Germline development from human pluripotent stem cells toward disease modeling of infertility

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Infertility caused by the disruption or absence of germ cells (i.e., sperm or egg) is a major and largely incurable medical problem. In vitro disease modeling using normal human germline cells is required to better understand the precise molecular mechanisms of infertility and to develop drugs to treat this condition. Recent advances in the differentiation methods of embryonic stem cells (ESCs) provide new avenues to generate germ cells in vitro. Furthermore, the discovery that induced pluripotent stem cells (iPSCs) can be created from a patient's adult somatic cells by introducing the combinations of several transcription factors (e.g., OCT3/4, SOX2, KLF4, and MYC) enables us to generate new and powerful in vitro human disease models. In this review, we summarize recent advances in the development of human germ cells from in vivo and in vitro cultured ESCs/iPSCs. Based on these studies, we propose strategies to develop in vitro disease models of infertility using human ESCs/iPSCs. Then, we also discuss the challenges that need to be addressed to harness the full potential of these models. These models will enable us to understand the precise molecular pathologies of infertility and will aid in the development of new treatments. (Fertil Steril® 2012;97:1250–9. ©2012 by American Society for Reproductive Medicine.)

Human infertility affects 10–15% of couples, with approximately equal likelihood from both partners. At present, treatments for infertility are limited. Instead, in vitro fertilization (IVF) techniques including intracytoplasmic sperm injection (ICSI) have been used to circumvent infertility problems. To develop true therapies to increase germ-cell numbers or to reconstitute reproductive organs, we require a deeper understanding of the biology of reproductive organ development (i.e., testis and ovary). A number of reports describing mouse models as well as human mutational screening and association studies reveal a high prevalence of genetic causes of severe infertility, including chromosomal aberrations and single gene mutations (1–6). The considerable differences in germline development between mice and humans, coupled with the inaccessibility of human gonads and reproductive organs, demand that in vitro models using human cells to recapitulate normal and pathological human germline development be created.

A promising strategy to generate in vitro human models is utilizing human pluripotent stem cells. Pluripotent stem cells have the characteristics of self-renewal and pluripotency, defined as the ability to proliferate while maintaining their differentiation potential and to differentiate into all three germ-layer cell types, respectively. Pluripotent stem-cell lines, human embryonic stem cells (hESCs) from blastocysts (7) and human embryonic germ cells (hEGCs) from primordial germ cells (PGCs) (8), were first established in 1998. Since the discovery of human pluripotent stem cells, researchers have made attempts to reprogram somatic cells to pluripotent stem cells. The year 2006 saw the first description of mouse induced pluripotent stem cells (miPSCs), which were generated by retrovirus-mediated transduction of four transcription factors (i.e., Oct3/4, Sox2, Klf4, and Myc) into mouse fibroblasts (9). Subsequently in 2007, human iPSCs (hiPSCs) were established by the transduction of either the same set of transcriptional factors (OCT3/4, SOX2, KLF4, and MYC) (10) or another set of transcriptional factors (OCT3/4, SOX2, NANOG, and LIN28) (11) into human fibroblasts. hiPSCs are similar to hESCs in morphology, gene expression, and epigenetic status of pluripotent cell-specific genes. Furthermore, they can differentiate into all three germ-layer cell types in vitro and form teratoma in vivo. Since hiPSCs can be

Received March 1, 2012; revised April 23, 2012; accepted April 25, 2012.
Y.H. discloses travel support from the Uehara Foundation. M.S. has nothing to disclose. S.Y. is a member of scientific advisory boards of iPearian Inc. and iPS Academia Japan Inc.
Supported by a grant from the Program for Biomedical Research at the University of California San Francisco (Y.H.); Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science and Ministry of Education, Culture, Sports, Science and Technology, Japan (M.S., S.Y.); a grant from the Leading Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan (Y.H., S.Y.); grants from New Energy and Industrial Technology Development Organization, Japan (S.Y.); a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, Japan (S.Y.); a grant from the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) of the Japanese Society for the Promotion of Science, Japan (S.Y.).
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Fertility and Sterility® Vol. 97, No. 6, June 2012 0015-0282/$36.00
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doi:10.1016/j.fertnstert.2012.04.037

generate from patients' somatic cells, numerous patient-specific hiPSCs have been established, leading to a number of new in vitro disease or transplantation models targeted for the study of various genetic diseases and regenerative medicine (12–15).

In this review, we summarize recent advances in the development of germ cells in vivo and from cultured hESCs/iPSCs. Since the development of mouse germ cells in vivo and in vitro has been extensively reviewed in other publications (16–21), here we focus on studies on human germline development and propose possible strategies to develop in vitro disease models of infertility using hESCs/iPSCs. To realize these strategies, several challenges must be overcome, and we will discuss the prospective challenges and possible solutions.

MOLECULAR SIGNATURES AND MECHANISMS OF HUMAN GERMLINE DEVELOPMENT IN VIVO

Regulations on the Study of Human Germline Development

Mammalian germline development has been most extensively studied using mouse embryos as model systems with numerous molecular biology and genetic techniques. In contrast, studies of human germline development are hampered by restricted access to appropriately aged material and the intractability of human tissues in culture. In the United States, federal and state laws and regulations govern the use of human embryos for research. Federal regulations permit funding for the study of human embryos provided that they were not obtained solely for research purposes (45 CFR 46.201-46.211). In the United Kingdom, the acquisition of material from first-trimester termination of pregnancy must proceed by similar guidelines as outlined in the US, which are based around the recommendations of the Polkinghorne Committee, a UK Government committee reported in 1989. These guidelines include the need to separate clinical and research consent, require that the donor receives no financial or commercial incentives, and make clear that donor research consent should be acquired remote from the planned laboratory experiments (22). In Japan, there are no official laws and regulations on the use of human embryos for research. The public statement made from the Japan Society of Obstetrics and Gynecology is applied to every research case. The derivation of germ lineage cells (gametes and their precursors) from pluripotent stem cells had been prohibited under Japan's stem cell research guideline by until 2008. Following the revision of it and the establishment of additional guideline in 2010, research protocols involving germ cell differentiation have been permitted only for researches into the mechanisms of development and regeneration or into the development of diagnostic, preventive, or regenerative medical procedures or products. However, fertilization via gametes derived from human pluripotent stem cells has been prohibited (23).

With these strict guidelines in place, recent studies have revealed the molecular profiles and characteristics of germ cells and their development in human reproductive systems. The schematic representations of human germline development in vivo and from hESCs/iPSCs are summarized in Figure 1.

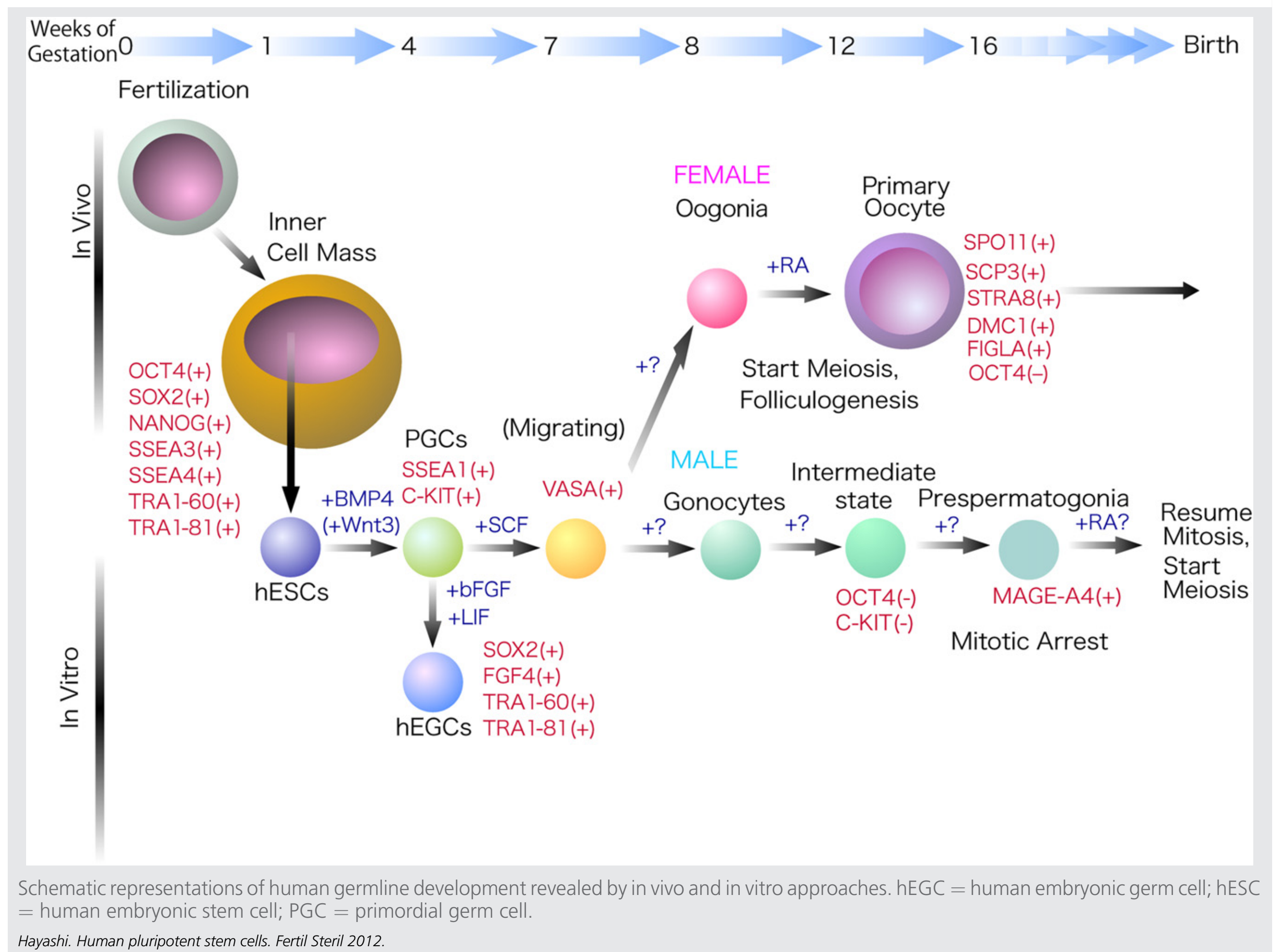
Differentiation and Characterization of Human PGCs

In human development, PGCs, differentiated from epiblast cells, are identifiable in the hind gut at 4 weeks of gestation and migrate to colonize the developing gonads by 7 weeks of gestation (24–27). Human PGCs can be isolated and confirmed by their migrating activity in vitro (28). In the presence of feeder cells, leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF), cultured human PGCs become hEGCs, which can maintain their self-renewal and pluripotency in vitro (8). hEGCs express alkaline phosphatase (AP), OCT4, SOX2, NANOG, stage specific embryonic antigen (SSEA) -3, SSEA-4, tumor rejection antigen (TRA)-1–60, and TRA-1–81 as the markers of pluripotent stem cells. Regarding the molecular signatures of human PGCs in vivo, they lack the expression of FGF4 and SOX2 compared with hESCs in vitro (29, 30). Human PGCs also lack the expression of TRA-1-60 and TRA-1-81 compared with hESCs or hEGCs in vitro (31, 32). In the human fetal embryos at different gestational stages, only a small percentage of the germ cells expressed C-KIT, NANOG, and OCT4, which represent as PGCs. The number of these cells correlated with an increase in the number of hEGC colonies derived in culture. From these findings, human PGCs in vivo can be characterized as C-KIT^{+}, SOX2^{-}, TRA1-60^{-}, TRA1-81^{-}, and FGF4^{-} compared with human pluripotent stem cell lines in vitro. As PGCs are isolated and cultured in specific culture conditions, they become hEGCs and may begin to express SOX2, FGF4, TRA1-60, TRA1-81. These signatures are summarized in Table 1 and will be useful to isolate and distinguish human PGCs differentiated from hESCs/iPSCs. From mouse studies, numerous genes are identified as specific and functional in PGCs (e.g., Fragilis [33], Stella/PGC7 [33, 34], Blimp1/Prdm1 [35], Nanos3 [36]). However, the expression and function of these orthologs in human PGCs remain unclear.

In migratory PGCs at 7 weeks of gestation, the VASA protein (DDX4; DEAD (Asp-Glu-Ala-Asp) box family of ATP-dependent RNA helicases) started to be expressed (37). This expression pattern may be different from mouse embryos in which the expression of the mouse Mvh gene was not detected in migratory PGCs before their arrival at the gonadal ridge, but rather was induced after direct interaction with gonadal somatic cells.

Mammalian epiblast cells acquire germ-cell fate in response to signaling molecules. From mouse studies, the formation and proliferation of PGC population is dependent on bone morphogenetic proteins (BMPs) 2, 4, and 8b (38–41). Regarding the molecular cues or inducers of human PGC from the epiblast, the addition of recombinant BMP4 dose-dependently increased the number of human PGCs after 1 week of culture (42). The efficiency of EGC derivation and maintenance in culture was also enhanced by the presence of recombinant BMP4 (42). BMP4 also promotes the differentiation of hESCs/iPSCs into PGC-like cells (43–45). Responsiveness to Bmp4 in mouse epiblast cells is ensured by Wnt3 expression (46); however, the role of Wnt3 in human germ cell development remains unclear.

FIGURE 1



Schematic representations of human germline development revealed by in vivo and in vitro approaches. hEGC = human embryonic germ cell; hESC = human embryonic stem cell; PGC = primordial germ cell.

Hayashi. Human pluripotent stem cells. Fertil Steril 2012.

Mouse studies revealed that the stem cell factor (SCF)/ C-kit receptor pathway is required for successful migration by preventing the apoptosis of PGC (47–49). As just discussed, C-KIT is expressed in human PGCs in vivo, but its function in human germline development is unclear. SCF expression by feeder cells is required for the germ cell differentiation from hESCs/iPSCs (43).

Human Germ Cell Maturation and Meiosis

Mouse studies revealed that the maturation and meiosis of germ cells are induced by retinoic acid (RA) signaling (50). Pre-meiotic female germ cells activated by RA signaling express pre-meiotic genes (e.g., Dmc1, Scp3, Spo11, and Stra8) and repress Oct4 expression and then enter meiosis. While female germ cells are susceptible to RA produced by gonadal somatic cells, male gonadal somatic cells highly express Cyp26b1, which encodes a P450 enzyme that catabolizes active all-trans RA into inactive metabolites, leading to suppress RA signaling activity in male germ cells (51). In mouse male gonadal somatic cells, sex determination factors, such as SOX9/SF1, may up-regulate Cyp26b1 expression, while an ovarian transcription factor, Foxl2, may suppress Cyp26b1 expression in female gonadal somatic cells (52). Male germ cells continue to express Oct4, but stop proliferating until the birth of the individuals. After birth, these cells resume proliferation and enter meiosis.

In human female ovary at around 8 weeks of gestation, PGCs become oogonia when it reaches the ovary. Oogonia can be distinguished by their more rounded outline and distinct increase in mitotic activity from migratory PGCs (53, 54). Regarding proliferation and apoptosis during germ cell maturation, BMP4 negatively regulates post-migratory PGCs (or oogonia) numbers in the human fetal ovary by promoting their apoptosis, although BMP4 is required to induce PGCs in earlier embryos (55). Conversely, ACTIVIN, which has a competitive role in SMAD signaling against BMP4, may be involved in the autocrine and paracrine regulation of germ-cell proliferation in the human ovary during the crucial period of development leading up to meiosis and folliculogenesis (56). Female germ cells undergoing folliculogenesis lose OCT4 expression and no longer proliferate. In contrast, VASA, C-KIT are expressed in germ cells at all developmental stages of oogenesis and folliculogenesis (57). FIGLA expression

TABLE 1

The list of gene markers for human pluripotent stem cell lines and germ cells.

Gene marker	hESC/hiPSC	hEGC	Early PGC	Migratory PGC	Oogonia/gonocyte
OCT4	+ (10, 11)	+ (OP)	+ (29, 30)	+ (31, 32)	± (57, 60)
NANOG	+ (10, 11)	+ (OP)	+ (29, 30)	± (31, 32)	ND
SOX2	+ (10, 11)	+ (OP)	− (29, 30)	ND	ND
SSEA1	− (7, 10)	+ (8)	+ (29, 30)	+ (31, 32)	ND
SSEA3	+ (7, 10)	+ (8)	ND	ND	ND
SSEA4	+ (7, 10)	+ (8)	+ (29, 30)	+ (31, 32)	ND
TRA1-60	+ (7, 10)	+ (8)	− (31, 32)	ND	ND
TRA1-81	+ (7, 10)	+ (8)	− (31, 32)	ND	ND
FGF4	+ (10)	+?	− (29)	ND	ND
VASA	− (37)	ND	− (37)	+ (37)	+ (37, 57)
C-KIT	− (OP)	ND	+ (29, 30)	+ (31, 32)	+ (57, 60)

Note: The number in parentheses indicates the number of references. hEGC = human embryonic germ cell; hESC = human embryonic stem cell; hiPSC = human induced pluripotent stem cell; ND = not determined or unknown; PGC = primordial germ cell.

Hayashi. Human pluripotent stem cells. Fertil Steril 2012.

is up-regulated during folliculogenesis (58). First γH2AX-positive meiotic cells appear at 11 weeks of gestation, when DMC1, SPO11, and STRA8 are expressed. In the organ culture of ovaries from 10-11 weeks gestation, the addition of RA significantly increases the meiotic cells. However, RA exposure did not induce meiosis in the organ culture of ovaries from 8–9 weeks of gestation, suggesting that the competence to respond to RA is developmentally programmed. The mRNA level of an RA-synthesizing enzyme, ALDH1A1, specifically increases in human ovaries when meiosis begins. Indeed, ALDH inhibition by citral prevented the appearance of meiotic cells (59).

In human fetal testis, male PGCs continue to differentiate in the following sequence classified by Gaskell et al. as gonocytes ($OCT4^{+}$/$C\text{-}KIT^{+}$/$MAGE\text{-}A4^{-}$/mitotically active); intermediate germ cells ($OCT4^{-}$/$C\text{-}KIT^{-}$/$MAGE\text{-}A4^{-}$/mitotically active); and pre-spermatogonia ($OCT4^{-}$/$C\text{-}KIT^{-}$/$MAGE\text{-}A4^{+}$/mitotically inactive). The pattern of expression of MAGE-A4 (melanoma antigen A4) is of particular interest since this protein is expressed in testis cancer cells and in spermatogonia. In first trimester, most germ cells show a gonocyte phenotype; however, from 18 week of gestation onward, pre-spermatogonia are the most abundant cell type in the testis (60). A pre-meiotic gene, STRA8 expression remains very low in the testis until 20 weeks gestation at least, but could be partially up-regulated by the addition of RA to organ culture system. However, the presence of RA is not sufficient to cause widespread meiosis-associated gene expression in the organ culture system. Furthermore, unlike in mouse fetal testis, the expression of CYP26B1 mRNA is not up-regulated in human male gonadal somatic cells (61). These results suggest that mechanisms other than CYP26B1-mediated metabolism of RA may exist to inhibit germ cells from entering meiosis in human fetal testis.

GERMLINE DIFFERENTIATION FROM PRIMATE AND HUMAN ESCs/iPSCs

In 2004, Clark et al. first reported the spontaneous differentiation of germ cells in embryoid bodies (cell aggregations which permit random differentiation) from hESCs (62). These differentiated cells express germline-specific RNA and protein markers (e.g., VASA and SCP3). Subsequent findings on germline differentiation from human pluripotent stem cells can be classified in the following ways.

Toward Efficient Differentiation into Germline by Specific Culture Conditions

Because mammalian germline development is dependent on signaling molecules and the gonadal microenvironment, culture conditins are critical to promote the differentiation of pluripotent stem cells into germ cells in vitro. In addition, Bucay et al. observed that putative germline development from hESCs is accompanied by the development of Sertoli-like support cells (63). To mimic a suitable microenvironment for the development of germ cells, co-culture systems or conditioned medium have been used for differentiating hESCs/iPSCs. Co-culture with human fetal gonadal stromal cells (64), mouse Sertoli cells (65), mouse embryonic fibroblasts (66), or porcine ovarian fibroblasts (45) increased the efficiency of germ cell-like differentiation from hESCs/iPSCs. These results confirm that specific trophic factors are required to develop germline from pluripotent stem cells. A disadvantage of these co-culturing system is that it is difficult to know which trophic factors affect differentiation and how. As well, the presence of feeder cells can impede biochemical and cellular assays and scalable differentiation.

Numerous cytokines and signaling molecules have been used in the differentiation of germ cells from hESCs/iPSCs. Recombinant BMP4 protein and other BMPs are added to cultures to promote PGC-like differentiation from hESCs/iPSCs (43–45). RA has been used to stimulate meiosis (45, 67). Basic FGF (66, 67), LIF (67), SCF (secreted from feeder cells) (43), and forskolin, an adenyrate cyclase activator (45, 67), are also used to enhance germline differentiation from hESCs/iPSCs. However, the efficient differentiation of germ cells from hESCs/iPSCs has not been achieved, and the precise effect of these molecules has not been examined. Because germline development is a multi-step differentiation process, step-wise differentiation protocols based on the precise effect of each molecule are required to achieve the efficient differentiation of germ cells from hESCs/iPSCs.

Forced Differentiation by the Manipulation of Gene Expression

Manipulation of gene expression can regulate the cell lineage decisions in differentiating pluripotent stem cells. Overexpression of RNA-binding proteins, DAZL (deleted in azoosperma-like) and/or VASA promotes PGC formation in differentiating hESCs/iPSCs. On the other hand, overexpression of the related genes, DAZ and BOULE promotes meiosis and the development of haploid gametes (68, 69). These methods driving pluripotent stem cells into germline by overexpression of key regulators could be an efficient platform to generate germ cell lines from hESC/iPSCs; however, it remains to be determined how similar the observed meiosis is to the physiological process in vivo.

Purification of Germ Cells Differentiated from Human Pluripotent Stem Cells

Even though efficient differentiation using specific culture conditions may be achieved, we should also be able to isolate and purify the specific cell types. Cell-surface markers specifically expressed in germ cells have been used to cell sorting. To purify PGC-like cells from differentiating hESCs/iPSCs, sorting with specific antibody for SSEA1 (70), SSEA1 and C-KIT (64, 71) , and CXCR4 (63) have been used. Protocols that purify $CD9^{+}/CD49F^{++}/CD90^{-}$ cell populations have been reported to enrich the pool of spermatogonia-like cells (67). Using mESCs/iPSCs, PGC can be isolated using intrinsic cell-surface markers, integrin-β3 and SSEA1 (72); however, it remains unclear that these cell surface markers can be used to sort hESCs/iPSCs. In addition, although cell sorting based on these cell-surface markers is useful and convenient, the expression of these markers is not exclusive to germline cells.

Another method to purify the specific cell types is using a reporter system based on specific gene expression. A VASA reporter has been used to purify a migratory PGC-like cell population (or germ cell populations in later stages) from hESCs/iPSCs (68, 73). Germline-specific reporter systems, together with purification approaches based on the detection of cell-surface markers, are highly useful to quantitative evaluations of germ-cell differentiation. Because germ-cell development is a multi-step process, different reporter systems tailored to each developmental stage should be created.

Germline Development from hiPSCs

Since the development of hiPSCs in 2007, several reports demonstrated that hiPSCs are able to differentiate into germ cells. Park et al. first showed that PGC-like cells can be differentiated from hiPSCs (64). BMP4 enhances the differentiation into VASA-positive PGC-like cells from fetal- and adult-derived hiPSCs in a similar manner to hESCs (74). Haploid cells resulted from meiosis are consistently obtained from hiPSCs lines generated from different tissues (67). Meiosis was induced in differentiating hiPSCs by overexpressing DAZL and/or VASA in a similar manner to hESCs (69). These studies indicate that hiPSCs have almost the same ability to differentiate into germ cells as hESCs. In the future, it will be interesting to examine patient-specific iPSCs that are defective in their ability to generate germ cells, by comparing their differentiation capacity with that of normal hESCs/iPSCs.

Germline Differentiation from Non-Human Primate ES/iPSCs

Non-human primate ESCs/iPSCs is also useful for assessing the functionality of the germ cells differentiated from pluripotent stem cells. In 2007, Teramura et al. demonstrated that cynomolgus monkey ESCs can differentiate into PGC-like cells (75). The differentiation can be enhanced by conditioned medium from testicular or ovarian cells or by adding recombinant BMP4, RA or SCF (76). The self-renewal of differentiated PGC-like cells can be prolonged by LIF treatment (77). Further studies, including functional assays in vivo, would be interesting to examine their developmental potential and molecular mechanisms of primate germline development as a substitute model for human germ cell differentiation from pluripotent stem cells.

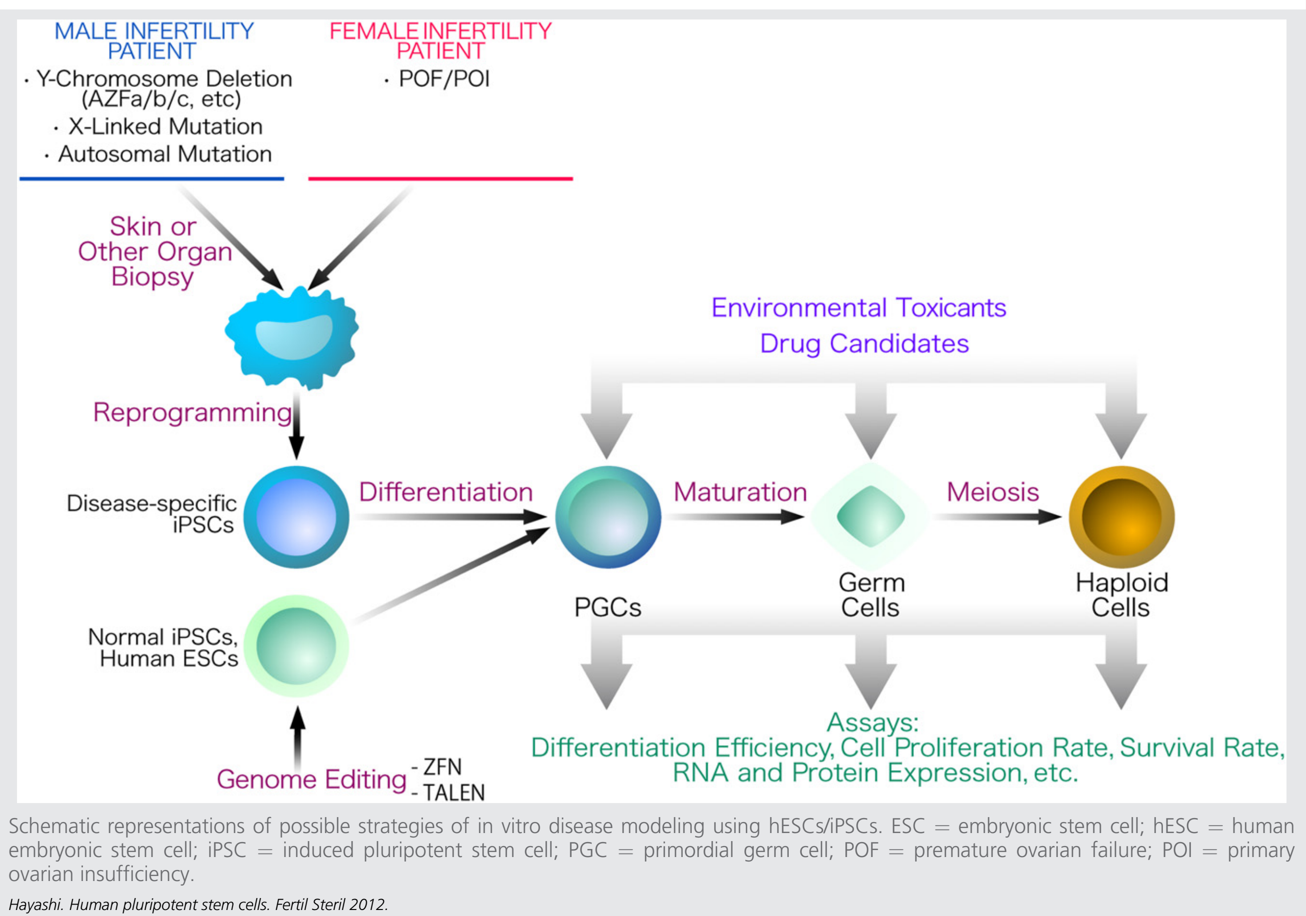
DISEASE MODELING USING HUMAN PLURIPOTENT STEM CELLS

Based on the studies reviewed above, we propose targets for potential disease modeling and drug screening using hESCs/iPSCs. We summarize the possible strategies in Figure 2.

Male Genetic Infertility

Infertility or subfertility affects 15% of couples, with a male contribution to the fertility problem in close to 50% of the couples. In case of male infertility, up to 20% are caused by azoospermia, defined as a lack of sperm in semen. Azoospermia can be categorized as: pre-testicular azoospermia (accounting for 2% of men with azoospermia, due to a hypothalamic or pituitary abnormality); testicular failure or non-obstructive azoospermia (49% to 93% of cases, with the term testicular failure suggesting a complete absence of spermatogenesis); and post-testicular obstruction or retrograde ejaculation (7% to 51% cases, caused by normal spermatogenesis but obstructive azoospermia or retrograde ejaculation). In testicular failure or non-obstructive azoospermia, molecular genetic techniques have unveiled a number of genetic mutations, including micro-deletions of the Y chromosome q arm. These micro-deletions, which remove the AZoospermia Factor (AZF) regions, are the most frequent genetic causes of azoospermia. AZF regions follow a certain deletion pattern, with three recurrently deleted non-overlapping subregions in proximal, middle, and distal Yq11, designated AZFa, AZFb, and AZFc, respectively (78). The AZFa region contains USP9Y and DBY genes. The AZFb region contains unique genes (i.e., HSFY, eIF-1Y, SMCY) and others that are shared with the AZFc region (i.e., one copy of BPY2 and CDY and two copies of DAZ). Moreover, two gene families with multiple copies on the Y chromosome have their active copies in the AZFb region, including RBMY and PRY genes. AZFc region contains DAZ gene and CDY gene families. Unfortunately, these Y-chromosome micro-deletions cannot be directly modeled in mice because candidate genes on the

FIGURE 2



Schematic representations of possible strategies of in vitro disease modeling using hESCs/iPSCs. ESC = embryonic stem cell; hESC = human embryonic stem cell; iPSC = induced pluripotent stem cell; PGC = primordial germ cell; POF = premature ovarian failure; POI = primary ovarian insufficiency.

Hayashi. Human pluripotent stem cells. Fertil Steril 2012.

human Y chromosome are frequently located on X chromosome or on autosomal chromosomes in the mouse genome. For example, the DAZ gene family, located on the Y chromosome in humans, is not present on the mouse Y chromosome (79). Its autosomal homologs are present in all mammals. In addition to the Y chromosome, certain autosomal chromosomal abnormality are also linked to male infertility, including aneuploidy, X-chromosome abnormality, or Robertsonian translocations (defined as rearrangements that occur in the five acrocentric chromosome pairs, namely 13, 14, 15, 21, and 22.) (80). Furthermore, many attempts at finding mutations in infertile humans by using known mouse infertility genes have been unsuccessful (1). To date, there are no established methods to promote spermatogenesis from non-obstructive azoospermia patients. Modeling of azoospermia using hESCs/iPSCs with the relevant chromosomal abnormalities would greatly improve our understanding of the underlying pathology and would help in the design of treatments.

Female Genetic Infertility

Primary ovarian insufficiency (POI)—also known as premature ovarian failure (POF) is a major cause for female infertility. The cause of POI/POF can be classified according to whether autoimmune disorders, abnormal hormone regulation, viral infection, surgery, chemotherapies or radiotherapies, or genetic mutations are involved. A number of studies using genetically modified mice or genome-wide associations have linked several ovarian genes to POI/POF, such as BMP15, BMPR1B, DAX1, FMR1, FOXL2, POF1B, and SF1; however, it is unknown how these genes are involved in the pathology of POI/POF (81). Modeling of POI/POF using hESCs/iPSCs with the known genetic mutations would be an excellent way to examine the pathology of POI/POF and to the development of treatments.

Environmental Factors

For male fertility, numerous environmental toxicants adversely affect spermatogenesis and can lead to low sperm count, abnormal sperm morphology and poor semen quality (82). Ovarian function in women can also be compromised by exposure to them. Ovarian toxicants can directly cause ovarian failure by extensive follicular destruction or by interference with steroid hormone action (hypothalamus and/or pituitary) (83). However, in spite of these findings, the precise effects of environmental factors on their pathology of infertility are largely unknown. Organ culture systems of human fetal gonads have been used to examine the toxicity of environmental factors (e.g., cadmium [84], uranium [85], and irradiation [86]). Since hESC/iPSC developmental models could

be examined in scalable, high-throughput, and direct manners, these models can be applied to monitor and examine the effect of these environmental chemicals. In fact, Kee et al. demonstrated that the number of PGC-like cells differentiated from hESCs is diminished by exposure to polycyclic aromatic hydrocarbons (PAHs), a family of toxicants commonly found in air pollutants released from gasoline combustion or tobacco smoke. PAHs act through the aromatic hydrocarbon receptor (AHR) and BAX signaling pathways in the hESC-based differentiation system (87). These results were consistent with studies using PGCs in human fetal testis (88).

CHALLENGES TO DEVELOP DISEASE MODELS USING hESCs/iPSCs

The development of genetic disease models in pluripotent stem cells for drug screening applications is hampered by current technology. Here, we discuss the challenges and possible solutions of disease modeling with hESCs/iPSCs. These discussions are useful not only for reproductive tissues, but for general disease modeling using hESCs/iPSCs.

Inefficient Differentiation and Purification Technologies

To develop the differentiated hESC/iPSC model for high-throughput drug development studies, robust and scalable differentiation and purification methods are required. However, current protocols to differentiate and purify human germ cell are inefficient. As shown in Figure 1, the differentiation of pluripotent stem cells into germ cells is a multi-step process that requires exposure to specific stimuli at discreet stages of development, to achieve directed and convergent differentiation. For the purification of germ cells, several different reporter systems, which represent the different developmental and sexual stages of germline development, must be developed.

Cell Line Variability of Cultured Pluripotent Stem Cells

To make disease model using pluropotent stem cells, a major ongoing issue relates to the inherent cell-line variability of these cells. hESC lines have a high differentiation propensity (89); therefore, hiPSC must be similar in this way. Mouse iPSCs derived from different adult tissues varied substantially in their propensity to form teratomas, as measured by the transplantation of secondary neural spheres into the striatum of NOD/SCID mice. The likelihood of teratoma formation was correlated with the persistence of undifferentiated cell populations within the neural spheres (90). Thus, patient-specific hiPSC lines even from the same patient may have the cell-line variability to cause off-shot effects. The cause of this variability remains unknown. Furthermore, in many cases, it is difficult to obtain patient-specific tissue samples from enough donors with the same type of mutations. Thus, the limited number of cell lines or donors currently available affects our ability to understand cell-line variability. To overcome this problem, we may need to develop other sophisticated strategies in conjunction with patient-specific hiPSCs. As options, we can use genome editing technology, such as zinc-finger nucleases (ZFN) (91) or TAL-effector nucleases (TALEN) (92) in hESCs/iPSCs. For patient-specific hiPSCs, Soldner et al. demonstrated that ZFN genome editing technology could produce isogenic hiPSCs that carry point mutations with genetic forms of Parkinson's disease (93). These technologies could help us to develop more robust genetic disease models using hESCs/iPSCs.

Inconsistency Between In Vivo Development and In Vitro Differentiation of Pluripotent Stem Cells

In the differentiation culture systems from pluripotent stem cells, we sometimes observe the different cell behaviors and patterns of gene expressions from in vivo development. These represent the inconsistency between in vivo development and in vitro differentiation of pluripotent stem cells. For example in germ cell development using hESCs/iPSCs, haploid cells can be induced just 14 days after induction by over expression of DAZL, BOULE and DAZ (68). These results might indicate the limitation of the in vitro developmental models using hESCs/iPSCs; however, even these culture artifacts (e.g., incredible speed of germ cell maturation) could be the advantages of the in vitro models after careful and precise considerations. It is important to examine which part of in vivo development can be recapitulated in disease models using hESCs/iPSCs.

Alternative Source 1: Germline Stem Cells Derived From Adult Testis or Ovary

One alternative way to obtain germ cells might be isolating (and expanding) adult germline stem cells from reproductive organs instead of by differentiating germ cells from pluripotent stem cells. In mice, male germ stem cells can be cultured while maintaining their self-renewal and functionality (94). Izadyar et al. reported that spermatogonial stem-like cells, which express SSEA4, C-KIT, NANOG, and OCT4, exist in adult human testis and that these cells can be isolated based on the expression of cell-surface markers (95). Virant-Klun et al. reported that ovarian stem-like cells, which express SSEA4, OCT4, NANOG, and C-KIT, exist in the ovarian surface epithelium from adult POF patients and can be isolated (96). Very recently, White et al. reported that mitotically active oogonial stem cells (OSCs) can be purified by sorting with VASA expression presented on the cell surface from adult mouse ovaries and human ovarian cortical tissues (97). The OSCs can be expanded for months on MEF feeders and can spontaneously generate 35- to 50-mm oocytes, as determined by morphology, gene expression and haploid (1n) status. Injection of the OSCs into human ovarian cortical biopsies leads to formation of follicles containing oocytes 1–2 weeks after xenotransplantation into immunodeficient female mice. These findings suggest that reproductive-age women may possess rare mitotically-active germ cells that can be propagated in vitro as well as generate oocytes in vitro and in vivo. Together, deriving germ cells from adult reproductive organs could be an alternative way to make In vitro disease models; however, there are caveats with this

approach as well. One, access to the adult reproductive organs for biopsy is very limited, making harvesting these cells difficult. Second, the robust purification and expansion enabling scalable differentiation of human germline stem cells are still difficult.

Alternative Source 2: Trans-Differentiation or Reprogramming into Germ Cells from Somatic Cells

Another alternative way to obtain germ cells might be to convert other cell types to germ cells instead of by differentiating germ cells from pluripotent stem cells. Hua et al. reported that that there are a subset of cells in human fetal bone marrow that express germ cell markers (98). Drusenheimer et al. also reported that a small population of bone marrow cells expressed the early germ cell markers OCT4, FRAGILIS, STELLA, and VASA and the male germ cell-specific markers DAZL and STRA8 (99). Huang et al. reported that human umbilical-cord Wharton's jelly-derived mesenchymal stem cells (HUMSCs) could form "tadpole-like" cells after induction with different reagents. These cells expressed germ cell-specific markers OCT4, C-KIT, CD49F, STELLA, and VASA in the culture conditions with all-trans RA, testosterone and testicular-cell-conditioned medium prepared from newborn male mouse testes (100). These approaches might be attractive to generate germ cells from somatic cells for therapeutic application of reproductive medicine and in vitro disease modeling; however, it is not yet confirmed that these trans-differentiated cells have the functionality of germ cells. Furthermore, Eggan et al. showed that no evidence that bone marrow cells, or any other normally circulating cells, contribute to the formation of mature, ovulated oocytes, using transplantation and parabiotic mouse models to assess the capacity of circulating bone marrow cells to generate ovulated oocytes (101). Therefore, whether trans-differentiation into germ cells from somatic cells is a viable strategy for in vitro disease modeling is still in doubt.

CONCLUSIONS

The development of hiPSCs has opened up new avenues to generate in vitro disease models of infertility using patient-specific stem cells. The advances of the technology to manipulate the differentiation of hESCs/iPSCs into germ cells will allow us to understand underlying disease mechanisms, and to develop new drugs of infertility. However, we have faced several challenges in our efforts to develop in vitro disease models and the applications for drug screening using hESCs/iPSCs, including low efficiency of directed differentiation methods and cell-line viability. Nevertheless, reproductive diseases and infertility are still highly promising areas with in vitro disease models using hESCs/iPSCs, as we discussed in this review. We hope that these models will be utilized to cure infertility for couples desiring parenthood.

Acknowledgments: We thank the members of our laboratory for valuable scientific discussions and administrative support. We thank Dr. Naoki Nagata for critical review of this article; Gary Howard and Anna Lisa Lucido for writing assistance; John Carroll for assistance with graphics; and Rie Kato and Karena Essex for administrative support.

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