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Embryonic Stem Cells and the Germ Cell Lineage

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1. Introduction

Stem cells possess the unique ability to either propagate by self-renewal or to differentiate to mature tissues under the influence of appropriate molecular cues. This remarkable feature, also termed "stem cell potency" has been the focus of both a historical and current research spotlight on stem cells, and particularly the potential for human stem cells in regenerative medicine and organ transplantation. There are several classes of stem cells, all varying in degrees of pluripotency. Depending on the developmental stage from where they originate, stem cell potencies range from totipotency (ability to transform into all cell types), pluripotency (most cell types), multipotency (many cell types), oligopotency (few cell types) to unipotency (one cell type) (Smith, 2001). Among these, embryonic stem cells (ESCs) represent the prototypical pluripotent stem cells.

Embryonic stem cells are localized to the inner cell mass of the developing mammalian blastocyst and can give rise to all future cell types and tissues of the organism. Additionally, a small population of embryonic stem cells is allocated to the germline (primordial germ cells). The evolution of these germline stem cells is different from the remaining cells which undergo gastrulation and give rise to the three germ layers (endoderm, mesoderm and ectoderm) during development (Ewen & Koopman, 2010). The survival, gonadal migration and proper epigenetic programming of primordial germ cells (PGCs) are major, distinct and early events that have an impact on future fertility and the successful transmission of genetic information from parent to offspring.

Finally, stem cells found in neonatal tissues, amniotic fluid, cord blood, and adult tissues are categorized as adult stem cells and are usually multipotent, oligopotent or unipotent. Adult stem cells are clinically important due to the absence of ethical and federal restrictions on their use. Furthermore, they are valued for their relative abundance and accessibility in somatic tissues. In contrast, human embryonic stem cells have been the focus of substantial research and discussion because of their unique potential to differentiate into almost all body cell types and tissues and the relative ease with which they are propagated in cell culture. The search for an alternative source of pluripotent stem cells other than from human embryos is a much sought after goal in the stem cell research community for several reasons. First, diversity in stem cells sources should be explored to better understand and evaluate their various clinical utilities. Second, immunological matching of stem cells to recipients is necessary to avoid rejection in stem cell-based regenerative therapies. Hence, the advent of induced pluripotency and nuclear reprogramming strategies have been well

received as alternatives to derive human stem cells (Yamanaka & Blau, 2010). Finally, the derivation and use of stem cells require adherence to moral, ethical and political guidelines that are constantly changing. This chapter will review the formation of the human germline from embryonic stem cells and the subsequent derivation of gametes from mouse and human pluripotent stem cells, with an emphasis on the male germline.

1.1 Mouse and human embryonic stem cells

The first forays into understanding pluripotency in the mammalian embryo began with rather simple experiments in which pieces of mouse germ cell tumors known as teratocarcinomas were grown in culture and a variety of pluripotent cell types emerged (Solder, 2006). One cell type, termed Embryonal Carcinoma Cells (ECCs), produced all three germ layers as well as the original tumorigenic cells when isolated and transplanted into host mice. This established for the first time the pluripotent nature of embryonic cells. In the mouse pre-implantation embryo, embryonic stem cells (mESCs) first form within the inner cell mass (ICM). In studies by Evans and Kauffman and others, cells of the ICM were isolated from late blastocysts and grown in culture (Evans & Kaufman, 1981; Martin, 1981). These cells proliferated and demonstrated the capacity of self-renewal. The subsequent addition of Leukemia Inhibitory Factor (LIF) promoted the self-renewal of mESCs in culture by acting through the Stat3 pathway (Ying et al., 2008). Further studies showed that the ICM-derived cells could, if reintroduced into an embryo lacking an ICM, recolonize and form all three germ layers of the embryo, including the germline (Bradley et al., 1984). MESCs are characterized by the strong and persistent expression of important pluripotency genes such as *Oct4, Sox2, Nanog* and *Tcf,* among others. Thus, in mice, the pluripotent nature of cells derived from the inner cells mass was established relatively rapidly and easily than in the human.

In the human, embryonic stem cells (hESCs) are derived in a very similar manner to mouse ESCs from explants of the Inner Cell Mass (ICM) of *in vitro* fertilized blastocysts. Thomson *et al.* first isolated hESCs from pre-implantation human blastocysts and analyzed their morphology and expression of pluripotency genes (Thomson et al., 1998). Although they express high levels of pluripotency genes, hESCs share a morphology that is more consistent with that of early epiblast stem cells of the mouse (mEpiSCs). Specifically, they are "flattened." In addition, they depend on basic FGF and Activin signaling for self-renewal, possess large nuclear to cytoplasmic ratios, and respond less favorably to single cell clonability in contrast to mESCs (Vallier et al., 2005, 2009). While the TGFβ/Activin/Nodal signaling pathways are undoubtedly important for hESC pluripotency, the molecular mechanisms underlying these observations have yet to be explained. Meanwhile, the transcription factors Oct4, Nanog and Sox2 are clearly all involved in hESC pluripotency and newer molecules such as the WNTs (Wingless-Type MMTV Integration Site Family Members) have been implicated to drive hESC self-renewal through canonical signaling pathways (Sato et al., 2004). The functions of Oct4, Sox2 and Nanog are regarded as indispensable for hESC self-renewal as their expression typically reduces when hESCs differentiate. Finally, hESCs and mESCs are similar in that they both express a reportoire of cell surface proteins that distinguish them from other cell types, including SSEA-3, SSEA-4, Tra1-60 and Tra1-81, markers that are routinely used for characterization of ES and iPS cell lines.

Despite the fact that mESCs and hESCs are derived from seemingly equivalent epiblast cells in the blastocyst, there remains the question of whether hESCs possess as 'naïve' a state of pluripotency as do mESCs. This is because mESCs in culture behave more like the early

mouse epiblast and demonstrate unrestricted potential for differentiation (Hanna & Jaenisch, 2010). Meanwhile, hESCs share some properties with mouse epiblast stem cells (pluripotency) but may be more restricted than mESCs (Vallier & Pedersen, 2009). This may be the case because current hESC derivation methods from ICM could actually select for cells in a different developmental stage than that from the mouse ICM (Nichols & Smith, 2011). Additionally, it has been observed that ESCS and embryonic germ cells (EGCs) share several biomarkers making it difficult to distinguish between the embryonic, pluripotent cells and primitive germ cells. The intimate relationship that exists between germline and embryonic stem cells in mice and humans is critically important to understand in the future, and will vastly improve current and future efforts to derive 'artificial' gametes.

1.2 Nuclear reprogramming and induced pluripotency

A set of landmark studies conducted by Yamanaka and others recently revealed that just four factors normally produced in the embryo are sufficient to reprogram differentiated, non-embryonic cells into pluripotent stem cells. This important finding bypasses many of the ethical issues associated with the use of human embryos, and also evades potential problems with immune rejection that might affect patients upon stem cells transplantation. The four Yamanaka reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, when introduced into differentiated cells in excess amounts, reverses cell fate and by inducing these cells to revert back to a progenitor-like or ESC-like state. It is believed that this is a stochastic process whose mechanisms lie in the ability of these four transcription factors to induce epigenetic restructuring of the genome, thereby enabling expression of previously silenced genes (Hanna & Jaenisch, 2010). These artificially created pluripotent stem cells are termed induced pluripotent stem cells (iPSCs) and were first described by Takashaki and Yamanaka in rats in 2006 (Takahashi & Yamanaka, 2006). The same researchers also demonstrated that they could derive iPSCs from adult human cells, a finding subsequently confirmed by others (Nakagawa et al., 2008; Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007).

A major concern with iPS cells is the level of genomic disruption that occurs with viral delivery of the reprogramming factors using retroviral or lentiviral vectors. Theoretically, these viral vectors, by integrating randomly into the host genome, may cause chromosomal translocations or transform reprogrammed cells into malignant cells. There are now several initiatives underway that seek to understand the degree of genome-wide mutations and changes in gene function that occur in iPSCs (Panopoulos et al., 2011). The art of generating pluripotent cells now involves making iPSCs with reprogramming proteins using techniques that both avoid the use of embryos and the downstream hazards of viral vectors. Additionally, it is now possible to employ just the reprogramming proteins of these factors without the use of viral vectors to create iPSCs (Zhou et al., 2009). Several gene delivery systems that have been described include employing excisable polycistronic cassettes (Sommer et al., 2010; Somers et al., 2010), *piggy*BAC introduction (Yusa et al., 2009), transfection and/or nucleofection of plasmids (Okita et al., 2008) and most recently, mRNA and miRNA delivery (Warren et al., 2010). There is also evidence that small chemicals can be used as agents to induce reprogramming in somatic cells (Zhu et al., 2010). Finally, direct reprogramming methods wherein cells from one lineage are directly trans-differentiated to another lineage (e.g. fibroblast to neural-like lineage) are gaining in popularity. Specifically, terminally differentiated cells like skin fibroblasts can be exposed to a cocktail of factors that simultaneously enable reversal of cell fate and induce differentiation to the second lineage

(Ieda et al., 2009; Uhlenhaut et al., 2009; Vierbuchen et al., 2010). The use of human, patient-specific iPSCs in regenerative medicine represents an exciting clinical avenue for the future, and will likely find application to human germ cell development.

1.3 Ethical and technical concerns in the use of stem cells

Among of the many ethical, technical, and safety concerns associated with the clinical application of stem cells, the primary concern surrounds the stem cell source. Human embryonic stem cells have generated the most controversy because the procedure of deriving a hESC line involves the sacrifice of a donated human embryo, even though the donated embryos most likely will be discarded (reviewed in Greely, 2006; Scott & Reijo Pera, 2008). Therefore, alternative sources of stem cells, such as iPSCs, which might achieve similar therapeutic goals, could be very valuable and bear fewer ethical issues than embryonic stem cells. Since one of the standard measures of a pluripotent stem cell is that it reliably forms teratomas when transplanted into animal models, careful long-term analysis and follow-up after transplantation will be needed in animal models before stem cells or directed differentiated cells are applied to humans. Yet, hESCs still represent the best model system with which to study early human development because of their differentiation potential and epigenetic programming and therefore remain the standard for studying stem cells properties. However, their suitability for transplantation studies is questionable mainly because of the risk of immune rejection from host human tissues (Swijnenburg et al., 2008). In contrast, patient-autologous iPSCs are considered more suitable for clinical transplantation due to better immunological suitability, but their differentiation profiles, epigenetic status, and potential for tumor formation are far less well characterized than are hESCs (Hyun et al., 2007). Therefore, it is imperative that studies with hESCs continue to allow better definition of the true potential of all other stem cell sources, including human iPSCs. Fortunately, there is currently considerable research devoted to deciphering the molecular mechanisms that underlie the cell fate reprogramming process. In parallel, many laboratories are now developing iPSC-based disease models for human degenerative diseases and cancers. These *in vitro* studies should provide the research community with insight into the mechanisms of disease progression and illuminate avenues of therapeutic potential. Consequently, hESCs will likely remain the "gold standard" stem cell type for developing basic scientific and clinical therapeutic protocols, and to which iPSCs and adult stem cells will be carefully compared for their therapeutic potential.

2. Mammalian germline formation

2.1 Primordial germ cell specification and candidate genes controlling germline establishment

The establishment of early germ cells and their successful maturation are complex processes, and require frequent changes in physiology, location and transcriptional profile of involved cells. Germline establishment in mammals occurs via "inductive" signaling, in contrast to lower organisms such as flies and worms where the germ cell identity is transmitted via the inheritance of germ "plasm" (McLaren, 1999, 2000; Saitou et al., 2002). In rodents and humans, the first glimpses of primordial germ cell (PGC) formation are observed in the embryo after implantation and gastrulation, when the epiblast, endoderm, mesoderm and ectoderm are first established (Matsui & Okamura, 2005). At this time, in response to molecular cues including Bone Morphogenetic Proteins (BMP4 and BMP8a) from the yolk sac, a population of pluripotent stem cells is segregated from the ICM and set physically

apart from the extra-embryonic ectoderm or yolk sac of the embryo (**Figure 1**) (Lawson et al., 1999; Ying 2000, 2001). While BMPs provide the inductive signal to epiblast stem cells to supply PGCs, it is not clear what signals control the size of the PGC founder population or what molecules signal the termination of PGC specification. BMPs are crucial to specification of PGCs due to their activation of the ALK2 receptor and Smad-1/5 signaling pathways, as evidenced in mice (Hayashi et al., 2002). In the human embryo, the effects of BMPs on germline specification are unclear because there is very limited access to early embryonic samples. From work in our laboratory, it appears that similar BMP protein pathways are activated in the human embryo during gastrulation that direct PGC specification (Clark & Reijo Pera, 2006, Kee et al, 2006).

Additional mechanisms that may assist germline specification are the activation of pathways that either promote PGC survival and/or inhibit molecules that promote somatic differentiation of PGCs (Ewen & Koopman, 2010). As such, mouse and human PGCs retain expression of several biomarkers of pluripotency, including Oct4, Nanog and Sox2 which underlies their close resemblance to other pluripotent stem cells (Clark & Reijo Pera, 2006; Medrano et al., 2010; Nicholas et al., 2009). Interestingly, the transcription factor Sox2 is expressed on both mouse and human embryonic stem cells but unlike in the mouse, human Sox2 expression is diminished when PGCs migrate to the fetal gonad (Perrett et al., 2008). Coincident with the timing of germline specification in the mouse (E7.5), PGCs near the extraembryonic ectoderm begin to express germ-cell specific markers such as Blimp-1 (*Prdm1* in human), Stella (*Dppa3* in human), E-Cadherin, and Dazl and harbor alkaline phosphatase activity. Blimp-1/Prdm-1 is a transcriptional repressor whose activity is restricted to the germ lineage and appears to be critical for maintaining a PGC fate. There is strong evidence to suggest that in the mouse, Blimp-1 actively represses the somatic fate of PGCs by inhibiting expression of key somatic regulators during development (Ohinata et al., 2005; Hayashi et al., 2007). It is unclear whether Prdm-1 carries out a similar function in human germline formation. Once cell-cell communication has been established, the *Fragilis* (IFITIM-1 in human) and *Stella/ Dppa3* genes promote further development of PGCs and may do so in a similar fashion as Blimp-1 in mice (Saitou et al., 2002).

Migration of germ cells to the gonad begins at E8.5 in the mouse and during weeks 4 to 6 of human gestation (first trimester). At this stage, PGCs accumulating at the base of the allantois exit the extraembryonic ectoderm and begin migration to the developing gonads, also known as the genital ridge. During migration, PGCs also proliferate by undergoing mitosis and express a new set of biomarkers, including the CXCR4 receptor in mice and the proto-oncogene c-KIT and its ligand, KIT in both mice and humans (Molyneaux & Wylie, 2004; Gomperts et al., 1994). The *DAZ* gene homologue, DAZL is also expressed on migrating PGCs. In the mouse, migratory PGCs display pseudopodia that may assist in movement through the hindgut and it is plausible that human germ cells behave similarly since they are also observed in the hindgut during migration. Various somatic tissues interact with PGCs during the migratory path to the gonad. It is likely therefore, that these tissues express molecules and factors that guide or "cue" the PGCs and help maintain their survival. In the mouse, several candidate molecules have been identified, including receptors such as β-1 Integrin and extracellular matrix components such as Collagen I (Chuva de Sousa Lopes et al., 2005; De Felici et al., 2005). Although migration of PGCs is less well understood in human development, germ cells have been histologically observed during the late first trimester, when they undergo migration to the hindgut (Fujimoto et al., 1977; Gaskell et al., 2004; Goto et al., 2004). Male and female PGCs have been isolated from

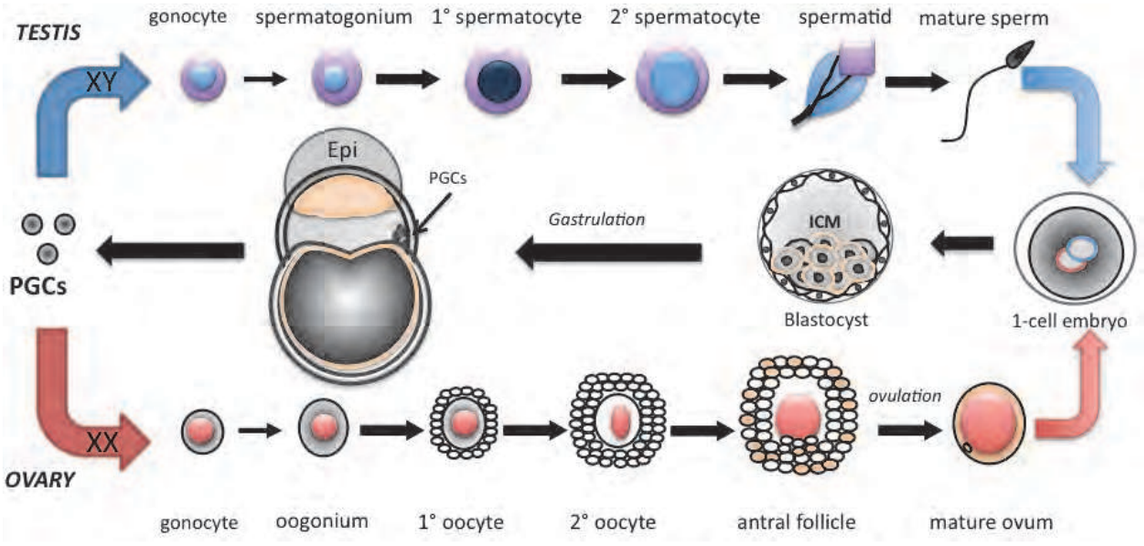

Fig. 1. Developmental Cycle of Mammalian Germ Cells.
Life cycle of the mouse and human embryo following fertilization, progressing through gastrulation and producing the germline. The germline develops in the gonads and transmits genetic information to the next generation, thus completing the cycle. Fertilization of oocytes by sperm promotes the formation of a 1-cell zygote that undergoes cell division and cleavage to form a blastocyst. The outer layer of blastocyst gives rise to the trophectoderm while the inner cell mass (ICM) contains embryonic stem cells (ESCs). During gastrulation (E7.5 in mouse; Day 15+ in human), the blastocyst cavitates and develops the three germ layers and the epiblast. The primordial germ cells (PGCs) are specified and localize near the extra-embryonic ectoderm, at the base of the allantois. Once PGCs are specified, they migrate to the fetal gonads and undergo sex-specific developmental to male and female gonocytes. Subsequently, male gonocytes undergo spermatogenesis while female gonocytes enter meiotic prophase I and begin oogenesis. Adapted from Schuh-Huerta et al., 2011.

10-week old fetuses and were observed to express Alkaline Phosphatase (AP), a marker of PGCs (Goto et al., 2004). The morphology of human PGCs also resemble the rounded shape of mouse PGCs. Female-specific germ cells have also been visualized at the ultrastructural level during gonadal development in human fetuses (Motta et al., 1997). Finally, recent studies by Kerr et al. with human fetal gonads (testis and ovary) provide a detailed analysis of pluripotency and germ cell-specific markers. The germ cells of the fetal testis are Oct4 +/Nanog +/ c-Kit+ from week 7 to 15 after which these cells become localized to the testis periphery. Meanwhile, the presumptive gonocytes in the week 15 testis show strong expression of Pumilio2 (PUM2), VASA and DAZL and express low to no pluripotency markers (Oct4, Nanog, c-Kit, Tra1-60, Tra1-81) (Kerr et al., 2007). In the human fetal ovary, as in the testis, the expression of pluripotency markers peaks by week 8 and then declines after week 9, as oocytes enter meiosis (Kerr et al., 2008). Interestingly, the cell surface markers SSEA-1 and SSEA-4 are co-expressed on the female germ cells from week 5 onwards although only SSEA-1 is restricted to the germ cell lineage.
Upon arrival at the genital ridge, germ cells express another germ-cell specific marker, VASA, a cytoplasmic protein that is implicated in translational regulation. The gene

encoding VASA expression, *DDX4* (*Mvh* in mouse) is highly conserved among species and is expressed exclusively in both male and female pre-meiotic germ cells (Gustafson & Wessel, 2010). This finding underlines the importance of the VASA protein in germline function and makes it an attractive candidate for further study. Along with VASA, other factors produced are germ cell nuclear antigen-1 (GCNA-1) and E-cadherin. The sex-specific character of the developing gonad is controlled by the chromosomal constitution of gonadal somatic cells. In particular, the *SRY* gene expressed on the Y chromosome in mammals is thought to be an essential regulator of various downstream targets including the *Sox9* gene that controls male gonadal development (McLaren, 1995, 2003). Once within the gonad, germ cells associate with Sertoli cells to form testis cords and this interaction induces the expression of VASA in post-migratory PGCs. VASA expression is induced in both male and female PGCs and persists until these cells enter meiosis and after which its levels diminish (Castrillon et al., 2000; Toyooka et al., 2000).

During germline development, an extensive remodeling of the epigenetic landscape occurs. This takes place during embryogenesis and during PGC migration to the gonad (Nicholas et al, 2009). The first wave of epigenetic remodeling occurs during implantation of the blastocyst and involves the erasure of all DNA methylation at CpG islands except those at imprinted gene loci. This transition is observed in all cells of the embryo, including the primitive germline. In female PGCs, there is another level of epigenetic change in the form of random X inactivation wherein one copy of the X chromosome pair is silenced. The second wave of epigenetic remodeling occurs when PGCs migrate to the primitive gonads and their paternal or maternal imprinted loci undergo a gradual process of erasure (Hajkova et al., 2002; Yamazaki et al., 2003). This phase occurs only in germ cells and may help to prime them for sex-specific DNA remethylation, when their developmental programs are established (Durcova-Hills et al., 2006). In the mouse, the re-establishment of imprints takes place prior to birth in the male prospermatogonia (E15) and only after birth in oocytes (Lucifero et al., 2002). In addition to DNA methylation changes, male and female germ cells also undergo post-translational histone modifications and RNA-mediated silencing (Reviewed in Tasler, 2009 & Nicolas et al., 2009).

The successful passage of germ cells through meiosis is a unique and highly rigorous process. However, between male and female embryos, the timing of meiosis is different. Male germ cells are restricted from entering meiosis while female germ cells enter meiosis within the embryo. Although the mechanisms for these contrasting behaviors are unclear, it appears that the gonadal cells provide the signal for (or against) meiotic entry (Brennan & Capel, 2004; Ewen & Koopman, 2010). One signal could be retinoic acid (RA) produced in the fetal ovary, which in turn induces *Stra8*, a key regulator of meiotic entry. In female germ cells destined to become oocytes, mitotic divisions cease and meiotic prophase begins with the correct stimuli (Borum, 1961); eventually oocytes arrest during Meiosis I prior to fetal birth and will only resume meiosis upon receiving hormonal signals during adulthood (Peters, 1970). Meanwhile, male germ cells transition from primordial status to the gonocyte stage, stop proliferating and remain quiescent in the fetal seminiferous tubules until after birth. The post-natal gonocytes then commit to a spermatogonial stem cell (SSC) fate and amplify through self-renewal or enter meiosis to initiate spermatogenesis. In both male and female germ cells, the synaptonemal complex proteins (SCPs) SCP-1, SCP-2 and SCP-3 are critical components of the meiotic machinery during chromosomal segregation (Chuma et al., 2001; Parra et al., 2004; Yuan et al., 2000). The completion of meiosis signals that germ cells have matured into haploid male and female gametes. At this stage, oocytes exclusively

express GDF9 and spermatocytes express TEKT1. However, one feature that distinguishes human and mouse germline differentiation is the synchronization of meiotic entry, as in human fetal gonads, one can observe both pre-meiotic and meiotic germ cells in close proximity (Anderson, 2007).

Significant efforts have been made to culture mouse and human PGCs and gonocyes *in vitro.* In the case of mouse germ cells, the addition of endogenous factors known to affect germ cell development such as BMPs, RA, LIF and Forskolin have produced mixed results in maintaining PGCs in culture. For example, adding LIF enhanced PGC survival but the observations with the use of other factors not as clear. PGCs may also behave erratically in culture (showing low survival rates and non sex-specific behavior) because of the lack of a normal somatic environment (Childs et al., 2008). Studies of PGCs by Shamblott et al., Turnpenny et al. and Tu et al. with fetal human gonocytes resulted in a mixture of cellular phenotypes. Some gonocytes appeared rounded while others took on an elongated or 'spindly' appearance. In addition, they appeared to have different proliferation rates (Shamblott et al., 1998; Turnpenny et al., 2003; Tu et al., 2007). Currently, it is not at all clear that these cells resemble their germ cell counterparts *in vivo,* but improvements in culture conditions and the cellular microenvironment will certainly help in this regard.

2.2 Spermatogenesis and early oogenesis

As delineated earlier, mammalian germ cells populate the testis and ovary during development in a incredibly dynamic manner. During early prenatal mice and human embryo development, PGCs migrate to the primitive gonad (gonadal ridge) and associate with Sertoli cells to form primitive testicular cords (Brennan et al., 2004). Within the testicular cords, the primitive germline stem cells (now termed gonocytes) remain in the testis as the gonad differentiates. Eventually, Sertoli cells, peritubular myoid cells and gonocytes form more compact structures known as the seminiferous tubules. When the gonocytes migrate to the periphery of the tubules, they transform into prospermatogonia and then into spermatogonia (Gondos & Hobel, 1971). In the fetal testis, prospermatogonia enter mitotic arrest, a feature observed at E12.5 to E14.5 in the mouse. At the molecular level, during entry into meiosis, both male and female human gonocytes express DAZL proteins and *Vasa* transcripts and downregulate OCT3/4 expression (Anderson et al., 2007). Interestingly, it is the early migrating germ cells that share similar properties with embryonic stem cells and testicular germ cell tumors (Ezeh et al., 2005). From what is known, the development of gonocytes in the fetal ovary follows a similar path in that OCT3/4 expression is reduced while VASA, Germ Cell Nuclear Antigen (GCNA) and DAZL are expressed (McLaren, 2003). In contrast to the male gonocytes, the female gonocytes receive sex-specific signals from the fetal gonad to enter meiotic prophase. After initiating meiosis, the female gonocytes will develop into primordial follicles and subsequently into primary follicles at puberty. A key difference between mouse and human systems is the timing of primary follicle formation: the mouse achieves this stage at birth while in the human ovary, this occurs at puberty (Bukovsky et al., 2005). There is some speculation whether this difference in follicle development is due to autocrine signals produced from the oocyte itself or from the ovarian environment (Hutt & Albertini, 2007). A plausible hypothesis is that the immediate environment of early germ cells determines whether they are commit to spermatogenesis or oogenesis. The most obvious source of signals are the mesonephros, primitive Sertoli cells in the testis, and primitive Granulosa cells in the ovary.

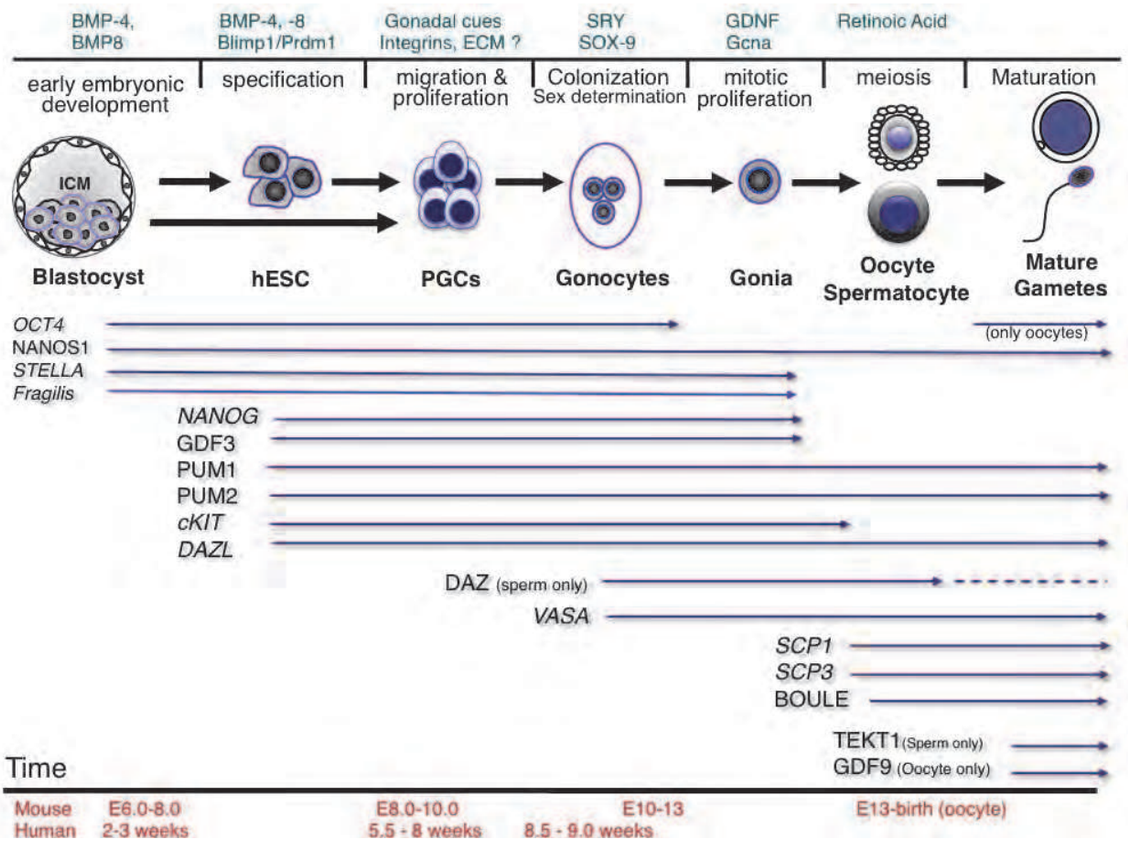

Fig. 2. Timeline of Germline Specification and Germ Cell Marker Expression. A temporal representation of the stages of human and mouse germline differentiation *in vivo*. At each cellular stage, important molecular and somatic signals controlling that stage are indicated above the diagram. Specific germ cell molecular markers are indicated on left with arrows depicting the duration of development during which they expression has been observed. Genes that are italicized are present in both mouse and human germ cells. At the bottom, an approximate timing of each stage during mouse or human germline development is indicated. Adapted from Schuh-Huerta & Reijo Pera, 2011.

The seminiferous tubule serves as the sperm production center, where approximately 123 x 10^6 spermatozoa are produced from germ cells daily, or about 1000 sperm/second (Amann et al., 1980; Rooij, 2009). Developing germ cells are arranged along the basement membrane in a highly ordered sequence and extend into the lumen of the tubule. At the most basal portion of the tubules lies the spermatogonial stem cell (SSC) population, closely associated with the adjacent Sertoli cells. Morphologic analysis of the various germ cells reveals at least 13 recognizable germ cell types in the human testis (Heller & Clermont, 1963, 1964). Each cell type is thought to represent a different step in spermatogenesis. From the least to the most differentiated, they have been named dark type A spermatogonia (Ad); pale type A spermatogonia (Ap); type B spermatogonia (B); preleptotene (R), leptotene (L), zygotene (z) and pachytene primary spermatocytes (p); secondary spermatocytes (II); and Sa, Sb, Sc, Sd_1, and Sd_2 spermatids (**Figure 3**). The early, type A spermatogonia are the most interesting germ cell type from a stem cell point of view (Rooij, 2009). In fact, time-course studies using GFP-based reporters with early type A, type Ad and type Ap spermatogonia in the mouse

revealed that the early type A cell has the ability to divide, self-renew, and give rise to the Ad and Ap sun-populations (Nakagawa T. et al., 2007). These observations provide evidence for the existence of SSCs in the testis and their clonal behavior is protypical of other adult stem cells.

It is currently thought that pale type A (Ap) spermatogonia in the basal, stem cell niche of the seminiferous tubule divide at 16-day intervals and differentiate to type B spermatogonia, which then become spermatocytes (Clermont, 1972). The ability of SSCs within the testis stem cell niche to undergo stem cell renewal is governed by several known factors. The growth factor-receptor kit ligand/c-kit receptor system and the niche factor glial cell line-derived neurotrophic factor (GDNF) are important in this process (Oatley & Brinster, 2008). In fact, spermatogenesis in the rat is dependent on c-Kit receptor activity, whereas spermatogonial stem cell renewal may be c-kit independent (Dym, 1994). GDNF appears to provide a significant stimulus to self-renewal of SSCs through receptors for GDNF on SSCs such as c-Ret and GFR-1α(Meng et al., 2000). Despite this, our knowledge of other receptor-ligand systems that control human SSC renewal is limited at this point. During spermatogenesis, the cytoplasm between spermatogonial daughter cells remains conjoined after mitosis, forming cytoplasmic bridges between adjacent cells (Ewing et al., 1980). Cytoplasmic bridges are thought to be important for synchronized cellular proliferation, differentiation, and possibly regulation of gene expression. Thus, SSCs and early spermatogonia in the adult testis are critical for germ cell renewal and differentiation into sperm and raise important questions about the source of proliferative and differentiation signals for spermatogenesis. The majority of stages in mouse spermatogenesis delineated above are translatable to the human germ cell development pathway except for differences in the timing of each stage during development.

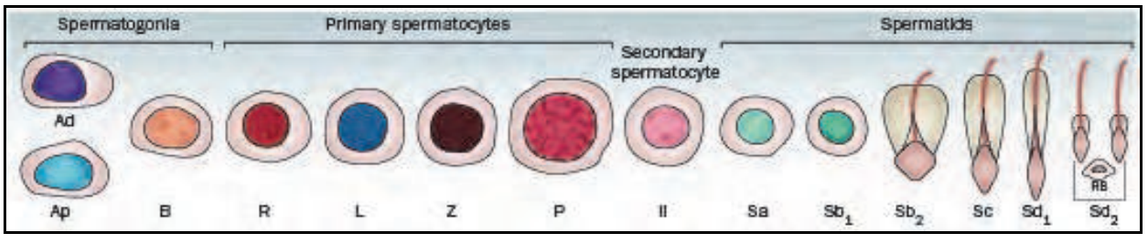

Fig. 3. Cells of the Human Germ Line Sequence. See text for details. With permission from Kee et al, 2009.

3. Germline stem cells

Under the premise that male and female gametes must be replenished, several investigators have queried the existence of elusive germline stem cells. Additionally, the pluripotency potential of embryonic germ cells and their similarity to mESCs has fueled the speculation about the existence of spermatogonial stem cells and their true differentiation potential both *in vivo* and *in vitro*. There is compelling data proving the existence and plasticity of testicular germline stem cells in the mouse and more recently the human testis. The exploration of germline stem cells in the human ovary has been confusing because of findings that challenged the long-held notion that females are endowed with a finite supply of oocytes. For instance, Johnson and colleagues observed that a small population of germline stem cells (GSCs) reside in the ovarian surface epithelium in the mouse and can undergo self

renewal of the follicular pool within 24 hours (Johnson et al., 2004). However, these results have not been corroborated (Telfer et al., 2005). Johnson et al. reported that bone marrow stem cells enter the ovarian follicular pool through the vasculature and could provide a source for ovarian stem cells (Johnson et al., 2005). Despite the proof of successful transplantation in this study, they failed to demonstrate meiosis in the oocytes generated from ovarian stem cells or bone marrow-derived stem cells (Telfer et al., 2005). Furthermore, it was not clear whether these ovarian stem cell-derived oocytes could be artificially fertilized. Based on these findings, the existence of germline stem cells in the adult ovary remains unknown.

3.1 Testicular stem cells in animal models

Evidence for stem cells in the adult testis was first provided by Brinster and colleagues in 1994 when they transplanted a putative SSC population into mice and observed complete spermatogenesis with transplanted cells (Brinster RL & Zimmermann JW, 1994). Recent research has sought to characterize the true stem cell potential of early spermatogonia within the seminiferous tubule. Kanatsu-Shinohara et al first reported the generation of pluripotent stem cells from the mouse testis (Kanatsu-Shinohara et al., 2004). In their study, a population of cells termed germ line stem cells (GSCs) were isolated from neonatal mouse testis and propagated in culture (Kanatsu-Shinohara et al., 2003). Subsequently, they found that cell colonies with morphology quite distinct from GSCs, termed ESC-like cells, also appeared in culture. The formation of both GSCs and ESC-like cells depended on a GDNF signal and only ES-like cells expanded in regular mESC media. The expression profile and imprinting profiles indicated that the ES-like cells were more like ESC than GSCs. The definitive test of pluripotency or multipotency in stem cells is to transplant them and assess their ability to form all three germinal layers. In the case of spermatogonial stem cells or their transformed cell types, the ability to form germinal layers and sperm was tested following transplantation. Interestingly, transplantation of ESC-like cells into seminiferous tubules gave rise to teratomas while GSC transplants led to spermatogenesis without teratomas. When ESC-like cells were transplanted via subcutaneous delivery, teratomas containing all three germ layers formed. These ESC-like cells also had the potential to develop into various other cell types, including mature germ cells, showed chimera formation and germ line transmission. More recently, a study by Simon et al. revealed that when putative mouse SSCs were recombined with organs of fetal or neonatal origin, these cells had the ability to transdifferentiate into cells of all three germ layers as well as uterine, prostatic and skin epithelia (Simon et al., 2009). Thus, these experiments in rodents reveal that GSCs and ESC-like cells have very different stem cell characteristics. Unlike with neonatal testis, the authors were unable to generate ESC-like cells from adult mouse adult testis.

A major finding was reported in 2006 by Guan et al. wherein they successfully derived pluripotent stem cells from the adult mouse testis (Guan et al., 2006). With the introduction of a *Stra8*-eGFP reporter in which GFP expression was regulated by *Stra8* promoter activity, ES-like cells were separated from a heterogenous population of testicular cells. When these isolated ES-like cells were grown on mouse embryonic fibroblasts or cultured in medium containing LIF, they evolved into a subpopulation of cells that the authors termed multipotent adult germline stem cells (maGSCs). The pluripotency potential of maGSCs was verified by RT-PCR, *in vitro* differentiation to all three somatic germ layers, and by

successful teratoma formation *in vivo*. Another transplantation study by Nakagawa et al. in 2007 employed the *Neurog3* (*Ngn3*) promoter to induce GFP expression and thereby enrich for two populations of undifferentiated spermatogonia (Nakagawa T. et al., 2007). The researchers took advantage of the expression of NGN3 on undifferentiated spermatogonia and, from their sorting studies, the first population of cells maintained the SSC progenitor pool through self-renewal while the second population revealed a pool of SSCs that diminished with age. More importantly, the study by Nakagawa and colleagues suggested that the population of SSCs in mice testes is ~2000 cells, substantially less than previous estimates of ~35000 cells (Nakagawa T. et al., 2007). A population of pluripotent stem cells in the adult mouse testis was again confirmed by Ko et al. in 2009. The authors showed that GSCs obtained from the adult mouse testis traverse through unipotency before conversion into multipotent ESC-like cells (Ko et al., 2009). They concluded that that the number of GSCs and culture duration are defining factors in deriving pluripotent stem cells from the germline: they termed these cells germline-derived pluripotent stem cells (gPSCs). In characteristic fashion of ESC-like cells, gPSCs demonstrated in vitro differentiation to all three somatic cell layers, teratoma formation and germ line transmission. Thus, several reports have documented the production of multi- or pluripotency germline stem cells in the mouse.

In parallel to these studies of mice multipotent germline stem cells, several groups have reported a collection of genes and biomarkers that are non-exclusively expressed by mouse SSCs (Reviewed in Caires et al., 2010). These include receptors and downstream facilitators of GDNF action such as GFR1α and RET proteins (Naughton et al., 2006). The gene *Ngn3* is also highly expressed on undifferentiated spermatogonial stem cells (Yoshida et al., 2004). The signaling activities of the PI3 Kinase family acting through AKT proteins are also important for SSC self-renewal because blocking these pathways suppresses GDNF activity and reduces spermatogenesis in mice (Oatley et al., 2006). Other molecules expressed on SSCs are *Oct4, Bclb6, Etv5, Lhx1* and *Nanos2* (Caires et al., 2010). In the future, we hope to employ these markers to more accurately isolate and definitively characterize putative SSC-like cells.

3.2 Testicular stem cells in the human

Based on the success in the adult rodent testes, several investigators have attempted the same types of studies in the human. In a landmark study, Conrad and colleagues devised a method by which they could harvest adult spermatogonial stem cells and subsequently reprogram them into pluripotent cells (Conrad et al., 2008). They employed the use of adult testis tissue from which they harvested a mixed cell population. The single cell suspension was then cultured *in vitro* in the presence of GDNF, as mouse studies have shown this growth factor to be critical for self-renewal of SSCs. They then purified SSCs by magnetic-activated cell separation (MACS) with CD49f bound beads. Further selection on collagen and laminin plates led to a highly enriched population of spermatogonial cells. When cultured in the same media used for hESCs and supplemented with LIF, the spermatogonial cells gradually arranged in multilayered, clustered colonies and displayed features consistent with pluripotent stem cells. The researchers termed these cells human adult germline stem cell (haGSC). Extensive characterization of haGSCs was then carried out by analyzing gene and protein expression and conducting microarray analysis for genome-wide changes in expression. Epigenetic reprogramming was assessed and various

pluripotency markers were measured. Finally, the cells were karyotyped and a teratoma formation assay was successful in immunodeficient mice (Conrad et al., 2008). Overall, the authors observed significantly different expression profiles in haGCSs compared to the parent spermatogonial cells, and haGSCs acquired expression profiles similar to hESCs. Although a complete analysis of a novel cell population in the human testis, several caveats to this research exist. Entire testicles were used to derive spermatogonial cells, bringing into question the feasibility of this approach for clinical use (Kee et al., 2010). In addition, the global gene expression patterns of plurpotency markers obtained from these haGSCs have been challenged as being more closely associated with fibroblasts than hESCs (Ko et al, 2010). Nevertheless, the above experiments underline the concept that adult germline stem cells and by extension PGCs may share basic properties with hESCs.

To address the issue of limited availability of starting material, an alternative protocol for generating human pluripotent stem cells was reported by Kossack et al., 2009. Their approach required only a testis biopsy as starting substrate. The biopsies were enzymatically digested and testicular cells released in suspension were grown in basic media until small colonies of cells began to form. Selection for cells in colonies was performed by manually dissecting out colonies and then propagation on mouse embryonic fibroblasts (MEFs) in hESC culture conditions, supplemented with bFGF. Under these conditions, the spermatogonial cells acquired a hESC-like morphology and physical characteristics, and were termed human multipotent adult germline stem cells (hMGSCs). Careful analysis revealed that hMGSCs were karyotypically nomal, expressed both pluripotency and early germ cell markers and contained a generally hypomethylated DNA pattern consistent with hESCs rather than somatic cells. Albeit spontaneously differentiating to cells of endodermal, mesodermal, and ectodermal lineages, hMGSCs did not successfully form teratomas *in vivo.* Therefore, despite a simpler methodology using limited substrate, this study suggested that hMGSCs, unlike true hESCs, might be only multipotent and not fully pluripotent. A third study also used testicular tissue fragments as a cell source from which to separate spermatogonial stem cells (Golestaneh et al., 2009). Using 1g of testicular tissue, much larger than routine testis biopsy, they also derived pluripotent stem cells after isolated seminiferous tubules were enzymatically digested and grown in hESC media (supplemented with bFGF and TGF-β but not grown on MEF feeders). After several passages, they observed medium-sized colonies of ~500 cells which were termed ESC-like cells. As expected, these cells expressed pluripotency markers and they were able to differentiate to endoderm, mesoderm, and ectoderm. Similar to the finding of Kossack et al, only small teratomas formed from the transplanted ES-like cells, and only when large cell numbers (~$2x10^6$) were used. Thus, this study also suggests that the pluripotency of these ES-like cells may not be identical to true hESCs.

As eluded to above, a major limitation of deriving SSCs is the requirement for generous amounts of human testicular tissue as starting substrate. Protocols need to be refined to allow pluripotent stem cell generation from the smallest amount of testis tissue substrate to make this technology feasible for clinical use. Another limitation is the low efficiency of isolating SSCs from testis tissue and of generating pluripotent stem cells from SSCs. Optimization of culture conditions may reduce these inefficiencies in the future. Despite this, research over the last decade has uniquely highlighted the stem cell potential of SSCs derived from the human testis and their remarkable similarity to the differentiation potential of ESCs. A major drawback has been the inability of the SSCs to consistently

produce large teratomas. This likely indicates that SSCs are only multipotent and not pluripotent (Kee et al., 2009), a distinction that has implications for the therapeutic potential of SSCs. Will they only be useful for obtaining sperm or will they have a broader potential for cell-based therapies in unrelated target tissues? Finally, the relatively invasive procedure for isolation of adult human germline stem cells from the testis compared to derivation of iPSCs may favor the latter approach as a preferred methodology for clinical use.

3.3 Azoospermia and genetic control of spermatogenesis

Human infertility is remarkably common, affecting 10-15% of couples. Most commonly, infertility is caused by defects in germ cell development (de Kretser, 1997; Skakkebaek et al., 1994). Microdeletions in the *AZF (Azoospermia Factor)* region of the human Y chromosome are the most common cause of human infertility and Non-Obstructive Azoospermia (NOA) in men. The associated clinical phenotypes are typically azoospermia or severe oligospermia, and on diagnostic testis biopsy reveal the complete absence of the germ cell lineage, to maturation arrest of spermatogenesis, to the production of a small number of germ cells (Gonzalves et al., 2005). The *AZF* region is highly repetitive and consists of numerous palindromic regions thought to have arisen out of repeated rounds of gene duplication and inversion (Kuroda-Kawaguchi et al., 2001; Skaletsky et al., 2003). The highly repetitive nature of the *AZF* region makes it particularly prone to homologous recombination between direct repeats and resulting in deletions. There is now a fairly comprehensive chromosomal map of the *AZF* regions with three specific regions currently identified, *AZFa, AZFb* and *AZFc,* that represent multi-gene segments on the Y chromosome (Kuroda-Kawaguchi et al., 2001). The most commonly deleted of these three is the *AZFc* region, resulting in phenotypes that range from complete absence of the germ cell lineage (termed Sertoli Cell Only [SCO] syndrome) to oligospermia (Reijo Pera, 1995, 1996). In spite of their deletion frequency, the function of genes that map to the Y chromosome *AZFc* are still poorly understood. This region harbors six multi-gene families including the *RBMY, PRY, VCY2, CDY1* and *DAZ* genes (Kuroda-Kawaguchi et al., 2001). Intriguingly, all of the protein-coding *AZF* genes are expressed exclusively in germ cells and some testicular cells, suggesting their importance in germ cell development (Navarro-Costa et al., 2010). Although precise functions have not been pinpointed, the predicted sequences and protein structures encoded by these genes provide a glimpse into their potential function. For instance, DAZ and RBMY genes are thought to be involved in RNA binding and transport, thereby positioning them as regulators of mRNA translation during spermatogenesis (Collier et al., 2005; Kee et al., 2009; Lee et al., 2006). Meanwhile, CDY and BPY2 genes are likely regulators of chromatin and genome organization (Caron et al., 2003; Lahn et al., 2002). Our overall understanding of infertility genetics will be greatly advanced by studying the molecular contribution of genes such as DAZ to meiotic recombination, DNA repair, epigenetic reprogramming and the development of cancer and other clinical aberrations.

4. From embryonic stem cells to germ cells

Our knowledge about mouse embryo and germline development from mESCs has allowed for *in vivo* and *in vitro* modeling of germline development. However, very limited access to early-stage human embryos and especially primordial human germ cells has greatly reduced the ability to conduct similar investigations into human germline development, let

alone modelling it *in vitro.* This section reviews the highlights of experiments conducted during the last decade using *in vitro* model systems of mouse and human germline development from mESCs and hESCs (Table 1).

Source of cells	Germline Differentiation Strategy	Endpoint			Reference
		Cell Types Formed	*Gametes*	*Fertilization*	
mESC - XY, XX	Spontaneous differentiation in adherent culture and selection with *OCT4-GFP* reporter	Follicle & Oocyte-like cells, parthegenote-like cels	N	ND	Hubner *et al.*, 2003
mESC - XY	BMP-releasing EB formation, selection with *Mvh-GFP* reporter	*Mvh*-positive PGCs, formed sperm when transplanted *in vivo*	N	ND	Toyooka et al., *2003*
mESC - XY	EB formation with RA addition, selection of SSEA-1 positive cells	Haploid spermatids capable of fertilization	Y	Y	Geijsen *et al.*, 2004
mESC - XY	EB formation with testis-conditioned medium	Immature oocyte-like cells, oocyte gene expression	N	ND	Lacham-Kaplan *et al.*, 2006
mESC - XY	EB formation and Adherent culture differentiation	Follicle & Oocyte-like cells	N	ND	Novak *et al.*, 2006
mESC - XY	EB formation, selection with *Stra8-GFP* and subsequently with *Prm1-dsRed;* RA addition	Haploid, SSC-like cells which showed motility and form sperm	Y	Y, ICSI	Nayernia *et al.*, 2006
mESC - XY	EB formation with RA addition	Oocyte-like and Sperm-like cells	N	ND	Kerkis *et al.*, 2007
mESC	Eb formation and co-culture with ovarian granulosa cells	Oocyte-like cells, oocyte genes expressed	N	ND	Qing *et al.*, 2007
mESC - XX	EB formation and Adherent culture differentiation, selection with *Gdf9-GFP* reporter	Follicle-like structures, oocyte genes expressed	N	ND	Salvador *et al.*, 2008
mESC - XY	EB formation with RA and Testosterone addition	Putative male germ cells	Y	ND	Silva et al., 2008
hESC - XX, XY	EB formation	Immature male/female germ cells	N	ND	Clark *et al.*, 2004
hESC - XX	EB formation with BMP addition	VASA+ germ cells	N	ND	Kee *et al.*, 2006
hESC - XX	EB formation and Adherent culture differentiation	VASA+ Follicle-like structures	N	ND	Chen *et al.*, 2007
hESC - XY	Adherent culture on MEF feeders	Immature germ cells	N	ND	West *et al.*, 2008
hESC	Low-density adherent culture differentiation with Laminin substrate	CXCR4+ germ cells and sertoli cells	N	ND	Bucay *et al.*, 2008
hESC -XX	EB formation and Adherent culture differentiation, selection of SSEA-1 positive cells	Immature germ cells with PGC-like imprinting status	N	ND	Tilgner *et al.*, 2008
hESC - XX, XY hiPSC - XY	Co-culture of hESCs and hiPSCs with human fetal gonadal cells, selection with SSEA-1 and c-Kit.	Double-positive germ cells with PGC-like imprinting status (only with hESCs)	N	ND	Park *et al.*, 2009
hESC - XX, XY	Overexpression of DAZ, DAZL, BOULE and Adherent culture differentiation +/- BMPs	Haploid germ cells expressing germline markers and PGC-like imprinting	Y	ND	Kee *et al.*, 2009
hESC - XY	Adherent culture differentiation on KITL KO / WT feeders with addition of BMP4	Early germ-like cells expressing VASA and OCT4	N	ND	West *et al.*, 2010 a
hESC - XY	Adherent culture differentiation on MEF feeders	VASA+/OCT4+ clonally expanded early germ-like cells	Y	ND	West *et al.*, 2010 b
hESC - XX, XY hiPSC - XX, XY	*VASA-GFP* reporter or Overexpression of DAZ, DAZL, BOULE and Adherent culture differentiation + BMPs	Haploid, sperm-like germ cells expressing germline markers and PGC-like imprinting	Y	ND	Panula *et al.* 2010
ND = Not Determined		Y = Yes			
ICSI = Intracytoplasmic Sperm Injection		N = No			

Table 1. A Summary of the Recent Efforts to Derive Gametes from ESCs and iPSCs. Note: mESC – mouse embryonic stem cells; hESC – human embryonic stem cells; EB – embryoid body; BMP – bone morphogenetic protein; Mvh – mouse Vasa homolog; RA – retinoic acid; ICSI – intracytoplasmic sperm injection; MEF – mouse embryonic feeders.

4.1 Derivation of germ cells from mouse and human embryonic stem cells

The mouse has proven to be a robust and dependable system for analysis of germline development. Studies conducted *in vivo* during mouse early development have greatly informed *in vitro* investigations and have generated promising prospects for creating gametes from stem cells. The earliest *in vivo* evidence for embryonic stem cell-derived germ cells was suggested from experiments performed by Bradley *et al.* in 1984. They generated germline chimeras after injecting mESCs into blastocysts (Bradley et al., 1984). This work suggests that mESCs, if cultured under the right conditions, could be differentiated to PGCs. We now know that mESC share many developmental features and markers with PGCs which further supports the use of mESCs as a starting point. This is most convenient as mouse PGCs are difficult to grow and differentiate *in vitro* due to limited viability in culture (Resnick et al., 1992; Farini et al., 2005). Thus, we have learned a lot over the two decades about mouse embryonic and germ cell development to guide us as we more forward.

The first report on the creation of putative germ cells *in vitro* was reported in 2003. In this study, mESCs were transfected with a reporter Green Fluorescent Protein (GFP) under the control of an *Oct4* enhancer sequence (Hubner et al., 2003). The withdrawal of LIF, a factor that promotes self-renewal in mESCs, promoted PGC development and resulted in an increase in GFP-positive cells. More importantly, the GFP-positive cells assumed a follicle-like appearance and showed overall structural and molecular similarities to oocytes. Tooyaka et al. used a similar, reporter-based approach to derive male germ cells that resembled mouse spermatozoa (Tooyaka et al., 2003). They utilized the mouse VASA homologous gene promoter *Mvh* to drive GFP expression in mESC, which then spontaneously differentiated to embryoid bodies (EBs) in the absence of LIF. The putative PGC population when transplanted into a mouse neonatal testis, differentiated to give rise to spermatozoa. These two landmark studies set the stage for further investigation of deriving gametogenesis from mESC.

In 2004, Geijsen et al. derived male gametes from EBs after culture in the presence of retinoic acid (RA), a key paracrine factor produced in the testis (Geijsen et al., 2004). Additionally, by selecting cells that only expressed SSEA-1 and Oct4, the authors were able to enrich for mature, post-meiotic germ cells that were then capable of forming blastocytes after injection into oocytes. It was later shown by Kerkis et al. that the divergence of male and female germ cell programs during spontaneous differentiation of EBs *in vitro* was dependent on the length of culture time (Kerkis et al., 2007). Several attempts at oocyte or follicle derivation from mESC have been made using the EB-based approach with various culture and attachment conditions all of which yielded oocyte-like cells that lacked critical structural features such as the zona pellucida or expression of meiotic molecular machinery (Lacham-Kaplan et al., 2006; Novak et al., 2006). Other studies have employed slightly different approaches to derive putative oocytes from mESCs. Qing et al. obtained PGCs from cultured EBs and then co-cultured them on a layer of fetal ovarian granulosa cells and observed an increase in the meiotic marker SCP3 and upregulation of oocyte-specific genes such as *Gdf9*. The GDF9 positive cells probably resembled an immature oocyte as they lacked a zona pellucida and did not express mature oocyte proteins such as ZP4 (Qing et al., 2007). In an alternative approach developed by Salvador et al., a *Gdf9*-driven GFP reporter assay was introduced into mESC. The mESCs were then differentiated to EBs and GFP-positive cells were isolated from the EBs to isolate PGCs (Salvador et al., 2007). In summary, these studies may have derived primitive oocytes from mESCs, but failed to accomplish meiotic entry in the final cell products.

An alternative approach to deriving female germ cells was employed by Nicholas et al. by using a germ cell-specific reporter, *ΔPE:Oct4: GFP*. This reporting system was previously employed by Hubner et al. to separate GFP-expressing ESCs, PGCs and primary spermatogonia in the mouse (Nicholas et al., 2009). After verifying the expression of GFP on these subsets of cells, the authors spontaneously differentiated mESCs carrying this reporter to EBs, then FACS-sorted GFP positive cells, and observed a higher expression of germ cell and oocyte markers in the selected cells. They then assessed whether ESCs could give rise to mature oocytes *in vivo* by first forming aggregates of the GFP-positive cells with fetal ovarian tissue and then transplanting them to the kidney capsule. In doing so, they observed mature oocytes of confirmed ESC origin, some of which resembled primordial follicles with a surrounding layer of granulosa cells. An important insight from this work was that the timing of germline development, particularly with oocyte maturation, is a major parameter in deriving gametes. Furthermore, it highlights that the use of

transplantation in combination with *in vitro* culturing may greatly improve the success of gamete production from mESCs.

The first study to show virtually complete gametogenesis *in vitro* was conducted by Nayernia et al. in 2006. A two-stage approach was employed to first derive pre-meiotic PGCs from mESCs with a *Stra8* driven GFP reporter induced with RA addition (Nayernia et al., 2006). GFP-positive cells were then separated by Fluorescence Activated Cell Sorting (FACS), grown in RA-supplemented medium and transfected with a second reporter, *Prm1-dsred*. The second reporter enriched for PGCs that had undergone meiosis and formed red-fluorescing, haploid cells. After further RA treatment, the haploid sperm-like cells were then injected into mouse oocytes by Intra-Cytoplasmic Sperm Injection (ICSI) to test the functionality of the derived germ cells. Indeed, blastocysts formed and viable offspring born that eventually succumbed to developmental complications and died prematurely. Potentially, the epigenetic reprogramming of the derived germ cells may not have been complete. Nevertheless, these efforts provided functional proof of gametogenesis *in vitro.*

Additional studies demonstrating *in vitro* spermatogenesis have employed organ culture systems as far back as 1960. Since then, there has been tremendous progress in the study of germ cell development, particularly in the mouse. This is best exemplified by a very recent study by Sato et al. in which functional mouse sperm were generated *in vitro* by employing a novel organ culture method. Specifically, they improved on existing organ culture methods by placing neonatal (mouse pup) testis fragments in FBS-soaked agar plugs in culture (Sato et al., 2011). With these plugs, they controlled the gas:liquid interface and also added exogenous factors. Their study conclusively showed through an analysis of reporter expression before and after meiosis, that functional, haploid spermatozoa were generated that could fertilize oocytes and give rise to viable, healthy pups. In addition, the generated pups were also shown to be naturally fertile as adults. In summary, attempts to generate germ cells from mESCs have yielded cells that resemble sperm or oocytes in gene expression, ploidy, epigenetic reprogramming and fertilization ability. However, these studies have also revealed inregularities in the duration of meiosis in culture, in the morphological features of oocytes, in the viability of offspring generated and in the germline transmission of exogenous genes to the offspring (Chuva de Sousa Lopes et al., 2010).

The evidence for germline differentiation from hESCs is more complex; as such literature is more preliminary than that from the mouse. Furthermore, doubts regarding the extent of pluripotency in hESCs vs. mESCs and the ethical constraints of testing the functionality of human germ cells are legitimate considerations with this research. Despite this, there is a tremendous need to understand human germline formation, the genes that control spermatogenesis and oogenesis and from a clinical standpoint, a need for *in vitro* derived functional gametes. Efforts to derive human germ cells began with a study by Clark et al. in 2004 in which spontaneously differentiated hESCs were used to create embryoid bodies (EBs) (Clark et al., 2004). Within the EB mixed cell population, they identified the levels of several known germ cell markers such as DAZL, STELLAR and c-KIT. Additionally, they observed that VASA levels were induced within a putative PGC population and other markers of meiosis were also upregulated. Collectively, their data indicated that PGCs had formed but had not entered meiosis. An important facet of their study was the identification of germline-associated genes such as DAZL and c-KIT as expressed in undifferentiated hESCs, which reminds us that hESCs may not be truly equivalent to cells of the ICM (Clark et al., 2006). The methods of Clark et al. were optimized in a follow-up study in which BMP-4, -7 and -8 were added exogenously during EB formation from hESCs and higher VASA

expression was observed in the putative PGC population (Kee et al., 2006). Therefore, similar to the mouse model, the role of BMPs in human germline specification may be important. More recent studies using mESCs *in vitro* by Young et al. have illustrated how BMP2 and BMP4 but not BMP8b are important inductive agents for specifying the germ cell lineage in EBs (Young et al., 2010). These results help to clarify our understanding of BMP action in the germline as the goal of deriving haploid germ cells is pursued.

Chen et al. and Tilgner et al. also derived human PGCs from substrate EBs in independent studies. They observed that their EBs contained small structures that resembled ovarian follicles and that tested positive for c-KIT and low amounts of VASA protein (Chen et al., 2007). They also observed these structures in hESCs that were spontaneously differentiated in adherent cultures, suggesting that EBs are not essential for germ cell differentiation, at least in the female lineage. Meanwhile, Tilgner et al. used a more sophisticated strategy to separate germ cells from EBs and adherent cultures through FACs sorting for cells expressing the surface marker SSEA-1, a protein associated with very early germ cells (Tilgner et al., 2008). Despite evidence of complete epigenetic reprogramming in these early germ cells, the authors did not observe meiosis. In 2009, Park et al. built upon the findings of Tilgner et al. by isolating differentiated cells enriched in SSEA-1 as well as c-KIT expression. However, they used an interesting differentiation strategy and co-cultured hESC colonies with stromal cells from the human fetal gonad (Park et al., 2009). In this model, the paracrine factors produced from fetal stromal cells would ideally resemble those made *in vivo*, thereby theoretically inducing primitive germ cells to differentiate into PGCs. Their efforts were successful, yielding SSEA-1 /c-KIT positive PGCs that expressed numerous germ cell markers such as VASA, PRDM1 and DAZL and revealed evidence of partial epigenetic reprogramming.

Based on the findings of spontaneous differentiation of hESCs to PGCs, researchers have entertained the possibility that a small population of cells within a typical hESC population is pre-destined to become germ cells. Based on this hypothesis, several groups have explored whether simply optimizing culture conditions of hESCs can promote or induce germline differentiation. Stice and colleagues first reported that by the simple addition of basic FGF (bFGF) to hESCs growing on inactivated MEF feeder cells, they could produce approximately 69% VASA-positive cells with increases in expression of germline and meiotic markers (West et al., 2008). These data seem rather optimistic given that most other groups have reported much lower germ cell numbers under similar culture conditions. Concurrently, Bucay et al. modified their culture conditions by lowering the confluency of hESC colonies and observed a subpopulation of cells expressing CXCR4, a membrane receptor implicated in PGC migration (Bucay et al., 2009). Upon purification, the CXCR4-expressing cells could be grown on a laminin substrate and gave rise to putative PGCs and Sertoli cells. In recent studies, West et al. explore the effect of modulating KIT ligand (KIT-L) and BMP4 activities in culture of hESCs and observed that these are required for early germ-like cell (GLC) derivation (West et al., 2010a).

A major milestone in germline differentiation of both male and female human PGCs is the initiation and completion of meiosis. Deriving haploid gametes *in vitro* is a significant feat and one that researchers have struggled with for years in human germline differentiation. Kee and colleagues derived human haploid gametes for the first time in 2009 by introducing three major genes of the Deleted-in-Azoospermia (DAZ) family, DAZ, DAZL, and BOULE in hESCs and then differentiating the cells spontaneously in adherent culture conditions (Kee et al., 2009). Although a low number of haploid (1n) cells were observed (~2%) seven

days after differentiation, they expressed numerous germ cell-specific markers and meiotic spreads indicated the presence of SCP3, a key member of the synaptonemal complex that forms during meiosis. These data also reinforce the importance of the DAZ family of genes for early (DAZL) and late (BOULE, DAZ) primordial germ cell formation. Since this work was published, another group has demonstrated meiosis in *in vitro* derived PGCs: West et al. had previously shown that they could isolate early VASA-positive germ-like cells (GLCs) and were able to subclone at least three populations of these cells from their mixed cultures and propagate them through at least 50 serial passages (West et al., 2010b). These cells were then be differentiated with FBS into a more homogenous population and the majority of cells entered meiosis (~71%) while also expressing germline and meiotic markers (West et al., 2010b). Taken together, these studies offer alternative and compelling strategies for derivation of PGCs from hESCs. While one (West et al., 2010b) suggests that GLCs are a natural clonal subpopulation of hESCs that can be propagated *in vitro*, the other (Kee et al., 2009) argues in favor of an active, gene-driven induction of germline differentiation.

4.2 Germline differentiation from human induced pluripotent stem cells

Ongoing advances in reprogramming of human somatic cell to pluripotent stem cells (iPSCs) or direct reprogramming of somatic cells to specific cell lineages, are a legitimate avenue for autologous, patient-derived stem cell therapies. By applying similar genetic approaches used for human ESCs, iPSCs can now be directed to give rise to cells of the germ cell lineage. We summarize here some of the recent efforts in this area. The use of human iPSCs (hiPSCs) to derive gametes was first shown by Park et al. in a study that also compared the derivation efficiency to hESCs (Park et al., 2009). In short, they grew hiPSCs on a feeder layer of fetal gonad stromal cells and subsequently isolated SSEA-1 + / c-KIT + cells which they characterized as a putative PGC population. These human induced PGCs (hiPGCs) expressed several germline markers but did not show evidence of imprinting erasure, a key feature of early PGCs. Further evidence for the creation of haploid hiPGCs from male and female hiPSCs was provided by Panula et al. recently wherein they used a GFP reporter under the control of the *Vasa* promoter to select for putative germ cells from spontaneously differentiated cultures (Panula et al., 2010). The *Vasa*-GFP transduced hiPSCs were differentiated adherently in feeder-free conditions in the presence of BMPs, giving rise to approximately 5% GFP-positive PGCs. These PGCs expressed a number of germ cell markers such as *Prdm1A, ACTC, Gata6, Pelota* and *IFITM1*. More importantly, the GFP-positive hiPGCs expressed the meiotic marker SCP3 in both punctate and elongated patterns, indicative of early and late meiosis, respectively. Interestingly, when compared to PGCs derived from both male or female hESCs, the efficiency of hiPGC derivation appears higher, suggesting that hiPSCs may be a better starting substrate for germline differentiation. In summary, these two investigations provide encouraging results about the ability to use hiPSCs to derive gametes *in vitro*. Although the differentiation potential of hiPSCs may vary greatly from one cell line to another, they appear to have strong potential as a genetic model system to study human germ cell development.

4.3 Clinical need and applications for *in vitro*-derived gametes

Currently many men suffer from non-obstructive azoospermia as described earlier that results in complete sterility. In addition, in the U.S. alone there are 55,000 childhood cancer survivors annually, many of whom were unable to bank sperm before receiving sterilizing

treatments. Similarly, women have a finite supply of reproductively competent oocytes while others undergo premature ovarian failure, making childbearing difficult or impossible. Alternative options for parenthood in these cases are not always simple or inexpensive. Although sperm donation is economical, oocyte donation is quite expensive and the use of donated embryos is unusual (Nicholas et al., 2009). For men, recent strategies

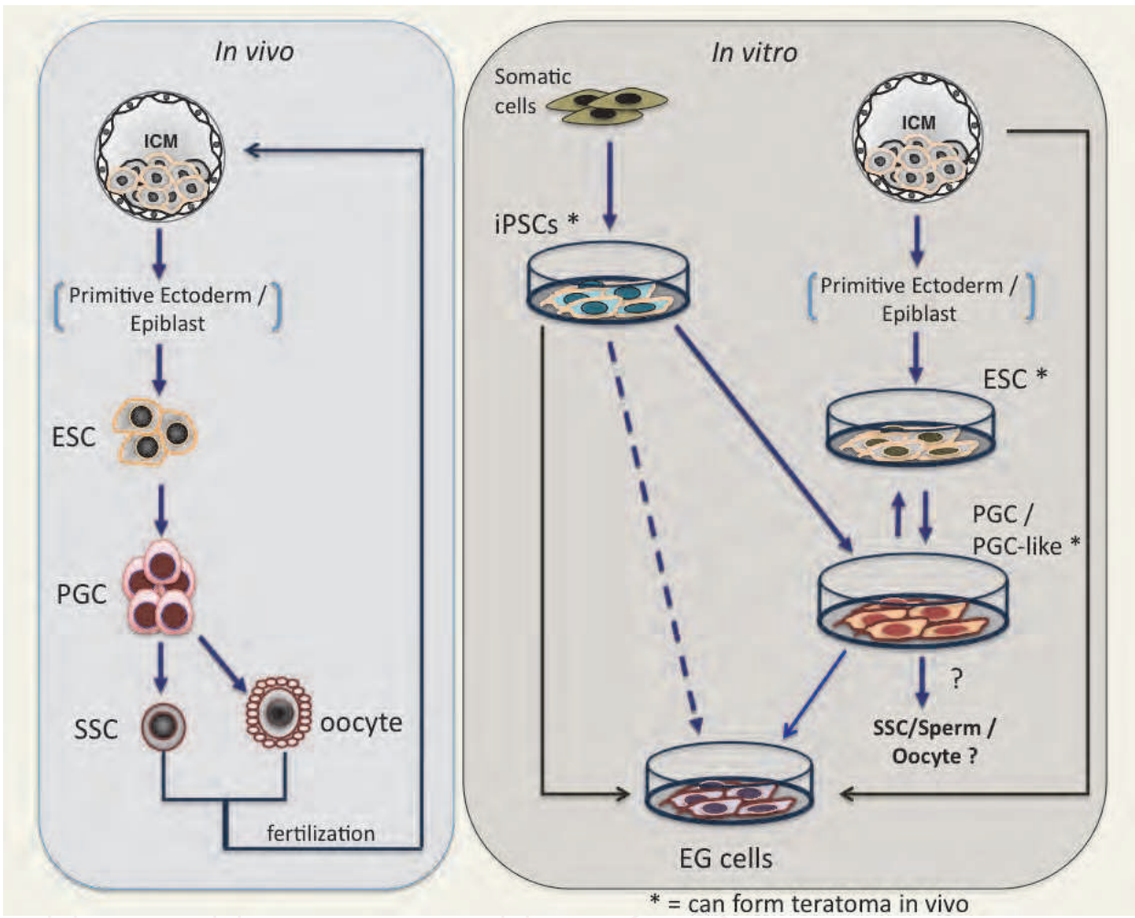

Fig. 4. Comparison of *in vivo* Germline Development and *in vitro* Germ Cell Derivation from ESCs and iPSCs.A depiction of *in vivo* development of germ cells and gametes from the mammalian embryo showing that embryonic stem cells (ESCs) originate from primitive ectoderm, by way of the inner cell mass (ICM). Primordial germ cells then migrate to gonads where they undergo self-renewal in the form of spermatogonial stem cells (SSCs) in the testis and oocytes in the ovary. On the right is a schematic of the cell lineages derived *in vitro*. ESCs cultured in dishes can be differentiated to PGCs via methods summarized in Table 1. PGCs cultured *in vitro,* at least in the mouse, can transform to embryonic germ (EG) cells. There is also evidence a subpopulation of hESCs cultured *in vitro* behave like EG cells. Finally, somatic cells can be reprogrammed with addition of Oct4, Sox2, Klf4 and c-Myc transcription factors to induce a stem cell-like fate. iPSCs can then be induced to form PGC-like cells or EG-like cells. Finally, ESCs, PGCs and iPSCs can all form teratomas when injected *in vivo* demonstrating their pluripotency.

such as extracting low numbers of testis sperm after prior localization with Fine Needle Aspiration (FNA) mapping, offer the hope of fatherhood for some candidates (Beliveau et al., 2011; Natali et al., 2011).

Although such strategies are gaining in popularity, the pregnancy success rates remains at about 30-40% using sophisticated in vitro fertilization (IVF) techniques (Centers for Disease Control [CDC], 2008). In addition, with these reproductive technologies, there remain important safety concerns about de novo sex chromosomal disorders, birth defects and imprinting-related disorders such as Beckwith-Wiedeman syndrome in offspring (Childs et al., 2008). Therefore, there is an obvious clinical need for high quality, *in vitro*-derived gametes. However, the prospect of employing current protocols and reagents to derive human gametes from hESCs appears unlikely at this time. On the other hand, the use of iPSCs and alternative stem cell sources such as adult stem cells, hold great promise in thisregard. In the foreseeable future, patients with genetic disorders affecting fertility could theorectially donate somatic cell biopsies that could then be reprogrammed into iPSCs and genetically modified to normal and subsequently utilized for gametogenesis *in vitro*.

However, there are numerous challenges to the application of iPSCs for clinical use (Hanna et al., 2010). For one, the exact level of authenticity and experimental proof required for the safe use of iPSC and hESC derived gametes in the clinic is entirely unknown. To address this issue, there are at least three major areas that the research community *can* and *should* address before taking *in vitro*-derived gametes to the clinic. Firstly, the chromosomal composition of the iPSCs must be identical to the original patient from where they were derived. There is now sufficient data detailing the gene mutations and copy number variations arising in iPSCs during reprogramming and clonal expansion. It would be wise to carefully screen for these variation in patient-derived samples (Reviewed in Panopoulos et al., 2011). Moreover, frequent chromosomal aberrations arise during meiotic recombination so it would be necessary to examine the fidelity of meiosis in gametes *in vitro*. Secondly, it is important to check for the proper erasure and subsequent re-establishment of maternal and paternal imprints in PGCs derived from both iPSCs and hESCs. There is published work with hESCs and iPSCs that has attempted to characterize imprinting status and DNA methylation patterns in these derived cells; the preliminary evidence seems to indicate that *in vitro* derived hiPGCs appear to undergo partial to full imprinting erasure (Kee et al., 2009; Panula et al., 2010). Whether the correct imprints are restored later on during germ cell development remains unknown. Thirdly, the most infallible method to test full functionality of *in vitro*-derived gametes is to test their ability to fertilize, form a blastocyst and viable offspring. In the mouse, this has been tested despite the premature death of offspring (Nayernia et al., 2006). One obstacle is how can we overcome ethical boundaries and challenges to do similar tests with human gametes? Perhaps for now, the primary medical application of human stem cell-derived gametes would be to use them as a reproductive toxicological screen for pharmaceutical compounds and other chemicals.

5. Conclusions

In the last decade, much progress has been made in our understanding of mouse and human embryology and embryonic stem cells. We have learned that complex genetic pathways that underlie the pluripotency aspects of hESCs and mESCs also underline our

inability to easily control and direct these cells toward a specific lineage. In addition, the advent of diverse strategies to 'reprogram' somatic cells has opened up an almost limitless access to patient-specific stem cells that if used correctly, could provide very powerful cell-based therapies. Scientifically, the biology of germline development has always been an popular area of exploration for embryologists and stem cell biologists, especially because germ cells are unique in their ability to transmit genetic information from one generation to the next. If we can understand how germ cells develop from ESCs at the cellular and molecular level, we can apply this information to the derivation of male and female gametes. The goals of gamete formation include: 1) to address immediate health issues such as infertility in men and women due to sperm and egg dysfunction or absence; 2) to produce gametes that can be fertilized and produce an almost limitless source of mammalian embryos and ESCs for the study of specific diseases; 3) to devise methods with which to target hereditary and non-hereditary germline mutations or chromosomal abnormalities during the earliest stages of germ cell production.

The studies summarized in this review have shown that multipotent ES-like SSCs can develop into somatic cells of all three germ layers, but their ability to differentiate to the germline remains unclear or untested. In addition, it is believed that SSCs may only be multipotent and will be useful only for autologous transplantation and maybe for only a few tissue types. In this regard, one promising avenue for SSCs is differentiation down the spermatogenic pathway. Finally, there is a limited expansion potential of SSCs compared to iPSCs or hESCs and the imprinting status of these cells is less well defined than that of hESCs. A similar approach as used by Kee et al. in 2009 to overexpresss DAZ genes in hESCs could potentially be applied to differentiate SSC's to spermatids or sperm *in vitro*. Alternatively, one could envision that the research on *in vitro*-derived human oocytes could be similarly advanced and lead to a better understanding of the concept that there are renewable ovarian stem cells in humans. In conclusion, the potential of ESCs and iPSCs to be used for cell-based therapy is now being realized. Major challenges, both scientific and clinical, still exist in deriving germline cells that look and behave as reliably as their natural counterparts. With further advances in technology and the elucidation of new pathways in gerrmline function in the mouse and human systems, we predict that these challenges can be met and overcome in the future.

Keypoints:

- Mouse and human embryonic stem cells provide the ideal substrate for germline differentiation, both *in vivo* and *in vitro*.
- The mammalian germline is established as primordial germ cells immediately prior to gastrulation in mice and humans, and undergo similar physiological and molecular events.
- Induced pluripotent stem cells (iPSCs) and adult stem cells may provide alternative sources of pluripotent and multipotent stem cells with less potential for host rejection and decreased tumorigenicity.
- Testicular stem cells with multipotent and even pluripotent potential have been isolated in mouse models and, more recently, in humans.
- The *in vitro* derivation of early male and female germ cells is now achievable from mESCs, hESCs and human iPSCs with the application of various reporter systems and through embryoid body (EB) formation.

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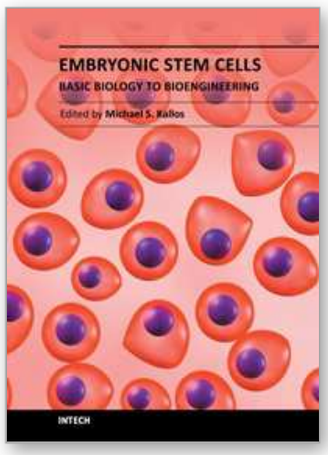

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Embryonic stem cells are one of the key building blocks of the emerging multidisciplinary field of regenerative medicine, and discoveries and new technology related to embryonic stem cells are being made at an ever increasing rate. This book provides a snapshot of some of the research occurring across a wide range of areas related to embryonic stem cells, including new methods, tools and technologies; new understandings about the molecular biology and pluripotency of these cells; as well as new uses for and sources of embryonic stem cells. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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