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GRAND THEME REVIEW

Transcriptional control of human gametogenesis

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ABSTRACT: The pathways of gametogenesis encompass elaborate cellular specialization accompanied by precise partitioning of the genome content in order to produce fully matured spermatozoa and oocytes. Transcription factors are an important class of molecules that

function in gametogenesis to regulate intrinsic gene expression programs, play essential roles in specifying (or determining) germ cell fate and assist in guiding full maturation of germ cells and maintenance of their populations. Moreover, in order to reinforce or redirect cell fate *in vitro*, it is transcription factors that are most frequently induced, over-expressed or activated. Many reviews have focused on the molecular development and genetics of gametogenesis, *in vivo* and *in vitro*, in model organisms and in humans, including several recent comprehensive reviews: here, we focus specifically on the role of transcription factors. Recent advances in stem cell biology and multi-omic studies have enabled deeper investigation into the unique transcriptional mechanisms of human reproductive development. Moreover, as methods continually improve, *in vitro* differentiation of germ cells can provide the platform for robust gain- and loss-of-function genetic analyses. These analyses are delineating unique and shared human germ cell transcriptional network components that, together with somatic lineage specifiers and pluripotency transcription factors, function in transitions from pluripotent stem cells to gametes. This grand theme review offers additional insight into human infertility and reproductive disorders that are linked predominantly to defects in the transcription factor networks and thus may potentially contribute to the development of novel treatments for infertility.

Key words: gametogenesis / transcription factors / infertility / germ cell / germ cell tumors / gene mutations / transcriptional profiling / single-cell RNA-sequencing / pluripotent stem cells / in vitro differentiation

Introduction

Human embryo development, like that of other organisms, is characterized by a series of cell-fate transitions from one cell type to another, starting from pluripotent stem cells (PSCs) and progressively specifying different lineages including extra-embryonic tissues, germ cell and somatic cell lineages. The primordial germ cells (PGCs) arise early in development as a small group of embryonic cells that will ultimately give rise to sperm and oocytes, and pass on genetic information to subsequent generations (Waters and Trainer, 1996; Donovan, 1998; Tang *et al.*, 2016; Kobayashi and Surani, 2018). The correct functioning of lineage specification is obviously critical; dysfunction during gametogenesis may lead to defects in germ cell development and/or function underlying diverse genetic fertility syndromes (Krausz and Riera-Escamilla, 2018; Xavier *et al.*, 2021). In this review, we use the term 'specification of cell fate or identity' in reference to when a cell is committed to differentiate down a specific pathway if left in its normal environment.

Germ cell development is dependent on the regulators of gene expression that function at multiple levels, including transcription factors that orchestrate expression at the transcriptional level by binding to enhancer or promoter regions of target genes. Following embryonic genome activation, a series of transcription factors sequentially regulates the activity of a host of genes involved in cell fate decisions, including PGC specification and migration, sex determination, meiosis and germ cell maturation. Concurrently, developmentally regulated protein expression is also proceeding with coordination by RNA-binding proteins, beginning at fertilization with the translation of maternally inherited mRNA and continuing throughout germ cell development, as evidenced by the number of RNA-binding proteins defined as markers of late stages of germ cell lineages (Clark and Reijo Pera, 2006; Makar and Sasaki, 2020).

PGCs exhibit many properties of classic pluripotent cells, including the property of pluripotency itself, and yet they are committed to the germ cell lineage (Kuijk *et al.*, 2011). The prime example or archetype of a pluripotent cell type, namely embryonic stem cells (ESCs), maintain their undifferentiated state via the activity of a defined set of transcription factors, coordinately regulating those genes necessary for reinforcing the pluripotent state, and suppressing lineage-specific genes that would otherwise drive differentiation (Kim *et al.*, 2008; Niwa, 2009; Ng and Surani, 2011). PGCs appear to employ a subset of members of this set of genes while also adopting a distinct subset or circuitry of transcription factors to define their identity and complete three crucial developmental events: repress somatic programs; reacquire pluripotency; and reprogram genome-wide epigenetics. For example, although human PGCs (hPGCs) are committed to the germ cell lineage, they share expression of a subset of pluripotency genes with human ESCs (hESCs), notably *OCT4* (also known as *POU5F1*, POU class 5 homeobox 1) and *NANOG* (Kehler *et al.*, 2004; Hoei-Hansen *et al.*, 2005); however, other key pluripotency genes, such as *SOX2* (SRY-related HMG box-containing gene 2), are not expressed in hPGCs (Perrett *et al.*, 2008). Co-expression of pluripotency transcription factors, as well as lineage specifiers, distinguishes hPGCs from all other human embryonic cell types as well as mouse PGCs (mPGCs) (Tang *et al.*, 2015). To maintain cell identity, hPGCs likely require a precise regulation/balance of pluripotency-related and lineage-specific transcription factors to repress somatic differentiation and concurrently activate germ cell programs.

A continuum of *in vivo* and *in vitro* models, based on human, mouse and non-human primate cells, has been explored and leveraged to study germ cell development, including the formation of PGCs, and their specification from PSCs or ESCs (Li *et al.*, 2020; Saitou and Hayashi, 2021). While mouse models are extraordinarily useful given their genetic malleability and ability to probe *in vivo* development of engineered cells, the genetics of germ cell development has both similarities and differences between species (Sasaki *et al.*, 2016; Kojima *et al.*, 2017; Stirparo *et al.*, 2018). For example, efforts to define a core set of transcription factors sufficient for PGC specification have succeeded in driving or even actively directing mouse cells further down the germ cell lineage than what has been achieved in human cell models (Niwa, 2009; Magnúsdóttir *et al.*, 2013). Indeed, it is likely that the microenvironment of the mouse gonad provides as yet undefined signals to induce germ cell differentiation of PGCs; moreover, xenotransplantation and co-culture with somatic cells have provided a superior microenvironment for further development of *in vitro*-derived PGC-like cells (Dominguez *et al.*, 2014; Durruthy Durruthy *et al.*,

2014; Ramathal *et al.*, 2014). Finally, recent analyses of *bona fide* germ cells in developing human embryos have provided insight into transcription factor expression as well as their interactions and functions during development (Otte *et al.*, 2017; Wen and Tang, 2019; Estermann and Smith, 2020; Li *et al.*, 2020; La *et al.*, 2021). Further analyses of these experiments are likely to add to our library of transcription factors potentially required for later stages of PGC function and germ cell development.

Methods

PubMed database was used to search articles and reviews with the following main keywords: human gametogenesis; transcription factors; infertility; germ cell; germ cell tumors; infertility; gene mutations; single-cell RNA-sequencing; pluripotent stem cells; *in vitro* differentiation; and other key terms related to these subjects. The search period included all publications until now (November 2021).

An overview of human gametogenesis

A number of reviews have contrasted, analyzed and discussed gametogenesis across species including humans. Two excellent recent examples are the reviews of Li *et al.* (2020) and that of Saitou and Hayashi (2021). In these reviews, *in vivo* and *in vitro* development are compared and contrasted, and differences between the processes across species are also highlighted. Here, we briefly provide an overview of human gametogenesis that distills details in specification, migration, sex determination and male- and female-specific development and then we focus on transcription factors and their functions and associated pathologies. Recent reviews and this work largely concur on major aspects while providing different content; this is indicative of the field of gametogenesis *in vivo* and *in vitro* maturing toward a common set of foundational developmental and genetic principles.

Human germ cell specification

In vivo, hPGCs are first identified in the posterior region of the yolk sac, and begin to migrate to the genital ridge about 4 weeks post-conception (McKay *et al.*, 1953; Motta *et al.*, 1997; Culty, 2009; Leitch *et al.*, 2013). Data from studies in the mouse indicate that signaling via bone morphogenetic proteins (BMPs) released from the extraembryonic ectoderm and proximal endoderm, including BMP4, BMP8b and BMP2, is essential for PGC specification (Lawson *et al.*, 1999; Ohinata *et al.*, 2009). Analysis of human fetal ovary also demonstrates that the expression of BMP2 and BMP4 may regulate the survival and migration of hPGCs (Childs *et al.*, 2010). In addition to BMPs, WNT (Wingless-related integration site) signaling, which is an evolutionarily conserved pathway in embryonic development, is required to activate the expression of many transcription factors that are indispensable in the specification of PGCs (Aramaki *et al.*, 2013). Finally, it is notable that a recent study of non-human primates demonstrates that cynomolgus monkey PGCs (cyPGCs) originate from the dorsal amnion instead of the posterior epiblast as seen in murine development (Sasaki *et al.*, 2016), suggesting the potential for distinct environmental cues for primate PGC specification versus other mammals.

Human germ cell migration

Following specification, hPGCs gradually proliferate as they also gain motility and initiate migration at 4–5 weeks (Pereda *et al.*, 2006; Mamsen *et al.*, 2012; Gomes Fernandes *et al.*, 2018). Despite significant differences in terms of migration rates and distances traveled, PGC migration in all species has conserved elements (Pereda *et al.*, 1998; Richardson and Lehmann, 2010; Grimaldi and Raz, 2020) including: first, the acquisition of motility/initiation of migration; second, directed migration; and third, termination of migration at the developing gonad.

In terms of acquisition of motility and initiation of migration, once PGCs are specified, specific molecular pathways direct the detachment from neighboring cells and the extracellular matrix as a prerequisite to motility. For example, studies in different organisms indicate that downregulation of the cell–cell adhesion protein, E-cadherin, initiates the migration process of PGCs.

Directed migration is regulated by attractive and repulsive cues. Following initiation of migration, PGCs require cues for directionality. PGCs from different organisms migrate along different paths while interacting with diverse cell types and the extracellular matrix. Immunohistochemistry and electron microscopy studies suggest that hPGCs preferentially migrate along autonomic nerve fibers and Schwann cells from the dorsal hind gut mesentery to the developing gonad (Mollgard *et al.*, 2010; Mamsen *et al.*, 2012). The migration is accompanied by a wave of chemical cues expressed by the surrounding somatic cells. Appropriate migration and survival of PGCs are instructed by both an intrinsic transcriptional program and external guidance cues. Stem cell factor, lipids and c-KIT (receptor tyrosine kinase) as well as G protein-coupled receptor signaling are implicated as attractive guidance cues for PGC migration to the genital ridge (Molyneaux *et al.*, 2003; Hoyer *et al.*, 2005). Similar to mouse PGCs (Hayashi *et al.*, 2007; Saitou and Yamaji, 2012), migratory hPGCs maintain a gene expression program characteristic of pluripotency, with sustained expression of pluripotency factors such as *OCT4* and *NANOG*. hPGCs also maintain a broad developmental potential, retaining the capacity for both germ cell and somatic cell differentiation.

Concerning termination of migration at the developing gonad, although there is no evidence for sex-specific differences during PGC migration, once PGCs arrive at the target gonad, motility is lost as the PGCs acquire sex-specific properties to contribute to gonad formation with somatic cells. Studies in mouse PGC development indicate that a change in cell adhesion may play a role in reduced mobility (Bendel-Stenzel *et al.*, 2000; Di Carlo and De Felici, 2000). However, the set of proteins responsible and their precise modes of action have yet to be identified and characterized in full. PGCs that fail to exit the nerve branches at the gonadal site may continue to migrate to other organs, such as the abdomen, adrenal glands, heart, lungs, and central nervous system. If they are not eliminated by apoptosis, these stray germ cells may give rise to germ cell tumors (Mamsen *et al.*, 2012).

Sex determination

Upon arriving at the genital ridge, PGCs interact with somatic cells and form the bipotential gonads. Sex determination of the gonad is a process by which the bipotential gonads differentiate into either testes or ovaries at gestational weeks 6–7 onward (Baker, 1963; Jorgensen *et al.*, 2012). Interestingly, sex determination of germ cells is dependent on external signals from the somatic environment rather than solely on the sex chromosome composition (XX or XY). Studies in mouse models confirmed this mechanism by demonstrating that XY germ cells can develop into oocytes in female chimeric embryos and XX germ cells can develop into prospermatogonia in male chimeric embryos (Ford *et al.*, 1975; Burgoyne *et al.*, 1988; Palmer and Burgoyne, 1991; Patek *et al.*, 1991). In the XX testis, the XX germ cells enter spermatogenesis and become prospermatogonia; however, they are eliminated before differentiation into spermatogonia. In the XY ovary, the XY germ cells enter meiosis and continue to differentiate as the primary oocytes; however, their fertility depends on species, genetic background and causes of sex reversal (Taketo-Hosotani *et al.*, 1989; Heard and Turner, 2011). The developmental fate of the bipotential gonad is dependent on a delicate balance of pro-testis and pro-ovary pathways in the supporting somatic cell lineage. To initiate male differentiation to the testis, the pro-testis pathway, characterized by the *SRY (Sex-determining Region on the Y chromosome)-SOX9 (SRY-related HMG bOX-containing gene 9)-FGF9 (Fibroblast Growth Factor 9)* gene network, needs to be activated to induce differentiation of the somatic cells into the male-specific Sertoli cells, and simultaneous repression of the ovarian pathway. In females, continuous activation of pro-ovary pathways, characterized by the RSPO1 (R-Spondin1)-WNT4-β-catenin signaling pathway, promotes differentiation of somatic cells to granulosa cells, leading to ovarian development. Once the somatic sex of the gonad is determined, sexual development of the rest of the embryo can progress. In males, the testes produce testosterone and anti-Müllerian hormone (AMH) to induce the formation of other organs in the male reproductive system and promote degeneration of the Müllerian duct. In females, the ovaries produce estrogen, which triggers development of the uterus, oviducts and cervix from the Müllerian duct. In response to somatic sex-determining cues, germ cells in female embryos initiate oogenesis and enter meiosis before birth. In contrast, male germ cells enter a mitotic arrest and do not enter meiosis until after birth.

Female germ cell development

Most of what we know of female germ cell development *in vivo* derives from studies in mice and rats with similarities observed in human fetal development, as well. After arriving at the genital ridge, female germ cells continue to proliferate through mitotic divisions with incomplete cytokinesis, to form oogonia cysts. In response to retinoic acid signals, oogonia cells then start meiosis and differentiate into primary oocytes (Bowles *et al.*, 2006; Koubova *et al.*, 2006). Meiosis initiates with prophase I stage, which is classically divided into five distinctive sub-stages based on the conformation of chromosomes: leptotene (prophase begins, chromosome start to condense), zygotene (synapsis begins), pachytene (crossing over), diplotene (synapsis ends) and diakinesis (prophase ends, nuclear membrane disintegrates). Primary oocytes arrest at the dictyate stage and become quiescent until sexual maturation. Around this time, the germ cell cyst breaks down, and the majority of oocytes that are not surrounded by somatic cells succumb to apoptosis and/or autophagy (Goldsmith, 1990; Pepling and Spradling, 2001; Escobar *et al.*, 2010). Surviving oocytes are assembled into primordial follicles with pre-granulosa cells; the primordial follicles are the reservoir of germ cells for the entire female reproductive life. At birth in humans, there are approximately 400 000 primordial follicles, and this number gradually declines with age (Block, 1953; Forabosco *et al.*, 1991; Gougeon, 1996). During a woman's reproductive life, approximately 400 follicles will undergo ovulation. With the onset of puberty, oocyte meiotic maturation is initiated by hormone stimulation, particularly by LH signaling molecules (Mehlmann, 2005). LH releases oocytes from meiotic prophase arrest and induces them to complete the first meiotic division and produce the first polar body. The second meiotic division begins immediately but pauses at metaphase, where the oocyte remains arrested until fertilization. The second meiotic division is triggered by the penetration of the sperm, and the second polar body will be formed at the same time.

Male germ cell development

Upon arriving at the genital ridge of a male embryo, male fetal germ cells (FGCs) will not enter meiosis *en masse*. Instead, at this stage in normal testis development, somatic cells and FGCs begin to differentiate into seminiferous tubules with germs cells in the center and Sertoli cells at the periphery (Wilhelm *et al.*, 2007). Somatic cells will provide the niche for developing FGCs. Spermatogenesis starts in early puberty, and it is a continuous cellular differentiation process that can be classified into four distinctive stages:

- Mitotic proliferation and maturation to generate spermatogonia (SPG). Spermatogonia are composed of three subtypes of cells: Type A (dark) cells (spermatogonia stem cells (SSCs) that do not undergo active mitosis), Type A (pale) cells (SSC that undergo active mitosis and divide to produce Type B cells), and Type B cells, which undergo growth and become spermatocytes.
- Two rounds of meiotic division to form haploid spermatocytes (SPC).
- Morphological transformation of spherical SPCs to elongated spermatids (SPT), a process also referred to as spermiogenesis.
- Final maturation of SPT to spermatozoa and release into the lumen of the seminiferous tubules, with the sperm passing through the epididymis to undergo final maturation (Clermont, 1972).

These four processes are interdependent and regulated by the somatic niche of the seminiferous tubules that is composed of three major cell types: Sertoli, peritubular and Leydig cells. It is estimated that the entire process of human spermatogenesis takes about 74 days (Heller and Clermont, 1964; Amann, 2008).

Intrinsic expression pattern of transcription factors in *bona fide* developing germ cells

Considering the complexity of the development pathways of germ cells in humans and the relation to the processes outlined above, it is

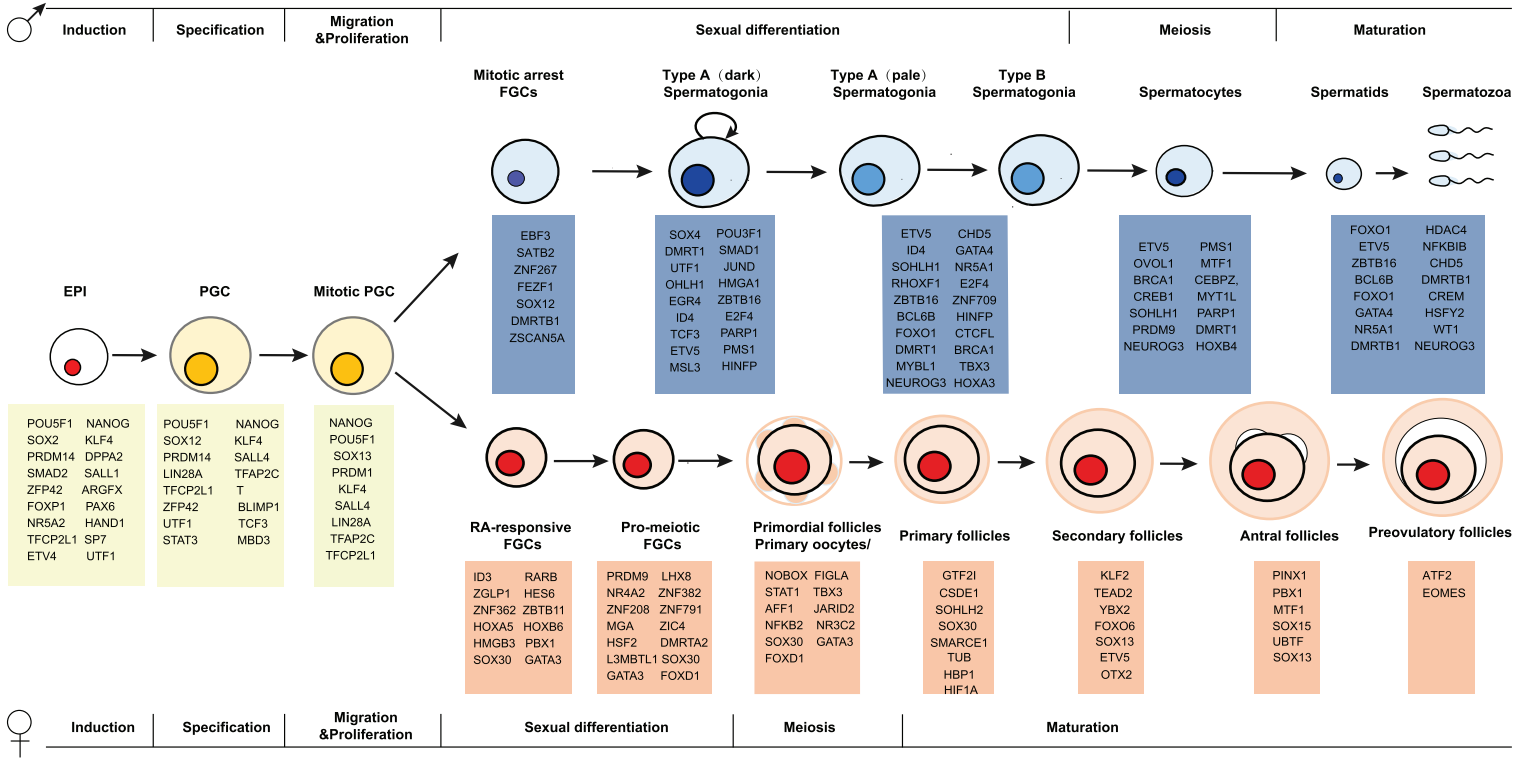

Figure 1. Transcription factors expressed/upregulated in developing human germ cells identified by scRNA-seq. EPI, epiblast; FGC, fetal germ cells; PGC, primordial germ cells; scRNA-seq, single-cell RNA-sequencing.

Table I Transcription factors expressed in developing human germ cells, determined by single-cell RNA-sequencing.

Developmental stage	Cell types analyzed	Transcription factors	Indicated functions of gene products in human reproduction or development	References
EPI	Human EPI, hESCs	*ESRRB, KLF17, KLF4, KLF5, SOX2, NANOG, ZFP57, FOXP1*	FOXP1, SOX2, NANOG and KLF4 are involved in the creation of pluripotency in EPI cells. ESSRB: maintain ESCs with OCT4 and SOX2.	Yan *et al.* (2013)
	Human preimplantation Embryos	*ESRRB, NANOG, POU5F1, SOX2, PRDM14, NR5A2, TFCP2L1, KLF17, SMAD2, SMAD4, ETV4*	PRDM14: essential for pluripotency and germ cell formation. NR5A2: important for embryonic development. SMAD: cell proliferation, apoptosis, and differentiation.	Blakeley *et al.* (2015)
	Human embryonic cells	*ARGFX, PRDM14, SOX2, NANOG, KLF17*	KLF17: involved to spermatid differentiation and oocyte development. ARGFX: related to pre-implantation embryo.	Petropoulos *et al.* (2016)
	Human pre-implantation embryos	*PRDM14, TFCP2L1, ZFP42, ARGFX, ESRRB, DPPA2*	TFCP2L1: important for establishment and maintenance of pluripotency in ESCs. ZFP42: involved in the reprogramming of X-chromosome inactivation during the acquisition of pluripotency and ESCs self-renewal. DPPA2: associated with developmental pluripotency	Stirparo *et al.* (2018)
	Human preimplantation embryos	*NANOG, PRDM14, SOX2, SOX21, SALL1, HAND1, SP7, PAX6, UTF1, ELF5*	SALL1: transcriptional regulation of PSCs. HAND1: acts as a transcriptional repressor of SOX15. UTF1: involved in differentiation of embryonic carcinoma and ESCs. ELF5: involved in tumorigenesis.	Zhou *et al.* (2019)
PGC	Human 4–19 weeks of gestation (WG) PGCs; gonadal somatic cells	**Early PGC:** *NANOG, POU5F1, SALL4, KLF4, ZFP42, TFAP2C, T, SOX15, SOX17* **Mitotic PGC:** *NANOG, POU5F1, SALL4, SOX17, SOX15, SOX13, PRDM1, PRDM14, TFAP2C, TFCP2L1, KLF4* **Male PGC mitotic arrest:** *EBF3, SATB2, ZNF267, FEZF1, SOX12, DMRTB1, ZSCAN5A, SOX30, GATA3, FOXD1* **Pro-Meiotic FGC:** *ZNF208, YBX1, ZNF791, PRDM9, LHX8, NR4A2, ZNF382, MGA, ZIC4, HSF2, DMRTA2, L3MBTL1, SOX30, GATA3, FOXD1*	EBF3: inhibits cell survival through the regulation of genes involved in cell cycle arrest and apoptosis. ZNF267 and ZSCAN5A: involved in transcriptional regulation. FEZF1:(GO) annotations related to this gene include RNA polymerase II proximal promoter sequence-specific DNA binding. DMRTB1: gene Ontology (GO) annotations related to this gene include DNA-binding transcription factor activity and sequence-specific DNA binding. ZIC3: this nuclear protein probably functions as a transcription factor in early stages of left-right body axis formation.	Guo *et al.* (2015) Li *et al.* (2017)
	Human prenatal germline cells	*SOX17, SOX12, KLF6, LEF1*	KLF6: transcriptional activator. LEF1: participates in the WNT signaling pathway.	Gkountela *et al.* (2015)
	Wk4–Wk9 human embryos	*NANOG, OCT4, KLF4, TFCP2L1, T, SOX17, TFAP2C, BLIMP1, UTF1, PRDM14*	KLF2: activates cell transcription	Tang *et al.* (2015)
	Human fetal tissues from first and second trimester	*NANOG, POU5F1, SOX4*		Vértesy *et al.* (2018)

Continued

Table I Continued

Developmental stage	Cell types analyzed	Transcription factors	Indicated functions of gene products in human reproduction or development	References
PCG (cont.)	Prenatal gonads from 4 to 16 weeks post-fertilization	**PGC:** *POU5F1, NANOG, PRDM1, SOX17, TFAP2C*		Chitiashvili *et al.* (2020)
Male	SSEA4+hSSCs and c-KIT+spermatogonia from whole adult human testis	**PGC:** *NANOG, POU5F1, SALL4, TCF3, KLF4, STAT3, MBD3, DMRT1* **SSC:** *DMRT1/6, BCL6, ID4, SALL4, ETV5, TCF3, KLF4, KLF2, STAT3, MBD3* **SPG:** *NR6A1, SOHLH2, TCF3, KLF4, KLF2, STAT3, DMRT1*	DMRT1/6: plays a central role in spermatogonia by inhibiting meiosis in undifferentiated spermatogonia and promoting mitosis, leading to spermatogonial development and allowing abundant and continuous production of sperm. BCL6: downregulated during maturation of dendritic cells by selective stimuli such as bacterial lipopolysaccharide. ID4: implicated in regulating a variety of cellular processes, including cellular growth, senescence, differentiation, and apoptosis. SALL4: plays a key role in the maintenance and self-renewal of embryonic and hematopoietic stem cells. NR6A1: may be involved in the regulation of gene expression in germ cell development during gametogenesis.	Guo *et al.* (2017)
	Spermatogenic cells from immature and adult male mice and adult men	**SPG:** *ETV5, ID4, SOHLH1, RHOXF1, ZBTB16, BCL6B, FOXO1, DMRT1, SOHLH2, NEUROG3, MYBL1, CHD5, GATA4, NR5A1* **SSC:** *ETV5, ID4, SOHLH1, RHOXF1, ZBTB16, BCL6B, FOXO1, DMRT1* **SPC:** *ETV5, SOHLH1, SOHLH2, NEUROG3* **SPT:** *ETV5, ZBTB16, BCL6B, FOXO1, NEUROG3, HSFY2, WT1, GATA4, NR5A1, DMRTB1*	RHOXF1: maybe involved in reproductive processes. Modulates expression of target genes encoding proteins involved in processes relevant to spermatogenesis.	Hermann *et al.* (2018)
	Testicular cells from donors with normal spermatogenesis and one with non-obstructive azoospermia (NOA)	**SPG:** *MYBL2, E2F4, ZNF709, HINFP, DMRTB1, CTCFL* **SSC:** *JUND, HMGA1, ZBTB16, POU3F1, UTF1, SALL4, FGF2, SMAD1, SMAD5, SMAD9, ID1, ID2, ID4, TCF7L2, SSRP1, SOX4, CLOCK, RHOXF1, TFDP2, PARP1, PMS1, E2F4, HINFP, DMRT1* **SPC:** *OVOL1, OVOL2, BRCA1, CREB1, PMS1, MTF1, CEBPZ, MYT1L, PARP1* **SPT:** *HDAC4, NFKBIB, CHD5*	OVOL2: plays a critical role in maintaining the identity of epithelial lineages by suppressing epithelial-to mesenchymal transition	Wang *et al.* (2018)
	Testicular cells from there healthy donors: 17,24 and 25 years old	**SPG:** *DMRT1, DMRTB1, SOHLH1, SOHLH2, BRCA1, TBX3, HOXA3* ***SSC:*** *UTF1, SOHLH1, EGR4, ID4, TCF3, ETV5, DMRT1, MSL3* **SPC:** *DMRT1, DMRTB1, DMRTC2, DMRT3, SOX4, SOX5, SOX30, SOHLH1, SOHLH2, HOXB4, HOXC6, PRDM9* **SPT:** *DMRTB1, CREM, SOX30*	CREM: plays a role also in human spermatogenesis and that the absence of the CREM switch can be associated to spermatogenic arrest. TBX3: acts as a negative regulator of PML function in cellular senescence.	Guo *et al.* (2018)

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Table I Continued

Developmental stage	Cell types analyzed	Transcription factors	Indicated functions of gene products in human reproduction or development	References
Male (cont.)	Testicular samples from obstructive azoospermia or non-obstructive hypergonadotropic azoospermia	**SPG:** *RHOXF1*	RHOXF1: the encoded protein is likely a DNA-binding transcription factor that may play a role in human reproduction.	Laurentino *et al.*, (2019)
	Neonatal and adult human testicular cells	**SSC:** *EGR4* **SPG:** *SOHLH1/2, DMRT1*	EGR4: the functional loss of EGR4 blocked spermatogenesis, leading to a significant reduction in spermatozoa production. SOHLH1: plays a pivotal role in the transition of germ cells from primordial to primary follicles and in the differentiation of spermatogonia. SOHLH2: plays a pivotal role in the transition of germ cells from primordial to primary follicles and in the differentiation of spermatogonia.	Sohni *et al.* (2019)
	Pre- and peri-pubertal human testicular samples were obtained from four healthy boys aged 7, 11, 13 and 14 years	**SPG:** *UTF1* **SPC:** *PRDM9*	PRDM9: mutations in PRDM9 may cause idiopathic infertility in human males. Expressed highest in testis.	Guo *et al.*, (2020)
	Human testis tissues from 3 embryonic stages, 3 fetal stages and 1 young infant stage	**PGC:** *POU5F1, NANOG, TFAP2C SOX17* **SSC:** *EGR4, MSL3*	MSL3: plays a role in chromatin remodeling, in X inactivation and transcriptional regulation.	Guo *et al.* (2021)
Female	Human 4-26-week fetal germ cells	**RA-Responsive female FGC:** *ID3, RARB, ZGLP1, HES6, ZNF362, ZBTB11, HOXA5, HOXB6, HMGB3, PBX1, SOX30, GATA3, FOXD1* **Oogenesis:** *NOBOX, FIGLA, STAT1, TBX3, AFF1, JARID2, NFKB2, NR3C2, SOX30, GATA3, FOXD1*	ID3: luteinization, oogenesis, oocyte maturation. RARB: receptor for retinoic acid. ZGLP1: germ cell development HES6: members of this gene family regulate cell differentiation in numerous cell types. ZNF362, ZBTB11: may be involved in transcriptional regulation. HOXA5, HOXB6: provides cells with specific positional identities on the anterior-posterior axis. HMGB3: plays a fundamental role in DNA replication, nucleosome assembly and transcription. PBX1: may have a role in steroidogenesis, sexual development and differentiation. PRDM9: the zinc finger array recognizes a short sequence motif, leading to local H3K4me3, and meiotic recombination hotspot activity. LHX8: plays a role in tooth morphogenesis, oogenesis and in neuronal differentiation. NR4A2: encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. ZNF382: may play roles in differentiation, proliferation and apoptosis.	Li *et al.* (2017)

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Table I Continued

Developmental stage	Cell types analyzed	Transcription factors	Indicated functions of gene products in human reproduction or development	References
Female (cont.)			MGA: functions as a dual-specificity transcription factor, regulating the expression of both MAX-network and T-box family target genes. ZIC4: members of this family are important during development. HSF2: activates heat-shock response genes. DMRTA2: may be involved in sexual development. L3MBTL1: probably plays a role in cell proliferation. NOBOX: oogenesis. FIGLA: functions in postnatal oocyte-specific gene expression. STAT1: induces a cellular antiviral state. TBX3: regulates developmental processes. AFF1: implicated in human childhood lymphoblastic leukemia. NFKB2: the endpoint of a series of signal transduction events. JARID2: stem cell differentiation and normal embryonic development. NR3C2: functions as a ligand-dependent transcription factor that binds to mineralocorticoid response elements.	
	Human fresh ovarian tissues from 7 female donors ranging from 24 to 32 years (yr), with a median age of 28 yr	**Primordial follicle:** *SOX30* **Primary follicles:** *GTF2I, CSDE1, SOHLH2, SMARCE1, TUB, HBP1, SOX30, HIF1A* **Secondary follicles:** *KLF2, YBX2, FOXO6, SOX13, ETV5, TEAD2, OTX2* **Antral follicles:** *PINX1, PBX1, MTF1, SOX15, UBTF, SOX13* **MII oocytes of preovulatory follicles:** *ATF2, EOMES*	In the oocytes, the expressions of GTF2I, CSDE1, SOHLH2, SMARCE1, TUB, HBP1, SOX30 and HIF1A were upregulated in primary follicles, indicating that these TFs may play a critical role in the transition from the primordial to the primary stage. KLF2, YBX2, FOXO6, SOX13, ETV5, TEAD2 and OTX2 were overexpressed in the oocytes from secondary follicles compared to those from primary follicles, implying that they are likely the regulators of the primary-to-secondary stage transition. PINX1, PBX1, MTF1, SOX15, UBTF, SOX13 and POU2F1 had higher expression levels in the oocytes of antral follicles compared to those of secondary follicles, indicating possible regulatory roles in the cytoplasmic and nuclear maturation of oocyte at the antral stage. ATF2 and EOMES were abundantly expressed in the MII oocytes of preovulatory follicles, indicating their potential roles in the unique transcription networks.	Zhang *et al.* (2018)
	Ovarian cortex samples from 21 patients	**Oocytes:** *FIGLA, PRDM1*	FIGLA: involved in continued oocyte survival as primordial follicles form in the human.	Wagner *et al.* (2020)
	In vivo and *In vitro* matured human metaphase II (MII) oocytes	**Oocytes:** *FIGLA, SOHLH2*	SOHLH2: involved in follicle development, initiation of primordial follicle growth, primary follicle growth, and germ cell development.	Ye *et al.* (2020)

EPI, epiblast; FGC, fetal germ cells; hESCs, human embryonic stem cells; PGC, primordial germ cells; PSC, pluripotent stem cells; SPG, spermatogonia; SSC, spermatogonia stem cells; WNT, Wingless-related integration site.

clear that given the rarity of germ cells in developing human embryos and the poor resolution of germ cell isolation methods, it is not possible to profile development and gene expression patterns at all stages. In addition, bulk RNA-seq or microarray analysis cannot resolve the heterogeneity within germ cells, which is essential for understanding the precise trajectory in which development occurs (Raser and O'Shea, 2005; Plass *et al.*, 2018). Transcriptome profiling at the single-cell level (i.e. single-cell RNA-sequencing: scRNA-seq) has been used to overcome this limitation by comprehensively measuring mRNA levels within all individual germ cells at a given developmental stage, and has been applied to diverse biological systems to begin to explore the potential molecular mechanisms for development (Junker and van Oudenaarden, 2014; Raj *et al.*, 2018; Genga *et al.*, 2019; Han *et al.*, 2020). Since 2013, a handful of reports have characterized the transcriptional dynamics during human germ cell development by analyzing human fetal and adult tissues using scRNA-seq. These studies were recently reviewed (Li *et al.*, 2020). Here, we focus on transcription factors that potentially act as master regulators to activate the unique gene expression program for each specific stage of germ cells (Fig. 1; Table I). We note that expression of a gene does not imply function; moreover, it is highly likely that genes are expressed at stages other than those that have been assayed and/or only briefly during development.

Transcription factors upregulated in germ cell specification and migratory hPGCs

The gene expression patterns of migrating and mitotic PGCs are similar in male and female germ cells. There is continued expression of transcription factors associated with pluripotency and ESCs, such as *POU5F1/OCT4, NANOG* and *PRDM14 (PR/SET domain 14)* (Guo *et al.*, 2015), although at different levels relative to pre-implantation epiblasts (EPI) (Yan *et al.*, 2013; Blakeley *et al.*, 2015; Guo *et al.*, 2015; Petropoulos *et al.*, 2016; Li *et al.*, 2017; Stirparo *et al.*, 2018; Zhou *et al.*, 2019). Concurrently, however, transcription factors that are diagnostic of germline cells, such as *PRDM1(PR/SET domain 1)* and *TFAP2C (Transcription Factor AP-2 gamma)*, and somatic lineages, such as *BRACHYURY (T)* and *EOMES* (eomesodermin), are also expressed in the same cells (Guo *et al.*, 2015; Tang *et al.*, 2015). A recent finding has also demonstrated that during hPGC specification, the classic endodermal transcription factor marker protein, SOX17 (SRY-related HMG box-containing gene 17), is required for hPGC commitment in an *in vitro* model of hPGC differentiation (Irie *et al.*, 2015). Moreover, scRNA-seq data of human gonadal PGCs *in vivo* confirmed the presence of SOX17 in early migrating and mitotic PGCs, consistent with its essential role in hPGC function (Guo *et al.*, 2015).

Transcription factors upregulated during male sex determination

To shed light on the critical transcriptional regulation in sex determination, scRNA-seq analyses were performed on both germ cells and their gonadal niche cells in multiple studies (Guo *et al.*, 2015; Li *et al.*, 2017; ChitiashviLi *et al.*, 2020; Guo *et al.*, 2021; Zhao *et al.*, 2021). Male sex determination initiates with activation of the Y chromosome-specific transcription factor, SRY, a dominant determinant for testis differentiation (Berta *et al.*, 1990; Gubbay *et al.*, 1990; Koopman *et al.*, 1990; Sinclair *et al.*, 1990; Kashimada and Koopman, 2010). Transcription factors that regulate SRY function and have been shown likely to be required for male sex determination include WT1(Wilms' tumor gene), NR5A1(nuclear receptor subfamily 5 group A member 1), GATA4(GATA binding protein 4), FOG2(FOG family member 2) and CBX2 (chromobox2) (Sekido and Lovell-Badge, 2008). Once SRY is activated, it acts by upregulating the expression of SOX9, which then activates a cascade including AMH, prostaglandins and steroidogenic genes, to promote complete organogenesis of the testis in humans and suppress the pro-ovary pathways (Koopman, 2001; Kozhukhar, 2012). SRY is also a direct target of the WT1. WT1 is a zinc finger containing DNA-binding protein that activates the expression of SRY in the initial sex determination process in humans (Shimamura *et al.*, 1997; Hossain and Saunders, 2001; Matsuzawa-Watanabe *et al.*, 2003). Other transcription factors that are essential for early testis differentiation include NR5A1, a highly conserved nuclear receptor transcription factor that interacts with SRY to regulate SOX9 expression during the differentiation of Sertoli cells (Sekido and Lovell-Badge, 2008; Rotgers *et al.*, 2018; Stevant and Nef, 2019). GATA4, a zinc finger transcription factor, also cooperatively interacts with NR5A1 to regulate downstream genes critical for testis differentiation (Viger *et al.*, 2008). Similarly, FOG2, a zinc finger cofactor, is suggested to be involved in testis determination through interaction with Gata4, potentially by modulating the activity of GATA4, and regulating the expression of SRY and SOX9 (Zaytouni *et al.*, 2011). CBX2, a component of the polycomb group (PcG) complex of regulatory proteins, has been reported to act in testis determination by activating the expression of NR5A1 and SRY and repressing genes involved in fetal ovarian development (Biason-Lauber *et al.*, 2009). Transcription factors belonging to the doublesex and mab-3 related transcription factor (DMRT) family, including DMRT1, 2 and 3, are found to be evolutionarily conserved sex-determining transcription factors. DMRT1 is a male-specific transcription factor gene which functions at multiple stages during male germ cell and Sertoli cell development to support spermatogonial development by antagonizing FOXL2 (forkhead box L2) activity and repressing the oogenesis program (Matson *et al.*, 2011). Mouse models found that *DMRT1*-mutant mice fail to develop functional testes, and continued expression of DMRT1 is necessary to prevent female reprogramming in the postnatal testis (Matson *et al.*, 2011). Mutation of DMRT transcription factors causes abnormal testicular formation and feminization (Ottolenghi and McElreavey, 2000).

Transcription factors upregulated during female sex determination

Female sex determination is regulated by transcription factors associated with RSPO1-WNT4-β-catenin signaling pathways. FOXL2 is considered a gatekeeper transcription factor for ovarian identity (Uhlenhaut *et al.*, 2009; Pannetier and Pailhoux, 2010) and promotes ovary development by blocking testis development through transcriptional repression of *SOX9* (Crisponi *et al.*, 2001; De Baere *et al.*, 2002; Udar *et al.*, 2003; Nallathambi *et al.*, 2007; Hersmus *et al.*, 2008; Shah *et al.*, 2009; Auguste *et al.*, 2011). Consistent with its critical role in

ovarian cell function, somatic mutations in *FOXL2* are found in nearly all cases of adult granulosa cell tumors of the ovary (Jamieson and Fuller, 2012). Other genes, such as *NR5A1*, may regulate anti-testis gene expression in the ovary; in 46, XX individuals, NR5A1 synergizes with β-catenin to upregulate the expression of anti-testis genes (e.g. *DAX1/NR0B1(Dosage-sensitive sex reversal-Adrenal hypoplasia congenita critical region on the X chromosome, gene 1)*) and possibly pro-ovarian genes (Gummow *et al.*, 2003; Hossain and Saunders, 2003; Jordan *et al.*, 2003; Mizusaki *et al.*, 2003).

Transcription factors upregulated in male germ cell development

Upon arriving at the genital ridge of a male embryo, germ cells arrest mitotically and transcription factors involved in cell cycle arrest, such as EBF3 (EBF transcription factor 3), are specifically upregulated (Guo *et al.*, 2015). Several groups have profiled the transcriptional trajectory across the entire spectrum of human adult spermatogenesis. Transcription factors mainly involved in repressing gene expression (e.g. E2F4 (E2F transcription factor 4), HMGA1 (high mobility group AT-hook 1)) are enriched in SSCs, consistent with their slow proliferation rate. After progressing to the differentiating SPG, cell cycle activation-associated genes, such as KIT and KI67, are significantly upregulated to ensure active proliferation and differentiation. Later, transcription factors involved in meiotic sex chromosome inactivation, homolog synapsis and meiotic recombination, such as OVOL1 (Ovo like transcription repressor 1), SOHLH1 (spermatogenesis and oogenesis specific basic helix-loop-helix 1) and DMRT1, are upregulated to initiate the meiotic gene expression program (Guo *et al.*, 2017, 2018; Hermann *et al.*, 2018; Wang *et al.*, 2018; Sohni *et al.*, 2019). As SPC complete their differentiation into SPT, nearly all these transcription factors are downregulated as the overall level of transcription gradually declines (Wang *et al*, 2018), with the exception of CHD5 (chromodomain helicase DNA binding protein 5), which is highly enriched in early SPT (Wang *et al.*, 2018). This is probably because of its involvement in the process of condensation of spermatid chromatin by regulating histone hyperacetylation and the replacement of histones by transition proteins in chromatin (Li *et al.*, 2014).

Transcription factors upregulated in female germ cell development

After arriving at the genital ridge, female germ cells rapidly lose expression of pluripotency transcription factors, for example, POU5F1/OCT4 (Rajpert-De Meyts *et al.*, 2004; Stoop *et al.*, 2005). Oogonia cells then undergo three sequential stages instructed by stage-specific transcription factors to generate fertilization-competent oocytes: the retinoic acid-responsive stage, the meiotic prophase stage and the folliculogenesis stage. Li *et al.* provided a thorough study to identify master transcription factors for germ cell development in the fetal stage (Li *et al.*, 2017) using the ARACNe (algorithm for the reconstruction of accurate cellular networks) algorithm. ARACNe identifies master regulators of development by correlation of expression of transcription factors and their target genes across various cell types. Their analyses indicate that *ZNF208 (ZiNc Finger protein 208)*, *YBX1 (Y-BoX-binding protein 1)* and *ZNF791* might be critical for the female mitotic phase, whereas *HES6* (*HES family BHLH transcription factor 6*), *MAEL* (Maelstrom spermatogenic transposon silencer 6), *ZGLP1* (Zinc finger GATA-Like protein 1), *ZNF362, ZBTB11 (ZiNc Finger and BTB domain containing 11), HOXA5 (HomeobOX A5), HOXB6, HMGB3 (High Mobility Group box3) and PBX1 (PBX homeobox 1)* are the potential transcriptional regulators in the retinoic acid-responsive phase. Meiotic recombination transcription factor proteins LHX8 (LIM homeobox 8), together with NR4A2, ZNF382, MGA (MAX dimerization protein), RLF (RLF zinc finger), ZIC4 (Zic Family Member 4), PAXBP1 (PAX3 and PAX7 binding protein), HSF2 (heat shock transcription factor 2), DMRTA2 (DMRT like family A2) and L3MBTL1 (L3MBTL histone methyl-lysine binding protein 1), are implicated in shaping the gene expression program for meiosis in the meiotic prophase (Guo *et al.*, 2015). Then cells start to express master transcriptional regulators, such as NOBOX (NOBOX oogenesis homeobox) and FIGLA (factor in germline alpha, also known as FIGLα or FIGα), to initiate the unique transcription network for folliculogenesis (Li *et al.*, 2017; Wagner *et al.*, 2020; Ye *et al.*, 2020). Human folliculogenesis is a complex process comprising five key stages (primordial, primary, secondary, antral and preovulatory follicles). The development of follicles is considered to be associated with highly dynamic transcriptional regulation (Aquila and De Amicis, 2014). Zhang *et al.*, explored the dynamic transcriptomes of the human oocyte, together with the neighboring granulosa cells across the entire process of follicular development, and identified potential master transcription factors for each stage using the ARACNe algorithm (Zhang *et al.*, 2018). Interestingly, once cells begin follicular development, the DNA methyltransferases DNMT1, DNMT3A and DNMT3B are highly expressed at all stages of oocyte development, suggesting that maintaining a high level of DNA methylation is essential for oocyte maturation.

Transcription factor mutations associated with human infertility

Despite enormous progress in human reproductive physiology, the underlying causes of diverse reproductive diseases, especially infertility, remains obscure. However, whole-exon sequencing or whole-genome sequencing analyses has identified thousands of gene mutations or variants that may be related to human infertility. These results suggest that most human reproductive diseases that were previously categorized as idiopathic may be of genetic origin. We have summarized mutations that were identified within transcription factors associated with human reproductive diseases in Table II.

Transcription factor mutations associated with disorders of sex development

Disorders of sex development (DSD) are defined as congenital conditions with a mismatch between sex chromosomes and gonadal/anatomical sex. DSD are generally classified into three categories: Sex chromosomes DSD; 46, XX DSD; and 46, XY DSD. Sex

Table II Transcription factor mutations reported to be associated with human infertility.

Disease	Associated transcription factors	Description	References
Disorders of sex development			
Swyer syndrome	*SRY*	Mutations in the *SRY* gene are the cause of 15% to 20% of cases of Swyer syndrome.	Arboleda *et al.* (2014); Baxter and Vilain (2013)
Sex reversal	*SOX9* *SOX3*	Copy number variants or mutations in the regulatory regions of the genes lead to human sex reversal.	Croft *et al.* (2018a); Croft *et al.* (2018b); Gonen *et al.* (2018); Vetro *et al.* (2015); Laumonnier *et al.* (2002)
Denys-Drash syndrome	*WT1*	Heterozygous mutations in the zinc finger domain of WT1 gene cause Denys-Drash syndrome.	Pelletier *et al.* (1991)
Frasier syndrome	*WT1*	A mutation in a splice donor site in WT1 leads to Frasier syndrome.	Klamt *et al.* (1998);
Gonadal dysgenesis	*NR5A1* *GATA4* *FOG2* *CBX2* *DMRT1/2*	Mutations in these transcription factors are associated with gonadal dysgenesis.	El-Khairi and Achermann (2012)
Cryptorchidism	*HOXD13, SOX2, ESR1, NR5A1, ZNF214, ZNF215, ARX*	Single gene mutations are associated with cryptorchidism.	Tannour-Louet *et al.* (2010)
Male infertility			
NOA	*DMRT1, PRDM9, ESR2, AR, KDM5D, NR0B1, NR5A1, SOX9, NPAS2, PGR*	The paper screened OMIM database and identified genes related to human male infertility- and NOA -	Wang *et al.* (2018)
NOA	*SOHLH1*	SOHLH1 mutations are associated with loss of testicular reproductive capacity.	Nakamura *et al.* (2017)
NOA	*SOX8*	SOX8 mutations were found at increased frequency in oligozoospermic men as compared with fertile/ normospermic control populations.	Portnoi *et al.* (2018) Choi *et al.* (2010)
SCOS, MA	*YBX1, YBX2*	YBX1 and YBX2 protein was markedly downregulated in SCOS and MA samples.	Alikhani *et al.* (2017)
Female infertility			
POI*	*FOXL2*	Foxl2 appears predominantly in the ovary and was first identified as mutated in a syndrome involving risk of POI.	Schlessinger *et al.* (2010) Crisponi *et al.* (2001) De Baere *et al.* (2005) Lakhal *et al.* (2008) Gersak *et al.* (2004) Harris *et al.* (2002)
POI*	*LHX8*	Preferentially expressed in germ cells and critical for mammalian oogenesis.	Qin *et al.* (2007)
POI*	*FOXO3A, FOXO1A*	Potentially causal mutations for POI.	Watkins *et al.* (2006) Lakhal *et al.* (2008)
POI*	*FIGLA*	Two plausible mutations in the FIGLA gene were identified among 100 POI cases (2%), whereas none were present among 304 ethnically matched controls.	Zhao *et al.* (2008)
POI*	*AIRE*	Mutations in AIRE gene are likely cause polyglandular syndrome, which is associated with POI.	McLaren *et al.* (2003)
POI*	*NOBOX*	Homeobox mutation causes POI.	Qin *et al.* (2007)
POI*	*SALL4*	Two novel variants (c.541G>A (p. Val181Met) and c. 2449A>G (p. Thr817Ala)) might be POI-associated gene variants.	Wang *et al.* (2009)
POI*	*WT1*	Two novel heterozygous mutations p. P126S and p. R370H were identified to be involved in POI.	Wang *et al.* (2015)

Continued

Table II Continued

Disease	Associated transcription factors	Description	References
POI*	*ESR1*	ESR1 gene variants are associated with both age at natural menopause and premature ovarian failure.	Qin *et al.*, (2012) Weel (1999)
POI	*TP63*	The combination of TP63 and BMP15 alterations contributes to the ovarian dysgenesis and early onset POI.	Bestetti *et al.* (2021)
POI	*LHX8, NOBOX, FOXL2, SOHLH1, FIGLA*	Combined functional and bibliographic analyses identified several novel or recurrent deleterious heterozygous mutations in POI patients.	Bouilly *et al.* (2016)

*MA, maturation arrest; NOA, non-obstructive azoospermia; POI, premature ovarian insufficiency (also known as premature ovarian failure); SCOS, Sertoli cell-only syndrome.

chromosome DSDs include 45, X Turner Syndrome, 47, XX Y Klinefelter Syndrome and 45,X/46,XY gonadal dysgenesis.

46,XX DSD includes disorders of ovarian development and disorders of the synthesis of congenital adrenal hyperplasia. 46,XY DSD includes disorders of testicular development, defects in testosterone biosynthesis, and impaired testosterone action (Lee *et al.*, 2006). The estimated frequency of DSD is approximately 1 in 2000–5500 newborns (Hughes *et al.*, 2007), and the frequency is as high as 1:200 to 1:300 if all genital congenital anomalies, including cryptorchidism and hypospadias, are considered (Nordenvall *et al.*, 2014). Genetic screening has identified many gene mutations associated with DSD, accounting for nearly 50% of the causality of cases; a few of the mutations are found in transcription factors, as described below.

SRY is the founding member of the SOX class of transcription factors, several of which play critical roles at multiple stages of germ cell development, including SOX8 (Portnoi *et al.*, 2018), SOX9 (Vining *et al.*, 2021) and SOX17 (Irie *et al.*, 2015; Sybirna *et al.*, 2019). DSD are most commonly associated with mutations in *SRY* gene or malfunction of the SRY protein (McElreavy *et al.*, 1992). For example, mutations in the *SRY* gene are the cause of 15–20% of cases of Swyer syndrome, which is characterized by failure in the development of the sex glands (Baxter and Vilain, 2013; Arboleda *et al.*, 2014). Mutations within the DNA-binding HMG-domain of SRY often lead to gonadal dysgenesis (McElreavey and Fellous, 1999).

SOX9 is a direct target of SRY and is essential for Sertoli cell development in testis formation. Copy number variants or mutation in non-coding regulatory regions upstream of the SOX9 gene lead to human sex reversal, including XY male to female DSD and XX female to male (Vetro *et al.*, 2015; Gonen *et al.*, 2018; Croft *et al.*, 2018a,b).

SOX3 (SRY-related HMG box-containing gene 3) is a gene closely related to SRY and SOX9. Loss-of-function mutations of SOX3 gene are linked with mental retardation and growth hormone deficiency (Raymond *et al.*, 1999; Laumonnier *et al.*, 2002). *De novo* duplication of SOX3 gene or its upstream regulatory region has been reported in DSD 46, XX male sex reversal (Sutton *et al.*, 2011; Moalem *et al.*, 2012; Haines *et al.*, 2015; Vetro *et al.*, 2015; Grinspon *et al.*, 2016).

WT1 is a zinc finger transcription factor known to be associated with kidney cancer. Heterozygous mutations in the zinc finger domain of *WT1* gene cause Denys-Drash syndrome, characterized by renal failure and 46, XY gonadal dysgenesis. A mutation in a splice donor site in WT1, which results in the loss of a specific isoform of WT1, leads to Frasier syndrome, which is characterized by 46, XY gonadal dysgenesis (Pelletier *et al.*, 1991; Klamt *et al.*, 1998; Hossain and Saunders, 2001).

NR0B1/DAX1 (nuclear receptor subfamily 0, group B, member 1/DSS-AHC critical region of the X chromosome, gene1) encodes an orphan nuclear receptor. Duplication of *DAX1* has been reported to be associated with 46, XY DSD (Baumstark *et al.*, 1996; Sanlaville *et al.*, 2004).

NR5A1 is associated with a wide range of reproductive anomalies, including 46, XY gonadal dysgenesis (El-Khairi and Achermann, 2012).

GATA4 is often linked to congenital heart defects. However, a recent study identified a familial case of a heterozygous mutation in the conserved N-terminal zinc finger domain of GATA4. Three of the family members present 46, XY DSD (Lourenco *et al.*, 2011). A 35-kb deletion downstream of *GATA4* was also discovered in a 46, XY complete gonadal dysgenesis patient with no evidence of heart disease (White *et al.*, 2011).

FOG2 is suggested, by human sequencing analysis, to play roles in testis determination. Two cases of 46, XY gonadal dysgenesis, are reported to bear translocations that included the FOG2 locus on chromosome 8 (Finelli *et al.*, 2007; Tan *et al.*, 2012). Missense mutations in the FOG2 gene are also identified in two independent cases of 46, XY gonadal dysgenesis (Bashamboo *et al.*, 2014).

CBX2Presence of 46, XY gonadal dysgenesis in a girl is reported to be associated with loss-of-function mutations in the human CBX2 gene (Biason-Lauber *et al.*, 2009).

DMRT1/2 deletion of chromosome 9 (9p), which contains DMRT1 and DMRT2 genes, is associated with 46, XY DSD. It is suggested that gonadal dysgenesis may result from the combined hemizygosity of DMRT1 and DMRT2 (Raymond *et al.*, 1999; Ledig *et al.*, 2012; Buonocore *et al.*, 2019).

Transcription factor mutations associated with male infertility

Many male infertility syndromes result from large chromosomal deletions, translocations or aneuploidies, often involving the sex chromosomes. Klinefelter syndrome (karyotype: 47, XXY) is the most common chromosomal aberration, detected in up to 14% of infertile males with azoospermia. Characterization of deletions in the Y chromosome, which lead to male infertility, allowed identification of the

founding member of the *DAZ* (deleted in azoospermia) family of RNA-binding genes required for spermatogenesis (Reijo *et al.*, 1995). More recently, many genetic infertility syndromes have been associated with single-gene mutations, some of which are mentioned above in the context of their role in germ cell development. While mutations in any individual gene contribute to a small number of infertility cases, the overall importance of transcriptional regulation in the appropriate development of germ cell lineages is underscored by the number of these syndromes that are characterized by transcription factor mutations.

DMRT1 is infrequently mutated or deleted in patients with nonobstructive azoospermia (NOA) (Lopes *et al.*, 2013; Tewes *et al.*, 2014), defined as no sperm in the ejaculate owing to failure of spermatogenesis and the most severe form of male infertility.

DAX1/NR0B1 *DAX1* mutations cause X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism (Muscatelli *et al.*, 1994; Zanaria *et al.*, 1994; Jadhav *et al.*, 2011), human syndromes which are characterized by hormonal imbalances leading to azoospermia. *DAX1* mutations have also been identified in sporadic cases of NOA, with pathogenic mutations leading to impaired function of the protein (Wang *et al.*, 2018).

NR5A1 regulates a large number of steroidogenic enzymes and other genes critical for male germ cell development. Mutations in *NR5A1* are associated with several male infertility syndromes including cryptorchidism (Tannour-Louet *et al.*, 2010), which is a condition in which one or both of the testes fail to descend from the abdomen into the scrotum. Characterization of the *NR5A1* gene in infertile males found missense mutations in 1–4% of men with azoospermia to severe oligozoospermia. Oligozoospermia is characterized by low sperm count, usually defined as fewer than 15 million sperm per millilitre of semen.

SOHLH1 encodes a germ cell-specific transcription factor acting in both males and females that is required for spermatogonia differentiation, spermatocyte production and correct testis morphology in mouse models (Ballow *et al.*, 2006; Barrios *et al.*, 2012; Suzuki *et al.*, 2012; Rossi, 2013; Toyoda *et al.*, 2014), as well as oogenesis (Pangas *et al.*, 2006; Toyoda *et al.*, 2014; Shin *et al.*, 2017). Mutations that are found in a subset of patients with NOA impair the transcriptional activity of SOHLH1 (Choi *et al.*, 2010; Nakamura *et al.*, 2017), likely contributing to the defect in normal spermatogenesis in these patients.

HSF2 encodes a testis-specific transcription factor required for spermatogenesis and seminiferous tubule formation in male mice (Wang *et al.*, 2003, 2004). An investigation of *HSF2* in patients with idiopathic azoospermia identified deleterious mutations in less than 1% of patients. However, one of these mutations caused not only loss-of-function of the transcriptional activity of the protein, but also a dominant-negative effect on the wild-type allele, underscoring a precise requirement for this pathway in spermatogenesis (Mou *et al.*, 2013).

TAF4B (TATA box-binding protein-associated factor 4B) is predominantly expressed in the testis relative to other organs in the body. A non-sense mutation that results in truncated TAF4B proteins is identified as a disease locus in two unrelated consanguineous families suffering from azoospermia and oligozoospermia (Ayhan *et al.*, 2014). The truncated protein has reduced DNA binding activity and weakened interaction with TAF12, which is essential for DNA binding at the core promoters of a subset of genes (Gazit *et al.*, 2009).

ZMYND15 (zinc finger MYND-Type containing protein 15) acts as a histone deacetylase-dependent transcriptional repressor essential for spermiogenesis and male fertility. A mutation that leads to premature termination of the protein is associated with azoospermia. The truncated domain of the protein is implicated in signal transduction (Yan *et al.*, 2010).

Transcription factor mutations associated with female infertility

There is growing evidence that genetic mutations are present in as many as 10% of female infertility conditions, including ovulatory disorders (e.g. Kallmann syndrome), chromosomal abnormalities (e.g. Turner's syndrome), endometriosis, pelvic adhesions, tubal abnormalities and hyperprolactinemia. We summarize mutations in transcription factors that are associated with a small subset of female infertility conditions, including premature ovarian insufficiency (POI), also known as premature or primary ovarian failure, (characterized by a loss of ovarian function before the age of 40 years), and uterine leiomyomata, a benign smooth muscle tumor in the uterus.

FOXL2 is one of several forkhead domain-containing transcription factor genes involved in female germ cell development (Gersak *et al.*, 2004). It is expressed in ovarian follicular and stromal cells and acts as a lineage-determining regulator of ovarian differentiation. *FOXL2* was first identified as containing the causative mutation in blepharophimosis, ptosis and epicanthus inversus syndrome, a facial development syndrome characterized by POI (Crisponi *et al.*, 2001). Subsequently, *FOXL2* mutations have been identified in other female infertility syndromes, including sporadic cases of POI (Harris *et al.*, 2002; De Baere *et al.*, 2005; Nallathambi *et al.*, 2007).

FIGLA is a female-specific transcription factor that acts early in oocyte development to initiate the expression of key genes required for folliculogenesis (Li *et al.*, 2017; Wagner *et al.*, 2020; Ye *et al.*, 2020). *FIGLA* is a germ cell-specific basic helix-loop-helix transcription factor required for follicle formation in mice (Soyal *et al.*, 2000; Hu *et al.*, 2010). Studies of women with POIhave identified mutations in *FIGLA*, which disrupt its interaction with transcriptional co-regulators (Zhao *et al.*, 2008; Bouilly *et al.*, 2016).

NOBOX is a homeodomain-containing transcription factor which has also been shown to be required for folliculogenesis and oocyte-specific gene expression in mouse models (Rajkovic *et al.*, 2004). Mutations of *NOBOX* have been found in up to 6% of sporadic cases of POI in women. The resulting amino acid substitutions in the homeodomain or transactivation domain lead to impaired transcriptional activity (Qin *et al.*, 2007; Bouilly *et al.*, 2016).

NR5A1 is essential for both male and female germ cell development. Mutations in *NR5A1* are associated with POI (Philibert *et al.*, 2010).

SALL4 (SAL-like 4) encodes a putative zinc finger transcription factor that plays an important role in the maintenance of pluripotent stem cells and the development of oocytes. A genetic study focused on Chinese women with non-syndromic POIhas identified two

probable gene mutations associated with the occurrence of POI (Wang *et al.*, 2009).

FOXO1A/3A (forkhead box o1A/3A) is expressed in the ovary and thought to play roles in ovarian development. Causal mutations were identified in POI patients, although the pathological role is yet undetermined (Watkins *et al.*, 2006).

MED12 (medicator complex subunit 12) is a well-known causal gene for uterine leiomyomas. Approximately 60% of patients with uterine leiomyomas have somatic MED12 mutations in some form, including missense, insertion and deletion. Most of the mutations are localized to exon 2 of the MED12 gene, suggesting that this domain is the major functional domain contributing to the genesis of uterine leiomyomas (Halder *et al.*, 2015; Heinonen *et al.*, 2017; Ajabnoor *et al.*, 2018).

Transcription factors as diagnostic markers for germ cell tumors

Human germ cell tumors (GCTs) are neoplasms presenting in the gonads, primarily in the testes. The transcriptome of GCTs is highly similar to authentic FGCs; thus, GCT cell lines are frequently used as a model to study the function of FGCs (Irie *et al.*, 2015). GCTs can be broadly categorized into seminoma and non-seminomatous GCTs (Oosterhuis and Looijenga, 2005; Vasdev *et al.*, 2013). Seminoma GCTs grow and spread more slowly and are sensitive to chemotherapy and/or radiation therapy. Non-seminomatous GCTs are divided into four subtypes: embryonal carcinoma, yolk sac carcinoma, choriocarcinoma and teratoma. Compared with seminoma, non-seminomatous GCTs are very variable in phenotype and prognosis. Non-seminomatous GCTs tend to grow faster, have an earlier mean age at the time of diagnosis, and have a lower 5-year survival rate (Litchfield *et al.*, 2016; Costa *et al.*, 2017; Shen *et al.*, 2018). Identification of molecular signatures to differentiate subtypes of GCTs is therefore crucial for determining prognostication and subtype-based selection of treatment. Thus, a number of studies have been conducted to identify signature genes for each subtype, and transcription factors are promising to be useful as distinct biomarkers for different categories of GCTs (Alagaratnam *et al.*, 2011; Litchfield *et al.*, 2017).

GCTs are thought to originate from FGCs since pluripotency transcription factors are highly expressed in the precursor lesion of GCTs. Master transcription factors for pluripotency, namely OCT4, NANOG, SOX2 and LIN28 (Lin-28 homolog A), are key markers of certain types of GCTs, implicating their roles in maintenance of these malignant cells in the growth of this tumor (Skakkebaek, 1972, 2002; Looijenga *et al.*, 2003; Cheng *et al.*, 2004; Hart *et al.*, 2005; Hoei-Hansen *et al.*, 2005; Cheng *et al.*, 2007; West *et al.*, 2009; Gillis *et al.*, 2011). Clinically, these pluripotency factors are emerging as diagnostic markers for both testicular and ovarian GCTs (Gillis *et al.*, 2011). Immunohistochemistry studies in primary samples have suggested OCT4 and NANOG as sensitive and specific markers for identifying GCTs (Jones *et al.*, 2004; de Jong *et al.*, 2005; Richie, 2005; de Jong and Looijenga, 2006; Jung *et al.*, 2006). However, these two transcription factors alone do not provide the specificity necessary to distinguish between seminomatous and non-seminomatous tumors (Ulbright and Young, 2005). Recent gene expression profiling and immunohistochemistry analyses have suggested that the combination of expression patterns of multiple transcription factors may serve as a feature to differentiate seminomatous and subtypes of non-seminomatous GCTs (Santagata *et al.*, 2007). For example, seminomas are found to be positive for OCT4 and NANOG and negative for SOX2, whereas embryonal carcinomas are positive for all three pluripotency markers. Besides pluripotency transcription factors, other crucial transcriptional regulators of FGC development are also indicated as diagnostic markers for GCTs. For example, the expression pattern of SOX17, a critical regulator of hPGC specification, can also distinguish seminoma from embryonal carcinoma when combined with SOX2 (Nonaka, 2009). Immunohistochemistry of TFAP2C, another essential transcription factor for germ cell development, has also been evaluated for the diagnosis of multiple subtypes of GCTs (Pauls *et al.*, 2005).

Core transcriptional network for hPGC specification identified by *in vitro* differentiation

Although both mutations linked to infertility and gene expression in various stages of human germ cell development contribute to identification of genes that act at specific stages of development, functional analysis is necessary to validate their developmental roles and pinpoint underlying mechanisms. Recent developments in stem cell biology and gene editing provide an opportunity to recapitulate human germ cell development *in vitro* and to functionally dissect genetic requirements. A key step in developing *in vitro* gametogenesis is identifying and characterizing genetic determinants in a robust model for germ cell specification. While it is clear that *in vitro*-derived germ cells lack important characteristics of authentic FGCs (notably, the ability to efficiently develop into gametes *in vitro*), *in vitro* gametogenesis provides a viable system to explore the core transcriptional machinery for germ cell specification.

Strategies to recapitulate human germ cell development *in vitro*

Numerous studies have contributed to protocols for directing germ cell differentiation from hPSCs, starting from both hESCs and human induced pluripotent stem cells (hiPSCs). Currently, human primordial germ cell-like cells (hPGCLCs) can be induced using rationally designed cocktails of growth factors and small molecules that have emerged over the years (Kee *et al.*, 2006, 2009; Easley *et al.*, 2012; Irie *et al.*, 2015; Sasaki *et al.*, 2015; Sugawa *et al.*, 2015; Jung *et al.*, 2017; Yamashiro *et al.*, 2018; Murase *et al.*, 2020), or induced by ectopic expression of genes associated with germ cell development, especially transcription factors (Kee *et al.*, 2009; Qiu *et al.*, 2013; Yu *et al.*, 2014; Irie *et al.*, 2015; Medrano *et al.*, 2016; Panula *et al.*, 2016; Jung *et al.*,

2017; Fang et al., 2018; Kojima et al., 2021). To induce maturation and more advanced differentiation *in vitro*, hPGCLCs are often co-cultured with somatic cells (Park et al., 2009; Lin et al., 2014; Yamashiro et al., 2018). In addition, xenotransplantation assays have been extensively applied to promote *in vivo* maturation of *in vitro*-derived germ cells. Two recent review articles have summarized progress and strategies of *in vitro* gametogenesis (Li et al., 2020; Saitou and Hayashi, 2021).

Identification of transcription factors involved in hPGCLC specification by genetic studies

Delineation of the conditions and factors required to promote *in vitro* PGC differentiation have set the stage for genetic studies that can probe the function and hierarchies of transcription factors during hPGC specification (Fig. 2). Several transcription factors which are usually involved in lineage specification during embryogenesis have been reported to be repurposed in PGCs to form a specific transcriptional network that may act to safeguard human germ cell fate by maintaining pluripotent status while repressing differentiation. In response to WNT (Kojima et al., 2017) and ACTIVIN signals, the mesoderm specifier *EOMES* activates *SOX17*, an endoderm specifier, which in turn upregulates *PRDM1*. Deletion of *EOMES* in hPSCs significantly impacts their competence toward hPGCLC differentiation (Chen et al., 2017; Kojima et al., 2017). *SOX17* can also be induced directly by BMP signaling to activate germ cell programs: conversely, *SOX17*-null hESCs cannot undergo hPGCLC specification (Irie et al., 2015; Tang et al., 2015). The trophoblast marker *GATA3* is an immediate effector of the BMP pathway and regulates *SOX17* and *TFAP2C*. Accordingly, *GATA3* null mutations significantly decreased hPGCLC induction efficiency in response to BMP signals (Kojima et al., 2021). *PRDM1* is a transcriptional repressor that acts as one of the key signature genes for germ cell fate (Ohinata et al., 2005; Irie et al., 2015; Kobayashi et al., 2017). *PRDM1* function is tightly controlled by

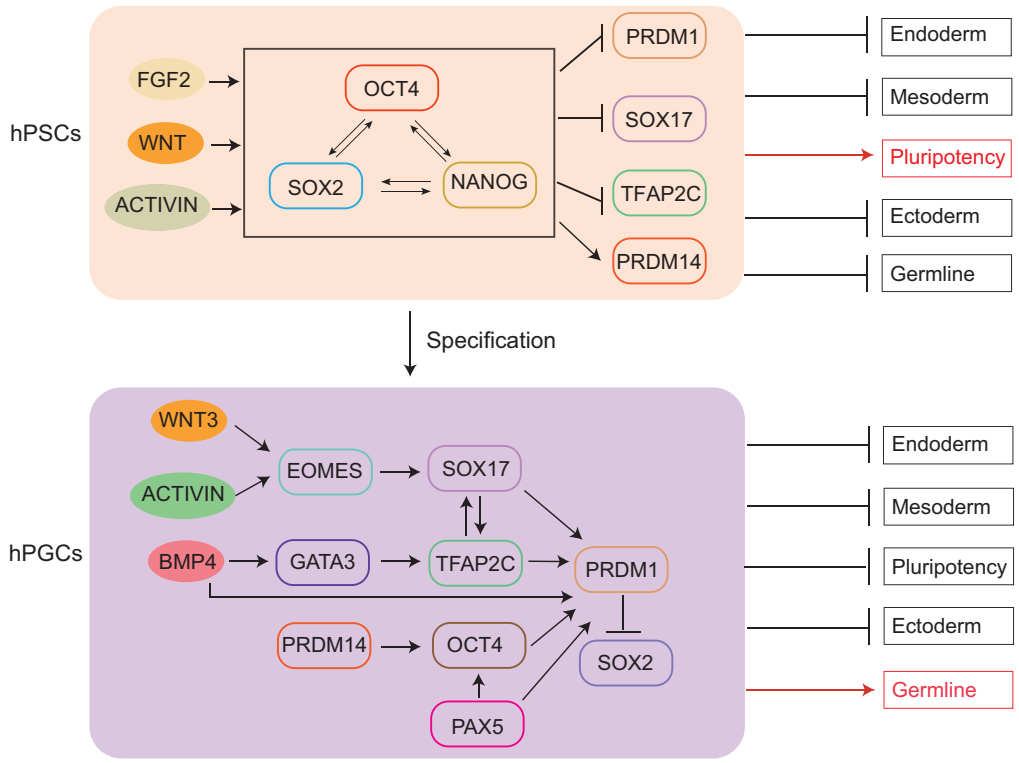

Figure 2. The core transcriptional network in human pluripotent stem cells and primordial germ cells. Arrows with pointed tips represent activation, and arrows with vertical line tips represent inhibition. In pluripotent stem cells, FGF2, WNT and ACTIVIN signaling pathways are essential to activate the gene expression program for pluripotency. In response to the signals, OCT4, SOX2 and NANOG are activated and form a core transcriptional network that suppresses the somatic and germline gene expression program. Once human pluripotent stem cells (hPSCs) start to differentiate toward germline, WNT3, ACTIVIN and BMP4 signals activate EOMES and GATA3, which then activate the expression of a few transcription factors essential for germ cell development, including SOX17, TFPAP2C and PRDM1. Moderate expression of pluripotency transcription factor OCT4 is also critical for human germ cell development. Upon differentiation, the expression of OCT4 is gradually reduced, and the expression of its functional partner in hPSCs, SOX2, is diminished. Instead, OCT4 partners with PAX5 in human primordial germ cells (hPGCs) to activate the expression of PRDM1.

multiple transcription factors to repress somatic differentiation during the process of hPGCLC specification; in *PRDM1*-deficient or *PRDM1*-knockdown cells, germline differentiation potential is significantly impaired, and somatic lineage genes are de-repressed (Lin *et al.*, 2014; Sasaki *et al.*, 2015). Part of the role of PRDM1 protein is to suppress *SOX2* expression and consequently inhibit neuronal differentiation directly. A study that examined hPGCLC specification in *TFAP2C*$^{-/-}$ cells found that *TFAP2C* acts upstream of *PRDM1* and plays a dominant role in repressing somatic programs in hPGCLCs (Kojima *et al.*, 2017). Another study used single-cell sequencing in *TFAP2C*$^{-/-}$ cells during hPGCLC specification and confirmed that *TFAP2C* functions upstream of both *PRDM1* and *SOX17*, acting to prevent cells from adopting somatic fates and thus safeguard germ cell fate (Chen *et al.*, 2019).

In addition to transcription factors that act in specifying lineages in development, pluripotency transcription factors are important in germ cell development. One of the unique features of hPGCs compared with other cell types of the body during development is that they share with hPSCs the expression of several pluripotency genes, including a pluripotency master regulator *OCT4/POU5F1*. In both mouse and human embryo development, *OCT4* is initially expressed in all blastomeres of the embryo; subsequently, expression is restricted to the pluripotent stem cells of the inner cell mass. During gastrulation *OCT4* level is maintained in epiblast cells and after gastrulation *OCT4* expression is confined exclusively to germ cells (Scholer *et al.*, 1990; Scholer, 1991; Yeom *et al.*, 1996; Nichols *et al.*, 1998; Pesce and Scholer, 2001). Mouse embryos depleted of *OCT4* fail to form an inner cell mass and the cells are committed to the trophoblast lineage (Nichols *et al.*, 1998); However, conditional knock out of *OCT4* in mouse PGCs leads to apoptosis of PGCs rather than cell fate change to the trophectodermal lineage (Kehler *et al.*, 2004), suggesting that *OCT4* is playing distinct roles in these two distinct cell types. To dissect the roles of OCT4 in hPSCs and hPGCs, Fang *et al.* (2018) compared genome-wide binding of OCT4 in hPSCs and hPGCs (the latter from human fetal testis samples). They discovered that OCT4 repressed neuronal differentiation in both hPSCs and hPGCs, while it regulated a unique set of genes during germ cell differentiation by switching partners from SOX2 to PAX5 (paired box 5). In hPSCs, OCT4 and SOX2 interact and form a protein complex to cooperatively bind and regulate target genes in order to activate or maintain pluripotency (Herr and Cleary, 1995; Nichols *et al.*, 1998; Wegner, 1999; Niwa *et al.*, 2000; Avilion *et al.*, 2003). As hPSCs begin to differentiate toward a germline fate, the expression of SOX2 is diminished and OCT4 switches functional partners to PAX5 as germ cells are specified. The PAX5-OCT4 complex functions in activation of *PRDM1* expression and other genes implicated in PGC specification. *PAX5* null mutations have significantly reduced *PRDM1* expression and impaired germ cell potential in hPSC xenotransplants *in vivo*. Hence, the PAX5-OCT4-PRDM1 proteins function as a genetic switch in the transition from a pluripotent state to germline (Fang *et al.*, 2018). *PRDM14*, another human pluripotency gene, is also critical for the acquisition and maintenance of the hPGCLC-competent state (Sybirna *et al.*, 2020); it functions to activate *OCT4* expression and to upregulate *PRDM1* (Chia *et al.*, 2010). Loss of *PRDM14* function results in significantly reduced efficiency of *in vitro* differentiation and an aberrant transcriptome of the resultant hPGCLCs (Sybirna *et al.*, 2020).

Overexpression of *SOX17* leads to the generation of hPGCLCs without BMP induction, suggesting that SOX17 is at the top of the transcriptional hierarchy for hPGCLC specification and is sufficient for human germ cell fate acquisition (Irie *et al.*, 2015). Although a major role of SOX17 is to activate *TFAP2C* and *PRDM1*, forced expression of *TFAP2C* could not generate hPGCLCs, even in conjunction with *PRDM1* overexpression (Kobayashi *et al.*, 2017). These results suggest that while *TFAP2C* is indispensable for hPGCLC specification, it is insufficient on its own for germline induction. Recent work reported that the GATA family of transcription factors (*GATA2/3*), combined with *SOX17* and *TFAP2C*, act as a minimum requirement to replace BMP signaling and confer germ cell fate on incipient mesoderm-like cells (iMeLCs) (Kojima *et al.*, 2021). In total, these genetic studies of hPGCs *in vitro* begin to allow the construction of a network of transcription factors that are involved in hPGC specification and maturation (Fig. 2).

Murine and human germ cells are characterized by evolutionarily distinct transcriptional networks

Prior to recent advances in stem cell biology and sequencing technologies, our understanding of germ cell development relied almost solely on animal models. However, germline commitment occurs within a limited window of embryo development, when the morphology of embryos and the timing of germ line specification diverges significantly between different species, including mice and humans (Sybirna *et al.*, 2019) (Fig. 3). Notably, mouse embryos develop as an egg cylinder, and mouse PGCs (mPGCs) are clustered in the proximal epiblast around the time of primitive streak formation (Tam and Behringer, 1997; Anderson *et al.*, 2000; McLaren, 2003). Human embryos present as a bilaminar disc and, based on the studies in non-human primates, hPGCs probably arise prior to primitive streak formation from the dorsal amnion, which is physically separate from the posterior epiblast (Behringer *et al.*, 2000; Rossant, 2015; Sasaki *et al.*, 2016; Kobayashi *et al.*, 2017). Given such major developmental differences in terms of timing, shape and cell origin, it is not surprising that the intrinsic transcriptional network required for PGC emergence also has divergent components and functions. In mPGCs, the Prdm1, Tfap2c and Prdm14 proteins constitute a core transcriptional network that is essential for PGC specification *in vivo* (Ohinata *et al.*, 2005; Yamaji *et al.*, 2008; Weber *et al.*, 2010) and sufficient to induce germ cell fate *in vitro* (Magnúsdóttir *et al.*, 2013; Nakaki *et al.*, 2013). In contrast, the expression of *PRDM14* in humans is strongly downregulated from hPSCs to hPGCs (Sugawa *et al.*, 2015), and *SOX17* has instead emerged as the critical determinant for hPGC specification (Irie *et al.*, 2015). Pluripotency genes *OCT4* and *NANOG* are re-expressed in both mPGCs (Murakami *et al.*, 2016) and hPGCs (Guo *et al.*, 2015; Tang *et al.*, 2015), and *SOX2* is absent in hPGCs (Lin *et al.*, 2014) although

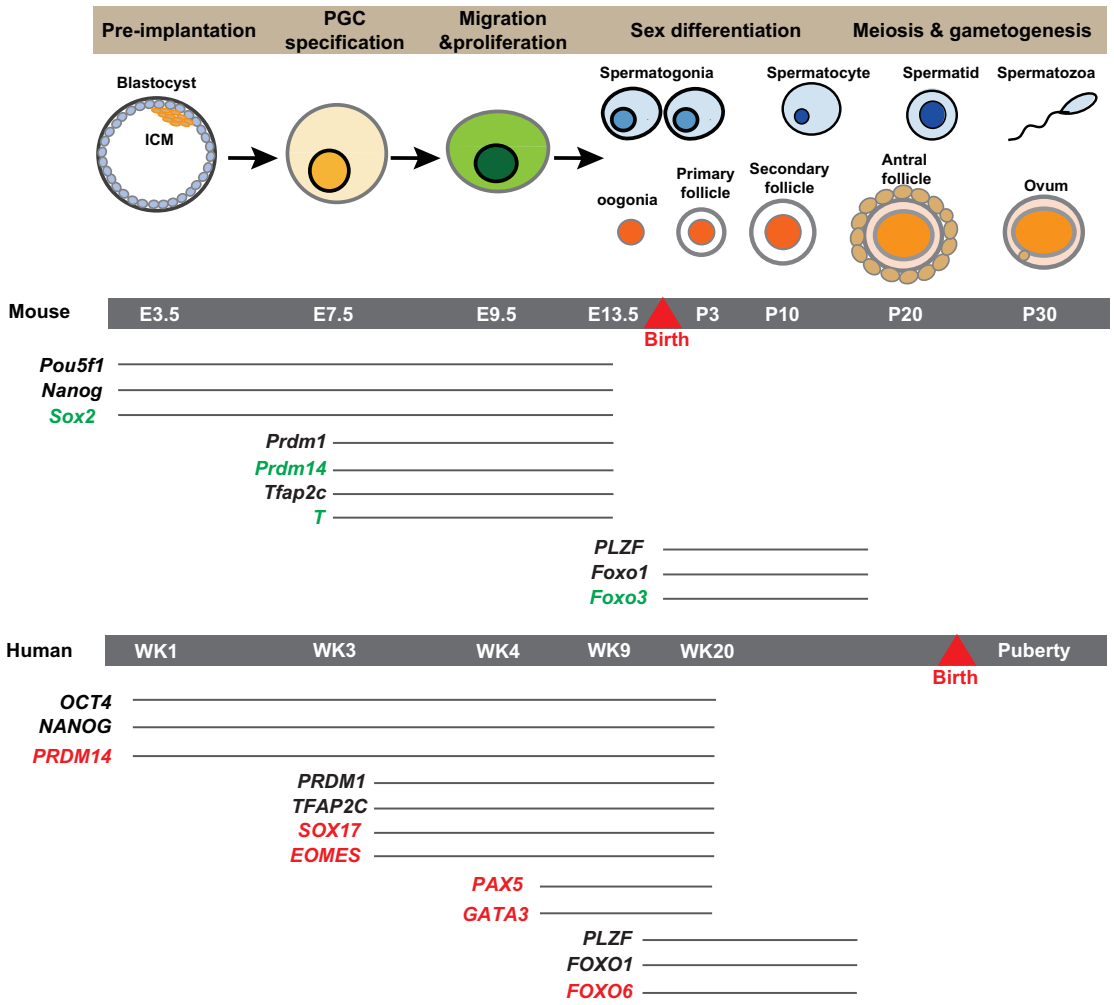

Figure 3. Differences in transcription factor expression between murine and human germ cells. E, embryonic day; ICM, inner cell mass; WK, week. Green marks the genes specifically expressed in mouse germ cell development, and red marks the genes specifically expressed in human germ cell development.

it is required for mouse PGC survival and proliferation (Campolo *et al.*, 2013). In addition, the mesoderm specifier gene *T/Brachyury* is essential for robust activation of *Prdm1* and *Prdm14* in mPGCs (Aramaki *et al.*, 2013) but this role is replaced by another mesoderm gene, *EOMES*, in hPGCs (Chen *et al.*, 2017; Kojima *et al.*, 2017). The co-expression of pluripotency genes and lineage specifier genes persists to the sex determination stage when PGCs differentiate to spermatogonia and oogonia in both mice and humans.

The expression of pluripotency genes also differs during germ cell development in mice and humans. In male mice, expression of *OCT4* persists as cell fate transits from PGCs to the undifferentiated SPG stage, and expression is downregulated once cells enter meiosis (Pesce *et al.*, 1998; Tadokoro *et al.*, 2002). However, *OCT4* expression, specifically isoform *OCT4A* (translated from transcript variant 1), is more restricted during male development in humans, being confined to hPSCs and hPGCs. In female mice, the expression of *OCT4* is downregulated by the onset of meiotic prophase and then re-activated after birth in oocytes within primary follicles and at the onset of folliculogenesis (Pesce *et al.*, 1998; Anderson *et al.*, 2007). In humans, the number of *OCT4* positive cells peaks by gestational week 8 and diminishes after week 9, as oogonia enter meiosis (Kerr *et al.*, 2008). *NANOG* has a similar expression pattern to *OCT4* (Hoei-Hansen *et al.*, 2005). Once FGCs arrive at the gonads and progress toward meiosis, pluripotency-related transcription factors undergo significant downregulation and are diminished in all adult reproductive tissues.

The FOXO subclass of the forkhead box transcription factors are key regulators of mouse reproduction (Brosens *et al.*, 2009). *FOXO3* is required to suppress primordial follicle activation in females as *FOXO3*-null female mice display age-dependent infertility (Castrillon *et al.*, 2003; Hosaka *et al.*, 2004), and *FOXO1* is essential for SSCs

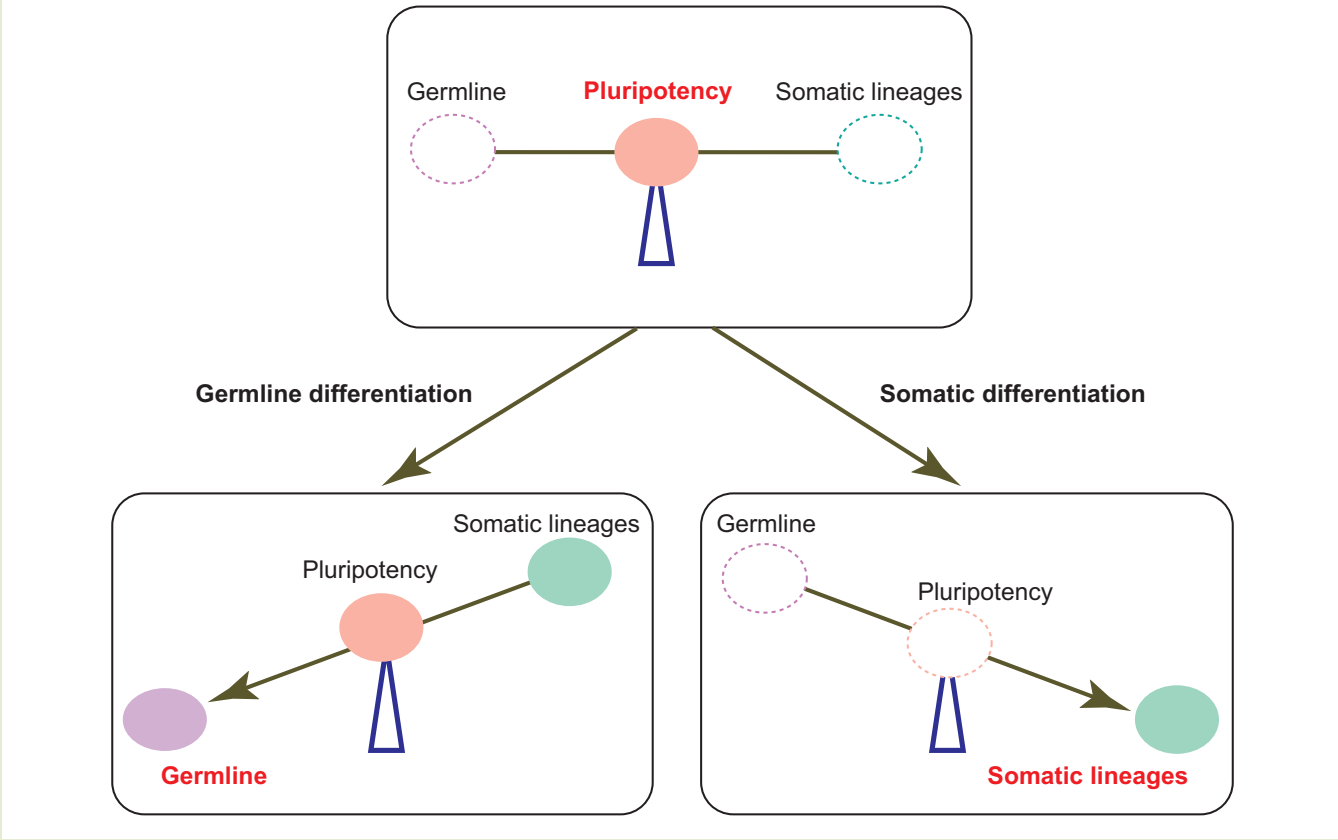

Figure 4. Proposed model for intrinsic transcription forces that drive human germline and somatic differentiation. Solid ovals represent gene expression programs that are activated, and dotted ovals represent gene expression programs that are silenced.

maintenance and the initiation of spermatogenesis in males (Goertz *et al.*, 2011). While *FOXO1* expression in spermatogonia and granulosa cells is conserved between humans and mice (Richards *et al.*, 2002; Liu *et al.*, 2013), *FOXO3* is not expressed in primordial oocytes in humans (Tarnawa *et al.*, 2013), suggesting that other members of the FOXO transcription factor family may replace its function. *FOXO6*, which has been identified as an upregulated gene in human oocytes by scRNA-seq, may be a potential candidate for this substitution of *FOXO3* function.

A unique transcriptional network defines human germ cells

The maintenance of cell identity in FGCs requires the repression of somatic lineages in concert with the activation of germ cell programs (Figs 2 and 4). Of note, human FGCs are defined by a unique transcriptional network that comprises germ cell-specific genes together with somatic lineage specifiers and pluripotency genes. How these transcription factors, which are master regulators for various cell types, function differently from their canonical roles in driving germ cell fate is an intriguing and fundamental question in the field of human germ cell developmental genetics. One hypothesis is that transcription factors work in different protein complexes to perform cell-type-specific roles. In support of this, Fang *et al.* (2018) observed that OCT4 switches partners from SOX2 in hPSCs to PAX5 and PRDM1 proteins in human FGCs (Fang *et al.*, 2018). While continuing to repress differentiation toward ectoderm, importantly in human FGCs, OCT4 shifts its binding from pluripotency-related genes to germline-specific genes to activate germ cell fate, coincident with the switch in activity of the respective OCT4 complexes.

An alternate but not necessarily mutually exclusive hypothesis is that the delicate balance of different transcription factors defines germ cells. Lineage specifiers that belong to the three germ layers and trophectoderm (SOX17, EOMES, PAX5 and GATAs), as well as pluripotency proteins (OCT4, NANOG), are all expressed in human FGCs and function as drivers of the germ cell lineage, as demonstrated by diverse functional studies. Thus, these master regulators of differentiation and pluripotency must be regulated to modulate their canonical functions (Fig. 4). For example, while the *SOX17* gene encodes a classical endoderm specifier and plays a critical role in normal hPGC specification, over-expression of *SOX17* beyond a PGC-competent window favors

the expression of endoderm genes rather than direction toward a germ cell fate (Kobayashi et al., 2017). In response to hPGC specification, the dosage and actions of these transcription factors must be balanced so that cell identity extends beyond pluripotency but is not co-opted toward any specific somatic lineages. It is likely that other germ cell determinants are activated to inhibit further somatic lineage differentiation and reinforce commitment of cells to the germline. For example, as a transcriptional repressor, PRDM1 activity in hPGCs represses expression of somatic lineage genes (Irie et al., 2015; Sasaki et al., 2015). In support of this, a recent study demonstrated that high-dosage overexpression of *SOX17* in hPSCs leads to aberrant expression of endoderm markers, which could be rescued by simultaneously providing a comparable dose of PRDM1 protein (Kobayashi et al., 2017). Given the importance of *PRDM1* in hPGCs, its activation may be safeguarded by multiple transcription factors to assure its appropriate expression to maintain germ cell identity.

The gap between hPGCLCs and *bona fide* hPGCs and beyond

Despite advances in our knowledge of transcription factor function in PGC specification, *in vitro*-derived hPGCLCs do not progress further down the germ cell lineage efficiently and do not readily enter or complete meiosis to produce functional germ cells. Accordingly, gene expression analysis of *in vivo* hPGCs from developing human embryos has revealed clear differences with hPGCLCs (Table III) (Gkountela et al., 2013, 2015; Tang et al., 2015; Chen et al., 2018; Sybirna et al., 2020). Most notably, late-stage hPGC markers, such as *DAZL* (deleted in azoospermia), *VASA/DDX4* (DEAD-box helicase 4), and *PIWIL1* (Piwi like RNA-mediated gene silencing 1), are not activated in hPGCLC models (Irie et al., 2015) suggesting a lack of activation of necessary transcription factors to induce the transcriptional program of later germ cell stages *in vitro*. Indeed, a time-course analysis of early- versus late-gestation cyPGCs found that the gene expression signature of hPGCLCs is more similar to early-stage PGCs than later-gestation PGCs, which have been most commonly profiled from human samples (Sasaki et al., 2016). Several transcription factors enriched in expression in 'late' cyPGCs, such as *RNF17* (ring finger protein 17) and *KRBOX1* (KRAB Box domain containing 1), lack expression in hPGCLCs, and are known to function in late-stage germ cell development.

The wealth of expression data now available from hPGCs *in vivo*, as well as different models of hPGCLC generation, might enable the unbiased identification of new transcription factors that may drive hPGC specification beyond the current state achievable *in vitro* (Gkountela et al., 2015; Chen et al., 2018). We have identified transcription factors whose expression is induced in hPGCs or hPGCLCs and found several notable transcription factor classes present *in vivo* that are not activated *in vitro* (Table III). For example, DMRT-family transcription factors are known to play a role in sex determination in many organisms, and both DMRT1 and DMRTC2 are found in hPGCs (Guo et al., 2015), but are not expressed in hPGCLCs. Consistent with their well-known role in embryonic development, many homeobox-containing genes, including both classic Hox cluster transcription factors as well as other homeodomain-containing transcription factors, such as ALX4 (ALX homeobox 4), EMX1/2 (empty spiracles homeobox 1/2), ESX1 (ESX homeobox 1), SIX1/2 (Sineoculis homeobox homolog 1/2) and LHX2/8 (LIM-homeodomain protein 2/8), are present in hPGCs but not hPGCLCs. This enrichment of homeodomain transcription factors is somewhat surprising given that repression of homeobox genes is a well-known feature of developing PGCs, so this may reflect contamination from somatic cells. Finally, some members of other transcription factor classes, such as PAX and SOX-domain transcription factors, are active in *in vivo* but not *in vitro* models of PGCs. Different gene expression profiles could be linked to different developmental timing (or different development trajectories between *in vitro* differentiation or *in vivo* development) or aberrant gene expression. It is likely that future efforts to derive germ cell-like cells *in vitro* will require the expression or activation of transcriptional programs of one or more of these transcription factors, either by exogenous gene introduction or identification of upstream signaling pathways, which can be activated by the addition of extracellular factors.

In addition to identification of stage-specific gene expression profiles, it is equally important to compare the epigenetic status of *in vitro* derived germ cells with that of *bona fide* germ cells. Global erasure of DNA methylation, with the exception of some repetitive elements, is the hallmark of *in vivo* hPGC development (Leitch et al., 2013; Irie et al., 2015). Several transcription factors are involved in the epigenetic modeling of hPGCs. PRDM1 and SOX17 function in maintaining the epigenetic program of 5-methylcytosine (5mC) erasure from week 4 to week 9. Partial erasure of 5mC and enrichment of 5hmC is also observed in the *in vitro* differentiation system. In hPGCLCs, loss of PRDM1 inhibits the initiation of DNA demethylation while the expression of SOX17 activates PRDM1, which then sustains the epigenetic program toward global 5mC erasure (Tang et al., 2015). Comparisons of the epigenome of *in vitro* derived hPGCLCs to that *in vivo* provides instruction on the progress of generation of germ cells.

Studies incorporating xenotransplantation

Previous research in multiple different stem-cell based experimental systems has demonstrated that cell and tissue transplantation is the gold standard for testing cell identity and function (Hanna et al., 2007; Nelson et al., 2009; Weissman, 2012; Takahashi and Yamanaka, 2013). As noted in a recent review, transplantation may provide the first ability to demonstrate the feasibility of generating PGCLC-derived, fully mature gametes in primates, including humans (Saitou and Hayashi, 2021). Three types of studies have been reported with different transplantation strategies and are summarized below.

In the first studies, whole slices of testicular tissue were transplanted from non-human primates (*Macaca fascicularis*) to a subcutaneous region of the back or side of male nude mice (Liu et al., 2016). The results indicated that xenotransplanted testis tissue from young mammals, including mice and monkeys, is capable of undergoing full and complete spermatogenesis in xenografts. Moreover, monkey xenografts to nude mice were capable of generating sperm capable of

Table III Transcription factors reported to be expressed in *bona fide* hPGCs and *in vitro* derived hPGCLCs.

Bona fide hPGCs			*In vitro* derived hPGCLCs			Functions in reproduction based on Mouse Genome Informatics (MGI)
Gkountela *et al.* (2013)	Gkountela *et al.* (2015)	Chen *et al.* (2018)	Sasaki *et al.* (2015)	Chen *et al.* (2018)	Sybirna *et al.* (2020)	
ALX4	ALX4	ALX4			ALX4	Male sterility
BNC1	BNC1	BNC1			BNC1	Required for testis development
		CDX1	CDX1	CDX1	CDX1	
		DLX5		DLX5	DLX5	
DMRT1	DMRT1	DMRT1			DMRT1	Male sterility, disorganized seminiferous tubules
DMRTC2		DMRTC2				Male sterility
EMX2	EMX2	EMX2				Bipotential gonad marker
ESX1	ESX1	ESX1				Role in spermatogenesis
			GATA2/3/4	GATA2/3/4	GATA2/3/4	
	HOXA2	HOXA2				
HOXA3/4/5/7/9	HOXA3/4/5/7/9	HOXA3/4/5/7/9				
	HOXB3/4/5	HOXB3/4/5				
HOXB7		HOXB7				
HOXC4	HOXC4	HOXC4				
HOXC9		HOXC9				
HOXD3		HOXD3				
HOXD9	HOXD9	HOXD9				
			IRX1	IRX1	IRX1	
	IRX4	IRX4				
			IRX6	IRX6	IRX6	
KLF2	KLF2					
			KLF4	KLF4	KLF4	
			LHX1		LHX1	
LHX2		LHX2				
			MSX2	MSX2	MSX2	
NR2F2	NR2F2		NR2F2			
	OSR2	OSR2				
PAX5		PAX5				PGCs development
PAX8	PAX8	PAX8				Male infertility
RNF17	RNF17	RNF17			RNF17	Testis specific; regulates piRNA maturation
			RUNX3	RUNX3	RUNX3	
SIX1		SIX1				
			SOX15	SOX15	SOX15	
			SOX17	SOX17	SOX17	
			T	T	T	
TBX2		TBX2				
		TBX3	TBX3	TBX3	TBX3	
TBX5		TBX5				
			TCL1A	TCL1A		
			TFAP2C	TFAP2C	TFAP2C	

Continued

Table III Continued

Bona fide hPGCs			*In vitro* derived hPGCLCs			Functions in reproduction based on Mouse Genome Informatics (MGI)
Gkountela et al. (2013)	Gkountela et al. (2015)	Chen et al. (2018)	Sasaki et al. (2015)	Chen et al. (2018)	Sybirna et al. (2020)	
	TFCP2L1	TFCP2L1	TFCP2L1	TFCP2L1	TFCP2L1	
TLX2		TLX2				
ZEB1	ZEB1					

PiRNA, Piwi-interacting RNA.

producing monkey offspring when sperm derived from the xenografts testis tissues from juvenile wild-type (WT) and transgenic cynomolgus monkeys (*M. fascicularis*) were used for assisted reproduction. These results may inform future strategies for *in vitro* gametogenesis.

Second, in an elegant set of proof-of-concept experiments, *Rhesus macaque* testicular tissue that was cryopreserved as an experimental validation of fertility preservation for prepubertal human patients was used in autologous transplantation (Fayomi *et al.*, 2019). Prepubertal testicular tissue that had been cryopreserved was autologously grafted under the back skin and scrotal skin of castrated pubertal *R. macaque* monkeys. Results indicated that mature sperm were produced that were capable of fertilizing *R. macaque* oocytes and culminating in live birth. This suggests that in non-human primates there is the capability for full gametogenesis, from spermatogonia to mature sperm, through autologous grafting.

Third, in studies reported in several manuscripts, the ability of germ cells to engraft was used to assess validity and quantitative and qualitative aspects of *in vitro* gametogenesis. In these studies, the ability of hPGCLCs to engraft in mouse seminiferous tubules following differentiation *in vitro* was tested (Dominguez *et al.*, 2014; Durruthy-Durruthy *et al.*, 2014; Ramathal *et al.*, 2014; Medrano *et al.*, 2016; Fang *et al.*, 2018). Results demonstrated that the ability to differentiate and produce engraftable germ cells was dependent on the genetic composition of the parental iPSC lines used to produce PGCLCs. Thus, PGCLCs differentiated from iPSC lines with Y chromosome deletions that are linked to poor or no spermatogenesis were shown to have limited engraftment relative to PGCLCs without genetic abnormalities derived from fertile men; similar results were also observed with PGCLCs produced from lines with numerical sex chromosome abnormalities (Dominguez *et al.*, 2014; Durruthy-Durruthy *et al.*, 2014; Ramathal *et al.*, 2014). In contrast, over-expression of key germ cell-specific genes was associated with increased engraftment (Medrano *et al.*, 2016; Panula *et al.*, 2016; Fang *et al.*, 2018). Furthermore, these studies documented the migration of PGCLCs to the basement membrane of spermatogenic tubules and expression of spermatogonial markers. As expected, however, complete spermatogenesis was not observed owing to the evolutionary distance between mice and humans. Instead, as reported previously, in transplantation with human SSC the germ cells migrate to the seminiferous tubule basement membrane and proliferate to form chains and patches of spermatogonia that persist in the long term but do not appear to initiate or complete meiosis (Nagano *et al.*, 2002; Hermann *et al.*, 2010; Dovey *et al.*, 2013).

Conclusion and future perspectives

The identification and functional characterization of transcription factors implicated in human germ cell development may not only increase our understanding of the fundamental genetic basis of pluripotency and heredity, but also may help to dissect the causative mechanisms of germline diseases and provide potential strategies for treatment (Fig. 5). With recent advances in stem cell biology and genetic and epigenetic profiling, there is the ability to compare and contrast data across different species, including human-specific aspects of germ cell development. Transcription factors that are specifically expressed or upregulated at critical developmental stages have the potential to act as master regulators of germ cell development. In addition to methods such as scRNA-seq that provide cell type-specific transcriptome information, rapid development of other single-cell 'multi-omics' technologies will integrate various components of genomic and epigenomic information, including DNA methylation, histone modification, chromatin accessibility, RNA expression and protein abundance within the same cell, enabling an in-depth understanding of gene regulatory mechanisms. Furthermore, advances in single-molecule imaging allow us to track the dynamic processes of development within a single cell at high spatial and temporal resolution. Together with these new technologies, we can develop a more comprehensive set of hypotheses to test our understanding of cell fate decisions, identity and the function of cells in normal germ cell development, physiology and disease. Knowledge of key transcription factors required for directed stem cell differentiation promises to provide additional tools to directly probe the function of putative master regulatory transcription factors in germ cell development through gain and loss-of-function studies.

Successes in modeling mouse germ cell differentiation and gamete formation have thus far translated to human systems only in part, indicating gaps in knowledge of human-specific factors involved in mature germ cell development. Current methods of human germ cell differentiation *in vitro* still remain inefficient, asynchronous, and prone to variability, with a majority of the initial pluripotent cells differentiating toward various somatic lineages spontaneously. The efficiency of hPGCLC progression to more mature germ cells is also still very low (Tang *et al.*, 2016). Developing multiple, testable strategies to differentiate hPSCs toward functional oogonia/spermatogonia efficiently is essential to model the entire germline development and diseases.

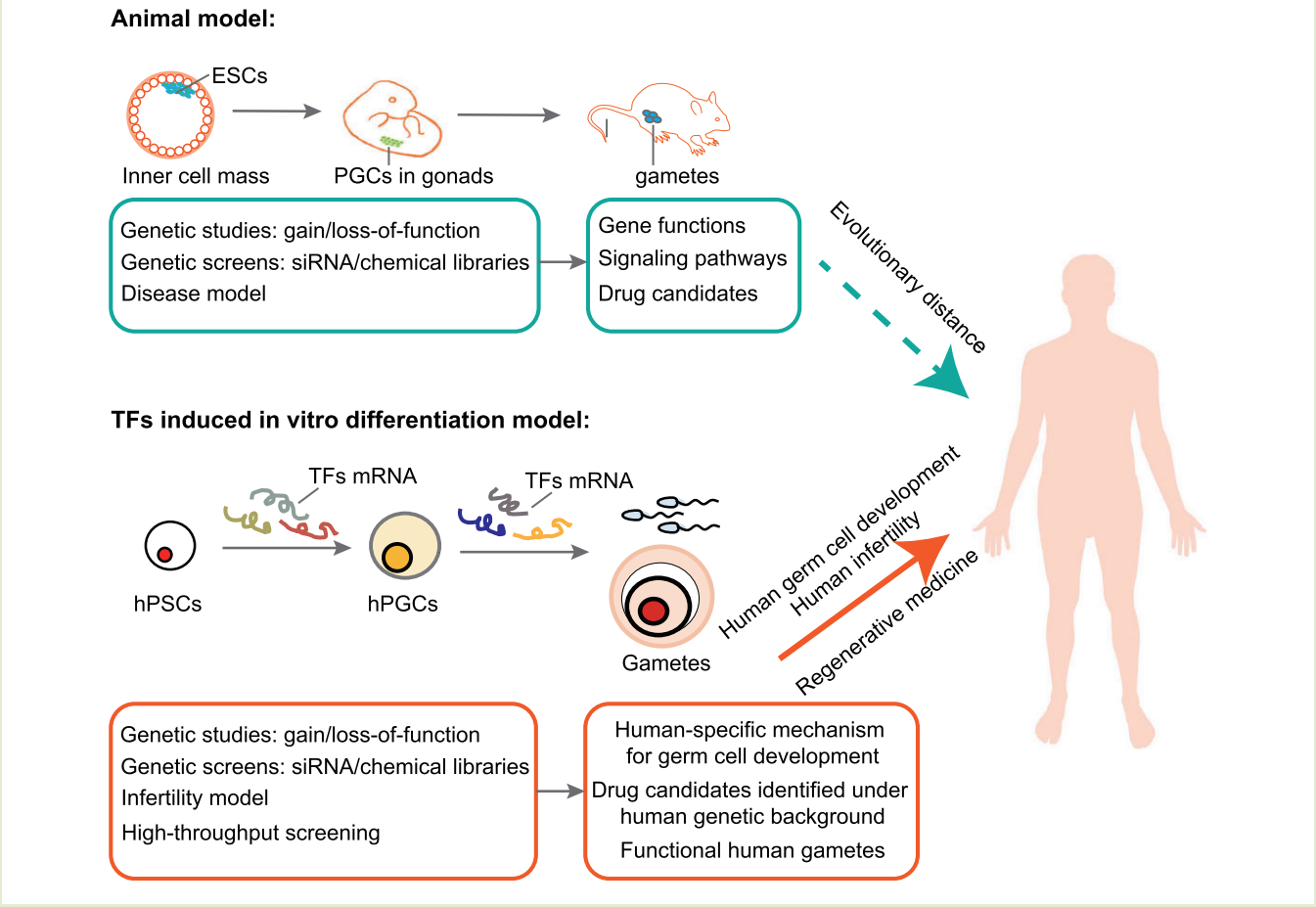

Figure 5. Improving human reproduction research through transcription factor-induced *in vitro* differentiation models. ESC, embryonic stem cell

In recent years, the biomedical community has witnessed the development of iPSC technology, which spawned a new era of controlling cell fate by modulating master transcription factors (Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007). The successes in mouse germ cell differentiation enhance the prospect of transcription factor-induced differentiation of hESCs to hPGCs, and beyond. However, multiple transcriptome analyses have revealed that human germ cells acquire a unique transcriptional network, which is distinct from that of mouse germ cells and from that of any other human cell type. The germ cell transcriptome is composed of a surprising combination of core germ cell-specific genes, somatic lineage specifiers, and pluripotency genes. We and others have hypothesized that these lineage specifiers cooperate with the pluripotency networks to act as the earliest molecular switch in the developmental transition from PSCs to the germ cell lineage (Fig. 4). Counteracting the activity of lineage specifiers, and continued transcription by pluripotency proteins, synergistically represses somatic lineages and activates germ cell programs to maintain germ cell identity. The identification and overexpression of these master transcription factors of human germ cells in hPSCs may enable faithful and efficient *in vitro* production of *bona fide* human germ cells and contribute to diagnosis and applications in human germ cell pathologies.

Numerous protocols over the years have attempted to mimic the spatial component of embryonic development by stimulation of differentiating cells in 3D aggregates. However, these methods can expose cells at different positions in the aggregates to varying levels of extracellular signals, leading to heterogeneity in differentiation. The ability to maintain cells in a uniform monolayer and induce cell fate change by overexpression of transcription factors could enhance differentiation efficiency while improving final purity. More importantly, this strategy would reduce the complexity and variability of current protocols and enhance the widespread availability of germ cell research, as well as reproducibility, between laboratories. Simplifying and increasing the efficiency of differentiation of germ cells will also allow previously challenging studies, such as high-throughput drug discovery and genetic screening assays, thereby accelerating our understanding of human germ cell biology, and expediting infertility research.

Finally, the ability to culture hPSCs and hPGCs indefinitely *in vitro*, in addition to providing an excellent model for germ cell research, could permit the repair of genetic defects in germ cell development by gene editing tools, for example, clustered regularly interspaced short palindromic repeats (CRISPR) technology. Note that *in vitro* hPSC differentiation-based methods or disease-modeling studies cannot diminish the importance of animal disease models for studying disease mechanisms or the downstream characterization and validation of putative drugs. However, the use of *in vitro* hPSC differentiation-based studies is providing additional tools for the identification of human-specific genes and pathways of development, and thereby accelerate the overall drug development process to treat human reproductive disease.

Data availability

The data underlying this article will be shared with all reasonable requests to the corresponding author.

Authors' roles

All authors contributed to the identification and critical evaluation of the relevant literature and reviewed all sections and critical discussion of the manuscript. F.F., P.J.I. and R.A.R.P. proposed the outlines, made the figures and drafted the main text. N.X., L.L. and L.D. performed the literature search and prepared the data for all the tables.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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