(induced)=0.001, *P*(non-induced)=0.022]. In contrast, LH did not alter the expression of *FSHR*, *STAR, INHA* or *LHCGR* in induced or non-induced cells (Study III, Supplementary Fig. S2D; Sepponen, unpublished results, Fig. 20). In conclusion, FSH-mediated cAMP signaling was activated upon differentiation, whereas LH did not stimulate changes in cAMP-

mediated signaling. Moreover, SF1 was able to induce the steroidogenic activity of differentiated cells, which was further augmented by FSH stimulation.

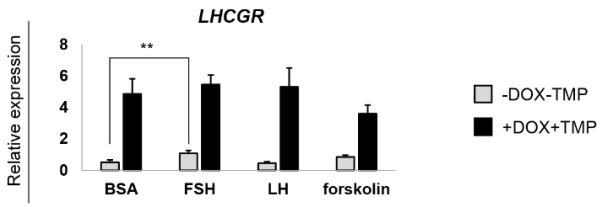

Figure 20. Relative expression of *LHCGR* in stimulated (100 ng/ml FSH/LH/10 µM forskolin) and unstimulated (BSA vehicle) cells at day 8 of differentiation in the absence (-DOX-TMP) or presence (+DOX+TMP) of *NR5A1* activation. SEMs are derived from three biological replicates and the expression levels were quantified relative to undifferentiated hiPSCs. Abbreviations: BSA, bovine serum albumin; DOX, doxycycline hyclate; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TMP, trimethoprim. Pairwise comparisons were calculated with Dunnett *t*-tests (2-sided) adjusted for multiple testing (**P<0.01). (Sepponen, unpublished results).

3. Transcriptional dynamics following SF1 induction

NR5A1 activation in the gonadal-like cells markedly altered the transcriptional pattern of gene expression on a large scale (Study III, Fig. 3C), explaining 18% of the variation in the transcriptomics of the induced and non-induced cells (Study III, Fig. 3A). Over 2600 DE genetic features (protein-coding and non-coding RNAs, *etc.*) were identified between SF1-induced and non-induced cells two days after the onset of induction, at differentiation day 6 (adjusted P<0.05, Study III, Fig. 3B). The number of DE genetic features increased on later days, including over 5000 DE genetic features at days 8 and 10 (adjusted P<0.05, Study III, Fig. 3B). The observed increase in their total number may be due to the accumulation of the initial effects of *NR5A1* activation in driving the dissimilar cell fates between induced and non-induced cells. Despite the marked changes induced by *NR5A1* activation, cell differentiation induced the greatest transcriptional changes, explaining approximately 70% of the variation in transcriptomes (Study III, Fig. 3A).

The biological processes primarily enriched among the genes demonstrating at least doubled expression levels ($\log_2$FC≥1) after *NR5A1* activation included male gonad development and steroid biosynthesis (Study III, Supplementary Fig. S3A). In addition to global changes in gene transcriptomics, individual genes exhibited dynamic expression changes in induced cells that were not detected in non-induced cells. The EBSeqHMM method that was used to monitor dynamic changes compares the expression levels between subsequent days of differentiation and estimates posterior probabilities for the direction of expression (upregulated, downregulated, or stable) accordingly. Among the dynamically DE genes, we focused on those that exhibited constant upregulation (which we termed U-U-U), constant downregulation (D-D-D), and either upregulation or downregulation followed by stable expression of the gene (U-S-S, D-S-S, respectively) exclusively in the SF1-induced condition. Within the top 20 DE genetic features (80 in total) allocated to one of these dynamic gene expression paths, we identified 27 genes that were associated with gonadal development or infertility in

the Harmonizome public collection of datasets involving the curated Comparative Toxicogenomics Database Gene-Disease Associations and the curated GO Biological Process Annotations datasets (Ashburner et al., 2000; Davis et al., 2009; Rouillard et al., 2016) (Study III, Fig. 4B).

Some of the dynamically expressed genes, such as testicular *TESC* (encoding Tescalcin) that was constantly upregulated in induced cells, were also significantly differentially expressed in induced and non-induced cells on specific differentiation days in pairwise comparison of samples using the DESeq2-method (Study III, Fig. 4B; Supplementary Data 1). According to the TRANSFAC Curated and Predicted Transcription Factor Targets datasets (Matys et al., 2003, 2006) in the Harmonizome collection of datasets and Ingenuity Pathway Analysis Knowledge Base (IPA, Qiagen), *TESC* is a predicted SF1 target that might require SF1 sumoylation, a specific subtype of post-transcriptional modifications, for its expression in SCs (Lee et al., 2011). In contrast, a protocadherin family member *PCDH8,* previously identified as a target of FOXL2 in mouse GCs (Georges et al., 2014), was dynamically downregulated and exhibited downregulation in SF1-induced cells using DESeq2 (Study III, Fig. 4B, Supplementary Data 1). As FOXL2 is required for maintaining ovarian identity of cells (Schmidt et al., 2004; Ottolenghi et al., 2005) and FOXL2 interacts with SF1 in transcriptional regulation during gonadal development (Kashimada et al., 2011; Takasawa et al., 2014), downregulation of *PCDH8* suggests repression of the ovarian pathway in differentiated SF1-induced cells.

However, some genes, such as *INHBB* (encoding inhibin βB subunit), were also dynamically expressed despite similar expression in pairwise comparisons between induced and non-induced cells (Study III, Fig. 4B). This indicates that, by revealing dynamic expression paths in a pool of cells, one can obtain novel insight into gene regulatory paths that could not have been generated by using more conventional bioinformatic methods. Overall, the transcriptional changes observed in this study following SF1 induction reveal how SF1 regulates its downstream targets in early gonadal-like cells.

4. New putative SF1 targets

SF1 activation in gonadal-like cells allowed us to identify novel SF1 targets, which have not been reported to be regulated by SF1 previously. During target candidate screening we specifically focused on those gene expression changes, which were more likely to derive from the nearly immediate effects of SF1, and therefore, the first time point (day 6 of differentiation) 2 days after the onset of SF1 induction was more carefully studied. Within the top 100 upregulated genes, we identified several novel target candidates of SF1. One of these was ferritin receptor *SCARA5/TESR* (Study III, Supplementary Fig. S3B), which is expressed in SCs and epithelial cells of mucosal surfaces in mouse (Sarraj et al., 2005; Jiang et al., 2006), and has been reported to be differentially expressed in the fetal male and female gonads after sex determination (Sarraj et al., 2005). Of the markers not previously associated with the gonads, we identified *ADAMTS14*, a gene encoding an ADAM family metalloprotease in trophoblast cells

(Lee et al., 2014) (Study III, Supplementary Fig. S3B). *ADAMTS14* is associated with various diseases such as cancer (Porter et al., 2004), osteoarthritis (Rodriguez-Lopez et al., 2009) and multiple sclerosis (Goertsches et al., 2005). Interestingly, several markers of the ADAMTS gene family are known to be associated with development or function of the testes or the ovaries (Russell et al., 2015; Aydos et al., 2019). However, ADAMTS14 has not previously been recognized among them.

In addition to differentially expressed putative target candidates on day 6, we identified a testicular marker *MAGEB1* among the top 100 differentially expressed genes on subsequent differentiation days (8 and 10) (Study III, Supplementary Data 1). According to our understanding, no regulatory interaction between MAGEB1 and SF1 has been previously reported to exist in the testes or the ovaries. In addition to being exclusively expressed in the testes, *MAGEB1* can be found in several tumors in humans (Lurquin et al., 1997). *MAGEB1* is located in chromosome Xp21 on the dosage-sensitive sex reversal region (Muscatelli et al., 1995), which is a 160-kb duplication sequence associated with 46,XY male to female sex reversal (Bardoni et al., 1994), and is therefore a candidate for DSD.

The expression kinetics of these candidate targets of SF1 were studied at several time points during differentiation in the presence or absence of activation of *NR5A1*. In SF1-induced cells, *SCARA5* was upregulated 24 h, whereas *MAGEB1* and *ADAMTS14* were upregulated 48 h after the onset of *NR5A1* activation (Study III, Fig. 5A). In non-induced cells, the genes were expressed at basal levels. SCARA5 and MAGEB1 were also detected in SF1-induced, but not in non-induced cells by immunocytochemistry at day 10 of differentiation (Study III, Fig. 5B). Due to the relatively short delay in gene expression changes of *SCARA5*, *MAGEB1* and *ADAMTS14* following the onset of SF1 induction, it is likely that these genes are activated directly downstream of SF1. However, to confirm this, SF1-induced transcriptional activation of these putative SF1 targets must be experimentally validated in future studies.

5. *NR5A1* activation with DOX and TMP

To induce adequate testicular differentiation, we tested various durations of *NR5A1* activation in differentiated cells. Figure 21 (Sepponen, unpublished results) presents the expression of selected markers in the conditions in which SF1 induction was started immediately after formation of the IM-like stage (at day 4 of differentiation). As the gene expression levels of *NR5A1*, testicular markers *AMH*, *SOX9,* and *INHA,* and steroidogenic marker *CYP11A1* indicate, the longest duration of *NR5A1* activation (6 days) most markedly induced their expression at days 8 and 10 of differentiation (Sepponen, unpublished results, Fig. 21). Therefore, 6-day induction was selected for the subsequent studies.

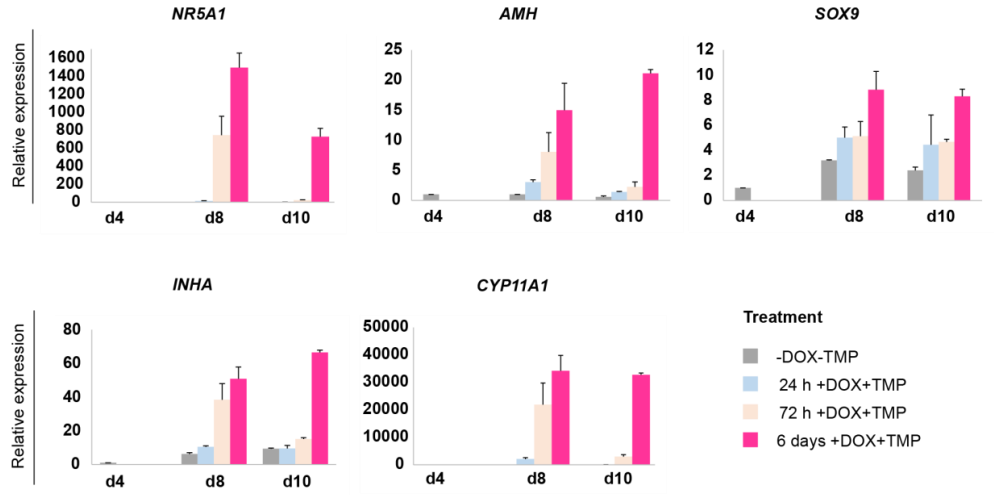

Figure 21. Expression of selected markers in response to different durations of *NR5A1* activation with DOX (1 µg/ml) and TMP (10 µM) in differentiated HEL46.11-DDdCas9VP192-NR5A1 clone 14 hiPSCs. Expression values represent average relative expression of two culture wells (d4, d8, d10) per time point in a differentiation experiment. Cells were differentiated according to the extended gonadal protocol M, and the expression values are relative to expression at day 4 of differentiation. Abbreviations: d, day of differentiation; DOX, doxycycline hyclate; TMP, trimethoprim (Sepponen, unpublished results).

In addition, various concentrations of DOX in the differentiating cells were tested. DOX at concentration 10 µg/ml was selected for the downstream differentiation studies based on its ability to induce high expression levels of *NR5A1* and testis markers, including *AMH* and *INHA* (Sepponen, unpublished results, Fig. 22). No obvious morphological differences in cell differentiation or excessive reduction in cell density were observed in the presence or absence of 10 µg/ml DOX and 10 µM TMP. However, in undifferentiated cells, substantial cell death was noted with a higher concentration of DOX (10 µg/ml), but not with a 10-fold reduced concentration of DOX (1 µg/ml), demonstrating different sensitivities of the undifferentiated and differentiated cells to DOX. This is in line with previous studies demonstrating that the undifferentiated hPSCs are more prone to clearance by apoptosis than the differentiated cells (Setoguchi et al., 2016) and that DOX induces apoptosis in *e.g.* cancer stem cells (Matsumoto et al., 2017).

It is possible that the induction chemicals, although promoting the expression of *NR5A1* and its target genes, led also to some unspecific effects in differentiating cells. For example, *FSHR* expression at day 8 of differentiation was downregulated in SF1-induced cells with a high concentration of DOX (10 µg/ml) compared with either non-induced cells or cells treated with lower DOX concentrations, and all tested concentrations of DOX reduced *FSHR* expression by day 10 compared with non-induced cells (Study III, Supplementary Fig. S2B and S2D; Sepponen, unpublished results, Fig. 22). SF1 has been reported to activate *FSHR* promoter in HEK293 cells transiently transfected with luciferase constructs driven by murine *FSHR* promoter fragments (Levallet et al., 2001). Interestingly, DOX- and TMP-mediated *NR5A1* activation did not induce *FSHR* expression in this study or in yet unpublished studies

performed to date by others in our laboratory using a related *NR5A1* activation approach introduced to H9 hESCs.

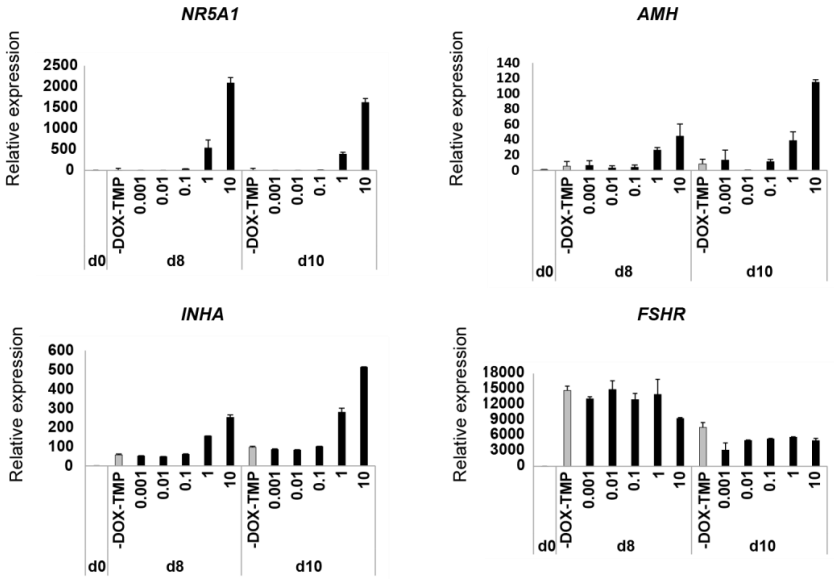

Figure 22. Expression of SC markers in differentiated HEL46.11-DDdCas9VP192-NR5A1 clone 14 hiPSCs in response to *NR5A1* activation using different DOX concentrations at 10-fold intervals (0, 0.001-10 µg/ml) and a constant concentration of TMP (0, 10 µM). Expression values represent an average of two culture wells per time point in a differentiation experiment and are relative to undifferentiated cells. Abbreviations: d, day of differentiation; DOX, doxycycline hyclate; TMP, trimethoprim (Sepponen, unpublished results).

6. Differentiation of cells expressing endogenous *FSHR*

FSHR expression levels were elevated with intermediate to high doses of BMP7 in a study performed using gonadal protocol M (Sepponen, unpublished results, Fig. 23). This demonstrated that a higher dose of BMP7 leads to a stronger induction of *FSHR* expression. Previously, BMP7 has been shown to increase mRNA expression levels of *FSHR* in neonatal mouse ovaries and in luteinized human GCs (Lee et al., 2004; Shi et al., 2010). While protocol M yielded the highest levels of gonadal marker gene expression levels of the 17 differentiation protocols tested in Study I (Study I, Fig. 1A), protocol D showed relatively high endogenous *FSHR* expression levels and it was selected for further studies comparing the biological properties of WT and mutated FSHR. To induce high endogenous expression of *FSHR,* an intermediate concentration (50 ng/ml) of BMP7 was applied in protocol D. As *FSHR* expression was endogenous in these cells, we exploited patient-specific hiPSC lines carrying inactivating mutation

in the *FSHR* for investigation of disease mechanisms and receptor functionality in both healthy and diseased states.

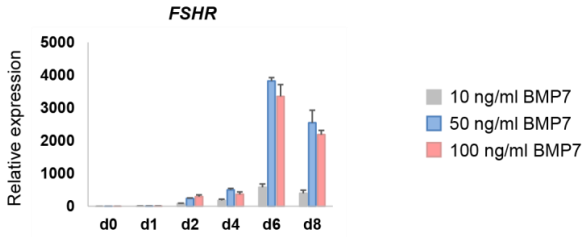

Figure 23. FSHR expression in cells differentiated with gonadal protocol M using different BMP doses. Bars ± SEMs represent an average of two technical replicates. Expression levels are relative to undifferentiated cells. Abbreviations: BMP, bone morphogenetic protein; d, day of differentiation (Sepponen, unpublished results).

Both conventional H9 stem cells (representing a healthy control cell line) and two patient-derived hiPSC lines (HEL127.6 and HEL128.5) presenting an A189V mutation, leading to inactivation of FSHR, were differentiated according to a slightly modified protocol D (described in detail in Study II, Materials and Methods). An increase in the relative expression of *FSHR* in differentiating cells in comparison with undifferentiated cells in all tested cell lines was first detected at day 2 of differentiation, and, depending on the cell line, the expression peaked between days 4 and 6 (Study II, Fig. 1A). In this study, both WT and A189V *FSHR* mRNA were expressed at roughly similar levels (Study II, Fig. 1A), which was anticipated based on similar levels of WT and A189V mutant *FSHR* mRNA detected previously in Northern hybridization studies following transient or stable *FSHR* transfection of mouse GCs (Rannikko et al., 2002). As expected, the undifferentiated cells did not express *FSHR* as demonstrated with a high cycle threshold-value of approximately 35 in qRT-PCR.

Within the differentiated cells population at day 8, approximately 8% of *FSHR* expressing cells were detected by a scRNA sequencing analysis (Study II, Fig. 1B). The method reveals gene expression in individual cells in contrast to conventional bulk sequencing, which yields information about the average gene expression within the whole sample consisting typically of millions of cells (Choi and Kim, 2019). The differentiated cells were recognized to be relatively homogeneous, demonstrated with closely located and partially overlapping cell clusters and ubiquitously expressed *FSHR* transcripts in all clusters. Based on the applied clustering tool, several of the cell clusters corresponded to gonadal endothelial cells, oogenesis phase fetal germ cells, mitotic arrest phase fetal germ cells, and fetal germ cells (Study II, Fig. 1B). Additionally, associations with fetal, stem cell or progenitor stages of cells related to gonadal, mesenchymal, or neural identity were recognized (Study II, Fig. 1B). As the annotation tools rely on datasets available at the time of analysis, the latter may be partially explained by the presence of a typically high number of genes in the datasets with an association with nervous system development. In conclusion, the cells differentiated with modified protocol D had gonadal-associated identity, but the exact identity remained elusive.

6.1. Assaying functionality of differentiated cells expressing WT and A189V *FSHR*

WT (H9) cells stimulated with FSH ligand for 1 h produced cAMP in a dose-dependent manner, while cells with a mutant receptor failed to respond to gonadotropin stimulation, as judged by measuring cAMP levels (Study II, Fig. 1C). The incapability of the mutant receptor to respond to gonadotropin stimulation by producing cAMP has been demonstrated earlier with a possible association with compromised receptor trafficking (Rannikko et al., 2002). As expected, both the WT and mutant receptor-bearing cells responded to forskolin stimulation by producing cAMP (Study II, Fig. 1C). Forskolin (7beta-acetoxy-8, 13-epoxy-1a, 6β, 9a-trihydroxy-labd-14-en-11-one) is a lipid-soluble cell membrane-penetrating diterpenoid extracted from the roots of the *Coleus forskohlii* plant, and it can directly activate adenylate cyclase, thereby increasing intracellular cAMP production (Seamon et al., 1981). The WT cells lyzed after an 8-h stimulation with FSH or forskolin had increased levels of *STAR*, *INHA,* and *DLK1* expression and reduced levels of *FSHR* expression compared with the vehicle control (Study II, Fig. 1D). A similar response to FSH regarding expression of *FSHR*, *STAR,* and *INHA* mRNA has been demonstrated in studies by others (Klaij et al., 1990; Themmen et al., 1991; Johnson and Bridgham, 2001), however, the non-canonical Notch ligand DLK1 has not previously been associated with FSH or its receptor. Although forskolin induced expression of *INHA*, *STAR,* and *DLK1* and downregulated *FSHR* also in cells expressing the mutant receptor, the expression of these genes was not altered in response to FSH stimulation (Study II, Fig. 1D). In conclusion, our results were in line with the previous reports demonstrating ablated cAMP signaling and downregulation of selected FSH target genes in cells expressing A189V FSHR.

6.2. Challenges in assessing FSHR expression

Our laboratory utilized various methods to investigate whether increased *FSHR* expression results in higher FSHR protein levels, but unfortunately, the expression of FSHR protein could not be shown experimentally. Several antibodies purchased from different commercial sources were tested and discovered to be unspecific in terms of antibody localization or staining outcome in undifferentiated versus differentiated hiPSCs. The unspecificity of some of these FSHR antibodies has been also reported by others in the field of reproductive biomedicine (based on personal communications). GFP -labeled or -unlabeled FSH failed to reveal any difference between cells expressing the WT or mutant receptors and, additionally, yielded a signal in undifferentiated cells, where *FSHR* was not expressed. Furthermore, cell lysates from differentiated WT and mutant cells and undifferentiated hiPSCs yielded bands of similar size and pattern in Western blotting, providing further evidence of the unspecificity of the tested antibodies. We attempted, in collaboration with Professor M. Varjosalo, to identify FSHR protein by using conventional mass spectrometry analysis from differentiated hiPSCs expressing either the WT or mutant receptor, and also from HEK293 cells overexpressing the WT receptor. Surprisingly, we failed to detect FSHR protein in the differentiated hiPSCs with this method. Instead, FSHR was identified in HEK293 cells overexpressing the WT receptor, although the total number of detected receptor

molecules was low (11-17 molecules per sample). Overall, major challenges were encountered in assessing FSHR expression despite of demonstrated *FSHR* expression in differentiated cells.

7. Novel protein-FSHR interactions

We applied a sophisticated method previously established by the Varjosalo Laboratory that enables identification of protein-protein interaction partners, interaction distances, and protein localization at the molecular level (Liu et al., 2018). In these studies, both stimulated and unstimulated WT and mutant FSHRs artificially expressed in tetracycline-conditioned transgenic Flp-In™ 293 T-REx cells were analyzed by AP-MS and by a complementary proximity-based labeling method BioID. Flp-In™ 293 T-REx cells were used instead of hPSCs, as the method has been established in the Flp-In™ 293 T-REx line, and to date major technical challenges have prevented its successful application to stem cells.

AP-MS reveals physically interacting proteins and BioID can be applied to identify proteins that lay within at distance of 10 nm from the protein of interest. The approach combining both methods employed MAC-tag labeling of the receptor. MAC-tag facilitated the AP-MS and BioID methods for implementation and was also expected to improve data reproducibility. Furthermore, we expected to gain information on the localization of the interacting proteins and proteins in close proximity to our protein of interest by comparing the obtained data with a reference molecular context repository (Liu et al., 2018). The advantage of employing the Flp-In™ T-Rex™ cell line in this approach was that after derivation of isogenic clones all cells are genetically identical, allowing constant protein expression, which can be controlled in a conditional manner. Additionally, the presence of merely a single copy of a transgene in their genome enables mimicking the endogenous levels of protein expression (Ward et al., 2011). As information regarding FSHR and its interaction partners has previously been gained mainly by overexpressing FSHR (https://thebiogrid.org/108770), the ability to identify interacting molecules in a more biologically relevant context was considered a major advantage of the method.

Several proteins associated with FSHR were identified for stimulated and unstimulated WT and mutant FSHRs by using AP-MS and BioID. To our surprise, all physically interacting proteins identified by AP-MS were not detected by using BioID. For example, the presence of FSHR CGA subunit, which was detected in stimulated cells by AP-MS, was not detected by BioID (Sepponen, unpublished results, Fig. 24; Study II, Fig. 2). Consequently, we chose to employ merely data obtained by AP-MS. With this method, 75 high-confidence protein-protein interactions were identified, of which 19 were detected specifically in WT receptors and 14 in A189V mutant receptors, with varying number of interacting proteins specific for stimulated and unstimulated conditions (Study II, Fig. 2). Most of the proteins were associated with the functional categories involving protein processing and trafficking or the endoplasmic-reticulum-associated protein degradation (ERAD) pathway, regulation of apoptosis and autophagy, or alternatively, mitochondrial localization (Study II, Fig. 2). Proteins associated with

protein processing and trafficking or the ERAD pathway were identified in both stimulated and unstimulated states, but FSH stimulation slightly increased the number of interacting proteins, especially in cells expressing WT-FSHR (Study II, Fig. 2). Although it can be speculated that the increase in the number of protein processing- and trafficking-related molecules in stimulated cells could be related to increased receptor trafficking, which involves *e.g.* members of the Rab GTPases (Sayers and Hanyaloglu, 2018) (Fig. 5) identified in the interactomics data in this study (Study II, Fig. 2), of those only Rab1 has been directly related to GPCR trafficking in previous studies (Wu et al., 2003; Filipeanu et al., 2004). Based on those mechanistic studies, Rab1 is involved in ER to Golgi and ER to plasma membrane transport, however, the studies were performed with GPCRs other than FSHR. Also, a retrograde transport protein SELIL1, which mediates transfer of misfolded proteins from ER into endosomes for their degradation (Sun et al., 2014), was identified among the proteins detected specifically in A189V FSHR-expressing cells (Study II, Fig. 2). As impaired receptor trafficiking to the cell membrane in the A189V FSHR has been suggested to explain differences between the functionality of WT and mutated receptors (Rannikko et al., 2002), the presence of a few additional trafficking-related proteins in WT cells compared with cells expressing the mutant receptor in our study provides limited support for the hypothesis.

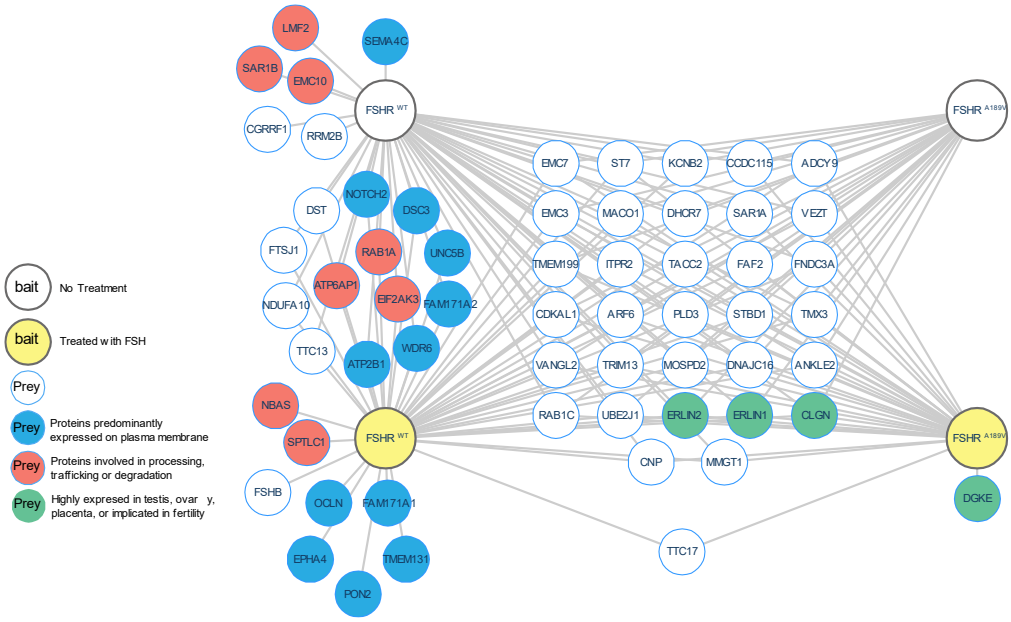

Figure 24. Protein-protein interactions of WT or A189V FSHR expressed in HEK293 cells in the presence (yellow bait nodes) or absence (white bait nodes) of FSH stimulation detected using BioID. Prey nodes, which represent proteins interacting with each of the FSHR baits, are colored according to their localization or function. Abbreviations: $FSHR^{WT}$, wild-type FSHR; $FSHR^{A189V}$, A189V mutant FSHR (Sepponen, unpublished results).

Interestingly, proteins associated with autophagy and regulation of apoptosis were mainly identified in cells expressing the mutant receptor (Study II, Fig. 2). Some of these, such as amyloid precursor protein and TGFβ receptor type-1, have previously been reported to have a role in gonadal function and reproduction (Chen et al., 2007;

Silva et al., 2015). Of the proteins detected in stimulated cells expressing either mutant or WT receptor, we identified IGF1R, which in addition to its role in gonadal development is also associated with fertility and FSH signaling in mammalian GCs and SCs (Baumgarten et al., 2014; Cannarella et al., 2018; Neirijnck et al., 2019). Overall, many proteins identified by the method were associated with male and female infertility, or both, using a web-based collection of databases (Rouillard et al., 2016) that includes curated Comparative Toxicogenomics Database Gene-Disease Associations datasets (Davis et al., 2009) (Study II, Fig. 2).

WT FSHR but not mutant FSHR was able to interact with FSH ligand subunits FSHB and CGA in the protein interactomic study performed with HEK293 cells (Study II, Fig. 2), which is in line with the phenotype exhibited by the differentiated hiPSCs. Surprisingly, only one of the 75 high-confidence protein-protein interactions, an anti-apoptosis protein DAD1, is currently known to directly interact with FSHR according to a published dataset (https://thebiogrid.org/108770) (Huttlin et al., 2017). Other previously recognized interaction partners of FSHR, such as G-protein-coupled receptor kinases, arrestins, G-proteins (Lazari et al., 1999; Ulloa-Aguirre et al., 2018a, Fig. 11), were not among the proteins interacting with FSHR in AP-MS. Proteins previously reported to interact with FSHR (that was artificially expressed in HEK293 cells), including APPL1, APPL2, 14-3-3 and FOXO1a (Cohen et al., 2004; Nechamen et al., 2004, 2007), were also not identified among the proteins interacting with FSHR. Although AP-MS revealed proteins interacting with FSHR in the previously published datasets (https://thebiogrid.org/108770), almost exclusively their interaction with FSHR was non-significant. In addition to DAD1, these proteins included hypertension and insulin resistance-associated UPK3BL1 (also known as UPK3BL), gap junction protein GJA1, a putative hepatocellular oncogene FAM189B (also known as COTE1), translation initiation factor subunit PPP1R15B, and lipid metabolism-associated FAM241B (also known as C10ORF35) (Huttlin et al., 2017). In the Harmonizome Comparative Toxicogenomics Database, DAD1, GJA1, FAM189B, and PPP1R15B were associated with male or female infertility, or both. A possible explanation for the discrepancy between our findings and the previously published datasets may be due to differences in the expression levels of FSHR. In our study, FSHR was expressed at lower level in the tetracycline-conditioned Flp-In™ T-Rex™ cells than in studies by others, in which the HEK cells typically overexpress FSHR as a result of transient transfection or viral transduction of the transgene. In the latter, unspecific binding may occur due to the non-physiologically high expression level of FSHR and large cell-to-cell variation, or both.

8. A189V FSHR receptor trafficking – an unresolved issue

The possibly impaired receptor trafficiking of the A189V FSHR (Rannikko et al., 2002) has not been experimentally proven; and to examine this, information on receptor localization would be required. As described above, we tried to elucidate localization of FSHR WT and mutant forms in unstimulated and stimulated states with several commercial antibodies, however, localization of the proteins remained elusive. Moreover, as the BioID approach was not applicable in this study, we could not benefit

from the molecular context map (Liu et al., 2018) in resolving the localization of the proteins. Although we could not clarify the mechanism behind disturbed A189V FSHR function, the interaction of FSH ligand subunits FSHB and CGA with the FSH-stimulated cells expressing the WT FSHR, and the absence of these interactions in cells expressing the A189V FSHR, indicates diminished binding of the FSH ligand to the mutant receptor compared with the WT. This may be explained by a low number of mutant receptors at the cell membrane and provides additional evidence for the hypothesis suggesting that receptor translocation at the cell membrane is adversely affected in A189V FSHR (Aittomäki et al., 1995; Rannikko et al., 2002).

9. Cell line-specific variation in differentiation outcome

Cells expressing bipotential gonadal markers were repeatedly generated with protocol M by using H9 hESC and HEL46.11-DDdCas9VP192-NR5A1 hiPSC lines (Study I, Fig. 1-5 and Study III, Fig. 1B). Morphologically, the differentiated cells of both hPSC lines at day 8 of differentiation were similar (Sepponen, unpublished results, Fig. 25). Both lines also exhibited induction of PS-like and IM-like stages during differentiation (Study I, Fig. 1D and Study III, Supplementary Fig. S1). Similarly, cells expressing *FSHR* were successfully differentiated with two distinct patient-derived hiPSCs in addition to a H9 line (Study II, Fig. 1A). It can be thereby presumed that our *in vitro* induction protocols are suitable for differentiating hPSCs of different origins.

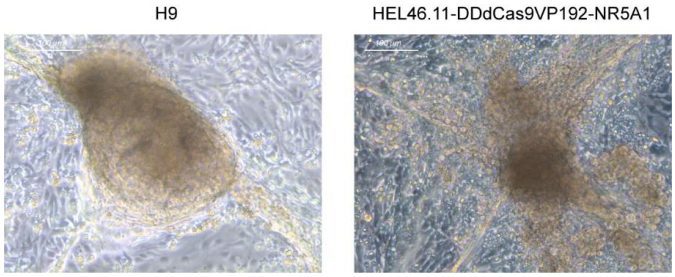

Figure 25. Morphology of differentiated hPSCs at day 8. Differentiation was performed according to protocol M. Scale bars 100 µm (Sepponen, unpublished results).

Nevertheless, it has been previously well-established that hPSC-lines are not identical in their differentiation capabilities (Mikkola et al., 2006; Osafune et al., 2008; Hu et al., 2010). In our studies, the variation detected in *GATA4* expression levels between differentiated H9 and HEL46.11-DDdCas9VP192-NR5A1 lines was more than 10-fold (Study I, Fig. 1-5 and Study III, Fig. 1B). In addition, differentiation by following the original protocol M into gonadal-like progenitors upregulated neural marker PAX6 in H9 cells (Study I, Fig. 1B and 3A-B), but its mRNA expression was not induced by differentiation of HEL46.11-DDdCas9VP192-NR5A1 cells in either the selected clone 14 or the pool of clones (Sepponen, unpublished results, Fig. 26). Additionally, *FSHR*, *INHA,* and *STAR* were differentially expressed between H9 and patient-derived cells in the absence of any additional supplementations such as hormonal stimulation (Study II, Fig. 1A and D). This indicates that the established protocols induce essentially similar

differentiation outcome with diverse cell backgrounds, but slight differences in gene expression patterns may occur.

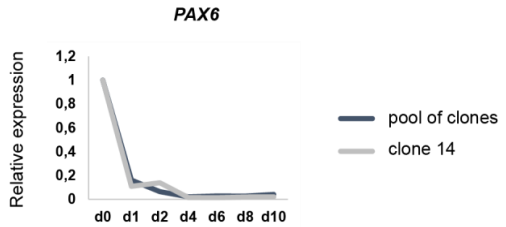

Figure 26. *PAX6* expression of differentiated cells derived clonally or comprising a pool of clones. Expression values are derived from a single differentiation experiment/cell line and are relative to undifferentiated cells. Cells were differentiated according to the extended gonadal protocol M. Abbreviations: d, day of differentiation; DOX, doxycycline hyclate, TMP, trimethoprim (Sepponen, unpublished results).

10. Culturing gonadal-like cells in 3D

Somatic cells of the gonads interact with each other and with germ cells (Fig. 2A-B). This interaction *in vivo* is taking place in a considerably different structural environment than the 2D monolayer culture of our *in vitro* studies. Culturing differentiating cells in a 3D microenvironment instead of a monolayer culture could better mimic the natural interactions between different cell types. We tested gonadal differentiation of HEL46.11-DDdCas9VP192-NR5A1 hiPSCs in the presence and absence of SF1 induction in a 3D spheroid culture. Cells were cultured in 2D until bipotential gonadal stage at day 8 of differentiation and transferred to microwells (Aggrewells), in which the number of cells was homogenized, enabling formation of regular-sized spheroids. The expression of bipotential gonadal markers (*GATA4*, *WT1*, *NR5A1*) and testicular markers (*AMH*, *INHA, CYP11A1*) in combined 2D and 3D cultures were upregulated following similar trends in gene expression as in 2D culture in induced and non-induced cells (Sepponen, unpublished results, Fig. 27). ROCKi Y-27632 has been previously reported to improve the formation of spheroids and to promote cell aggregation during 3D culturing of hiPSC-derived cells (Luo et al., 2018). The addition of ROCKi to 3D culture further increased the expression of the majority of the assayed gonadal markers (Sepponen, unpublished results, Fig. 27). The results indicate that gonadal protocol M can also be applied to 3D culture, allowing development of organoid culture models for gonadal differentiation and disease modeling in the future.

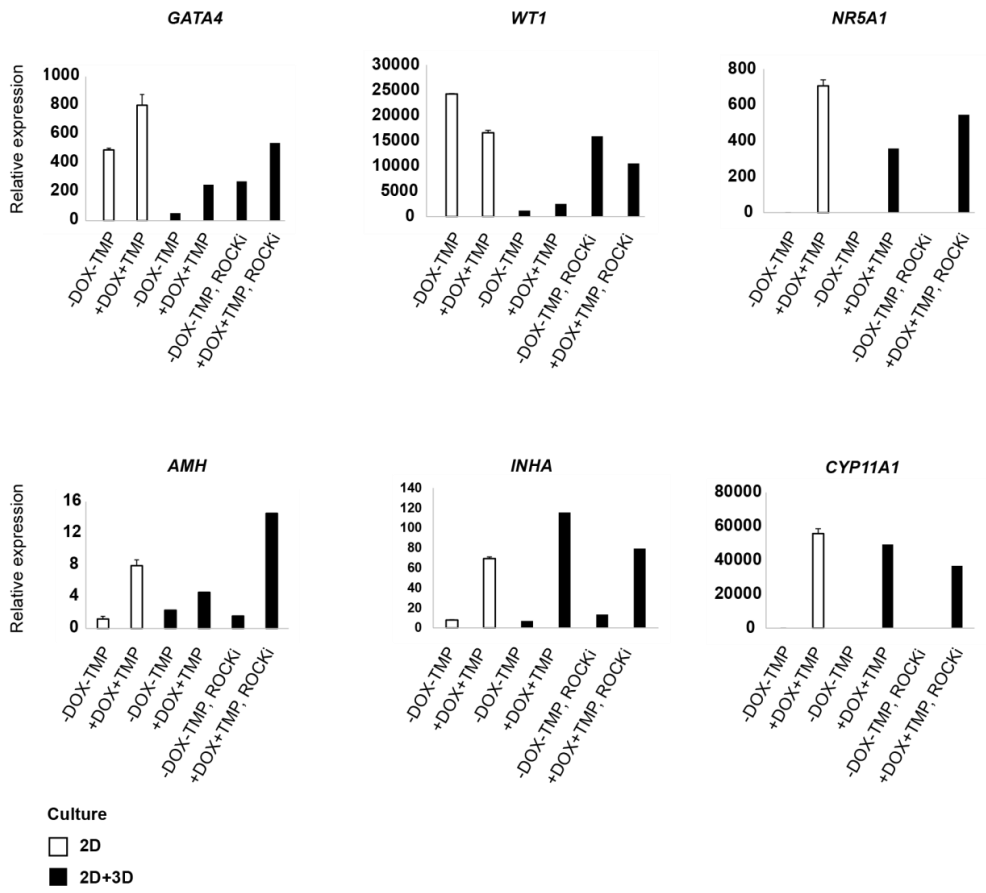

Figure 27. Expression of bipotential gonadal and testicular markers in HEL46.11-DDdCas9VP192-NR5A1 clone 14 hiPSCs at day 10 of differentiation in the presence and absence of 10 ng/ml DOX, 10 µM TMP, or 10 µM Y-27632 (ROCKi). Expression levels are relative to undifferentiated hiPSCs. Bars ± SEMs of cells cultured in 2D represent an average of two technical replicates on 12-well plates, bars of cells cultured in 3D represent single culture wells on a 24-well plate. Abbreviations: d, days; ROCKi, Rho-associated coiled coil forming protein serine/threonine kinase inhibitor (Sepponen, unpublished results).

11. Utilization of established culture models

Acquiring knowledge about the regulatory mechanisms and signaling cascades that take place in the human gonads at various fetal stages is difficult due to the scarcity of human material available, the technical challenges in maintaining primary cells and tissues in long-term culture, and ethical reasons considering human fetuses and their usage in research. Furthermore, data obtained from animals, typically rodents, corresponds poorly with human data. The model we have established to investigate early development of the human gonads is unique and provides a versatile tool for examining exogenous and genetic factors that may adversely affect gonadal development prior to sex determination. As we differentiated hPSCs of male and female origin into bipotential gonadal stage, the resulting cells exhibited a similar trend in the expression of bipotential gonadal markers, indicating that the culture model is reproducible and could also be used to differentiate additional hPSC lines into gonadal-like cells. For example, development of DSDs with different genetic backgrounds could be assessed with this model by using patient-derived stem cells. Additionally, the model provides a versatile tool for evaluating the impact of environmental toxicants or drugs on human fetal gonadal development and for assessing signaling mechanisms and transcriptional regulation of human gonadal development.

Furthermore, by combining differentiation of these gonadal-like cells and activation of endogenous SF1 expression at different stages of gonadal development, information about novel SF1 target genes and their interplay during gonadal development in humans can now be acquired *in vitro*. These new putative target genes may be associated with reproductive diseases or impaired fertility, and therefore, their role may extend beyond the developmental processes of the gonads.

We also demonstrated *in vitro* derivation of gonadal-like cells, which featured characteristics of fetal immature SCs. Previously, combined overexpression of *GATA4* and *NR5A1* has yielded SCs reported to be similar to adult primary SCs (Liang et al., 2019). To our knowledge, this was the first study describing differentiation of putative fetal human SCs via *NR5A1* activation, therefore highlighting the role of SF1 during development of gonadal-like cells. As we did not characterize any exogenous factors that could induce SF1 expression without artificial gene activation, the usability of our gonadal model is restricted to studies in which understanding of all natural signaling events induced in gonadal-like cell development is not critical. Furthermore, as comparisons of sequenced samples with the available public datasets predicted annotations for more than one somatic gonadal cell type, the precise identity of the differentiated SF1-induced cells remains unclear. In general, cell heterogeneity is a common issue encountered in PSC differentiation studies. Therefore, to identify the various cell types present in culture, the analysis of scRNA transcriptomics of gonadal-like cells could be advantageous.

We have focused only on a fraction of the putative SF1 target genes, and undoubtedly, more genetic associations remain to be identified in the high-quality RNA sequencing data set obtained from various time points during the differentiation. Moreover, we

mainly focused on protein-coding genes, but the RNA sequencing data displayed several other features, such as non-coding RNAs, *etc.*, which were differentially expressed between the induced and non-induced conditions. The role of epigenetic mechanisms, which refer to changes in gene expression without altering the nucleotide sequence and include regulatory non-coding RNAs, has become an increasingly important subject of research in recent years and includes mechanisms related to testicular function or infertility (Cheung et al., 2019; Joshi and Rajender, 2020). The RNA sequencing data presented in this study provide an excellent source for increasing our understanding of the epigenetic regulatory mechanisms in the developing human gonads and may aid in identifying the causes behind impaired gonadal development and infertility.

In addition to modeling gonadal development *in vitro*, we also generated cells of human origin, which endogenously express FSHR and are able to respond to FSH-mediated signaling by activating cAMP signaling cascade. Prior to our study, FSHR had been primarily examined in tumor cells and cell lines artificially stimulated to overexpress FSHR (Rannikko et al., 2002; Cohen et al., 2004; Kara et al., 2006). This is not ideal when physiological levels of FSHR are needed. The established protocol can be highly valuable for elucidating FSH action and for disease modeling in humans despite that the identity of differentiated FSHR-expressing cells remained elusive and therefore our *in vitro* model is not suitable for studying FSHR regulation cell type-specifically.

CONCLUSIONS AND FUTURE PERSPECTIVES

Proper gonadal development and function are crucial to achieve and maintain fertility. To acquire a better understanding of the signaling pathways and disease mechanisms affecting early human gonadal development, generation of models that utilize cells of human origin and mimic the distinct developmental stages of the fetal gonads *in vivo* is important. To date, only a limited number of such gonadal *in vitro* models exist, and in several of these models hPSCs have been used.

The testes and the ovaries have a common origin, as they both differentiate from the bipotential gonad. Previously, bipotential gonadal differentiation had not been demonstrated *in vitro* using hPSCs. Our data show that bipotential gonadal-like cells can be repeatedly and consistently generated from hPSCs of different origins by activating Nodal, Wnt, and BMP signaling pathways, and further differentiated to male gonadal-like cells by activating *NR5A1*. The established *in vitro* differentiation method aids in elucidating signaling mechanisms during early gonadal development. Moreover, the transcriptomics analysis of developing gonadal-like cells with and without *NR5A1* activation revealed genetic regulatory interactions in a dynamically changing environment, which may facilitate the development of *in vitro* models for studying reproductive system diseases and causes of infertility in the future.

The established gonadal model serves as a starting point for examining the differentiation of hPSCs into somatic gonadal cell types. It allows *e.g.* screening of growth factors, application of different monolayer and 3D culture environments, and establishment of gene reporter or activation studies to drive generation of the desired gonadal cell types. The model can be applied *e.g.* in differentiation of patient-derived cell lines into gonadal-like cells or for screening environmental toxicants, thereby elucidating cell type-specific effects behind genetic or exogenic causes that lead to disruption of gonadal development.

In addition, our data demonstrated that *FSHR*-expressing cells can be generated from hPSCs in a consistent and reproducible manner. The established protocol for *in vitro* differentiation serves as a valuable tool for studying FSHR biology in the presence of more physiological expression levels of the receptor. Moreover, the proteomics analyses of WT and A189V mutant FSHR revealed dissimilarities in ligand binding and delineated novel candidate molecules interacting with FSHR. This information may aid understanding of FSHR receptor signaling in general and in relation to the inactivating A189V mutation.

Taken together, this thesis comprehensively describes differentiation of gonadal-like cells and cells expressing endogenous *FSHR* using hPSCs. The exact identities of the resulting differentiated cells remained elusive, which suggests that more efficient targeting into specific cell types is required before the models can be used to mimic *in vivo* human gonadal cell development. In addition, deeper understanding of the transcriptional and signaling networks that induce distinct gonadal cell fates is needed.

Nevertheless, the work presented here provides a considerable amount of new knowledge about gene expression changes and signaling events during differentiation of gonadal-like cells. This information will surely be important in uncovering the causes of gonadal dysfunctions and infertility.

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