Reprod Med Biol (2013) 12:39–46
DOI 10.1007/s12522-012-0141-x

REVIEW ARTICLE

Induced pluripotent stem cells in reproductive medicine

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Received: 10 October 2012 / Accepted: 29 November 2012 / Published online: 14 December 2012

Abstract Despite recent advances in reproductive medicine, there are still no effective treatments for severe infertility caused by congenital absence of germ cells or gonadotoxic treatments during prepubertal childhood. However, the development of technologies for germ cell formation from stem cells in vitro, induction of pluripotency from somatic cells, and production of patient-specific pluripotent stem cells may provide new solutions for treating these severe fertility problems. It may be possible to produce germ cells in vitro from our own somatic cells that can be used to restore fertility. In addition, these technologies may also bring about novel therapies by helping to elucidate the mechanisms of human germ cell development. In this review, we describe the current approaches for obtaining germ cells from pluripotent stem cells, and provide basic information about induction of pluripotency and germ cell development.

Keywords Germ cells · Induced pluripotent stem cells · In vitro differentiation · Pluripotency · Reprogramming

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Introduction

Pluripotent stem cells have the potential to differentiate into cells of any lineage. Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass of blastocysts. ESCs have been used extensively to study mammalian development and human diseases, because these cells can develop into all three germ layers in mouse chimeras [1] and form teratomas [2]. Importantly, it has also been demonstrated that ESCs can differentiate into germ lineages (i.e., gametes) in vivo and in vitro. Detailed investigation of the earliest stages of germ cell development in humans is subject to practical and ethical limitations. Therefore, relatively little is known about the specification of human germ cells at primordial stages (primordial germ cells, PGCs) [3, 4]. Thus, in vitro model systems that can recapitulate the development of human germ cells and gametes will be extremely valuable as research tools.

From the first report of induced pluripotent stem cells (iPSCs) in 2007 [5], pluripotent stem cell technologies have grown with the goal of creating individualized, patient-specific stem cell therapies. These technologies may enable us to understand the causes of severe infertility at the level of individual patients. More importantly, the ability to produce germ cell replacements using the patients' own somatic cells will relieve many of the constraints associated with current methods for infertility treatment.

To generate patient specific germ cell in vitro, two keys steps are required: (1) induction of pluripotency in somatic cells; and (2) generation of germ cells from iPSCs. In the following sections, we describe current approaches used in each of these steps, and discuss the possibilities and challenges for regeneration of the reproductive system.

Induction of pluripotency: development of "MATERIALS" for germ cell production

To obtain stable germ cells from iPSCs, it is important to maintain the quality of iPSCs pluripotency. It is a well-known fact that germ-line competency levels differ dramatically among ESC and iPSC lines; therefore, screening of cell lines prior to differentiation may significantly improve the success of germ cell induction. In mice, some predictive markers such as Nanog [6] and Gtl2 [7] enable selection of germline competent cells. However, it is unclear whether these markers will be applicable to other species. Therefore, improvements in the induction methods for creating iPSCs may represent an additional avenue for producing germline competent cells. To find these novel induction methods, it will be important to elucidate the complete molecular mechanisms for establishing pluripotency.

Pluripotency is established and maintained by a core circuit of signaling molecules composed of Oct4, Sox2, Nanog, and related molecules such as Klf4, Esrrb, Tbx3, and cMyc [8, 9]. This core circuit controls expression of pluripotency maintenance genes and is involved in epigenetic modification. The original method for inducing pluripotency is based on introduction of four reprogramming factors: Oct4, Sox2, Klf4, and cMyc (also known as the Yamanaka factors) [10]. Oct4, a POU homeodomain transcription factor, is an essential factor for reprogramming, and is required for pluripotency of inner cell mass (ICM) cells and ESCs [11, 12]. Sox2 forms a complex with Oct4 to regulate the transcription of key pluripotency control genes, such as Oct4, Sox2, and Nanog [11, 13]. However, Sox2 is dispensable for establishment of pluripotency, as demonstrated by experiments where forced expression of Oct4 rescued the undifferentiated state of Sox2 null ESCs [11]. Furthermore, in artificial induction of pluripotency in mouse fibroblasts, TGFβ inhibitors can replace Sox2 [14]. The Krüppel-like zinc finger transcription factor Klf4 also plays an important role in the pluripotency core circuit by regulating the expression of Sox2 and Nanog [8, 15]. Finally, cMyc is a major oncogene that binds to promoters for cell-cycle activating genes and pluripotent state-specific microRNAs (miRNAs) [16]. cMyc can also contribute to pluripotency by activating histone acetyltransferases/demethylases, which function to remodel chromatin, helping other pluripotency-related transcription factors to access target genes [17]. However, chimeras derived from cMyc-iPSCs frequently develop tumors; therefore, replacement of cMyc with LMyc [18], or omission of exogenous Myc, is appropriate [19] since endogenous cMyc is already expressed at low levels in many somatic cells. Exogenous expression of these genes induces the following sequential reprogramming events. First, a change resembling mesenchymal-to-epithelial transition (MET) occurs as somatic cell-specific gene expression is downregulated. Next, early pluripotency markers such as alkaline phosphatase, SSEA-1, and Fbx15 become activated [20, 21]. Later on during reprogramming, Nanog and other pluripotency-related genes become activated. Once endogenous expression of pluripotency genes such as Nanog occurs, the cells can maintain pluripotency independent of exogenous factors [20–22] (Fig. 1).

Oct4, Sox2, Klf4 and Myc are not the only combination of factors that can generate iPSCs. Yu et al. reported that Klf4 and cMyc can be replaced with Nanog, another core member of the pluripotency maintenance circuit [23]. Lin28, a RNA binding protein, is involved in degradation of let7 miRNAs [24, 25]. Control miRNAs capable of inducing pluripotency are also available. Judson et al. observed a marked increase in reprogramming upon overexpression of the miR-290 and miR-302 clusters [26], which are known to accelerate cell cycling [27]. Meanwhile, Anokey-Danso et al. succeeded at generating iPSCs from both human and murine somatic cells by overexpression of the miR-302-367 cluster, which facilitates MET by induction of E-cadherin expression and inhibition of TGF-β signaling [28]. Using the same principles, Miyoshi et al. demonstrated that iPSCs could be generated by repetitive transient delivery of mature miRNAs from the

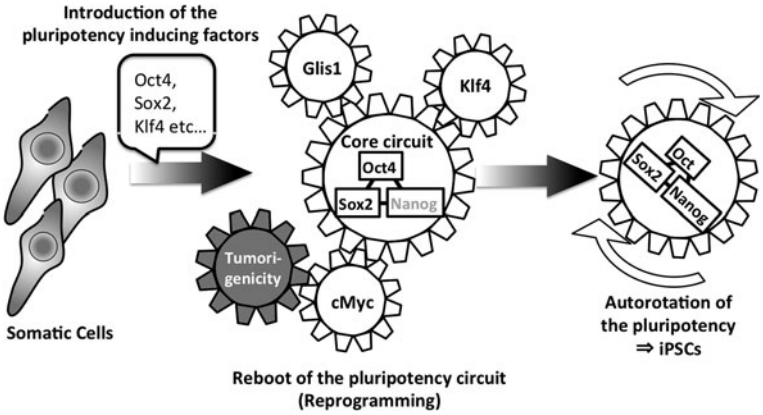

Fig. 1 Reprogramming of the somatic cells by exogenous gene expression

miR-200c, miR-302, and miR-369 families, which are all involved in MET [29]. On the other hand, Heng et al. reported that the nuclear receptor Nr5a2 can replace Oct4 [30]. Recently, Maekawa et al. re-screened genes identified from molecules enriched in unfertilized oocytes and zygotes that enhanced induction of pluripotency, and discovered a novel transcription factor, Glis1. This molecule promotes induction with high efficiency and reduces tumorigenicity. Interestingly, Glis1 not only interacts with the pluripotency maintaining core-circuit molecules Oct4, Sox2, and Klf4, but also interacts with some related proteins, such as several Wnt ligands, Lin28a, Myc, and Foxa2 [31]. Better combinations of reprogramming/induction genes may be discovered by elucidating the mechanisms of pluripotency. Understanding the factors that regulate pluripotency may help to control tumorigenicity and reveal new methods for controlling cell differentiation.

Specification of PGCs: understanding the origin of germ cells for development of successful induction methods

Elucidation of the mechanisms involved in production of "native" germ cells will be essential for development of technologies that generate germ cells from pluripotent stem cells in vitro. In particular, studies pertaining to primordial germ cells (PGCs), which give rise to oocytes and sperm, will reveal important information that can be used for germ cell production.

Primordial germ cells are derived from a subset of cells in the epiblast immediately after implantation. These cells undergo reprogramming that leads to their specification during the few weeks that they travel through the gonadal ridges [32, 33]. In the mouse, PGCs appear at 6.25 days post-conception (dpc). Germ-line competence can be identified in the first founder of Blimp1 (B-lymphocyte-induced maturation protein 1) expressing epiblast cells [33, 34]. PGCs are readily identified at 7.25 dpc as a Stella+/tissue nonspecific alkaline phosphatase (TNAP)+ cell population of approximately 40 cells in the developing yolk sac. The PGCs then migrate towards the future gonads (genital ridges) and start to express germ cell-specific genes, such as mouse *VASA* homolog (*MVH*) [4, 35]. From 11.5 to 12.5 dpc, PGCs undergo epigenetic reprogramming, including genome-wide demethylation, removal of parental imprints [36], histone modifications [37], and activation of the X chromosome [38, 39]. Finally, the PGCs enter mitotic arrest in males or prophase of meiosis I in females [40, 41].

In the mouse, germ cell competence of epiblast cells is induced in response to signals from the extra-embryonic ectoderm, including bone morphogenic proteins (BMPs) belonging to the transforming growth factor-β (TGF-β) superfamily. For example, when epiblasts are stimulated with a combination of BMP4 and BMP8b, PGCs are produced [42–45]. It is assumed that BMP2 functions in PGC specification, since inactivation of BMP2 results in fewer PGCs [42, 43, 46].

Of course, PGC specification is also strictly controlled at the level of gene expression. Certain genes such as *Blimp1*, *Stella*, *Fragilis*, *c-Kit*, *VASA* (*MVH* in mouse), *DAZ*, and *DAZL* play crucial roles in the appearance and development of PGCs. During the first step of germ cell determination, *Fragilis*-expressing cells appear and *Blimp1* expression is transiently induced [47]. *Fragilis*, a member of a larger family of interferon-inducible genes, encodes a transmembrane protein and is the first gene to mark the onset of germ cell induction. *Fragilis* may serve to increase the length of the cell cycle in PGCs. Blimp1 is a zinc-finger containing DNA-binding transcriptional repressor. This molecule functions as a master regulator in the foundation of the mouse germ cell lineage together with its partner molecule Prdm14. Expression of *Fragilis* is increased in migratory PGCs, and in turn induces expression of other germ cell-specific genes such as *Stella* and *VASA* [48]. Stella is a SAP-like domain and splicing factor motif-like structure-containing protein that may function in chromatin remodeling or RNA processing during the development of PGCs [49]. *VASA* is a widely conserved gene that encodes an ATP-dependent RNA helicase with a DEAD-box. It is capable of unwinding double-stranded RNA loops to promote the translation of germ line-specific genes [50, 51]. The tyrosine-kinase receptor c-Kit and its ligand, Stem Cell Factor (SCF), are also essential for maintenance of PGCs. SCF is expressed in Sertoli cells [52]. It is assumed that it functions in spermatogenesis. DAZ (deleted in azoospermia) is a RNA-binding protein that belongs to the DAZ family. Men with deletions encompassing the DAZ genes on the Y-chromosome show significant defects in germ cell generation, indicating that they are defective in the formation and maintenance of germ cells. In humans, the *DAZ* gene family encompasses genes such as *BOULE*, *DAZ-like* (*DAZL*), *PUM2*, and *DAZ* encoding translational regulators [4, 53]. DAZL is expressed throughout gametogenesis and is involved in the translational regulation of Vasa/MVH and synaptonemal complex protein 3 (SYCP3) in meiotic cells [54]. Haston et al. [55] showed that disruption of *DAZL* in mice affects multiple attributes of germ cell differentiation, including failure to erase and re-establish genomic imprints on PGCs. Loss of DAZL function in mice also decreases the number of post-migratory, pre-meiotic PGCs and reduces their ability to undergo normal meiosis [54].

Elucidating gene expression profiles and the functions of master genes for germ cell specification has at least two purposes: (1) By using our knowledge to increase the

expression level of certain genes, germ cells may be induced more effectively; and (2) By using these genes as monitors, we can evaluate precisely the methods for germ cell induction.

Recent advances in the germ cell differentiation from iPS cells

Many of the foundational studies on pluripotency and differentiation to germ cells involved ESCs. Toyooka et al. [56] discovered that mouse ESCs (mESCs) can differentiate into PGC-like cells that is capable of engraftment into testis and of forming sperm. Subsequently, Hubner [57] and Geijisen [58] demonstrated that ESC-derived germ cells can give rise to blastocysts. In 2006, fertilization of mouse oocytes with ESC-derived haploid cells induced in vitro resulted in generation of live offspring, although these expressed phenotypic abnormality and died prematurely [59]. Appearance of PGCs and haploid cells from human ESCs (hESCs) has also been observed [4, 60, 61]. The above studies were based on spontaneous differentiation of pluripotent stem cells, but germ cell differentiation is strongly dependent on signaling molecules and the gonadal microenvironment. Therefore, providing the correct culture conditions is critical for inducing germ cell differentiation from pluripotent stem cells in vitro. Co-culture systems and conditioned medium have been used to recapitulate the gonadal microenvironment for differentiation of germ cells. Co-cultures containing fetal gonadal stromal cells [62], Sertoli cells [63], or embryonic fibroblasts [64] increase the differentiation of pluripotent stem cells to PGCs. Although these co-culture systems may produce the desired results, induction with chemically or biologically defined factors is preferred, because it increases the safety of cells for clinical applications and improves the reproducibility of the differentiation process. Thus, supplementation of defined media with growth factors is usually the option for inducing differentiation. For example, BMP4 and BMP8b promote the differentiation of ESCs into PGC-like cells [43, 44, 65], and retinoic acid (RA) can be used to stimulate meiosis [66]. In addition, SCF [67], leukemia inhibitory factor (LIF) [66, 68], forskolin, GDNF (a cytokine found to support in vitro self-renewal of spermatogonial stem cells) [69], and adenylate cyclase activator [66] enhance germ line differentiation of pluripotent stem cells. Manipulation of gene expression can also be used to control lineage specification of differentiating pluripotent stem cells. For example, overexpression of *DAZL* and *VASA* promotes PGC formation from hESCs, and overexpression of *DAZ* and *BOULE* promotes the development of haploid germ cells [70].

Recently, several reports have demonstrated production of germ cells from iPSCs. Park et al. [62] first reported the production of PGC-like cells by co-culture with human fetal gonads. Panura et al. reported that BMP4 supplementation increases differentiation to *VASA*-GFP-positive PGC-like cells from hiPSCs. They went on to produce haploid cells by overexpressing *DAZ* [71]. Eguizabal et al. [66] also reported induction of haploid cells by culturing the iPSC-derived PGCs in forskolin, LIF, bFGF and an inhibitor of CYP26 (a P450 enzyme that catabolizes active all-trans RA into inactive metabolites). Medrano et al. [72] improved the rate of meiotic cell formation using plasmids to induce *VASA* and *DAZ* overexpression in hiPSCs. Meanwhile, Saitou and colleagues obtained fertile sperm and live mouse offspring from iPSCs by transplanting *Blimp1*-Venus-positive germ cells induced with BMP4/BMP8b/SCF/LIF and EGF-supplemented medium into infertile male mice [73]. Furthermore, they recently used iPSCs to reconstruct ovary-like tissues from Integrin-β3 +/SSEA1 + PGC-like cells and fetal gonadal cells. Once transplanted into the ovarian bursa of nude mice, these tissues generated oocytes that developed into live offspring [74].

However, despite these major advances, the conditions for producing germ cells that can develop into normal offspring entirely in vitro have yet to be discovered. At present, live offspring can only be obtained by transplanting immature germ cells that have been partially induced in vitro. So far, the function of these in vitro-generated germ cells has been demonstrated only in mouse. Germ cells develop in a species-specific manner under specific developmental periods, hormonal environments and structures of the reproductive organs. Therefore, we will have to examine the processes in animals that are more similar to humans. Non-human primate models may be required. In 2007, we demonstrated that cynomolgus monkey ESCs could differentiate into PGC-like cells [75]. Yamauchi et al. [76] improved the differentiation method by supplementing conditioned medium from testicular or ovarian cells with recombinant BMP4, RA, or SCF. Further studies, including functional assays in vivo, will reveal the molecular mechanisms that dictate primate germ line development and provide information that can be extrapolated to human germ cell differentiation. Recently, Hermann et al. demonstrated the feasibility of spermatogonial stem cell transplantation in a nonhuman primate alkaline chemotherapy-induced infertility model. They provided important evidence for donor spermatogenesis in both autologous and allogeneic transplant recipients [77]. These findings lay the groundwork for development of future pluripotent stem cell-based germ cell regeneration technologies.

Challenges to overcome for therapeutic utilization of iPSCs

Although iPSCs could open a new door for reproductive medicine, a number of significant hurdles clearly exist

before these cells can be used in clinical settings. The most important issue is whether the induced germ cells from iPSCs are "normal" enough to be transplanted as a conceptus. Recently, several groups reported that many iPS cell-lines contain somatic coding mutations, copy number variations, and aberrant epigenetic reprogramming [78–80]. Although there is conflicting evidence, iPSCs were found to retain epigenetic memories from their cell type of origin that influenced lineage specification [81]. Moreover, iPSCs from fragile X syndrome patients failed to reactivate the fragile X mental retardation 1 (FMR1) gene [82]. These genetic and epigenetic changes must not be carried over to the genome of the fetus. These findings suggest that the genetic quality of iPSCs may require more rigorous testing before they can be applied in clinical settings. There may be some cases where iPSC use may be inappropriate.

Another issue is the low efficiency of differentiation of germ cells from pluripotent stem cells. As described above, development of germ cells occurs through multiple differentiation processes within the surrounding germinal tissue. Therefore, step-wise differentiation protocols based on precise levels of signaling molecules such as cytokines, transcription factors, or cell adhesion molecules will be required to improve the differentiation methods. To date, an appropriate protocol for full term in vitro development of sperm or oocytes has not been discovered. However, in order to avoid tumorigenesis of transplanted pluripotent stem cells, it will be necessary to develop such a process. Thus, a comprehensive understanding of germ cell development factors will be essential. Furthermore, some differences are often observed in the gene expression patterns and timing of germ cell appearance from PGCs in vivo. Some researchers have observed haploid cell marker expressions at only 14 days after induction [68, 70, 75]. It is unlikely that this event reflects the precise developmental timing of germ cells and has motivated us to improve the systems used for differentiation.

The last issue relates to the differentiation propensity of pluripotent stem cells [83]. Miura et al. [84] reported that mouse iPS cell-lines produced from different tissues varied in propensity to form teratomas. In any case, patient-specific hiPSC lines obtained from different tissues of the same patient may produce different outcomes in terms of germ cell differentiation rate and quality. Furthermore, there are some cases in which germ cells cannot be produced because of mutations in the patient's genome. For these types of patient-specific iPSCs, Soldner et al. suggested a sophisticated strategy using zinc-finger nuclease (ZFN)-mediated genome editing. Using this technology, they established isogenic iPSCs possessing point mutations for genetic forms of Parkinson's disease [85]. It is possible that these technologies can also be applied to reverse genetic mutations in iPSCs that are linked to severe infertility caused by point mutations that result in abnormal germ cell generation [86] and/or function [87], or non-functional accessary cells [88].

Conclusion

The development of iPSCs has opened the potential to treat many intractable diseases. These iPSC technologies will allow us to manipulate the differentiation of patient-specific iPSCs to improve our understanding of diseases and assist in the development of new drugs and treatments. If the induction system enables the production of "functional" and "completely normal" germ cells, it will resolve many problems that cause infertility (Fig. 2). However, we are currently facing several challenges for clinical use of iPSCs, and we are just beginning to develop methods for generating germ cells in vitro. The process of germ cell formation is complicated. In normal gametogenesis, both sperm development and oocyte growth and maturation require support from Sertoli cells, theca, or granulosa cells.

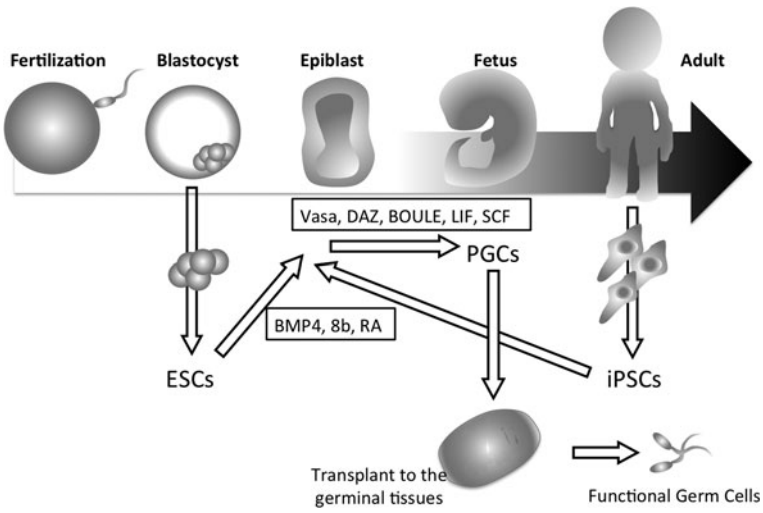

Fig. 2 Pluripotent stem cell-based germ cell generation

Understanding the supportive niche for germ cell development in vivo will be essential for obtaining "functional" germ cells at more advanced stages in vitro. Although many challenges remain, the generation of gametes using these approaches may improve the future of reproductive biology and medicine.

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