Review

Stem cells and germ cells: microRNA and gene expression signatures

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Summary. The study of primordial germ cell development *in vivo* is hampered by their low numbers and inaccessibility. Recent research has shown the ability of embryonic and adult stem cells to differentiate into primordial germ cells and more mature gametes and this generation of germ cells *in vitro* may be an attractive model for their study. One of the biggest challenges facing *in vitro* differentiation of stem cells into primordial germ cells is the lack of markers to clearly distinguish the two. As both cell types originate early in embryonic development they share many pluripotent markers such as OCT4, VASA, FRAGILIS, and NANOG. Genome wide microarray profiling has been used to identify transcriptome patterns unique to primordial germ cells. A more thorough analysis of the temporal and quantitative expression of a panel of genes may be more robust in distinguishing these two cell populations. MicroRNAs, short RNA molecules that have been shown to regulate translation through interactions with mRNA transcripts, have also recently come under investigation for the role they may play in pluripotency. Attempts to elucidate key microRNAs responsible for both stem cell and primordial germ cell characteristics have recently been undertaken. Unique microRNAs, either individually or as global profiles, may also help to distinguish differentiated primordial germ cells from stem cells *in vitro*. This review will examine gene expression and microRNA signatures in stem cells and germ cells as ways to distinguish these closely related cell types.

Key words: Stem cell, Pluripotency, Germ cell, Micro RNA, Stem cell migration

Introduction

Although the process of germ cell formation is well described for a variety of species, the molecular mechanism by which this occurs and the factors regulating it are still poorly understood. Primordial germ cells (PGCs) are derived from the pluripotent epiblast and are first visible as alkaline phosphatase (AP)-postive cells. They then proliferate rapidly, migrating to the genital ridge, which form the future gonads, colonizing and proliferating until male germ cells enter the G phase of the cell cycle and female germ cells begin meiosis (Sato et al., 2003). In order to elucidate the factors and mechanisms involved in PGC specification, an understanding of PGC formation is essential. Unfortunately, the study of PGC formation *in vivo* has proven to be difficult for a number of reasons. Their low numbers; their location deep within the developing embryo; and their migration during development makes *in vivo* analyses difficult to undertake (Chiquoine, 1954; Ginsburg et al., 1990; Lawson and Hage, 1994).

Several studies have shown the ability of embryonic stem (ES) cells to differentiate into PGCs as well as more mature gametes (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004; Nayernia et al., 2006b; Qing et al., 2007). In addition to ES cells, a number of somatic stem cell types have also been used to successfully generate germ cells and gametes (Dyce and Li, 2006; Dyce et al., 2006; Nayernia et al., 2006a; Danner et al., 2007). Using stem cells to provide an *in vitro* model for PGC formation will help further the understanding of germ cell development. One challenge in deriving PGCs from stem cells is that PGCs formed during *in vitro* differentiation express many of the same pluripotent markers expressed by stem cells, making their identification difficult. While no single expression marker can be used to positively distinguish PGCs from stem cells, microarrays and newer digital gene expression technologies have made transcriptome-wide expression profiles possible. Comparing global gene

expression patterns may help to distinguish these similar cell types. In the same vein as gene expression profiles, the recently discovered class of small RNA molecules, microRNAs (miRNAs), may also be a source of markers by which to distinguish PGCs from stem cells as miRNA expression is cell-type dependent (Hayashi et al., 2008; Foshay and Gallicano, 2009; Ren et al., 2009).

This review will investigate both miRNA- and gene expression-based comparisons between different stem cell types and PGCs to highlight possible methods to used to identify PGCs *in vitro*.

MicroRNA

MicroRNAs (miRNAs) are a type of small RNAs, a family of biological molecules that include small interfering RNAs (siRNAs), small nuclear and small nucleolar RNAs (sn- and snoRNAs) (Singh et al., 2008). MicroRNA is expressed in a time- and tissue-specific manner, and shows a great deal of conservation among a diverse range of species (Niwa and Slack, 2007). They consist of 18-25 nucleotides that originate from endogenous hairpin transcripts called primary miRNAs (pri-miRNA). The pri-miRNAs are processed by the RNase Drosha in the nucleus before being exported as ~70-nucleotide pre-miRNAs. In the cytosol, pre-miRNAs are further processed by another RNase, Dicer, into mature double-stranded miRNAs (van den Berg et al., 2008). After being separated, one strand becomes incorporated into the RNA-Induced Silencing Complex (RISC) that can then bind to the 3' untranslated region (UTR) of the target mRNAs and regulate their translation (Smalheiser, 2008). Nucleotides 2-8 of the miRNA, the "seed region", are considered to be the most critical region for selecting targets (Singh et al., 2008). Based on the degree to which the seed region complements the mRNA target, the mRNA transcript is either degraded, if the miRNA is highly complementary, or translationally repressed (van den Berg et al., 2008). Currently 706 and 547 miRNAs have been described in humans and mice, respectively (http://microrna. sanger.ac.uk). MicroRNAs are often transcribed as polycistronic clusters with multiple mature miRNAs sharing similar seed sequences and often redundantly targeting the same mRNA transcripts (Singh et al., 2008). While miRNAs are usually thought to inhibit translation, it has been recently shown that some miRNAs may enhance translation of the target mRNAs (Smalheiser, 2008). Because of their apparent post-transcriptional control over gene expression, miRNAs have recently come under investigation as another layer of control in determining cell fate in both differentiation and disease.

MicroRNAs expressed in ES cells have been examined extensively as their control over translation suggests that some miRNAs may regulate the pluripotency potential of ES cells. Several miRNA profiles of ES cells have resulted in ES cell-specific miRNAs, although few specific miRNAs have been studied in detail (Wang et al., 2009). Recent studies, however, suggest that those miRNAs previously believed to be ES cell-specific are not in fact expressed solely in undifferentiated stem cells (Houbaviy et al., 2003; Chen et al., 2007; Hayashi et al., 2008), but for clarity this nomenclature will be used in this review. The miR-290-295 cluster is one of the most studied ES cell-specific miRNA clusters and is conserved among placental mammals (Houbaviy et al., 2003, 2005). In the developing mouse zygote, the expression of the miR-290-295 cluster are among the first embryonic miRNAs to be detected (Tang et al., 2007) and miRNAs in this cluster are the most highly expressed in mouse ES cells (Wang et al., 2008). Interestingly, the pluripotency-associated transcription factors OCT4, SOX2, NANOG and TCF3 are reported to be associated with the promoters for miRNAs enriched in ES cells and connected with pluripotency, including those in the miR-290-295 cluster (Marson et al., 2008). Members of the miR-290-295 cluster have been shown to regulate the G1-S transition, which is mediated by constitutively active cyclin E-CDK2 complexes in mouse ES cells. MicroRNA-290-295 cluster members bind to the 3' UTR of the cyclin E-CDK2 inhibitor Cdkn1a, resulting in its downregulation. ES cells lacking the Drosha co-factor DCGR8 required for miRNA processing were arrested in the G1 phase (Smalheiser, 2008; Wang et al., 2008). Following transfection with miR-290-295 cluster miRNAs these *Dcgr8* knockout ES cells were able to resume the cell cycle, suggesting the importance of miR-290-295 cluster members in regulating ES cell proliferation.

A second cluster of ES cell-specific miRNAs, the miR-302-367 cluster, is also highly expressed in ES cells with no noticeable expression in somatic cells (Chen et al., 2007). Expression levels of five miRNAs in this cluster (miR-302a, miR-302b, miR-302c, miR-302d, miR-367) were negatively correlated to the time of differentiation from ES cells to embryoid body (EB) cells (Chen et al., 2007). MicroRNAs in the miR-302-367 cluster regulate cell growth, cellular metabolism, and transcription. This cluster also has several predicted targets associated with chromatin modification (Ren et al., 2009). Both the miR-290-295 and miR-302-367 clusters appear to have regulatory functions that have a direct impact on the pluripotency potential of ES cells. Tightly controlled regulation of the cell cycle, transcription, and epigenetic modification are essential for the maintenance of the controlled pluripotency and proliferation observed in ES cells.

The oncogenic miR-17-92 cluster is another cluster of miRNAs expressed in undifferentiated ES cells both *in vitro* and *in vivo* in the developing embryo, with its expression increasing at the onset of differentiation (Foshay and Gallicano, 2009). In both ES and EB cells, the levels of miRNA transcripts from this cluster is nearly twice that seen in somatic cells (Chen et al., 2007). One elucidated role for miR-20 and miR-93, both members of the miR-17-92 cluster, is participation in the

JAK-STAT pathway through interactions with *Stat3*. Both miR-20 and miR-93 have been shown to bind the 3' UTR of *Stat3* and decrease its expression in ES cells (Foshay and Gallicano, 2009). STAT3 is expressed in ES cells at high levels and becomes down-regulated at differentiation (Foshay and Gallicano, 2009). *Stat3* knockout mice are embryonic lethal with defects in visceral endoderm and mesoderm formation that suggest a role for STAT3 in the initial stages of differentiation. Mice deficient in miR-17-92 miRNAs die shortly after birth from lung and heart defects, implicating miR-17-92 family members in the regulation of differentiation (Foshay and Gallicano, 2009). Based on their interactions with Stat3 mRNA, it appears as though miR-17-92 cluster members are closely associated with the promotion and initiation of differentiation by down-regulating the expression of *Stat3*.

While the miR-290-295 and miR-302-367 clusters have been described as ES cell-specific, high levels of expression of both clusters, and the miR-17-92 oncogenic cluster, have also been found in PGCs and spermatogonia *in vivo* (Hayashi et al., 2008). PGCs isolated from mouse embryos using FACS-sorted Oct4-GFP-positive germ cells at E9.5-E11.5, as well as separately sorted male and female PGCs from E12.5 and E13.5, were then screened to assess expression of 214 known miRNAs (Hayashi et al., 2008). MicroRNAs belonging to the miR-17-92 cluster were the most highly expressed miRNAs in PGCs (Hayashi et al., 2008). The expression of the miR-17-92 cluster remained constant throughout the PGC developmental stage, with only a few members (miR-17-5a, -18, -19a, and -19b) decreasing in female PGCs after E12.5 as they prepare to enter the meiotic prophase. MicroRNA-290-295 cluster miRNAs also showed robust expression throughout PGC development. This cluster of miRNAs, like those of the miR-17-92 cluster, had some members (miR-291-5p and miR-292-3p) that were also down-regulated in female PGCs at E13.5 (Hayashi et al., 2008). These results suggest a shared pathway for pluripotency, as clusters thought to be specific for ES cells are expressed in developing PGCs. Interesting to note is the downregulation of certain pluripotent miRNAs only in female PGCs that are nearing meiotic prophase. In agreement with the observed functions in ES cells, in PGCs these miRNA clusters are strongly associated with mitotic cell cycle progression and proliferation. While the role of small RNAs in meiosis has been examined using *Dicer* knockouts (Murchison et al., 2007), no meiosis-specific miRNAs have been identified to date.

Another cluster of miRNAs that showed changing temporal expression in PGCs was the let-7 cluster, one of the earliest miRNAs discovered (Zhao and Rajkovic, 2008). Let-7 miRNAs are predicted to function as tumor suppressors by targeting the Ras/MAPK pathway, and they are likewise more highly expressed in differentiated cells compared to tumor cells and ES cells (Gu et al., 2008). An increase in let-7 expression has been associated with neural differentiation of ES cells (Rybak

et al., 2008). Increased in the expression of let-7 cluster miRNAs was observed only in male PGCs, suggesting they may also play a role in the differentiation of male germ cells (Hayashi et al., 2008). In newborn and adult mouse ovaries, the let-7 cluster of miRNAs was among the most highly expressed, although levels in specific cell types were not examined (Zhao and Rajkovic, 2008). This association with differentiated cell types makes let-7 miRNAs likely candidates for involvement in the differentiation of PGCs into more mature gametes.

Examination of miRNA expression in adult stem cells has revealed additional information about the role of miRNAs in pluripotency. Multipotent adult germline stem cells (maGSCs) are dedifferentiated pluripotent cells arising from a highly lineage-specified state, namely spermatogonial stem cells. MaGSCs have been shown to have miRNA profiles similar to those of ES cells (Zovoilis et al., 2008). The pluripotency markers *Oct4, Sox2, Zfp206,* and *Sall4* were expressed by both cell types and the expression of these genes was used to determine the differentiation state of the cells. Expression levels of the miR-290-295 and miR-302-367 clusters remained stable throughout culture and co-currently decreased when both maGSCs and ES cells were cultured in differentiating conditions (Zovoilis et al., 2008). The pluripotency of maGSCs and the expression of ES cell-specific miRNA clusters further supports the importance of the miR-290-295 and miR-302-367 clusters in maintaining a pluripotent state, although their precise roles differ. MicroRNA-290-295 cluster members were found to be expressed at a high level in untreated cells and were significantly down-regulated during differentiation, directly correlated with Oct4 mRNA expression. By contrast, the expression levels of miR-302-367 cluster miRNAs began to increase at the beginning of differentiation, suggesting that they are associated with the first stages of differentiation and not the maintenance of pluripotency (Zovoilis et al., 2008). Several other studies have also identified candidate miRNAs responsible for the differentiation of both mesechymal and neural stem cells in roles similar to those played by miR-302-367 cluster miRNAs but in cell-type-specific contexts (Cheng et al., 2009, Schoolmeesters et al., 2009). With a more thorough investigation into miRNAs specifically responsible for PGC development it seems likely that these miRNAs would be valuable in identifying PGCs derived from stem cells *in vitro*. Exogenous expression of these miRNAs could also help to guide pluripotent stem cells down a defined differentiation pathway to germ cell formation.

Gene expression

Three major theories of how PGCs are derived from ES cells *in vitro* have currently been suggested: ES cells may contain PGCs following isolation and *in vitro* culturing; ES cells may directly differentiate into PGCs; or PGCs may develop through an intermediate cell type

(Kehler et al., 2005). Due in part to the striking similarities of genes expressed by ES cells and PGCs it is difficult to determine which scenario is likely to be the correct model. Pluripotent markers such as OCT4 and SSEA1 are expressed in both cell types. Many other markers linked to germ cells such as VASA, FRAGILIS, DAZL, NANOG, c-KIT, and STELLA are also expressed in both ES cells and PGCs (Clark et al., 2004; Geijsen et al., 2004; Lacham-Kaplan et al., 2006). A comparison of the global expression of over 19 000 transcripts between ES cells and 12.5 dpc female PGCs found 1 586 genes up-regulated (>2 fold increase in expression) and 2 546 genes down-regulated (>50% decrease in transcript level) in PGCs (Mise et al., 2008). In the same study the correlation coefficient between ES cells and PGCs was found to be 0.424-0.612 as compared to 0.709-0.878 between different ES cell lines. This suggests a significant global diversity in gene expression profiles. Of particular note the expression of *Oct4* in *PGCs* was close to half that of ES cell lines, while conversely *Stella* was elevated in PGCs. Upon closer evaluation, genes positively regulated by OCT4 such as *Zfp42* and *Upp1* were down-regulated in PGCs. Comparing 30 up-regulated OCT4 target genes determined that 60% were down-regulated in PGCs. Interestingly only 23% of negatively regulated genes were up-regulated in PGCs and 10% were down-regulated. Markers traditionally linked to PGCs such as *Piwil2*, *Rnh2*, *Tdrd1*, and *Tex14* have also been detected in ES cells (Mise et al., 2008). A comparison of common pluripotent markers expressed in ES cells as they transit to PGCs shows the up-regulation of Vasa, and Dazl and the down-regulation of *Oct4*, *Klf2*, *Klf4*, and *Nanog* (Mise et al., 2008). The shared expression of many markers between ES cells and PGCs makes the identification of PGCs formed *in vitro* from differentiating ES cells difficult. The simple presence of a marker may not provide solid evidence of PGC formation and a more quantitative expression approach is required.

A recent study looking at the gene expression dynamics during germline specification has revealed that *Eras*, which is expressed in ES cells, is absent in PGCs (Yabuta et al., 2006). Nevertheless, the study confirmed the expression of many well-known pluripotent markers found in PGCs, such as *Sox2*, *Oct4*, *Stella*, and *Nanog*, in both cell types (Yabuta et al., 2006). This shared expression of genes is not surprising, as both cells are primitive cells and therefore would require the expression of similar group of genes for maintenance of differentiation potency. However, phenotypic differences do exist between the two cell types: PGCs cannot be cultured long term *in vitro*; do not form embryoid bodies; and cannot colonize the soma or germline following injection into blastocysts (Donovan and de Miguel, 2003).

It has been recently reported that a population of very small embryonic-like (VSEL) stem cells that are highly plastic and are capable of differentiating into several mesenchymal cell types as well as cells of all three germ layers following *in vitro* culture reside in somatic tissues (Kucia et al., 2006). These pluripotent cells also express the markers OCT4, NANOG, SSEA-1, REX1, and SSEA-4 (Kucia et al., 2006, 2007). Interestingly, the level of expression of these pluripotent markers in VSEL cells is similar to that seen in an established ES cell line (Kucia et al., 2006). Unlike ES cells, the VSEL cells also express early markers for neurons, cardiac muscle, or skeletal muscle suggesting that they may be a heterogeneous population. The extent to which VSEL cells relate to ES cells remains to be clearly defined but the similar expression of pluripotent markers hints at an embryonic origin. Similarly, the expression of OCT4 and other pluripotent markers have also been reported in somatic stem cells. For example, OCT4, SSEA-1, and stem cell antigen 1 (SCA-1) have been found in pulmonary stem cells (Ling et al., 2006). Stem cells derived from porcine skin grow as floating spheres, express OCT4, NANOG, and SOX2, and exhibit multiple lineage potentials (Dyce et al., 2004; Zhao et al., 2009). Using human embryonic stem cell conditions, a distinct population of stem cells were isolated from human hair follicles. Instead of expressing the squamous or melanocytic markers that are indicative of epithelial or melanocytic stem cells, these cells express NANOG and OCT4, grow as spheres, and are capable of self-renewal and differentiation into multiple lineages upon induction, suggesting they are a line of less committed stem cells (Yu et al., 2006). Stem cells isolated from adult rat pancreas have also shown potential for self-renewal and multilineage differentiation. These pancreas-derived stem cells are positive for AP, SSEA-1, OCT4 and NESTIN (Kruse et al., 2006). In addition, it has been reported that a subpopulation of the primitive stem cells can be isolated from amniotic fluid-derived mesenchymal stem cells. These primitive stem cells are also capable of clonal expansion, and express OCT4 and NANOG (Tsai et al., 2006). Multipotent adult progenitor cells (MAPCs) from the bone marrow have been isolated from several species (Reyes et al., 2002; Jiang et al., 2003; Zeng et al., 2006). These cells have the ability to proliferate without senescence and differentiate into mesodermal, neuroectodermal, and endodermal cell types (Jiang et al., 2002a). Cells with similar characteristics as MAPCs can also be isolated from postnatal mouse muscle and brain tissues as well as the bone marrow (Jiang et al., 2002b). MAPCs have been show to express the pluripotent markers OCT4, c-KIT, c-MYC, and KLF4 (Ulloa-Montoya et al., 2007). Interestingly, MAPCs did not express other pluripotent markers found in ES cells such as SOX2 and NANOG but did express the *Eras* gene.

Germ cell-like cell identification *in vitro*

The identification of PGCs formed *in vitro* from stem cells has proven difficult due to their shared expression of many pluripotent markers and miRNAs.

One of the earliest studies to show PGC formation from ES cells used an Oct4-GFP construct (Hubner et al., 2003). By removing the proximal enhancer and leaving only the distal enhancer, GFP expression could be traced to pre-implantation embryo and germ cells in the *in vitro* culture. By day 7 of differentiation, c-KIT-expressing Oct4-GFP-positive cells, and Oct4-GFP-positive cells that expressed VASA were identified. The expression of c-KIT is a common characteristic of migratory PGCs, while VASA expression is linked to post-migratory PGCs (Lacham-Kaplan, 2004). The meiotic markers DMC1 and SCP3 were not detected in the Oct4-GFP/VASA cell population suggesting the cells were pre-meiotic. By day 12 of differentiation the expression of Oct4-GFP was reduced while the expression of VASA increased. The cells expressing Oct4-GFP and VASA were present in colonies while the surrounding cells did not express the pluripotent markers, suggesting they were somatic cells. At day 16 the expression of the oocyte marker GDF9 was detected in suspended cell aggregates. Data supporting the idea that these aggregates were the formation of cumulus-oocyte-like complexes was the expression of the steroidogenic markers aromatase, CYP17, and StAR (Hubner et al., 2003). The oocyte-like cells (OLCs) generated expressed the oocyte markers ZP2, ZP3, FIGLA, and GDF9 as well as the meiosis markers SCP1, SCP3. The expression of FIGLA, GDF9, and ZP2, which are not expressed in ES cells, supports the formation of female gametes. The expression of these markers are thus more indicative of germ cell formation than the expression of ZP3, SCP1, and SCP3 as they have all been detected in undifferentiated ES cells (Kerkis et al., 2007). The lack of ZP1 expression is consistent with other studies in which the ZP proteins were not correctly expressed (Dyce et al., 2006, Lacham-Kaplan et al., 2006). However, differentiating ES cells to OLCs by co-culturing with granulosa cells has resulted in the expression of all three ZP transcripts (Qing et al., 2007).

In another study, the Oct4-GFP construct was also used to differentiate ES cells into germ cells and the ability of retinoic acid (RA) to rapidly induce ES cell differentiation and stimulate PGC proliferation was exploited (Geijsen et al., 2004). Cells isolated from EBs were cultured in the presence of RA for five days. Following this culture, a sub-population of SSEA-1-positive cells was identified. Large colonies that stained positive for AP that were surrounded by motile cells resembling migratory PGCs were also identified (Geijsen et al., 2004). However, several of the pluripotent markers used to identify the formation of PGCs, such as OCT4, STELLA, and DAZL, were present in the ES cells prior to differentiation (Table 1). To confirm PGC formation, the methylation status of the *Igf2r* gene was investigated. It was found that while all undifferentiated ES cell clones displayed the somatic methylation profile, most seven-day differentiated EB clones had a PGC-like unmethylated profile (Geijsen et al., 2004). By day 10, all EB germ clones had lost the

imprint on the *Igf2r* gene. This provided evidence that EB-derived PGCs display a similar phenotype and methylation pattern as *in vivo* PGCs. Confirmation of male germ cell formation was provided using markers not detected in the ES cells used such as GCNF, HAPRIN, LHR, and acrosin. However, the expression of HAPRIN has been detected by others in undifferentiated ES cells (Kerkis et al., 2007). In a similar study the male germ cells produced were transplanted into the testis of germ cell-depleted recipient mice. Following four months incubation, sperm was found in the lumen and successfully used for intracytoplasmic injection to produce live offspring (Nayernia et al., 2006b).

The later-stage PGC marker VASA has also been used to identify potential PGCs formed from ES cells (Toyooka et al., 2003). Around day 3, the VASA-positive cells appeared randomly distributed within the EBs, but gradually clustered together during differentiation. Similar results have been obtained using human ES cells where VASA expression was observed following 3 days of differentiation and clustered VASA-positive cells (Clark et al., 2004). This may be a functional example of the presence of migratory PGCs *in vitro*. While VASA was absent in the ES cell line used for differentiation, other germ cell markers such as OCT4, FRAGILIS, STELLA, and BMP8b were present both before and after differentiation. It was also found that the meiosis marker SCP3, which is expressed in some ES cell populations (Table 1), was expressed in cells after induced differentiation (Toyooka et al., 2003). Further evidence for migratory PGCs *in vitro* was provided by an increased VASA-positive population when ES cells were co-cultured with BMP4-expressing cells. BMP4 has previously been shown to play an essential role in germ cell formation (Lawson et al., 1999). The effect was seen within one day of co-aggregation with BMP4 producing cells. Finally, by transplanting VASA-positive (LacZ-labeled) cells co-aggregated with gonadal cells under the testis capsule, newly formed tubules were found with VASA-LacZ-positive cells present within the tubules. When unsorted EB-derived cells were transplanted with gonadal cells, LacZ-negative teratomas were formed (Toyooka et al., 2003).

In our study, the fetal porcine skin-derived stem cells expressed the pluripotent markers OCT4, FRAGILIS, and GDF9b prior to differentiation (Dyce and Li, 2006; Dyce et al., 2006). By analyzing the pluripotent marker expression at different time points during induced differentiation, it was found that the pluripotent markers were down-regulated early, and then up-regulated at a later stage. This may be due to the stem cells initially differentiating into precursor cells followed by the induction of PGC formation. The dynamic changes in marker gene expression levels in the time course study provides evidence that the differentiating cells may have undergone a transition from undifferentiated cells through germ cell formation stages. Further evidence of PGC and gamete formation is provide by the expression

of markers not detected in the undifferentiated stem cells such as DAZL, ZP3, SCP3, FSHR, and aromatase, as well as the morphologies of cumulus-oocyte complex-like and zona pellucida-like structures (Dyce et al., 2006).

OLCs have also been formed during the differentiation of a clonal pancreatic stem cell line (Danner et al., 2007). Prior to differentiation the clonal cell line expressed the pluripotent markers OCT4 and VASA as well as the later stage markers GDF9, and SCP3. While these markers were also present in the OLCs it is difficult to determine whether PGCs were formed. Further evidence was provided by the expression of the meiosis marker DMC1, which was not detected prior to differentiation but was present in the OLCs formed, as well as the typical size and morphology expected of oocytes. Bone marrow (BM) stem cells have also been show to form male germ cells *in vitro* (Nayernia et al., 2006a). This was accomplished by using a Stra8-GFP construct within the BM stem cells. As seen in Table 1, STRA8 was not expressed in the BM population. Following differentiation, a population of Stra8-GFP-positive cells was isolated and the expression of germ markers was compared to undifferentiated BM stem cells. While the expression of OCT4 was detected both prior to and after differentiation, only the differentiated cells expressed FRAGILIS, STELLA, VASA, DAZL, c-KIT, and STRA8 (Nayernia et al., 2006a). The absence of many of the pluripotent markers shared by ES cells and PGCs from the BM stem cells allow for a more precise identification of PGC-like cells with this cell population.

Perspective

There are multiple potential explanations as to why pluripotent cells and PGCs have similar gene expression patterns and miRNA profiles. First, PGCs belong to the initial embryonic lineage that segregates during development in mammals; both ES cells and PGCs are naïveté cells, which may be reflected in their similar expression signatures. Second, germ cells are responsible for generating offspring and transmitting genetic information to subsequent generations. They therefore have to maintain a high level of potency similar to pluripotent cells. Third, the origin and the extent to which pluripotent stem cell lines represent any specific *in vivo* embryonic cell type or if they instead reflect tissue culture artifacts is still under debate (Rossant, 2001; Buehr and Smith, 2003; Zwaka and Thomson, 2005). In fact, it was recently hypothesized that ES cells represent a family of pluripotent cell lines that originate from germ cells (Zwaka and Thomson, 2005). Early germ cells migrate multiple times and in

Table 1. Summary of markers detected during the differentiation of stem cells to PGCs and gametes.

Cell Type	Undifferentiated	Early	Mid/Late (gamete)	Cell Type Obtained	Ref
mESCs	NT	*Oct4, c-kit, Vasa*	ZP2, ZP3, SCP3, SCP1, GDF9, Figla, DMC1	OLCs	Hubner et al., 2003
mESCs	*Oct4, Stella, Fragilis, Dazl,* SSEA-1, ZP3	*Oct4, Dazl, MIS*	acrosin, haprin, LHR, AZ1, GCNF	Spermatids	Geijsen et al., 2004
mESCs	*Oct4, Fragilis, Stella,* BMP8b	*Vasa*	Vasa, Oct4, E-cadherin, SSEA1, c-kit, AP, SCP3	PGC-like	Toyooka et al., 2003
hESCs	*Oct4, GDF3, Nanog, Stella, Dazl, c-kit*	NT	GDF9, SCP1, Vasa, SCP3, MLH1	OLCs	Clark et al., 2004
mESCs	*Oct4, Vasa, c-kit, Stella, Dazl*	*Oct4, Vasa, c-kit, Stella, Dazl*	Fig1a, ZP3, Sry, Stra8	OLCs	Lacham-Kaplan et al., 2006
mESCs	*Oct4, c-kit, Fragilis, Stella, Vasa*	NT	GDF9, Figla, ZP1, ZP2, ZP3, SCP3	OLCs	Qing et al., 2007
mESCs	*Oct4, c-kit, Fragilis, Stella, Vasa, Stra8, DMC1*	NT	SCP3, acrosin, TP2, Prm1	Sperm	Nayernia et al., 2006b
mESCs	*Stella, Eras, Nanog, Sox2, Oct4, c-kit, Vasa, Myc*	*Hoxa1, Fgf8, T*	Nanos3, Sox2, Oct4, Dppa5, Fragilis, c-kit, Fgf8	PGC-like	Wei et al., 2008
mESCs	*Oct4, BMP8b, Vasa, Stella, Dazl, Stra8, Haprin, ZP3, SCP1, SCP3*	NT	GDF9, LHR, acrosin	OLCs, Sperm	Kerkis et al., 2007
Skin-derived stem cells	*Oct4,* GDF9b	*Oct4, GDF9b, Dazl*	SCP3, c-Mos, ZPA, ZPC, FSHR, Oct4, GDF9b, Dazl, Vasa	OLCs	Dyce et al., 2006
Pancreatic stem cells	*Oct4,* SSEA-1, SCP3	NT	Oct4, SSEA1, SCP3, DMC1, Vasa, GDF9	OLCs	Danner et al., 2007
BM stem cells	*Oct4*	NT	Oct4, Fragilis, Stella, Vasa, Dazl, c-kit, Stra8	male PGCs	Nayernia et al., 2006a

Undifferentiated: genes detected prior to inducing differentiation; Early: genes detected during the early stages of induced differentiation; Mid/Late: genes detected during the mid to late stages of induced differentiation; NT: not tested.

multiple directions before they reach the genital ridges. In mice, PGCs form in the proximal epiblast of embryonic day 5.5 to 6 (E5.5-6) embryos. They move out of the epiblast and become clustered at the base of the allantois in the extra-embryonic mesoderm at E7 (Ginsburg et al., 1990). By E9, PGCs have migrated back into the embryonic endoderm. There are four distinct phases of migration afterward: At E9.0, PGC move rapidly within the hind-gut endoderm. At E9.5, germ cells emerge from the hind-gut and invade the body wall moving dorsally, but do not target the genital ridges. At E10.5, PGCs migrate towards the genital ridges from widely divergent starting positions in the dorsal body wall and the base of the mesentery. At E11.5, most germ cells have finally arrived at the genital ridges, and their migration slows (Molyneaux et al., 2001). The significance of these active and complex movements of the early germ cells is not known. Do they really need to migrate to different components of the embryo to acquire the potential to develop to later stage germ cells? Germ cell development is a tightly controlled process and one would expect the early germ cells would try to protect their identity and specific differentiation path by preventing their exposure to other somatic signals as much as possible, and by taking the shortest path to migrate to the future gonad. In the above-mentioned study, *in vivo* mouse germ cell migration was observed using an Oct4-distal element-GFP reporter. The distal element of the *Oct4* regulatory region directs its expression to not only germ cells, but also to preimplantation embryo pluripotent cells (Yeom et al., 1996). Thus another possibility that may account for the multiple directions in PGC migration is that these so-called PGCs are not committed cells yet, but instead they may represent a mobile group of pluripotent cells that exist at this stage of development; these migrations may allow the opportunity to "seed" some of these pluripotent cells into somatic tissues for future tissue repair. This notion is in line with the above-mentioned hypothesis of a germ cell origin of ES cells. Also in accord with this view is a hypothesis that has received some experimental and clinical support. It is proposed that a small, dormant, and quiescent population of VSEL stem cells that are descendants of epiblast-derived stem cells, including some PGCs, are deposited during development and reside in somatic tissues. These cells may play important roles in tissue and organ regeneration later in life (Kucia et al., 2006, 2008; Dawn et al., 2008; Zuba-Surma et al., 2009). Interestingly, the skin-derived stem cells demonstrated to have germ line potential in our laboratory were primarily isolated from the dorsal skin. It is tempting to speculate that they may be the descendants of the primitive stem cells seeded in the dorsal body wall during the early stages of uncommitted PGC migration, remaining quiescent due to the inhibitory somatic environment within the skin, but able to exhibit germ cell potential when placed in an appropriate environment.

The striking similarity in gene expression profiles between pluripotent cells and early germ cells make it difficult to use marker-based approaches to discriminate the two cell types. It is therefore important to use other PGC signatures and later germ cell markers to identify and confirm PGC-like cells that have been derived from differentiated pluripotent cell types. Since meiosis is unique to germ cells, markers for meiosis are good candidates for later-stage germ cell identification. As with gene expression, there are currently no miRNAs that have been definitively shown to be expressed only in PGCs. Additionally, no meiosis-specific miRNAs have been identified, although given the prediction that ~30% of protein-coding genes are regulated by miRNAs (Filipowicz et al., 2008), chances are high that they do exist. DNA methylation is a major epigenetic modification that plays a key role in suppression of the imprinted genes (Li et al., 1993). Imprinting is required for differential expression of alleles that are dependent upon their parent of origin. During germ cell migration and maturation, the somatic status of imprinted genes is progressively erased (Yamazaki et al., 2003), and this process of imprint erasure is also a feature unique to PGCs. Imprint erasure therefore could be used as a hallmark for identifying migrating PGCs in differentiating cultures. It is hoped that further characterization of pluripotent and germ cell populations will help to provide more insights on the principal cellular potency and the mechanism of germ cell differentiation.

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