[Frontiers in Bioscience 15, 46-56, January 1, 2010]

In vitro derivation of germ cells from embryonic stem cells

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1. ABSTRACT

Embryonic stem cells (ESCs) have the potential to differentiate into cells of all tissue types, as demonstrated by their capability to contribute to the development of all germ layers in chimeras and to form teratomas. Of interest, however, is the lack of evidence of gametogenic gonadal tissues within teratomas, such as testicular tubules or follicles. Although this may reflect the fact that *in vivo* germ cell development and subsequent gametogenesis are complex biological processes, the generation of putative primordial germ cells (PGCs) has been reported during the differentiation of mouse and human ESCs in *in vitro* systems. In addition, mouse ESCs can even lead to the development of putative gametes, but the efficiency of this process needs to be improved and the functionality of the gametes remains to be proven. Interest in the generation of fully functional gametes from ESCs lies in an experimental model fundamental to the study of reproductive biology, a potential unlimited source of oocytes as a reprogramming bioreactor, and perhaps even in therapeutic applications to overcome human reproductive problems.

2. *IN VIVO* DEVELOPMENT OF GERM CELLS

Germ cells are a unique population of cells in mammals, transferring their genetic information from one generation to the next, and generating a fully functional organism by processes induced by their fertilization (1, 2). The production of gametes, - i.e., germ cells in their most differentiated state - is therefore one of the most important biological events in the propagation of a species. The earliest biological event in germ cell development is the formation of PGCs - the embryonic precursors of gametes - which begins around the time of gastrulation (1). In the mouse PGCs remain in a rather undifferentiated stage until the beginning of sexual differentiation, at 12.5 day post coitum (dpc). PGCs differentiate into prospermatogonia and oogonia at a late stage during the development of male and female mammalian fetuses, respectively. After birth, prospermatogonia further differentiate into spermatogonial stem cells (SSCs) (3), which are capable of undergoing self-renewal and serve as a reservoir of sperm precursor cells. Female germ cell differentiation, on the other hand, is characterized by the development of oogonia into primary

oocytes prior to birth, arrest of meiotic progression until puberty, and maturation of secondary oocytes into mature oocytes thereafter (4).

2.1. Development of primordial germ cells

The development of PGCs *in vivo* can be divided into three distinct biological processes: specification of PGCs in the proximal epiblast, proliferation of PGCs, and migration of PGCs into the fetal genital ridge (5). Each of these processes involves region- and stage-specific signaling pathways mediated either by the interaction between PGCs and somatic cells, or between PGCs themselves.

The specification of PGCs is regulated by complex molecular mechanisms in which multiple molecular signals occur between PGC precursor cells and neighboring somatic cells, as well as between the precursor cells themselves. Regional specification of all germ layers, including the germline in the epiblast, occurs shortly before the beginning of gastrulation. The proximal region of the epiblast, which responds to signals from extraembryonic ectoderm, contains a population of cells committed to develop into PGCs (6). Early events in the specification of PGCs are regulated by signaling molecules and transcription factors. In the mouse, at about 5.5 dpc, bone morphogenetic protein (BMP) 4 and BMP8b, both members of the TGF beta superfamily, are released from extraembryonic ectoderm and exert their functions in the epiblast (7-9). In response to these signals, a subpopulation of cells in the proximal epiblast region at 6.0 dpc express mothers against decapentaplegic (SMAD) 1 and SMAD5 or simply SMADs, and are committed to develop into PGCs (10, 11). About six cells of the epiblast at 6.25 dpc that express fragilis/Ifitm3 in response to BMPs will subsequently express B-lymphocyte-induced maturation protein-1 (Blimp1)/Prdm1. These Blimp1-expressing cells will acquire germ cell competence and separate from the somatic cells (6). Blimp1 has been identified as a key signaling molecule involved in the inhibition of PGC differentiation into the mesodermal cell lineage, which expresses Hoxb1 among other mesodermal marker genes (12). Consistent signals in the proximal region of the epiblast are required for the maintenance of predetermined PGCs and for further recruitment of PGCs until 6.5 dpc. About 40 Stella-positive cells, which comprise the founder PGC population, settle at the posterior end of the primitive streak at 7.25 dpc (6, 12, 13). PGCs become motile at about 7.5 dpc, start migrating into the hindgut by 8.5 dpc, and localize therein at 9.0 dpc. At 9.5 dpc, they leave the hindgut and begin migrating through the dorsal mesentery (14-17). Migratory PGCs enter the developing genital ridges at about 10.5 dpc (16, 17) and complete their migration by 12.5 dpc.

2.2. Factors required for survival and proliferation of PGCs after specification

Subsequent to the specification of PGCs, germ cells need to maintain germ cell competence and proliferate without differentiating into somatic cell lineages. It has been reported that the POU domain transcription factor Oct4 is required for the maintenance of germ cell competence as well as for the survival of PGCs (14). During specification of mouse PGCs, expression of Oct4 is restricted to the founder PGC population at 7.25 dpc. In female fetal germ cells, Oct4 expression is maintained until sexual differentiation at 13.5 dpc and then starts to decline (18). In male fetal germ cells, Oct4 expression is maintained even after sexual differentiation (18). Later, Oct4 expression in the adult testis is restricted to SSCs (19). It is worth mentioning that Oct4 is a key factor not only for the pluripotency and self-renewal property of ESCs (20), but also for the survival of germ cells (14, 21). It is also interesting to note that ESCs and PGCs share common gene expression profiles; representative pluripotency-related genes, such as *Oct4*, *Sox2*, and *Nanog*, are all expressed in both ESCs and PGCs (22, 23). However, unlike ESCs, PGCs are unipotent, differentiating only as gametes. Still, stemness is a property common to both ESCs and PGCs.

In contrast to female gonads, male gonads contain germline stem cells (GSCs)- also referred to as SSCs - which persist after birth. These are postnatal male germ cell precursors that self-renew and undergo spermatogenesis to develop into male gametes (24). It should be mentioned that there are other known somatic stem cells that exhibit stemness. However, only SSCs express Oct4, one of the ESC-specific marker genes (25). Adult female germ cell derivatives also express Oct4, although they are not actively proliferating (14, 26). Analysis of oocyte growth has revealed the upregulation of Oct4 expression *de novo* during the later stages of prophase I of meiotic division in female germ cells (27). These data suggest that downregulation of Oct4 in male and female germ cells might trigger meiotic commitment (27). The upregulation of Oct4 expression in oocytes at the completion of meiotic prophase I further suggests a specific involvement of this transcription factor in oocyte growth and in the acquisition of meiotic competence (27).

Activation of and downstream signaling from the c-kit tyrosine kinase receptor are essential for the survival and proliferation of PGCs during PGC migration (28). Defects in either the c-kit receptor or its ligand, e.g. stem cell factor/steel factor (SCF), can cause depletion of germ cells (29). The importance of c-kit signaling in PGC survival is also observed in *in vitro* cultures (30). When PGCs are cultured in medium without SCF, most of the cells do not survive, which reflects the scenario *in vivo*. But when PGCs are cultured in the presence of SCF, survival is prolonged and the cells proliferate. c-Kit mediated signaling is also important for growth and differentiation of both male and female postnatal germ cells (31-33). A number of studies in the mouse model have demonstrated an important role of SCF in proliferation and in prevention of apoptosis of differentiated spermatogonia, as well as in either initiation or maintenance of meiosis (33-35). In the female, c-kit signaling between ovarian somatic cells and oocytes is associated with follicular growth and involved in the meiotic process (31).

2.3. Sexual differentiation of PGCs

In the mouse, PGCs proliferate during migration but stop dividing at about 13.5 dpc. Forty PGC founder cells give rise to approximately 25,000 cells in the fetal

gonad at 13.5 dpc (1). PGCs are indistinguishable in both male and female gonads until 13.5 dpc. In male gonads, PGCs arrest in the G0/G1 stage of the mitotic cell cycle and develop into prospermatogonia (36), which differentiate to spermatogonia that, in turn, resume cell proliferation shortly after birth and begin the process of spermatogenesis. In female gonads, PGCs develop into oogonia that enter prophase I of meiosis, arrest in the diplotene stage at late gestation to complete meiosis after puberty and to develop into oocytes (4). It has been suggested that the different fate of PGCs in male and female mammalian development is regulated by retinoic acid (RA) (37, 38). In female fetal gonads, RA induces the expression of Stimulated by Retinoic Acid Gene 8 (*Stra8*), leading to the initiation of meiosis. In male fetal gonads, however, RA undergoes degradation by *Cyp26b1*, preventing PGCs from entering meiosis (37). It is interesting to note that expression of *Stra8* is found in premeiotic spermatogenic cells in postnatal testes, during puberty, concomitant with a reduction in *Cyb26b1* expression (39). Thus, RA may still function as a meiotic inducer molecule responsible for the initiation of the meiotic process, albeit at different time points during the sexual development of both sexes (18, 37, 39).

2.4. Generation of gametes from fetal and postnatal germ cells

The generation of gametes from fetal germ cells, either PGCs or sexually differentiated germ cells, has been reported by several studies in mouse. In fact, gametogenesis was observed when fetal gonads were transplanted underneath the kidney capsule or under the skin (40-42). These studies described the development of transplanted fetal male gonads into putative adult testicular tissue containing round spermatids within the testicular tubules, suggesting that fetal PGCs can differentiate into fertility-competent male germ cells. Upon transplantation of fetal male germ cells isolated from late-gestation embryos into testicular tubules of W/W mice, fully mature sperm could be obtained that were capable of fertilizing oocytes, subsequently giving rise to live offspring (43). Of interest is the fact that even PGCs at 10.5 dpc can still differentiate into fully mature sperm by testicular transplantation (43). Furthermore, SSCs, which are postnatal male germ cells, can develop into GSCs in *in vitro* culture (44, 45). These GSCs established *in vitro* have the capability to undergo self-renewal without differentiation and restore spermatogenesis upon transplantation into germ cell-depleted testis. One study demonstrated that competent oocytes could differentiate from fetal female germ cells by *in vitro* culture of 12.5-dpc fetal female gonads (46). Consistent with that study, our unpublished data showed that *in vitro* culture of fetal female gonads induced oogenesis, but would not bring about complete maturation of the oocytes. By contrast, a recent study reported the differentiation of fetal female germ cells into fully matured oocytes by *in vivo* culture of fetal gonads. Fully mature oocytes were obtained from 3-week *in vivo* cultured fetal gonads after transplantation underneath the kidney capsules (42, 47, 48). Fully mature oocytes were also obtained from primordial follicles by *in vitro* maturation culture (48), gaining developmental competence and giving rise to live pups subsequent to fertilization.

Overall, these results suggest that fetal or postnatal germ cells can differentiate into functional gametes by either *in vitro* or *in vivo* culture systems, suggesting that the derivation of PGCs or germ cells of later developmental stages from ESCs, such as SSCs in adult male gonads and primordial follicles in adult female gonads, may be the first step in the generation of gametes from ESCs.

3. *IN VITRO* DIFFERENTIATION OF ESCS INTO GERM CELLS

Several reports have recently demonstrated the generation of germ cells of different stages from ESCs. Depending on the *in vitro* differentiation system, these ESC-derived PGCs could further differentiate into gametes by either *in vitro* or *in vivo* culture. Furthermore, *in vitro*-derived postnatal germ cells, such as SSCs and primary follicular cells, could also successfully differentiate into male gametes and oocytes, respectively (49, 50).

3.1. Formation of germ cell precursors from ESCs

In vivo development of germ cells involves complex biological processes, which require specific environmental cues to support induction of germ cell precursors and maturation of the precursors into gametes. It is therefore remarkable that it is possible to obtain germ cells from cultured ESCs in an *in vitro* environment. Although it is still unclear whether such complex processes can take place in a Petri dish and whether they, in fact, mimic the *in vivo* scenario, it seems likely that distinct steps in germ cell development, required for the production of specific germ cell precursors, do take place. *In vitro*-derived germ cell precursors can be largely defined as several specific cells, these being PGCs, prospermatogonia, oogonia, and SSCs, depending on the timeline of germ cell development and sex determinants. PGCs are the earliest germ cell precursors in both male and female mammals. Several groups reported the formation of PGCs from ESCs *in vitro* (50-52). Our laboratory was the first to report the generation of putative PGCs of various stages in a monolayer ESC differentiation culture system. Identification of these cell populations was based on detection of expression of the germ cell-specific Oct4-GFP (gcOct4-GFP) transgene, along with that of stage-specific marker genes (*c-kit* and *Vasa*) (50). After 3 days of differentiation, we first detected early-stage PGCs (gcOct4-GFP+/c-kit+) and later, putative migratory PGCs (GFP+/c-kit+/Vasa-) and post-migratory PGCs (GFP-/c-kit+/Vasa+). Our results demonstrate the generation of PGCs from ESCs in a monolayer culture induced by a seemingly spontaneous differentiation process. We also observed that Vasa-positive cells detached from the monolayer and formed aggregates in the supernatant. These aggregates resembled late-stage oogonia, the precursors of oocytes, suggesting that these putative PGCs could further differentiate into presumptive female germ cell precursors.

Toyooka *et al.* used transgenic ESCs that contained the Lac-Z gene under the control of the Vasa

promoter and reported the generation of Vasa-positive putative PGCs from embryoid bodies (EBs). These PGCs were capable of undergoing germ cell maturation (51). Formation of PGCs in this EB culture system appeared to be faster than in monolayer differentiation cultures. Vasa-positive putative PGCs could be detected after only one day of EB differentiation. The formation of Vasa-positive cells could be facilitated by coaggregation of EBs with BMP4-producing cells. While direct treatment of the EB differentiation culture with BMP4 appeared to have no effect, close contact with BMP4-producing cells appeared to have a positive effect on the formation of Vasa-positive cells. By contrast, a recent study using human ESCs showed that direct treatment of differentiating human EB cultures with BMP4 led to a dose-dependent, positive effect on Vasa expression (53). The addition of BMP4 also led to an increase in SYCP3 expression, although the effect was modest. This study clearly shows that PGC differentiation from human ESCs is promoted by the addition of BMP4 to EB cultures, in contrast to PGC differentiation from murine ESCs. This difference may be due to the fact that human ESCs are more closely related to mouse epiblast cells than to mouse ESCs, as it has recently been suggested that human ESCs indeed have properties in common with mouse epiblast-derived stem cells (54, 55). As described previously, PGC specification occurs in cells of the epiblast, which respond to BMP4 signaling. Nevertheless, both studies demonstrate the generation of putative PGCs from ESCs, a process promoted by BMP4. Utilizing a different approach, Geijsen *et al.* reported the detection of putative PGCs that express SSEA1 in differentiating EB cultures, and claimed that PGC formation in EBs was promoted by treatment with RA (52). The effect of RA on proliferation or survival of PGCs isolated from embryos has been demonstrated in several studies (56). It is therefore possible that putative PGCs, which may have spontaneously differentiated into EBs, proliferated in response to RA, rather than RA having triggered the formation of PGCs. To characterize the generated cell population, SSEA1-positive cells were isolated and expanded under culture conditions used to derive embryonic germ (EG) cells. DNA methylation analysis in imprinted genes revealed that the expanded cells had lost their methylation pattern, suggesting that EB-derived SSEA1-positive cells were indeed PGCs.

More recently, Nayernia *et al.* reported the derivation of male germ cell precursors of a postnatal stage from ESCs (49). In their study, RA was used for the initial differentiation of ESCs into Stra8-GFP-positive cells, which were further cultured in RA-free culture medium to establish putative SSCs. Our own findings revealed that in ESCs treated with RA, Stra8 expression was elevated within 24 hours when analyzed by reverse transcriptase PCR (unpublished data). In the aforementioned study, the initial elevation of Stra8 by continuous treatment with RA for 10 days may have been favorable for the differentiation of ESCs into SSCs (49). These so-called SSCs could then be maintained as cell lines. However, the imprinting patterns of these putative SSCs were different from the paternal imprinting patterns in SSCs derived from the adult testis: H19 is fully methylated, while the Igf2r gene is unmethylated by analysis of bisulfite sequencing (unpublished data). In fact, the imprinted genes in the ESC-derived SSCs did not show proper paternal imprinting patterns in the H19, Igf2r, and Snrpn loci before treatment of RA (49). Nonetheless, the putative SSCs showed gene expression profiles similar to *in vivo* SSCs. Of interest is the induction of meiosis by RA treatment, along with changes in imprinting patterns, as demonstrated by the somatically imprinted H19 gene and the paternally imprinted Igf2r and Snrpn genes (49).

3.2. Strategies for the derivation of gametes from ESC-derived germ cell precursors

In vivo germ cell precursors readily mature into functional gametes (42, 43, 47). Therefore, the generation and further enrichment of germ cell precursors *in vitro* can be critical and beneficial for the *in vitro* derivation of mature gametes. Several strategies have already been used to generate gametes from germ cell precursors of various stages.

In our monolayer differentiation system, aggregates containing Vasa-positive cells, which comprised putative oogonia, were further differentiated under maturation conditions (Figure 1A) (50). After 2 weeks in culture, follicle-like structures had formed, which expressed key genes for female estrogen biosynthesis. After 26 days in culture, oocyte-like cells were released from the follicle-like structures, which contained an outer layer resembling the zona pellucida. Occasionally, blastocyst-like structures developed from oocyte-like cells, possibly due to parthenogenetic activation. It has been reported that parthenogenesis could be induced from *in vivo* preantral follicle-derived oocytes (57). Our results provide indirect evidence for the functionality of oocyte-like cells derived from ESCs, for they have the capability to undergo spontaneous parthenogenesis.

To induce maturation of ESC-derived PGCs into male gametes, Toyooka *et al.* first obtained aggregates of these putative PGCs with male fetal gonadal somatic cells, and subsequently cultured them *in vivo* under the testicular capsule (Figure 1B) (51). Seminiferous tubules formed within these aggregates and produced sperm-like cells. Even though that study did not provide direct evidence for the functionality of the putative sperm, these results demonstrate that fetal gonads can be artificially generated and that ESC-derived PGCs and their wild-type somatic cell counterparts can induce spermatogenesis.

It has been demonstrated that maturation of ESC-derived PGCs can be induced solely by *in vitro* differentiation culture (Figure 1B) (52). Geijsen *et al.* reported the *in vitro* derivation of haploid male germ cells from ESCs. EBs at day 11 of differentiation were found to express genes associated with spermatogenesis and gonadal somatic cells. Putative haploid male germ cells could be isolated by staining EBs with a FE-J1 surface marker characteristic of male meiotic germ cells. The male germ cells were further tested for functionality by intracytoplasmic injection into oocytes. Although a significant number of injected oocytes developed into

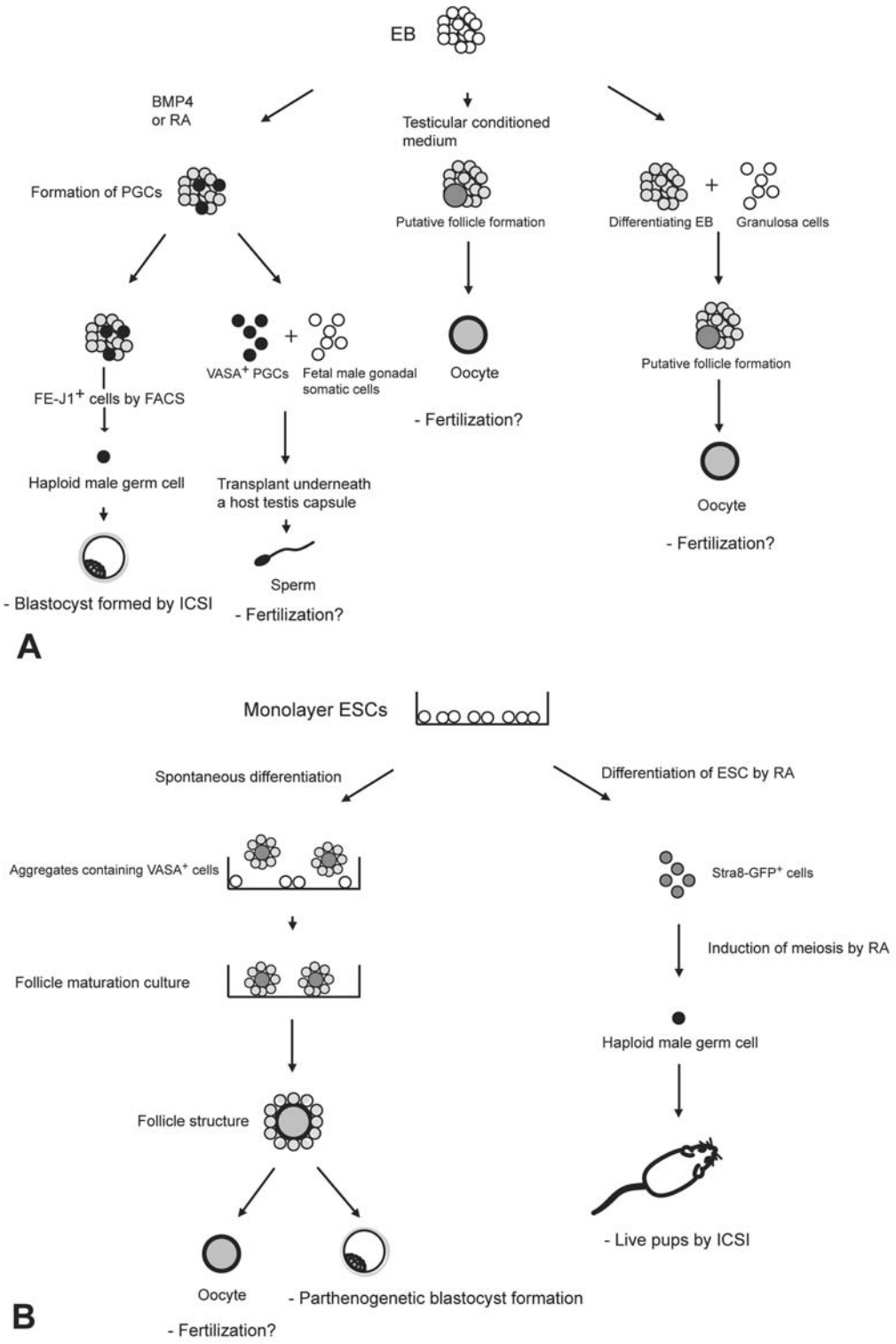

Figure 1. *In vitro* culture systems for differentiation of ESCs into germ cells. Both the monolayer culture system and the EB culture system can support the differentiation of either male or female germ cells based on the culture conditions. A) In the EB culture system, ESCs can give rise to male germ cells in the presence of RA and induce the differentiation of female germ cells in testicular conditioned medium or by co-culture with granulosa cells. B) In the monolayer culture system, female germ cells can spontaneously differentiate from ESCs and further mature in maturation culture, while RA can induce the differentiation of male germ cells.

blastocysts, live offspring and, thus, ultimate proof of complete spermatogenesis *in vitro*, could not be obtained. Nayernia *et al.* reported remarkable results on the generation of functional male gametes from ESCs using two germ cell marker genes: Stra8 (premeiotic germ cell marker)-GFP and protamine 1 (Prm1: postmeiotic germ cell marker)-DsRed (49). Stra8-GFP was used to establish SSCs from an ESC monolayer culture in the presence of RA and Prm1-DsRed served as a marker for sperm after further maturation of the established SSCs, again, in the presence of RA. Treatment with RA appears to lead to different outcomes depending on the germ cell stage. RA is known to promote PGC survival; however, Nayernial *et al.* (49) described induction of meiosis by RA in SSCs resulting in the generation of male haploid gametes that successfully fertilized oocytes, which subsequently gave rise to live offspring. RA has recently been identified as an effector molecule in meiotic initiation in female fetal gonads and, possibly, in male adult gonads as well (37, 38, 58). The study by Nayernia *et al.* is the first to demonstrate the generation of functional gametes from ESCs. In addition, those outstanding results suggest that meiotic processes can be completed in SSCs isolated from the testis or in SSCs established in culture by treatment with RA.

3.3. Direct differentiation of gametes from ESCs

The culturing of EBs with conditioned medium prepared from male adult gonadal tissues has been shown to directly result in the generation of putative female gametes. Of interest is the formation of follicle-like cells at the borders of EBs observed after 6-7 days in co-culture (Figure 1B) (59). Signals from testicular tissues, which are similar to those in ovaries, may, in fact, facilitate the differentiation of ESCs into female rather than male gonadal cells (59). Growth factors found in the testes are also present in ovarian cells and during oogenesis. Since one-day differentiated EBs are known to contain putative PGCs, albeit a small percentage, it is possible that these preexisting germ cells readily respond to gametogenic cues in the conditioned medium (59). The establishment of gametogenic cues in regular differentiation cultures appears to require a relatively long time in culture (50, 52). Therefore, conditioned media appear to be more effective and faster than monolayer and EB culture systems described above at supporting the generation of gametes directly from ESCs. Other groups have also reported the beneficial effects of unknown factors from adult gonadal tissues. The co-culture of EBs with ovarian granulosa cells was found to effectively induce the formation of oocyte-like cells within 10 days in differentiation culture (Figure 1B) (60). The identification of key molecules from adult gonadal tissues is likely to improve the generation of germ cells from ESCs and their subsequent maturation.

3.4. *In vitro* microenvironment of ESC and germ cell cultures

Given the fact that *in vivo* germ cell development and subsequent germ cell maturation are complex biological processes that require precise cues from the germ cells themselves and from surrounding somatic cells, the achievement of *in vitro* germ cell differentiation from both, mouse and human ESCs seems remarkable (49-52, 61). It is still unclear how the somatic microenvironment and a range of germ cell determinants that support germ cell formation, such as certain growth factors and hormone stimulus, are created in ESC differentiation cultures. Two culture systems, monolayer and EB culture, are routinely used for germ cell differentiation, both of which are also commonly used for the generation of cell types of other lineages (49-52, 59, 60). The monolayer culture system provides a microenvironment suitable for the differentiation of both male and female germ cells. Our laboratory showed that female germ cells could be generated by the differentiation of mouse ESCs grown in monolayer (50). After 12 days in culture, we observed follicle-like structures. We also detected estradiol in the culture medium, which we assumed was released by the follicles. These findings are suggestive of a microenvironment comprising female germ cells and their supporting gonadal somatic cells. Nayernia *et al.* were able to obtain male germ cells from ESCs in a monolayer culture, which, in contrast to our system, depended on treatment with RA (49). Within 10 days of RA treatment, putative SSC precursors were generated from ESCs and were further expanded and established as SSC lines.

Several other groups have used EB culture systems to generate germ cell precursors or to directly produce gametes. Germ cell formation in an EB culture system appears to occur faster than in a monolayer culture system. PGCs can be detected as early as day 1 in differentiating EBs (51). Although a small number of PGCs differentiate spontaneously, BMPs and RA were found to facilitate PGC formation in EBs (51, 52). When EBs were cultured in conditioned medium prepared from adult gonadal cells or when they were co-cultured with adult gonadal cells, putative female gametes were found to form in the EBs (59, 60). These results suggest that the EB culture system also supports the generation of both male and female germ cells, depending on the stimulus to germ cell formation added to the system. Therefore, the crucial cues in directing ESC differentiation toward either the male or female germ cell lineages appear to rely on the determinants present in the culture systems. The microenvironment, created in both the monolayer and the EB culture systems, can support the formation of germ cell precursors that subsequently respond to additional determinants to further drive differentiation toward either the male or female gamete lineages.

3.5. Dedifferentiation of germ cells into ESC-like cells

It has been reported that germ cells can undergo spontaneous conversion into pluripotent cells. However, more than a decade has passed since it was first shown that mouse PGCs could be converted into embryonic germ (EG) cells, these being equivalent to ESCs with regard to pluripotency (Figure 2) (62, 63). More recently, ESC-like cells were also generated after the derivation of SSCs from neonatal or adult testicular cells (Figure 2) (64, 65). The dedifferentiation of unipotent cells into pluripotent, ESC-like cells is an interesting phenomenon in germ cell biology. A descriptive explanation for this conversion is that germ cells and ESCs both exhibit common gene profiles and have self-renewing capacities both *in vivo* and

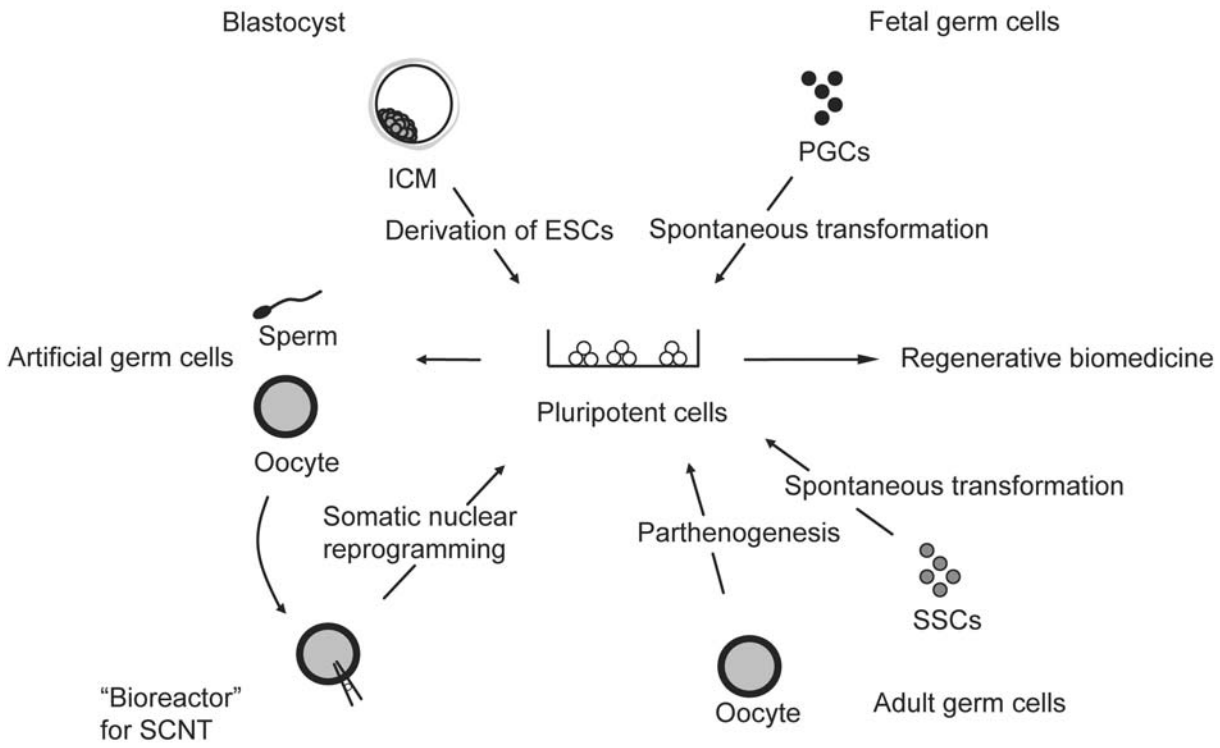

Figure 2. Relationship between germ cells and pluripotent cells. Germ cells are a unique population of cells. PGCs, which are fetal germ cells, can dedifferentiate into pluripotent cells (embryonic germ cells). Adult male germ cells can be spontaneously converted into pluripotent cells, while female germ cells can be used to generate pluripotent cells by parthenogenesis. If artificial oocytes, which would be suitable as a SCNT "bioreactor", can be generated, they will have a tremendous impact on therapeutic cloning in the generation of patient-specific pluripotent cells.

in vitro. The signaling pathways underlying these shared characteristics involved in the transformation of germ cells into ESC-like cells are still unknown.

Spontaneous cross-transformation between germ cells and ESCs has not been described to occur in any other somatic cells, unless specific factors that transform germ cells into ESC-like cells are artificially introduced (66). The fetal germ cell precursors PGCs can be converted into EG cells, which are pluripotent. Recent studies have reported that ESC-like cells could be obtained from either neonatal or adult testis (64, 65, 67). As no immediate evidence for direct dedifferentiation was provided, it is more likely that these pluripotent cells originated from SSCs residing in the testis. The latter hypothesis could be proven by demonstrating that established *in vitro* SSC lines can be directly transformed into pluripotent ESC-like cells.

4. ALTERNATIVE SOURCES FOR THE GENERATION OF GERM CELLS

Several recent studies have shown the potential of cells from alternative sources to be harnessed for the generation of putative germ cells (68-71). Dyce *et al.* demonstrated that stem cells isolated from porcine fetal skin were capable of differentiating into oocyte-like cells (68). When the skin stem cells were cultured in the presence of porcine follicular fluid, expression of germ cell-specific genes, such as *Oct4*, *GDF9b*, *Dazl*, and *Vasa*, was detected in a subpopulation of cultured cells, which later formed cell aggregates (68). These aggregates underwent further differentiation in oocyte-growth medium containing gonadotropin, forming oocyte-cumulus-like complexes and releasing putative oocyte-like cells. The results of this study suggest that adult skin stem cells, which may represent a suitable cell source for the generation of gametes, may have the capacity to develop into oocytes *in vitro*.

Recent studies have also described the use of other alternative sources for the generation of germ cells *in vitro*. Nayernia *et al*. demonstrated the ability of bone marrow stem (BMS) cells to differentiate into putative male germ cells (69, 70). The differentiation of mouse BMS cells into male germ cells was induced by RA treatment (69). About 3% of the BMS cells differentiated into male germ cells, which displayed gene expression profiles characteristic of germ cells. Some of the BMS cells that had been transplanted into seminiferous tubules of germ cell-depleted testes colonized the tubules and developed into spermatogenic cells. More recently, the same group demonstrated that human BMS cells were even capable of differentiating into male germ cells. Although direct

evidence for the functionality of BMS cell-derived human male germ cells was not presented, expression of early germ cell markers, such as Oct4, Fragilis, Stella, and *Vasa*, as well as male germ cell markers, such as *Dazl*, *TSPY*, *Piwil2*, and *Stra8*, was clearly shown (70). While these are preliminary findings, they do offer some direction on the utility of BMS cells as an alternate source of cells for the generation of germ cells.

Another recent study has demonstrated that pancreatic stem cells (PSCs) derived from adult rat pancreas are capable of differentiating into putative oocyte-like cells (71). PSCs spontaneously formed three-dimensional aggregates in suspension culture, which then released oocyte-like cells. Of interest is the expression of oocyte marker genes, such as *Oct4*, *SSEA-1*, *SCP3*, *DMC1*, *Vasa*, *CD9*, and *GDF9*, exhibited by these oocyte-like cells. PSCs, like BMS cells, have a multipotent phenotype and can thus differentiate *in vitro* into various cell types. The alternative cell sources for the derivation of germ cells described above are of a multipotent phenotype. While pluripotent ESCs can contribute to the formation of germ cells in chimeras, multipotent cells cannot. The description of *in vitro* germ cell formation from multipotent cells can therefore possibly be attributed to a subpopulation of multipotent cells that have undergone spontaneous dedifferentiation *in vitro* toward the pluripotent stage, from which germ cells may arise.

5. CONCLUSION AND PERSPECTIVES

To improve the process of generating germ cells from ESCs, it is crucial to carefully and efficiently monitor the processes of *in vitro* germ cell formation and, subsequently, to isolate germ cells from these cultures. As in any other differentiation protocol, identifying a specific cell population and isolating the desired cell population is a challenge. At present, this is accomplished by morphological examination and analysis of germ cell markers. Germ cells of different stages can be detected by the use of different germ cell markers. In order to derive and identify germ cells from ESCs, the use of efficient determinants in cultures must be improved, and markers with greater specificity need to be developed.

The study of human development is difficult since early and critical steps in germ cell development occur after implantation, after which point manipulation of embryos is rendered impossible due to ethical considerations. The *in vitro* generation of germ cell precursors and mature gametes from ESCs can provide models to study germ cell development and to develop reproductive technologies. Recently, an intriguing study reported the generation of ESCs from primates by means of somatic cell nuclear transfer (SCNT) technology, demonstrating the potential of SCNT for human therapeutic applications (72). However, the large number of oocytes used in that study and the ethical considerations concerning the use of human oocytes make this methodology unsuitable for human applications. Artificial oocytes from ESCs may at least function as a “bioreactor” to reprogram somatic nuclei (Figure 2). This prospect holds tremendous potential impact on therapeutic cloning in the generation of patient-specific pluripotent cells to be applied in regenerative medicine. So far, one report using a mouse model demonstrated that male gametes derived from ESCs were functional in that they were capable of giving rise to live pups, thus showing the potential utility of ESCs in the generation of functional gametes (49). Although several groups, including our own, have shown that female gametes can be generated from mouse ESCs, the functionality of these female gametes has not yet been demonstrated. In addition, it remains to be determined whether such oocyte-like cells can be obtained from human ESCs and whether they will be capable of reprogramming a somatic nucleus. Therefore, the current protocols need to be improved in order to obtain functional oocytes, at least for the purpose of SCNT.

The importance of investigating the relationship between germ cells and ESCs is emphasized by recent reports demonstrating that adult germ cells can be utilized to generate pluripotent cells (65, 66). Distinct from other somatic cells, germ cells appear to have the unique property of undergoing transformation into pluripotent cells, either spontaneously or by parthenogenetic activation. This attribute suggests that there are unique signaling mechanisms that mediate the transformation of germ cells and ESCs into each other. Once these mechanisms are uncovered, and our understanding of basic germ cell properties is enhanced, we can fully explore the potential usage of germ cells in the generation of pluripotent cells, and work toward the technological advancements necessary to differentiate functional gametes from ESCs.

6. ACKNOWLEDGEMENTS

This work was supported by the Max Planck Society.

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Abbreviations: ESCs: embryonic stem cells, PGCs: primordial germ cells, SSCs: spermatogonial stem cells, Blimp1: B-lymphocyte-induced maturation protein-1, BMP: bone morphogenetic protein, RA: retinoic acid, BMS: bone marrow stem, SCNT: somatic cell nuclear transfer

Key Words: Embryonic Stem Cells, Primordial Germ Cells, *In vitro* Gametogenesis, Sperm, Oocytes, Review

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