Germ Stem Cell

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Abstract: The definition of stem cell is "an unspecialized cell that gives rise to a specific specialized cell, such as a blood cell". Stem cells can differentiate into different cell types under appropriate in vitro and in vivo conditions. Embryonic stem cells are derived from the inner cell mass of blastocyst stage embryos. Somatic stem cells are generally believed to differentiate only into cells characteristic of the tissue wherein they reside. The germ cell is the only lineage that makes the genetic information across the generations in most multicellular organism's perpetuation. Mammalian gametes are derived from a founder population of primordial germ cells that are determined early in embryogenesis and set aside for unique development. Primordial germ cells are closely related to embryonic stem cells and embryonic germ cells. If genetic and epigenetic methodological limitations could be solved, therapeutic opportunities could be also considered. The differentiation of functional oocytes from stem cells may permit the success of human somatic cell nuclear transfer for reprogramming studies and for the production of patient-specific embryonic stem cells. Embryonic stem cell derived oocytes could ultimately help to restore fertility in women.
[Ma H, Yang Y, Sun Y, Ma M. **Germ Stem Cell.** *Stem Cell* 2010;1(4):21-41] (ISSN 1545-4570). http://www.sciencepub.net/stem. 5

Key words: DNA; life; stem cell; germ; differentiate

Abbreviations: ESC, embryonic stem cell; MSC, mesenchymal stem cell; PGC, primordial germ cell; SC, stem cell; SSC, somatic stem cell

1. Introduction

Life is a physical and chemical process (Hongbao Ma 2005). To the life, 2 points are most important: live and die. Conventionally, everybody of us thinks that all the life has a beginning as the birth and the end as the die. All plants and animals, including all the people must die. It is the stem cell (SC) gives the hope for life to maximum extend the living time arrangement and finally target the eternal (Ma Hongbao, 2009).

The definition of SC is an unspecialized cell that gives rise to a specific specialized cell, such as a blood cell. Embryonic stem cell (ESC) is derived from the inner cell mass of blastocyst stage embryos. Somatic stem cell (SSC) is generally believed to differentiate only into cells characteristic of the tissue wherein they reside. SC is the original of life. All cells come from SCs (Hongbao Ma, 2005).

SCs are undifferentiated cells that have the potential to self-replicate and differentiate into all kinds of cells, which are unique in their ability to undergo both self-renewal and differentiation. (Lester et al., 2004). ESCs are derived from the inner cell mass of the preimplantation embryo and retain their pluripotency. SCs have the capacity to develop into any cell type of somatic ectodermal, mesodermal, or endodermal lineages. ESCs can develop into the germ cell. The studies of germ cell development in culture provide unprecedented opportunities to understand the basis of the propagation of life. Advances in SC research have opened new perspectives for regenerative and reproductive medicine and the life sciences (Weissman, 2005).

Human mesenchymal stem cell (MSC) isolated from bone marrow stroma, peripheral blood, dermis, muscle and adipose tissue have the advantage of potential autologous transplantation ability. They can be differentiated into chondrogenic, osteogenic, adipogenic and myogenic lineages. The isolation of MSCs from adipose tissue can be as an alternative source. Adipose tissue is derived from embryonic mesoderm and contains a heterogeneous stromal cell population. Inductions of the cells into multiple mesenchymal lineages resulted in the expression of several lineage-specific genes, proteins and specific metabolic activity. The potential benefit of the multi-germline capacity of human MSCs seems to be a promising approach for allogenic cell therapy and human tissue engineering (Pansky et al., 2007). SCs can be obtained from the embryo at cleavage or blastocyst stages, but also from extra-embryonic tissues such as the umbilical cord blood obtained at birth, the placenta and the amniotic fluid. SC can also be obtained in adult body. These somatic SCs can be found in a wide range of tissues including bone marrow, blood fat, skin and also testis. Human umbilical cord blood is an excellent primitive source of noncontroversial SCs for clinical applications. It

was reported that in situ characterization to identify and localize a panel of some markers expressed by mesenchymal stromal cells (CD44, CD105, CD73, CD90) and CD146 in UC (Schugar et al., 2009). The predominant expression by UC-MSCs of genes engaged in the osteogenic differentiation and their tendency to differentiate into osteoblasts (Ciavarella et al., 2009). ESCs exposed to appropriate and specific conditions differentiate into cell types of all three germ layers and also into germline cells (Iwayama et al., 2005).

In males, sperm are produced continuously during the adult life. Male germ cells, the repository cells of the genome, comprise several successive developmental stages starting in the embryo and ending up with the spermatozoon. Gonocytes represent the fetal and neonatal stages preceding the formation of spermatogonial SCs. The option of storing mature sperm prior to treatment is a common practice (Ahringer and Kimble, 1991; Culty, 2009). SCs can be obtained from the embryo at cleavage or blastocyst stages, but also from extra-embryonic tissues such as the umbilical cord blood obtained at birth, the placenta and the amniotic fluid. SC can also be obtained in adult mammals from specific niches. These somatic SCs can be found in a wide range of tissues including bone marrow, blood, fat, skin and also the testis. The differentiated somatic cells could be reprogrammed into pluripotent state. Consequently, the reprogrammed somatic cells recapitulate the capacity to differentiate into specific cell lineages under appropriate culture conditions (Huang et al., 2009).

There are two pathways for germ cell fate: (1) preformation, to be embryonic cells that inherit maternal determinants from the egg go on to form the germ cell lineage; (2) epigenesis, pluripotent cells formed early in development are induced by signals from adjacent tissues to form the germ cell. The germ cell is the only lineage that makes the genetic information across the generations in most multicellular organism's perpetuation. Germ cell produces primordial germ cell (PGC) and PGS becomes the oocytes and spermatozoa. In non-mammalian species of fruitflies, nematodes and frogs, germ cells of both males and females are specified via the inheritance of germ plasm. Specification of germ cell fate is vital to development and heredity. In mice, germ cell fate is induced in a subset of pluripotent epiblast cells during early gastrulation. Reflecting the function of the germ line as the transducer of genetic information, germ cell specification integrates at least three key events: repression of the somatic program, re-acquisition of potential pluripotency, and ensuing genome-wide epigenetic reprogramming (Saitou, 2009).

Consistent with the function of germ cell lineage as the transducer of genetic information, PGC specification is an integration of at least 3 key events: (1) repression of the somatic program; (2) re-acquisition of potential pluripotency; (3) an ensuing genome-wide epigenetic reprogramming. More detailed knowledge of the mechanism of PGC specification will provide a critical foundation for induction of the germ cell lineage from pluripotent stem cells *in vitro* not only in mice but also in other mammals, including humans. Immature sperm cells derived from ESCs in culture could generate live offspring. Nuclear transfer-derived SCs may also be selected and directed into differentiation pathways leading to the production of specific cell types, tissues and, eventually, even organs for research and transplantation (Tecirlioglu and Trounson, 2007). The ESC technology offers great potential for new types of reproductive investigations including epigenetic modifications of the germline (Moore and Aflatoonian, 2007).

According to the World Health Organization, infertility is considered a disease affecting millions of people in Europe, where prevalence is 14% of couples in reproductive age. Based on the 2005 National Survey on Family Growth American report, there was a 20% increase in American couples experiencing impaired fecundity between 1995 and 2002. This may be related to a tendency to delay motherhood to the third decade of life due to professional and social reasons. As a consequence, oocyte quality in females is reduced. This article summarizes the current knowledge of germ cells.

2. General Description of Germ Stem Cells

Germ cells carry the genome onto the next generation. In females, the gonocyte surrounded by a cortical interstitial layer initiates meiosis and becomes a primary oocyte and follicle, thereby ending precursor proliferative potential. In males, the gonocyte surrounded by the fetal sex cord of the gonadal ridge arrests in G0/G1 of mitosis as a prospermatogonium, but retains a proliferative precursor potential. After birth, prospematogonia migrate to the basement membrane of the seminiferous tubule and differentiate into spermatogonial tem cells. Like adult SCs, SSCs can both self-renew and provide daughter cells, which differentiate into one or more terminal cell types. In the testis, the continuous production of sperm is maintained by a small population of SCs (Wei Leng, 2006). Fragilis is a transmembrane protein and part of a larger interferon-inducible family of genes that is evolutionarily conserved and has human homologues. Interferon-inducible proteins such as fragilis have an anti-proliferative function and may serve to increase

the length of the cell cycle in PGCs. The genes fragilis and stella have key roles in germ cell competency and development (Lange et al., 2008). Expression of some of these genes is wide spread in a variety of mouse immune tissues while others appear to be much more restricted (Smith et al., 2006).

Mammalian development commences with the totipotent zygote which is capable of developing into all the specialized cells that make up the adult animal. As development unfolds, cells of the early embryo proliferate and differentiate into the first two lineages, the pluripotent inner cell mass and the trophectoderm. Pluripotent cells can be isolated, adapted and propagated indefinitely in vitro in an undifferentiated state as ESCs. ESCs retain their ability to differentiate into cells representing the three major germ layers: endoderm, mesoderm or ectoderm or any of the 200+ cell types present in the adult body. Since many human diseases result from defects in a single cell type, pluripotent human ESCs represent an unlimited source of any cell or tissue type for replacement therapy thus providing a possible cure for many devastating conditions. Pluripotent cells resembling ESCs can also be derived experimentally by the nuclear reprogramming of somatic cells. Reprogrammed somatic cells may have an even more important role in cell replacement therapies since the patient's own somatic cells can be used for reprogramming thereby eliminating immune based rejection of transplanted cells. ESCs are pluripotent and they differentiate along somatic cell lineages. ESCs are totipotent as they can spontaneously generate trophoblast and trophoblast-SCs. ESCs can form germ cells which have a totipotent potential (Mitalipov and Wolf, 2009). Regenerative medicine and tissue engineering are searching for a novel SC based therapeutic strategy that will allow for efficient treatment or even potential replacement of damaged organs. The pluripotent stem cell, which gives rise to cells from all three germ lineages, seems to be the most ideal candidate for such therapies. Pluripotent SC could be extracted from developing embryos. However, since this source of SCs for potential therapeutic purposes remains controversial, SC researchers look for pluripotent SC that could be isolated from the adult tissues or generated from already differentiated cells. True pluripotent SC should possess both potential for multilineage differentiation in vitro and, more importantly, also be able to complement in vivo blastocyst development (Ratajczak et al., 2008).

During human ESC culture, markers of female germ cells are expressed in both XX and XY cell lines. Both male and female ESC lines to display female germ cell markers, since culture conditions may be sub-optimal and lack meiosis inhibition. When human ESCs are cultured the process of sex determination seems even more dysregulated as markers of both male and female germ cell development have been detected regardless of the sex of the cell line. Testis, for example, displays no intrinsic circadian rhythmicity and the molecular mechanisms of clock gene activation in male germ cells appear to differ from other tissues. Germ cells may develop according to an intrinsic clock (Cermakian et al., 2003). In 2007, Kucia et al identified a population of CXCR4(+) lin(-) CD45(-) cells that express SSEA, Oct-4 and Nanog in adult bone marrow. These cells are very small and display several features typical for primary ESCs such as: i) a large nuclei surrounded by a narrow rim of cytoplasm; ii) open-type chromatin; and iii) high telomerase activity. Germ lineage, in order to pass genes on to the next generation, has to create soma and thus becomes a 'mother lineage' for all somatic cell lineages present in the adult body. Germ potential is established after conception in a totipotent zygote and retained subsequently during development in blastomers of morula, cells form the inner cell mass of blastocyst, epiblast and population of PGCs. The authors envision that VSEL-SC are epiblast-derived pluripotent SCs and could potentially become a less-controversial source of SCs for regeneration (Kucia et al., 2007).

3. Endogenous Oocyte Development

Totipotent SCs have the potential to differentiate into every cell type. Renewal of totipotent SCs in the germline and cellular differentiation during early embryogenesis rely upon posttranscriptional regulatory mechanisms (Pagano et al., 2009). The mammalian germline is specified through inductive signaling during gastrulation. Mammalian germ cells lack definitive germ plasm but still contain homologs of germ plasm components such as *Nanos* and *Pumilio*. These components, in addition to epigenetic regulation, are thought to similarly suppress somatic development and activate germ cell-specific programs. In nonmammalian species, the germline is specified through germ plasm inheritance from the maternal oocyte just after fertilization (Saitou et al., 2003). A hallmark of germline cells throughout the animal kingdom is their ability to execute meiosis (Mukai et al., 2007). Mammalian germ cells have additional mechanisms to preserve germ cell identity in the midst of somatic cell embryonic development (Pagano et al., 2009).

After specification, PGCs migrate from their extragonadal origin to the developing gonad, or genital ridge, where they interact with gonadal somatic cells that will later determine their commitment to a sex-specific developmental program and maturation into functional gametes. It is a central

dogma in reproductive biology that oogenesis is completed before or just after birth and that the postnatal ovary is endowed by a fixed and non-renewing number of oocytes in mammals (Oktem and Oktay, 2009).

Germ cells in the fetal testis are protected from retinoic acid exposure and are inhibited from entering meiosis due to factors expressed by somatic cells in the fetal testis. Male germ cells do not enter meiosis until puberty. In the developing ovary, oocytes are required for follicle formation, and an absence of oocytes results in an absence of follicles. The lack of meiotic oocytes in the ovary can result in ovarian sex reversal, the formation of testicular-like tubules, and differentiation of Sertoli-like cells from granulosa cells. Meiotic oocytes are required to direct ovarian follicular maturation. Pluripotent cells can be isolated from the mammalian inner cell mass of the embryo at the blastocyst stage, and maintained in culture as undifferentiated. During normal embryo development, PGCs migrate into the aorta-gonad-mesonephros region (De Miguel et al., 2009). Apoptosis is prominent during follicular endowment, and apoptosis regulatory genes are involved in its regulation (Greenfeld et al., 2007).

To accomplish its future fate as an oocyte or sperm, a developing germ cell must erase the epigenetic program obtained in early embryogenesis, remove the genomic imprints inherited from the previous generation, and reestablish parental imprints in a sex-specific manner during spermatogenesis or oogenesis. This erasure and remodeling of epigenetic marks is termed epigenetic reprogramming and refers to DNA modifications that do not alter the sequence of DNA. Three main types of epigenetic mechanisms are known to exist and include DNA methylation, histone modification, and RNA-mediated silencing. Epigenetic information undergoes extensive reprogramming in the germline between generations (Katz et al., 2009).

DNA methylation is a vital epigenetic mark that participates in establishing and maintaining chromatin structures and regulates gene transcription during mammalian development and cellular differentiation. Differences in epigenetic patterns between individuals may contribute to phenotypic variation and disease susceptibility. Of the different epigenetic mechanisms, DNA methylation is the most widely studied and best-characterized epigenetic modulator. The genomic context determines the developmentally regulated epigenetic status at most nonimprinted regions of mammalian genomes (Schilling et al., 2009). Two waves of erasure and establishment of DNA methylation patterns occur during early embryogenesis. At the morula stage of development, the methylation of DNA is almost completely erased, with the exception of maternal and paternal DNA imprints. DNA methylation is subsequently reestablished during a period of *de novo* methylation, which takes place at the time of implantation. DNA methylation could indicate preferred sites for recombination, or methylation following recombination could inhibit further recombination, perhaps by being part of the enigmatic molecular pathway mediating crossover interference (Sigurdsson et al., 2009).

Histones are the main protein components of chromatin and are comprised of two classes, core histones and linker histones. Core histones are unique in that they have the capacity to undergo posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination and ADP-ribosylation (Lin et al., 2009). Although chromatin remodeling in female germ cells is not as dramatic as the DNA-protein structure changes that occur during the final stages of male germ cell development, certain histone modifications are known to be essential for achieving meiotic and developmental competency in the oocyte. Although phosphorylation is important for histone regulation during spermatogenesis, acetylation appears to be the predominant type of histone modification in oogenesis. Post-translational modification of chromatin is emerging as an increasingly important regulator of chromosomal processes. In particular, histone lysine and arginine methylation play important roles in regulating transcription, maintaining genomic integrity, and contributing to epigenetic memory (Ng et al., 2009).

Methylation of DNA might occur in a sequence-specific manner. Many organisms have a mechanism for down regulating the expression of non-synapsed chromosomes and chromosomal regions during meiosis (She et al., 2009; Sugimoto et al., 2007). Significant evidences have brought new insights on the mechanisms by which epigenetic machinery proteins regulate gene expression, leading to a redefinition of chromatin regulation in terms of modification of core histones, DNA methylation, RNA-mediated silencing pathways, action of methylation-dependent sensitive insulators and Polycomb/Trithorax group proteins. The possibility of reversing epigenetic marks, in contrast to genetic code, may provide new pharmacological targets for emerging therapeutic intervention (Santos-Reboucas and Pimentel, 2007).

Centrosomes had been discovered in germ cells and germ cells continue to provide excellent but also challenging material in which to study complex centrosomal dynamics. Asymmetric centrosome dynamics are also critical for SC division to maintain one daughter cell as a SC while the other daughter cell undergoes centrosome growth in preparation for

differentiation (Schatten and Sun, 2009). Mammalian oogenesis is characterized by three main developmental stages: the initiation of meiosis in the fetus, follicle formation during the perinatal period, and oocyte growth and maturation in the adult (Kimble and Crittenden, 2007). During the development of multicellular organisms, the processes of growth and differentiation are kept in balance to generate and maintain tissues and organs of the correct size, shape and cellular composition. The primitive oocytes begin to undergo atresia shortly after their entry into meiosis (Crittenden et al., 2003).

Oocytes secrete soluble factors that regulate the growth and differentiation of follicular cells, including maintenance of the distinctive cumulus cell phenotype (Gilchrist et al., 2001). Oogenesis is a complex process that is tightly regulated by both endocrine and locally produced factors. From follicle formation to ovulation, extensive cross-talk between the arrested meiotic oocyte and the granulosa cells is required for proper oogenesis. The oocyte directs granulosa cell proliferation, whereas the granulosa cells regulate oocyte maturation. During fetal development, the oocytes develop in groups, or cysts, and are connected, as in spermatogenesis, by intercellular bridges. The intercellular bridges enable the sharing of RNAs and proteins to facilitate oocyte maturation. The oocyte cysts are then surrounded by pregranulosa somatic cells during meiotic prophase and later break down at birth in mice. This breakdown allows for the infiltration of pregranulosa cells and formation of primordial follicles consisting of a single oocyte surrounded by a single layer of flattened granulosa cells. TNFα signaling is thought to be important for cyst break down and primordial follicle assembly, whereas estrogen and progesterone inhibit follicle assembly (Sun et al., 2008). The steroidogenic enzymes expressed in theca cells convert cholesterol into androgen, and androgen is then used by granulosa cells expressing aromatase to produce estrogen. Oocytes reach maturity during the antral follicle stage, and mature mouse oocytes of approximately 70–80 µm in size are capable of resuming meiosis upon ovulation. Although follicle-like aggregates expressing steroidogenic enzymes and producing estrogen have been generated from differentiating ESCs, the characterization of stepwise primordial to antral follicle maturation has not been reported (Kolle et al., 2007). The gap junction proteins connexin37 and connexin43 are required for ovarian folliculogenesis in the mouse. The oocytes do not need to express connexin43 in order to develop into meiotically competent, fertilizable gametes, but must express connexin37 for communication with granulosa cells, a requirement for oogenesis (Gittens and Kidder, 2005).

Prenatal oogenesis produces hundreds of thousands of oocytes, most of which are discarded through apoptosis before birth (Cushman et al., 2000; Hartshorne et al., 2009). It is possible that the recruitment signal may actually originate from the oocyte itself. The newborn ovary contains only a small fraction of the total oocytes that entered meiosis in the fetal ovary (Anderson et al., 2007). It is the remaining sister chromatids, together with the chromosomes contributed by the sperm that form a zygote. homologous chromosomes pair very efficiently, but the high frequency of complete, premature homologue separation found at diplotene suggests that mechanisms other than the pairing process could be more likely to lead to the high aneuploidy rate observed in human oocytes (Roig et al., 2005).

Spermatogonial SC ensures continuous production of mammalian male gametes. In rodents, the SSC are Asingle spermatogonia (Grisanti et al., 2009). It appears that precommitted and premeiotic fetal ovarian germ cells are unable to completely mature *in vitro* and require transplantation to produce mature oocytes (Nagano, 2007).

Pluripotent SCs are of paramount therapeutic interest, since they could be utilized: as in vitro models of disease, for pharmaceutical screening purposes, and for the regeneration of damaged organs. Over the years, pluripotent cells have been cultured from teratomas, the inner cell mass, and PGCs. Accumulating informations have partially decrypted the molecular machinery responsible for the maintenance of a very primitive state, permitting the reprogramming of differentiated cells. Although the debate is still open, an extreme excitement is arising from two strictly related possibilities: pluripotent cells could be obtained from adult tissues with minimal manipulations or very rare pluripotent cells could be identified in adult tissues (Beltrami et al., 2009). Oocytes can be fertilized to produce offspring, form teratomas in humans and mice after parthenogenetic activation, and reprogram terminally differentiated somatic nuclei after somatic cell nuclear transfer. Mature gametes are ultimately totipotent, but nascent PGCs require reprogramming during germ cell development to regain pluri/totipotency. It appears that postspecification mammalian germ cells may possess the potential to be artificially reprogrammed throughout the entirety of germ cell development until the naturally reprogrammed totipotent state of mature gametes is restored (Feng and Chen, 2009; Korkola et al., 2009; Mitalipov and Wolf, 2009).

4. Oocyte Differentiation from ESCs

The isolation of human ESC in 1998 has created the hope that SCs will one day be used to

regenerate tissues and organs, even though it is obvious that a number of hurdles will need to be overcome for such therapies to become reality. The cloning of "Dolly" in 1997, more than 40 years after the first frogs were cloned, combined with the very fast progress made in our understanding of the molecular processes that govern the pluripotency of ESC has lead to the ability of scientists to recreate a pluripotent state in fibroblasts and other cells from mouse, rat and man, named induced pluripotent SC (Verfaillie, 2009).

Global gene expression profiling has shown human ESC lines to be distinct from the inner cell mass. In fact, human ESC lines may be more closely related to epiblast because they are similar to mouse epiblast-derived SCs, and human ESCs may have different germ cell developmental potential than mouse ESCs (Machaca, 2007; Theurkauf, 1997).

The therapeutic potential for human ESC drives intense public and scientific interest (Gavrilov et al., 2009). The clinical utility of SC-derived oocytes would require autologous SCs for the treatment of non-germ cell autonomous causes of infertility and a combination of this with gene therapy for germ cell autonomous causes. Additionally, oocyte differentiation from autologous SCs would be useful for drug screening and toxicity testing. In contrast to men who have testicular spermatogonial SCs into adulthood that can yield pluripotent autologous SCs, it is thought that women do not have functional adult germline SCs as evidenced by oocyte depletion and limited reproductive potential. This dogma was recently challenged, but the existence of functional adult female mouse or human germline SCs in the bone marrow or peripheral blood remains controversial (Fu et al., 2009; Gavrilov et al., 2009; Hough et al., 2009; Smith and Wagner, 2009).

Pluripotency can be artificially maintained *in vitro* by culturing mouse ESCs with human ESCs. The artificial nature of this system poses a significant challenge for generating germ cells considering that endogenous PGCs may be reprogrammed when removed from their somatic niche *in vivo* and cultured *in vitro* on feeders with similar factors (Elkabetz and Studer, 2008; Rippon et al., 2008; Verfaillie, 2009).

The integration of X-inactivation with development is a crucial aspect of this classical paradigm of epigenetic regulation. During early female mouse development, X-inactivation reprogramming occurs in pluripotent cells of the inner cell mass of the blastocyst and in pluripotent PGCs (Do and Scholer, 2009; Navarro and Avner, 2009).

Application of embryonic and adult SCs in regenerative medicine will require efficient protocols for directing SC differentiation into well-defined lineages. The use of exogenous cytokines, growth factors, or extracellular matrix substratum, will obviously require extended durations of in vitro culture. With autologous adult SCs, this could delay transplantation to the patient, as well as alter the immunogenicity of the cultured autologous cells. Genetic modulation to direct SC differentiation would obviate prolonged durations of in vitro culture; but there are overwhelming safety concerns with regards to the application of recombinant DNA technology in human clinical therapy (Heng and Cao, 2005). SCs contain an intrinsically predefined, unidirectional differentiation program. This means that the developmental fate of a SC is dependent on the general potential of the cell as well as on microenvironmental cues, such as stimuli from growth factors (Banas et al., 2007; Bruserud et al., 2000; Piller, 2009; Schaeffer et al., 2009).

Undifferentiated ESCs grow as colonies with defined borders and are compact cells with prominent nucleoli. ESC differentiation is routinely achieved by withdrawing the pluripotency-maintaining factors from the culture media, removing the feeder layer, and allowing the ESCs to spontaneously differentiate into a heterogeneous mixture of cell types representing the three somatic germ layers and the germline. ESCs are primarily differentiated as either embryoid bodies in suspension, using hanging drops or untreated low-attachment plates, or as adherent monolayer cultures (Ko and Scholer, 2006; Saiti and Lacham-Kaplan, 2007). Spontaneous differentiation of ESCs into a diverse mixture of cell types is poorly understood and is most likely related to the unknown mixture of factors in the serum and media. To help direct the differentiation of a specific cell type, factors can be added to the differentiation media, with or without serum, to generate more homogeneous cultures (Hubner et al., 2006; Ko and Scholer, 2006; Nicholas et al., 2009; Sasaki et al., 2009; Vadivelu et al., 2005).

Germ cells were originally identified in human ESC-derived cultures by assessing the expression of germ cell-specific markers during differentiation (Brevini et al., 2007; Fadare and Zheng, 2009; Matsumoto et al., 2009; Walakira et al., 2008).

ESC technology should enable the generation of specific cell types for the study and treatment of human diseases. Therapeutic cloning provides a way to generate ESCs genetically matched to diseased individuals through nuclear reprogramming of the somatic genome. An alternative approach to reprogram the somatic genome involves fusion between somatic and pluripotent cells. Potential fusion partners with reprogramming activities include embryonal carcinoma cells, embryonic germ cells, and ESCs (Pralong et al., 2006). Cells isolated from both the embryoid bodies and genital ridge positive

controls expressed germ cell markers and are viable, suggesting that density gradient isolation might represent a feasible and clinically relevant approach for obtaining ESC-derived germ cells (Brevini et al., 2009; Lin et al., 2007; Mise et al., 2008; Nicholas et al., 2009).

ESCs, derivatives of totipotential cells of early mammalian embryos, have proven to be one of the most powerful tools for studying developmental and SC biology. When injected into embryos, ESCs can contribute to tissues derived from all three germ layers and to the germ line (Hubner et al., 2006).

Many challenges need to be overcome to achieve robust and functional oocyte differentiation from ESCs. The low efficiencies of ESC-derived oocyte-like cell maturation reported to date may be unavoidable because most endogenous oocytes undergo atresia during development. However, the optimization of methods to direct ESC-derived germ cell specification, oocyte commitment, and oocyte maturation may increase efficiencies and enable functional oocyte production. After endogenous germ cell specification in the early embryo, germ cells are set aside from the embryo proper and are isolated from initial somatic cell differentiation. Considering that meiotic progression to diplotene arrest may be required for subsequent follicle formation, it will be of utmost importance to optimize ESC-derived germ cell specification and/or oocyte commitment protocols to overcome the current obstacles to proper meiotic entry and progression during ESC differentiation (Mendenhall et al., 2001; Simonian, 2003).

Given the success of ESC-derived sperm and endogenous PGC maturation via transplantation and the particular importance of ovarian somatic cells in supporting endogenous oocyte development, appropriately staged ovarian granulosa cells may be required for the maturation of committed ESC-derived oocytes using coculture and/or transplantation methods (Hsieh et al., 2009; Kato et al., 2009; Lenartowicz et al., 2008; Nicholas et al., 2009; Whitten and Miller, 2007).

5. PGC Description

PGCs in mice have been shown to originate from some of the most proximal epiblast cells, to become identifiable during early gastrulation by their characteristic alkaline phosphatase activity. (de Las Heras et al., 2009; Houston and King, 2000; Rangan et al., 2009; Tani et al., ; Yabuta et al., 2006).

Most notably, PRDM1 has been found to be one of the most critical regulators for PGC specification. In the absence of PRDM1 activity (Kurimoto et al., 2008; Yabuta et al., 2006; Yamano et al.).

Specification of germ cell fate is fundamental in development and heredity. Recent evidence indicates that in mice, specification of PGC), the common source of both oocytes and spermatozoa, occurs through the integration of three key events: repression of the somatic program, reacquisition of potential pluripotency and ensuing genome-wide epigenetic reprogramming. Here we provide genetic evidence that Prdm14, a PR domain-containing transcriptional regulator with exclusive expression in the germ cell lineage and pluripotent cell lines, is critical in two of these events, the reacquisition of potential pluripotency and successful epigenetic reprogramming (Yamaji et al., 2008). Germ cell specification in mice, which generates PGCs, the common source of the oocytes and spermatozoa, from the epiblast, integrates three key events: repression of the somatic program, re-acquisition of potential pluripotency, and genome-wide epigenetic reprogramming The launch of the germ cell lineage in mice, therefore, is orchestrated by two independently acquired, PR domain-containing transcriptional regulators (Kurimoto et al., 2008). PGCs have been shown to undergo extensive epigenetic reprogramming, including genome-wide DNA demethylation, erasure of parental imprints, and re-activation of the inactive X-chromosome (Hayashi and Surani, 2009, Chuva de Sousa Lopes et al., 2008; Hayashi and Surani, 2009; Yabuta et al., 2006).

The formation of germ cells during embryogenesis bears the ultimate importance for the continuation of every species. It becomes evident that mechanisms governing germ cell fate specification are not well conserved across the animal kingdom. In most of the invertebrate and nonmammalian vertebrate species, certain maternally derived factors are key to the establishment of germ cell lineage. In contrast, mouse PGCs are induced from the pluripotent epiblast cells before and during gastrulation by the extraembryonic cell-derived signals (Ying et al., 2002). There have been several reports regarding the derivation of gametes from ES cells in culture (Fukunaga et al., ; Hiller et al., ; Yabuta et al., 2006).

In the last decade of intensive research, a major part of the framework for the signaling and transcription in PGC specification in mice has been established (Kimura and Brenner, 1997; Mistry and Cresci; Soodeen-Karamath and Gibbins, 2001).

6. Related Techniques

Mammalian spermatogenesis consists of three biologically significant processes: SC self-renewal and differentiation, meiosis, and haploid cell morphogenesis. Understanding the molecular mechanisms behind these processes might provide

clues to the puzzle of species preservation and evolution, and to treatments for male infertility. However, few useful in vitro systems exist to investigate these processes at present. To elucidate these mechanisms, in vivo electroporation of the testis might be a convenient option (Bowles et al., ; Kanatsu-Shinohara et al., 2004; Yomgogida, 2008).

(1) Specification of germ cells in mammals

Germ cells are the biological route for genetic transmission from one generation to the next. These cells constitute a very different cell population from somatic cells, with unique characteristics, and display a haploid chromosomal number after a delicate process of meiosis. PGCs in the female embryo do not complete meiosis, and are arrested at prophase I of the first meiotic cycle. Progress in meiosis occurs post-natally just prior to ovulation followed by another arrest in meiosis II, which is completed just after fertilization (Morimoto et al., 2009; Park et al., 2009; Van Haute et al., 2009; Yang et al., 2009).

(2) *In vitro* germ cells differentiation from ESCs

ESCs represent permanent cell lines that can be maintained in an undifferentiated state. In an environment that induces differentiation, they form derivatives of the three embryonic germ layers: mesoderm, ectoderm, and endoderm. The pups showed growth abnormalities, and most died during the first months of life, emphasizing the crucial concept that imprinting status of the derived gametes from SCs must be carefully studied (Aflatoonian et al., 2009; Teramura et al., 2009; Zovoilis et al., 2009). Taking a step back, scientists need to reconsider their strategies when related to *in vitro* gametogenesis by looking at the *in vivo* processes. During murine and human ESCs *in vitro* differentiation, markers of both male and female germ cells have been detected in culture regardless of the sex of the cell line. The cells may be responding to an external signal instead to an intrinsic program to enter meiosis. It is possible that retinoic acid present in the culture conditions propitiate meiosis commencement and gamete determination. (Hwang et al., 2008; Micallef et al., 2007; Vaags et al., 2009). Attempts to derive germ cells from human ES resulted in similar findings as described in mice (Lin et al., 2007; Mai et al., 2007; Teramura et al., 2007; Wianny et al., 2008).

(3) *In vitro* germ cells differentiation from somatic SCs

Spermatogenesis is a complex process that cannot be modeled in vitro. The somatic Sertoli cells within the seminiferous tubules perform a key role in supporting maturation of germ cells. intra-testicular injection of adenoviral vectors disturbs SC function in vivo and future work will therefore focus on the use of lentiviral delivery systems (Hooley et al., 2009). Germline SCs and mature gametes can be derived *in vitro* from multipotent SCs other than ESCs. In mice, teratocarcinoma and MSC and induced iPS are considered multipotent cells, which are able to differentiate into various cell types representative of all three germ layers. With the exception of iPS cells, the other cell types have been shown to differentiate into either germline SCs or early germ cells *in vitro* and to advanced male gametes *in vivo* following transplantation. Embryonal carcinoma cells derived from teratocarcinomas, like ESCs, are pluripotent and able to differentiate through embryoid bodies to germline SCs. When transplanted into sterile mice, EC-derived germline SCs are able to complete meiosis and spermatogenesis (Marques-Mari et al., 2009).

(4) Demanding biological descent: how to obtain patient-specific gametes

The growing demand for biological offspring for patients with impaired fertility has put its hope in scientific research and the obtaining of patient-specific differentiated gametes. Nuclear transfer can be defined as the creation of somatic embryos using the oocyte cytoplasm as the reprogramming conductor following transfer of somatic nuclei into enucleated mature oocytes (Galende et al., 2009; Kim et al., 2009a; Lee et al., 2009a; Utikal et al., 2009). Somatic cells can be reprogrammed to a totipotent state through nuclear transfer or cloning, because it has been demonstrated that the oocyte has the ability to reprogram an adult nucleus into an embryonic state that can initiate the development of a new organism. Therapeutic cloning, whereby nuclear transfer is used to derive patient-specific ESCs, embraces an entire new opportunity for regenerative medicine (Chang et al., 2009a).

(5) Epigenetic modifications in the germline

Genomic imprinting is a DNA modification pattern which is common and unique to all cells of an individual. This imprinting can be found at both genomic areas without a specific function and areas where the expression of specific genes is controlled. Thereby imprinting can control the proper expression levels of genes required for normal embryonic development and cell function, without changing the genome itself. In an organism the imprinting pattern is partially inherited from both paternal and maternal genomes. After fertilization the imprinting marks and epi-mutations are erased by the epigenetic machinery of the zygote, and then re-established in a new and unique pattern depending on the sex of the new

individual. Germ cells constitute the vehicles of genetic information and thereby of inheritance through generations. The epigenetic control mechanisms that govern maintenance and reprogramming of the germline in diverse organisms have gained increasing interest as they reveal essential regulatory pathways implicated in health and disease (Mishra and Sassone-Corsi, 2008). These epigenetic marks, which define the imprinting, include modifications in DNA and histones, especially methylation, acetylation, phosphorylation and ubiquinitation, DNA methylation being the best characterized of these mechanisms, which has been shown to have essential functions in the germline and the embryo development. These chromatin modifications provide a mechanism for the adequate expression and repression of genes and hence for their temporal or permanent inactivation. Epigenetic modifications are sequentially established and erased in the germinal lineage. PGCs undergo DNA demethylation of the imprinted loci as soon as they reach the gonadal ridge and the parental patterns are erased. Re-establishment of different imprints in germ cells of both sexes with new patterns according to the gender of the new individual is initiated during male and female gamete differentiation. During spermatogenesis, de novo methylation occurs before the onset of meiosis, while in oogenesis it occurs after the onset of meiosis. For the zygote to acquire totipotency extensive epigenetic reprogramming occurs. Shortly after fertilization the paternal genome undergoes rapid demethylation, while the maternal genome is slowly demethylated during the first cleavages. Then, around the time of implantation of the blastocyst there is a wave of de novo methylation to establish an individual specific pattern. Parthenogenesis, a mechanism by which an oocyte is activated without fertilization by a spermatozoid, has been described in some studies after differentiation of oocyte-like cells from SC in culture (Ambe et al., 2008; Devaux et al., 1989; Van den Bergh et al., 1995).

(6) *In vitro* differentiation of gametes from SC

Meiotic and post-meiotic markers are more reliable markers, but it has been demonstrated that the progression through the meiotic process is still a challenge in the *in vitro* differentiation of gametes. The transfection of ESC lines with marked or fluorescent proteins linked to specific gene promoters enables the visualization of the cells in which the specific gene of interest is expressed during the differentiation process (Maurin et al., 2009; Muruvi et al., 2009; Netzel-Arnett et al., 2009; Pires et al., 2009; Xu et al., 2009).

(7) Human stem cell culture

Information regarding the human ES cell lines, HSF-6, HSF-1 and H9 (NIH codes UCO6, UC01 and W-9, respectively) can be obtained at http://stemcells.nih.gov/stemcell/. Undifferentiated human ES cell colonies are cultured on irradiated CF1 mouse embryonic fibroblast feeder cells at 5% CO_2 in supplemented DMEM medium as previously described (Caldwell et al., 2009; Kim et al., 2009c; Oyer et al., 2009; Rodriguez et al., 2009; Slack-Davis et al., 2009).

(8) RNA and cDNA production

At days 0, 3, 7, 14 and 22, embryoid bodies are collected, centrifuged and resuspended in 600 ml RLT buffer for RNA extraction. Total RNA is extracted via the RNeasy system from three independently isolated samples from each of the three different human ES cell lines. cDNA is generated from 3 µg of total RNA using 250 ng of random hexamers under standard conditions with MLV reverse transcriptase. PCR is performed with 50 ng of the first strand cDNA reaction. Three inner cell masses are isolated from blastocysts by immunosurgery using antibody against human choriocarcinoma cells and guinea pig complement according to standard procedures. Total RNA from the three independently isolated mammalian inner cells mass is extracted using the PicoPure RNA isolation system followed by reverse transcription and cDNA production. Mammalian inner cell mass cell cDNA is concentrated using DNA Clean and Concentrator and resuspended in 15 µl water. cDNA from each mammalian inner cell mass is split into eight equal aliquots and used to assay expression of NCAM1, OCT4, NANOS, STELLAR, DAZL, VASA, SCP1 *and* GAPDH (Jones et al., 2009; Junttila et al., 2009).

(9) Quantitative PCR

Quantitative PCR reactions on human ES cell cDNA are performed in duplicate on each sample in the presence of 4.5 mM $MgCl_2$, 10 mM dATP, dGTP, dCTP and dTTP, 2 µM primers, 0.25 U Platinum *Taq*, 1×SYBR green, 1×fluorosceine and 2% DMSO. SYBR green PCR amplifications are initiated at 95°C for 5 min followed by 35 cycles of 95°C, 30 s; 60°C, 30 s; and 72°C, 30 s. Quantitative PCR on human mammalian inner cell mass cell cDNA is performed using the Assay-on-Demand technology for NCAM1, OCT4, VASA, DAZL, SCP1 and GAPDH and Assay-by-Design for NANOS *and* STELLAR according to manufacturer's specifications using FAM-490. Results are analyzed using an iCycler iQ^{tm}. For SYBR green, equal reaction efficiencies are verified via serial dilution of testis cDNA over a 100-fold range. All experiments included negative controls with no cDNA

for each primer pair. Primers are designed to span exons to distinguish cDNA from genomic DNA products on human ES cell cDNA (de la Lastra and de la Fuente, 2008; Hu et al., 2009; Page et al., 2007; Xie et al., 2009).

(10) Western blot analysis

Western blot analysis is performed according to standard procedures.

(11) Hypotonic immunohistochemistry for meiotic markers

Germ cells arise from a small group of cells that express markers of pluripotency including OCT4. In humans formation of gonadal compartments takes place during the 1st trimester (6-8 weeks gestation) (Anderson et al., 2007). Human ES cells at day 0 are digested for 15 min at 37°C with 1 mg/ml collagenase type IV/1 mg/ml dispase in knockout DMEM high glucose containing 20% knockout serum replacer, 1 mM glutamine, non-essential amino acids, 0.1 mM β-mercaptoethanol and 4 ng/µl FGF2. Colonies are gently dislodged, centrifuged at 1000 rpm, 5 min, and then resuspended in freshly prepared hypoextraction buffer pH 8.2 (30 mM Tris pH 8.2/50 mM sucrose/17 mM citric acid/5 mM EDTA/0.5 mM DTT/0.5 mM Pefabloc). Embryoid bodies and ES cell colonies are then lightly teased apart before further incubation in hypoextraction buffer for 30 min. ES cell colonies and embryoid bodies are then collected into 100 mM Sucrose before teasing apart using 20 gage needles to form a single cell suspension. The single cell suspensions are pippetted onto glass slides, previously treated with 1% paraformaldehyde in PBS pH 9.2 containing 0.25% Tween 20. The slides are then in 0.04% photoflo in distilled H_2O followed by incubation for 30 min in Antibody Dilution Buffer containing 10% Normal Donkey Serum/3% BSA/0.5% Tween 20. Slides are incubated overnight at 37°C with rabbit anti-human SCP3 and mouse anti-rat MLH diluted in ADB at 1/500 and 1/25, respectively. Slides are washed for 10 min in ADB followed by 24 h in ADB at 4°C. Slides are incubated with secondary antibodies (rhodamine-conjugated anti-mouse for MLH; FITC-conjugated anti-rabbit for SCP3; 1/100 dilution), 45 min at 37°C. Slides are washed four times in PBS and sealed under a coverslip with anti-fade mounting media (Borodin et al., 2009; Togni et al., 2009; Viana-Pereira et al., 2009).

(12) Immunohistochemistry on paraffin-embedded tissue sections

Contemporary pathology involves an emerging role for molecular diagnostics (van Maldegem et al., 2008). Human testis specimens are fixed by immersion in Bouin's fixative; embryoid bodies are fixed in 4% paraformaldehyde in PBS (pH 7.4), 1 h, processed to paraffin, and cut at 5 µm serial sections onto slides. Slides are blocked by incubation in PBS/0.1% BSA/0.3% Tween 20/10% normal goat serum as appropriate for 30 min. Slides are incubated with primary antibody overnight at 4°C (rabbit anti-human DAZL; 1/100; rabbit anti-human STELLAR: 1/1000 [antibodies made against peptide sequence RESVGAAVLREIEDE of human STELLAR; chicken anti-human VASA, 1/1000] in PBST before washing twice for 10 min in PBS. Sections are then incubated twice for 10 min in PBS before mounting under a glass coverslip with anti-fade mounting media. For avidin biotin immunohistochemistry, following incubation in primary antibodies, sections are incubated for 30 min at room temperature, with either biotin conjugated anti-rabbit to detect DAZL and STELLAR according to manufacturer's instructions, or biotin conjugated anti-chicken (1/200) to detect VASA. Sections are washed twice for 10 min in PBS followed by incubation for 30 min in ABC reagent (Lu et al., 2009; Shi et al., 2007).

(13) Whole mount immunohistochemistry

Whole-mount in situ hybridization is a technique used to localize and visualize specific gene transcripts in whole embryos by hybridizing labeled RNA probes complementary to the sequence of interest. A digoxigenin (DIG)-labeled riboprobe synthesized during in vitro transcription through the incorporation of DIG-labeled UTP is hybridized to the target sequence under stringent conditions, and excess unhybridized probe is removed during a series of washes. The location of the labeled riboprobe, and thus the mRNA sequence of interest, is then visualized by immunohistochemistry. This protocol outlines the steps involved in preparing Hawaiian bobtail squid embryos, hybridizing a DIG-labeled riboprobe in whole-mount embryos, and visualizing the labeled RNA colorimetrically using an alkaline-phosphatase-conjugated anti-DIG antibody (Lee et al., 2009b).

Undifferentiated human ES cells are grown on feeder layers for at least 4 days before removing the media and fixing in 4% paraformaldehyde in PBS (pH 7.4) for 15 min. Colonies are then washed twice for 10 min in TBS+0.05% Tween 20, followed by incubation in 0.1% Triton X in PBS for 10 min. Colonies are washed as above, incubated for 30 min with 4% normal donkey serum, and incubated with primary antibodies diluted in PBS (1/100, DAZL; 1/100, STELLAR; 1/1000, VASA; 1/30 SSEA1; 1/30 SSEA3; 1/30 SSEA4; 1/30 TRA-1-81; 1/30 TRA-1-60; 1/50 NCAM1; 1/50 KDR and neat AFP for immunofluorescence). For Avidin–biotin

immunohistochemistry antibodies are diluted (1/500, DAZL; 1/1000, STELLAR; 1/1000 VASA in PBS). All primary antibodies are incubated for 1 h at room temperature. Colonies are then washed twice in PBS and incubated with secondary antibodies as described above. Colonies are viewed using a Leica microscope fitted for immunofluorescence after mounting with anti-fade mounting media or PBS (Akhtar et al., 2009; Rasweiler et al., 2009; Sawada et al., 2008).

7. Clinical Significance, applications and future prospects of Germ Stem Cell

SC research offers great promise for understanding basic mechanisms of human development and differentiation, as well as the hope for new treatments for diseases such as diabetes, spinal cord injury, Parkinson's disease, and myocardial infarction. However, human SC research also raises sharp ethical and political controversies. The derivation of pluripotent SC lines from oocytes and embryos is fraught with disputes about the onset of human personhood. The reprogramming of somatic cells to produce induced pluripotent SCs avoids the ethical problems specific to ESC research. In any human SC research, however, difficult dilemmas arise regarding sensitive downstream research, consent to donate materials for human SC research, early clinical trials of human SC therapies, and oversight of human SC research. These ethical and policy issues need to be discussed along with scientific challenges to ensure that SC research is carried out in an ethically appropriate manner (Lo and Parham, 2009). If SC-derived oocytes could someday be safely used for reproductive purposes, a large and diverse group of infertile women would have the opportunity to bear genetically related offspring. Common causes of infertility that could be treated using SC-derived oocytes include premature ovarian failure, reproductive aging associated with delayed childbearing, and poor oocyte quality, which can occur even in young women (Revazova et al., 2008).

Mouse ESCs can differentiate into female and male germ cells in vitro. Primate ES cells can also differentiate into immature germ cells in vitro (Yamauchi et al., 2009). Seemingly, the germ cell differentiation process is dependent on spatial distribution of differentiating cells. Embryoid bodies represent a three-dimensional structure with a microenvironment that propitiates differentiation, although other cell types besides germ cells arise from them. Currently, one of the most critical steps after differentiation is selection and isolation of differentiated germ cells. Since ESCs and PGCs share some common markers, detection of post-migratory and meiotic markers is a useful method, as well as the use of transgenes with fluorescent reporter genes under the control of specific promoters of male and female germ cells. These have been the most employed strategies in the studies of germ cell differentiation developed to date. The problem is that methods based on gene modification and the use of retroviral vectors limit the use of gamete-like cells in future clinical treatments. Future translational application of these ESC-derived gametes in ART when functional gametes are not available from patients require still further investigation into gamete differentiation. Ultimately, the process needs to be reproducible and efficient. The focus of current and future studies should be on meiotic completion to avoid unwanted aneuploidies and the determination and establishment of accurate epigenetic modifications and imprinting status to provide reproductive hope to humans lacking gametes (Berruti, 2006).

Premature ovarian failure is typically defined by amenorrhea, hypoestrogenism, and hypergonadotropinism in a woman who is under 40 yr of age at the time of onset. Although the incidence of premature ovarian failure is not known with certainty, it has been estimated to occur at a frequency of approximately 1%. Because some women with apparent premature ovarian failure may ovulate or occasionally conceive after the diagnosis is established, the term "primary ovarian insufficiency" has been suggested to describe the condition more accurately. Primary ovarian insufficiency is a heterogeneous disorder that may result from a decrease in the initial primordial follicle number, an increase in follicle destruction, or a failure of the follicle to respond to gonadotropin stimulation. Known genetic causes include absence or structural abnormalities of the X chromosome, Fragile X syndrome, structural rearrangements of autosomes, point mutations of autosomal genes that are required for oogenesis and folliculogenesis, and abnormalities of pleiotropic Mendelian genes that may disturb ovarian function along with perturbation of other organ systems. Multiple autoimmune etiologies for primary ovarian insufficiency have also been described, and environmental causes are just beginning to be explored as well. For example, in the mouse, *in utero* exposure to the environmental contaminant bisphenol A has been convincingly associated with oocyte aneuploidy in female offspring. Ovarian function may also be lost via surgical removal of the ovaries due to ovarian cancer or as a result of chemotherapy for nongynecological malignancy. Many causes of ovarian failure remain idiopathic. Research involving SC-derived oocytes may not only provide a potential reproductive option for women with primary ovarian insufficiency, but

may also lead to a better understanding of its causes (Guo et al., 2009).

The rise in life expectancy over the last century, together with higher maternal and paternal ages and have highlighted the issue of reduced fertility with advancing age. Aging of the male reproductive system is incited by multi-factorial changes at molecular, cellular and regulatory levels, and individual characteristics are highly variable, although strongly influenced by lifestyle and environmental factors. Damage accumulated with age leads to progressive deregulation of the hypothalamic-pituitary-gonadal axis and of local auto/paracrine interactions, thereby inducing changes in target organs such as the testis, penis and prostate. Elderly human males produce less testosterone, have fewer motile sperm and a higher incidence of erectile dysfunction and prostate disorders, all of which contribute to lower fertility. Cellular aging can manifest itself at several levels. Aging cells progressively accumulate "waste" products, resulting in a decreased functionally. Changes to mitochondria are among the most remarkable features observed in aging cells and several theories place mitochondria at the hub of cellular events related to aging, namely in terms of the accumulation of oxidative damage to cells and tissues, a process in which these organelles may play a prominent role, although alternative theories have also emerged. Furthermore, mitochondrial energy metabolism is also crucial for male reproductive function and mitochondria may therefore constitute a common link between aging and fertility loss (Amaral and Ramalho-Santos, 2009). Childbearing at an advanced maternal age is strongly associated with an increase in the risk of fetal aneuploidy. An age-associated increase in aneuploidy has also been noted in human oocytes and in biopsied human embryos. Multiple factors could potentially account for genetic abnormalities in the oocyte, and it is likely that abnormalities may arise during multiple stages of oogenesis. SC-derived oocytes may eventually allow a better understanding of age-associated aneuploidy as well as a potential option for the creation of euploid oocytes (Atsalis and Margulis, 2008).

Assessment of oocyte maturity and quality at the time of retrieval is difficult as the egg is obscured by a large cumulus mass that hinders adequate scoring (Ebner et al., 2008). Fertility specialists commonly encounter infertile women with poor oocyte quality and resulting poor embryo development, particularly in couples who are characterized as having "unexplained" infertility before *in vitro* fertilization. In some cases, the problem with oocyte quality may be defined by recurrent low rate of oocyte maturation. In other cases, the recurrent problems with oocyte quality are characterized on the basis of abnormal morphological appearance of the oocytes and resulting embryos that develop with high rates of fragmentation and arrest before the blastocyst stage. There are no suitable techniques for improving oocyte quality either in the clinic or in the embryology laboratory. Problems with oocyte quality are poorly understood, and it is hoped that SC-derived oocytes may offer some understanding and eventually a treatment option for these infertile couples as well (Nikolaou, 2004).

Oocyte donation is very expensive due to the need for extensive screening of the donor, ovarian stimulation with injections of gonadotropin, oocyte retrieval, and financial compensation given to the donor. As an alternative to oocyte donation, some couples may be willing to consider embryo donation. Research utilizing oocytes derived from SCs could also provide valuable insight into the processes of both normal and abnormal oocyte development. It is quite plausible that this research will not only enhance our ability to address infertility, but will also help us to understand better some of the causes of abnormal offspring in spontaneous conceptions occurring among the general population. The clinical relevance of research involving SC-derived oocytes is unquestionable (Egashira et al., 2009; Tao and Del Valle, 2008). In diverse model organisms that include species of flies, worms, frogs, fish, salamanders, mice and non-human primates, as well as in humans, the expression of *DAZ* gene family homologs has been shown to be restricted entirely to the germ cell lineage (Chang et al., 2009b).

Embryoid bodies represent a three-dimensional structure with a microenvironment that propitiates differentiation, although other cell types besides germ cells arise from them. Currently, one of the most critical steps after differentiation is selection and isolation of differentiated germ cells. Since ESCs and PGCs share some common markers, detection of post-migratory and meiotic markers is a useful method, as well as the use of transgenes with fluorescent reporter genes under the control of specific promoters of male and female germ cells. These have been the most employed strategies in the studies of germ cell differentiation developed to date. The problem is that methods based on gene modification and the use of retroviral vectors limit the use of gamete-like cells in future clinical treatments. Future translational application of these ESC-derived gametes in ART when functional gametes are not available from patients require still further investigation into gamete differentiation (Chomez et al., 1996).

Human ES cell lines are the *in vitro* manifestation of cells of the mammalian inner cell mass derived from blastocysts. Previous reports have demonstrated that human ES cells are pluripotent and

capable of differentiating into a wide variety of somatic cell types during EB formation. However, germ cell differentiation from human ES cells has never been reported. In this study, we used a panel of germ cell-specific and germ cell-enriched markers, together with markers of somatic cell lineages, to assess the ability of three lines of undifferentiated human ES cells to form germ cells *in vitro*.

Directing embryonic stem (ES) cells to differentiate into functional motoneurons has proven to be a strong technique for studying neuronal development as well as being a potential source of tissue for cell replacement therapies involving spinal cord disorders (Soundararajan et al., 2007). The genetic similarity of human ES cells to the mammalian inner cell mass has never been addressed. It is difficult to determine *a priori* whether it would be likely or not for human ES cells and mammalian inner cell mass to differ significantly in gene expression (Inanc et al., 2008).

VASA expression during ES cell differentiation allows for identification of more mature stages of germ cells in humans (Eguizabal et al., 2009; Lavagnolli et al., 2009).

Previously, two independent studies demonstrated that mouse ES cells could form germ cells *in vitro*. Both groups took advantage of the ease of genetic manipulation of mouse ES cells and generated undifferentiated ES cell lines that could be sorted for a fluorescent GFP marker under the control of germ cell specific promoters (Denker et al., 2007). The derivation of germ cells from pluripotent SCs in vitro could provide an invaluable model system to study both the genetic and epigenetic programming of germ cell development in vivo (Eguizabal et al., 2009).

Discussion

Exactly how ESC cultures may mimic the somatic environment that encapsulates either developing oogonia (the follicle) or the sperm SC (seminiferous epithelium) is unclear. The appropriate growth factor and hormonal microenvironment required to support and sustain these complex niches probably depends to some extent on the type of culture system adopted for the differentiation process. ESCs are cultured usually in two basic ways. Monolayer adherent cultures of ESCs can be allowed to differentiate directly to form an appropriate niche or ESCs can be induced to aggregate to form embryoid bodies that form a more three-dimensional microenvironment. In conjunction with these two culture methods, ESCs can be initially co-cultured with feeder layers or conditioned-medium from feeder cells and various growth factor and serum supplementation (Falender et al., 2005; Goel et al., 2008; Nistal et al., 2006; Sakai et al., 2004).

In the mouse, it has been claimed that germ cells can be derived from bone marrow and peripheral blood cells and bone marrow-derived germ cells can repopulate the ovarian follicular reserve. Many studies in both mice and humans have shown that ES cells have the capacity to proliferate and to differentiate to numerous cell lineages. Using *in vitro* studies, mouse ES cells can differentiate into lineages that include hematopoietic, vascular, pancreatic, neural, and muscular and germ cells. Likewise, human ES cells can also differentiate to several lineages *in vitro*, including neural, pancreatic, muscular, endothelial, trophoblast and hematopoietic cells (Choi et al., 2009; Hester et al., 2009; Inanc et al., 2008; Martinez-Lorenzo et al., 2009).

Since undifferentiated human ES cells expressed a number of markers indicative of putative premeiotic germ cells, we tested whether germ cell specific proteins are also expressed in undifferentiated human ES cells. Western blot analysis of human fetal and adult testis tissue was used as a positive control since the transcriptional and translational profiles of these tissues have been well documented (Faipoux et al., 2008; Kuypers et al., 2009; Nervi et al., 2007).

The culture of spermatogonial cells under well-defined conditions would be an important method for elucidating the mechanisms involved in spermatogenesis and in establishing tissue regeneration in vivo (Creemers et al., 2002). The promiscuous nature of spontaneous ESC differentiation in culture makes it especially difficult to determine at present whether PGC and germ cell development follow similar program to those occurring *in vivo* or whether the conditions merely provide suitable conducive conditions that permit an intrinsic developmental process but not necessarily the same as the gonadal environment (Kim et al., 2009b). From their formation in the early embryo to their ultimate acquisition of functionality in the adult, germ cells must complete a long journey with many genetic and epigenetic requirements (Arany et al., 2009; Hsieh et al., 2009).

Acknowledgement

All the descriptions in this article are obtained from the materials published. Thanks all the scholars this article references.

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3/1/2010