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Developmental fates of the mouse germ cell line

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ABSTRACT The specification of mouse germ cell lineage takes place after a population of pluripotential cells is established, and cell communication among the pluripotential cells may be important for this process. Primordial germ cells (PGCs) first appear around the allantois at 7 dpc which are distinct from pluripotential cells in the early embryo because they can not colonize blastocysts. However, a portion of PGCs are transformed into pluripotential cells in the ectopic environment or in culture, suggesting that the developmental fate of PGCs may still be somewhat plastic. PGCs may be destined only for gametes after they enter into the mitotic arrest phase or the meiotic prophase in embryonic gonads, which may be regulated by intrinsic and/or environmental molecules. After fetal germ cells are mitotically arrested, a large number of germ cells undergo programmed cell death. Bcl-2 and its related molecules are involved in the determination of death or survival of fetal germ cells, as well as of spermatogonia in adult testis. The cell death of spermatogonia may be necessary either for eliminating impaired germ cells or for arranging optimal interactions between germ cells and their supporting cells. Although maturating germ cells seem to differentiate only to sperm cells, oocytes that complete the first meiotic division can give rise to pluripotential cells, suggesting that maternal molecules accumulated in oocyte may play a role in the restoration of pluripotency.

KEY WORDS: *primordial germ cell, pluripotency, cell lineage, apoptosis, spermatogonía*

Introduction

In many multicellular organisms, germ cells are the only cells which can give rise to successive generations, while all somatic cells are destined for death once they start differentiation. The segregation of germ cells from somatic cell lineage takes place during early embryonic development, and at the time of lineage restriction, the germ cell line may acquire the ability to maintain and express the pluripotency. Although the study of mechanisms underlying germ cell determination has been one of the most popular subjects in developmental biology, little is known about the molecular mechanisms of the allocation of mammalian pluripotential epiblast cells to the germ cell line.

After the allocation, primordial germ cells (PGCs) start to migrate from the base of the allantois toward genital ridges. At the same time, PGCs undergo active mitosis, during which, as has been well documented in mice, a number of environmental factors affect their growth and survival (Wylie and Heasman, 1993). For the period of their proliferation, PGCs may autonomously and instructively differentiate and change their character. For example, when mouse PCGs enter into genital ridges, their locomotive ability is lost and their morphology and adhesiveness are changed (Donovan *et al.*, 1986 ; Wylie and Heasman, 1993). In addition, they stop dividing at around 13.5 dpc and enter either mitotic arrest in the developing testis or meiotic prophase in the ovaries (Eddy *et al.*, 1981). Thereafter, many fetal germ cells as well as spermatogonial stem cells in adult testis undergo programmed cell death, with only a small percentage of germ cells successfully completing the differentiation into gametes (Allan *et al.*, 1987). The determination of proliferation, differentiation, or death of germ cells and the physiological meanings of their massive cell death is of great interest.

Although fetal and neonatal germ cells can differentiate only to gametes, mature oocytes can give rise to all cell lineage. Maternal molecules accumulated in oocytes as well as germ cell line specific molecules expressed throughout their development might be necessary for this pluripotency. However, the molecular basis of the pluripotency is still largely unknown. In this review, the possible mechanisms of the determination and completion of the fate of germ cells will be discussed.

Abbreviations used in this paper: PGC, primordial germ cell; dpc, days post coitum.

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The allocation of pluripotential cells to the germ cell line

In such organisms as *C. elegans*, *Drosophila*, and *Xenopus*, germ plasm (polar plasm) containing germ cell determinants is maternally accumulated and localized in eggs, and only blastomeres which inherit germ plasm develop to germ cells. Components of polar plasm in the *Drosophila* egg are synthesized in nurse cells, transported to oocytes through intercellular connections, and localized in the egg's posterior pole. The importance of the polar plasm for germ cell establishment has been shown by transplantation. That is, injection of polar plasm from unirradiated donor embryos has successfully reversed the sterility of irradiated eggs (Okada *et al.*, 1974), indicating that the germ cell formation is triggered by germ cell determinants already existing in eggs.

In mammalian embryos, in contrast, germ plasm has not been found, and the restriction of germ and somatic cell lineage occurs at later stages of development. In the case of mice, each blastomere is equipotent up to the 8-cell stage, after which there is a gradual restriction of the developmental potency of the cells. The blastomeres located on the inside of the embryo give rise to the inner cell mass made up of a population of pluripotential cells, whereas the outside cells give rise to the trophectoderm, which is the future placenta (Hogan *et al.*, 1994). The restriction of the inner cell mass and the trophectoderm lineage is the first differentiation event in mouse embryogenesis. After implantation takes place at around 4.5 dpc, the inner cell mass develops to a simple epithelium (primitive ectoderm, epiblast) surrounding the proamniotic cavity. When single cells of 4.5 dpc epiblast are injected into a blastocyst, they flourish in the blastocyst environment, populating not only all somatic cell lineage but the germ cell line as well (Gardner and Rossant, 1979). This indicates that epiblast cells are still pluripotential at the time of implantation, although it is unclear whether each cell is equally pluripotential (Fig. 1). By 5.5 dpc, epiblast cells lose the ability to colonize blastocysts (Gardner *et al.*, 1985), suggesting that epiblast cells change their character and require the new environment formed in the epiblast to develop further. However, the clonal analysis mentioned below has shown that their developmental fates are not determined at even later stages. In addition, analysis of X-inactivation mosaics showed that the allocation of epiblast cells to germ and somatic cell lineage took place after 5.5 dpc (McMahon *et al.*, 1983). Taken together, these results suggest that mouse fertilized eggs may first develop into a population of pluripotential cells from which all somatic and germ cells are then derived (Fig. 1).

The developmental fates of epiblast cells have been examined by clonal analysis. In these experiments, single epiblast cells were labeled with horse radish peroxidase (HRP) or fluorescent dye (Lawson *et al.*, 1991; Lawson and Hage, 1994). Labeled whole embryos were allowed to develop in culture and the labeled descendants were identified. The results indicated that the progenitors of germ layers or tissues were not randomly distributed but were localized in certain regions in the epiblast. The fates of the cells, however, were not restricted to a single tissue, and the boundaries of the different prospective tissues were extensively overlapping when the gastrulation started (Lawson *et al.*, 1991). Precursors of PGCs were localized in the proximal region of the epiblast and all clones which gave rise to PGCs also differentiated to the extraembryonic mesoderm (Lawson and Hage, 1994). The localization of the progenitors of each cell lineage suggests the importance of the local cues in the epiblast for lineage specification (Fig. 1).

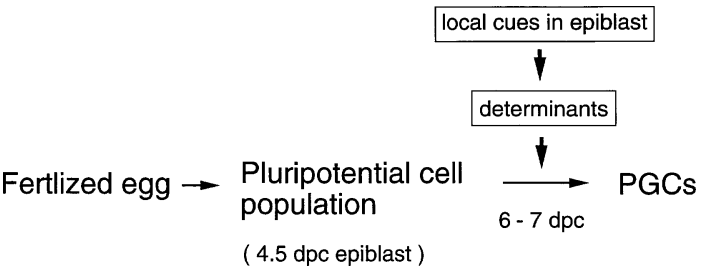

Fig. 1. Specification of the germ cell lineage in the mouse embryo. *The early epiblast consists of pluripotential cells. The allocation of epiblast cells to primordial germ cells takes place at the time of gastrulation. Local cell interaction in the proximal region of epiblast and/or cell movement accompanying gastrulation may be involved in the induction of germ cell determinants in a small number of epiblast cells.*

This idea has been further supported by the heterotopic transplantation of the epiblast cells (Tam and Zhou, 1996). The epiblast cells which expressed the *lacZ* gene were heterotopically transplanted to normal host embryos. After culturing the whole embryos, the LacZ expressing descendants were identified. The cells in the distal region of the epiblast normally differentiate to the ectoderm, whereas the proximal region corresponds to the prospective PGCs and extraembryonic mesoderm, as mentioned above. After the heterotopic transplantation, the grafted cells followed the fate characteristics of the new location. For example, when the distal cells were transplanted into the proximal region, they mostly differentiated to PGCs and extraembryonic mesoderm, and vice versa (Tam and Zhou, 1996).

The regionalization of cell fate might reflect either site-specific signals within the pre-gastrulating epiblast or the order of the morphogenetic events accompanying gastrulation, the latter of which may lead the cells into new positions providing specific signals for lineage specification. The allocation of epiblast cells to different lineage may occur as the cells ingress through the primitive streak, and it is supposed that the signaling molecules expressed in cells around the primitive streak are involved in mesoderm differentiation (Sasaki and Hogan, 1993). Because clonal analysis has indicated that PGCs and extraembryonic mesoderm are derived from common precursors (Lawson and Hage, 1994), it is likely that specification of the germ cell line may also take place around the primitive streak. Alternatively, local cell interactions occurring in the proximal region of the pre-gastrulating epiblast may be necessary before or at the time of the allocation to PGCs. In any case, yet unknown environmental signals might induce germ cell determinants in a small number of proximal epiblast cells; the allocation of epiblast cells to PGCs may occur after 6.5 dpc; and a cluster of PGCs identified by alkaline phosphatase activity first appear at around 7 dpc in the extraembryonic mesoderm (Ginsburg *et al.*, 1990; Fig. 1).

Developmental potency of primordial germ cells

After the allocation, how are the distinct fates of germ cells from somatic cell lineage ensured? The cell-fate determination in *C. elegans* provides a clue. During early cleavage, *C. elegans* em-

bryos undergo asymmetric divisions, which produce one daughter cell differentiating only to somatic cell lineage and another which gives rise to both germ and somatic cell lineages. The asymmetric distribution of cytoplasmic molecules into specific blastomere is thought to be involved in cell-fate specification. The germ line specific factor, PIE-1, is one such molecule (Mello *et al.*, 1996). PIE-1 is a nuclear protein containing potential zinc-finger motifs and is localized in germ line blastomeres (P1-P4). In pie-1 mutant embryos, the P2 blastomere does not produce germ cells, but rather undergoes somatic differentiation. Normally, somatic blastomeres express a large set of genes, while germ line blastomeres are transcriptionally inactive. In the absence of PIE-1, however, embryonically transcribed RNAs have been detected in both somatic and germline blastomeres (Seydoux *et al.*, 1996). These observations indicate that PIE-1 is required for the formation of germ cells due to its interference with gene expression in germ line blastomeres. PIE-1 might protect germ line blastomeres from factors which promote somatic cell development by interfering with their transcription. Although this kind of mechanism may not always be present in germ cell lines of other species, the *C. elegans* model might be considered a good example of the distinction between newly formed germ cells and somatic cell lineage.

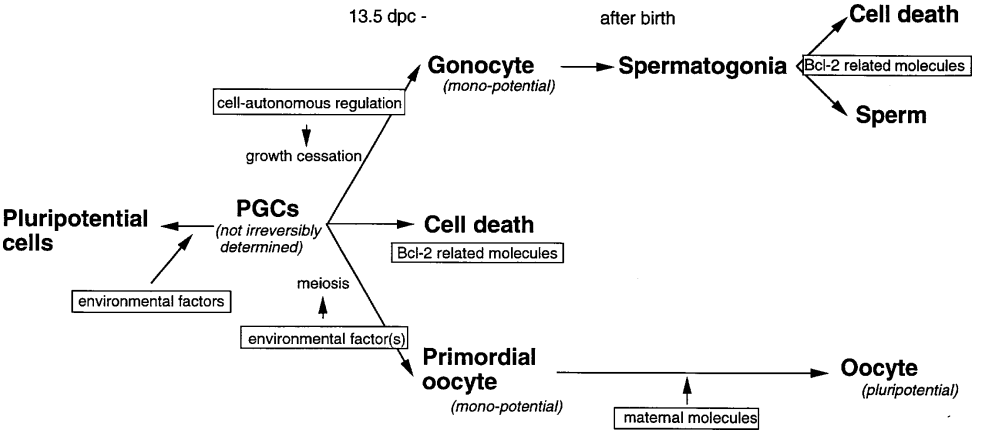

Fig. 2. A history of the mouse germ cell differentiation. *Formation of teratoma and EG cells from PGCs suggests that they are not irreversibly determined to germ cells. After growth cessation or entry into meiosis in embryonic gonads, fetal germ cells may become mono-potential and differentiate only to gametes. A large number of gonocytes and primordial oocytes in embryonic gonads, as well as spermatogonia in adult testes, undergo programmed cell death, which may be regulated by Bcl-2 related molecules. Oocytes seem to restore pluripotency after accumulation of maternal molecules in adult ovaries.*

The germ cell specification in *C. elegans* described above suggests that PGCs in vertebrates are also protected in some manner in order to realize their fates. In *Xenopus* embryos, prospective PGCs containing germ plasm asymmetrically divide up to the 8-cell stage, with one of the daughter cells that inherit germ plasm differentiating into PGCs and the other giving rise to somatic cells (Whitington and Dixon, 1975). At the early gastrula stage, the symmetrical division cycles start, and both daughter cells differentiate to germ cells. Therefore, final establishment of the germ cell line in *Xenopus* seems to take place at this stage. However, migrating PGCs at later stages still show plasticity with regard to developmental fates in the ectopic environment. When migrating PGCs are transplanted into the blastocoel cavity, they differentiate into a number of different cell types in growing host tadpoles (Wylie *et al.*, 1985). This suggests that the environment in the migration pathway of PGCs plays a role in realizing their fate.

As described above, the fate of *Xenopus* PGC is plastic and is dependent on environment. This may also be true in mammalians. If a mouse male genital ridge at 12.5 dpc is grafted under the kidney capsule, PGCs develop to teratoma containing various differentiated cells as well as undifferentiated stem cells (Stevens, 1984). Mouse PGCs also undergo similar transformation in culture. PGCs at 8.5-12.5 dpc give rise to pluripotential stem cells (EG cells) in the presence of membrane associated Steel factor, LIF and basic FGF in culture (Matsui *et al.*, 1992; Resnick *et al.*, 1992). Because PGCs are not pluripotent, as shown by the failed attempts to obtain chimera by injecting them into blastocysts, a portion of PGCs may revert to pluripotential cells that resemble early primitive ectoderm cells by action of certain environmental factors. Alternatively, the PGC population might contain a small number of pluripotential stem cells which were passed over by blastocyst injection but were expanded in the ectopic environment or by growth factors. In any case, the developmental fate of a portion of PGCs still seems to be plastic (Fig. 2). Even so, this plasticity may be lost after 13.5 dpc because the frequency of teratoma formation has been dramatically reduced when 13.5 dpc genital ridges are grafted (Stevens, 1984), and because the establishment of EG cells from 15.5 dpc germ cells has been unsuccessful (Labosky *et al.*, 1994). Mouse fetal germ cells might be finally destined only for gametes after 13.5 dpc when they enter either mitotic arrest in male gonads or meiotic prophase in female gonads (Fig. 2).

The growth arrest or initiation of meiosis may be a turning point in the fate of germ cells, but the question of their regulation remains. PGCs mimic their *in vivo* capacity of finite proliferation in culture (Matsui *et al.*, 1991), and a clonal culture of migrating PGCs has suggested that their growth arrest is autonomously regulated (Ohkubo *et al.*, 1996; Fig. 2). Although the mechanisms of the cessation of their growth are unknown, down regulated expression of growth factor receptors (Manova *et al.*, 1991) or the inactivation of their signal transducing molecules is likely to be involved in this process. Alternatively, machinery directly regulating the cell cycle progression in nuclei may be inactivated at the time of their growth cessation.

On the other hand, initiation of meiosis is thought to be controlled by both positive and negative environmental factors (Fig. 2). Normally, male germ cells do not enter meiosis until 10 days after birth. They will, however, proceed to meiosis and oogenesis in the fetal stage when they fail to reach the genital ridges and are thus located at ectopic sites, such as the adrenal gland (Zamboni and Upadhyay. 1983). Conversely, female germ cells in fetal testis in XX-XY chimera do not enter meiosis and develop as mitotically arrested T-prospermatogonia (Palmer and Burgoyne, 1991). These findings suggest that male genital ridges

provide factors to prevent both male and female germ cells from entering meiosis (Fig. 2). In addition, the presence of meiosis-inducing activity has also been suggested by organ culture experiments (Byskov, *et al.*, 1993). Meiosis of male germ cells was not induced in cultured fetal gonads, as it was in embryos, but conditioned media obtained from cultures of ovaries or testes, or follicular fluid can induce their meiosis. The meiosis-inducing activity may be present in ovaries and testes, as well as in other tissues, including the adrenal glands in embryos. Alternatively, both male and female fetal germ cells may have the potential to enter meiosis autonomously in the absence of the inhibitory influence in male fetal gonads. The competence of fetal germ cells for meiosis seems to be modified in a time-dependent manner. When isolated male PGCs at 10.5 and 11.5 dpc were aggregated with fetal lung cells and were cultured for 5-7 days, they entered meiotic prophase, while no meiotic cells were found in aggregates with 12.5 and 13.5 dpc PGCs (McLaren and Southee, 1997). These observations show that male germ cells can be rescued from the inhibitory influence by their removal from genital ridges at 11.5 dpc, but by 12.5 dpc male genital ridges may limit the ability of PGCs to enter meiosis. In addition, when 10.5 dpc PGCs were aggregated with fetal lung cells, meiotic germ cells did not appear after culturing for 3-days, but many meiotic cells of zygotene and pachytene stages were found after culturing for 7 days (McLaren and Southee, 1997), suggesting that migrating PGCs obtain competence for initiating meiosis after they undergo differentiation.

Programmed cell death of germ cells

Many fetal germ cells undergo programmed cell death after they are mitotically arrested or after entering meiotic prophase (Fig. 2). The causes and biological significance of these cell deaths are of interest. It has been postulated that germ cells which have chromosomal abnormalities in the course of proliferation may be eliminated by cell death. Alternatively, over-produced germ cells may commit suicide to obtain an optimal germ cell-supporting cell ratio (Allan *et al.*, 1987), although the real reasons for their death remain to be proven.

The intracellular cascades for cell death are likely to be conserved in various cell types, and Bcl-2 and related molecules are well-known as regulators of cell death of many cell types, including hematopoietic cells (Vaux *et al.*, 1988) and neuronal cells (Kane *et al.*, 1993) exposed to a variety of adverse conditions in culture. Although the expression of Bcl-2 and its related molecules in PGCs has not been determined, these expressions seem to be involved in PGC survival, since enforced expression of Bcl-2 and Bcl-xL in PGCs have been shown to inhibit their death in culture (Watanabe *et al.*, 1997; Fig. 2). The morphology of PGCs in the migratory phase changes from a polarized or a spread shape to a rounded shape in culture. The rounded PGCs soon undergo apoptosis and are eliminated from the culture. After the transient expression of bcl-xL in PGCs, a remarkable increase in rounded PGCs has been observed, with the total number of PGCs being about twice that of control transfection. This suggests that Bcl-xL inhibits apoptosis of rounded PGCs. Because Steel factor supports PGC survival (Dolci *et al.*, 1991; Godin *et al.*, 1991), it is of interest whether c-Kit signaling is linked to Bcl-xL. In contrast, there was no apparent increase in rounded PGCs by bcl-2 transfection, although the total PGC number was similar to that of bcl-xL transfectants (Watanabe *et al.*, 1997). The meaning of this difference is, however, currently unclear.

Large numbers of spermatogonia also undergo programmed cell death, and degeneration of type A2-A4 spermatogonia has been observed in rodents. Quantitative studies have shown that only 25% of the theoretically expected number of preleptotene spermatocytes is produced from type A1 spermatogonia (Allan *et al.*, 1987). Apoptosis of spermatogonia is also regulated by the pathway affected by Bcl-2 (Furuchi *et al.*, 1996; Fig. 2). In transgenic mice misexpressing Bcl-2 in spermatogonia and early spermatocytes, germ cell apoptosis detected by the terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling (TUNEL) method was inhibited compared with that in age-matched normal mice. By 4 weeks of age, a massive accumulation of spermatogonia and early spermatocytes was observed in all seminiferous tubules, and only a few later spermatocytes, but no spermatids, were found in the transgenic testis, though many spermatids and spermatozoa were present in the normal testis at this age. These observations indicate that ectopically expressed Bcl-2 inhibited normal spermatogonial apoptosis and differentiation of the surviving germ cells. In addition, a significant proportion of the accumulated germ cells were stained by the TUNEL method and shown to degenerate at 4 weeks of age, leading to vacuolization in some seminiferous tubules by 7 weeks (Furuchi *et al.*, 1996). These results suggest that atypical spermatogonia, forced to survive by exogenous Bcl-2, may be at a disadvantage for further survival and differentiation. In normal testis, healthy germ cells might be selected by apoptosis. Alternatively, the normal interaction between Sertoli cells and spermatogonia, which is known to be important for testicular germ cell development (Sharpe, 1993), may have been disrupted in the transgenic mice because of numerous spermotogonia in seminiferous tubules. The number of spermatogonia may be regulated to obtain an optimal germ cell: Sertoli cell ratio (Allan *et al.*, 1987) by the pathway affected by Bcl-2. Because Bcl-2 and Bcl-x are not expressed in spermatogonia (Hockenbery *et al.*, 1991; Krajewski, *et al.*, 1994), other molecules of the Bcl-2 family should be physiological regulators for spermatogonial cell death. It has recently been reported that bax-deficient mice have a testicular abnormality similar to that of the bcl-2 transgenic mice (Knudson *et al., 1995*). Bax is structurally related to Bcl-2 and binds directly to Bcl-2. Bax counters the effect of Bcl-2 on cell survival and the ratio of Bcl-2 to Bax is the critical determinant of death or survival of cells (Oltvai *et al.*, 1993). Bax may associate with another Bcl-2-related molecule, and together the two proteins may determine cell survival or death in spermatogonia (Fig. 2).

Restoration of the pluripotency in the germ cell line

Mouse fetal germ cells might become monopotential at around 13.5 dpc, and thereafter start maturating to gametes at the expense of massive cell death. Oocytes regain pluripotency during their maturation, but how can they accomplish this process, and what kinds of molecules ensure the pluripotency? Female germ cells go through meiotic prophase in the fetal ovaries, and primordial oocytes are arrested at the diplotene stage. The oocytes then start growing to accumulate maternal molecules at any time after birth. They are fully grown by about 2 weeks after the initiation of growth and subsequently resume the first meiotic division and are

released from ovaries. Ovulated oocytes are arrested again at the second meiotic metaphase, and fertilization triggers the completion of meiosis. The accumulation of maternal molecules may play a key role for the restoration of pluripotency (Fig. 2). Spontaneous ovarian teratomas composed of many kinds of tissues are often found in strain LT/Sv mice. These teratomas result from parthenogenetic cleavage of ovarian oocytes which complete first meiotic division (Eppig *et al.*, 1977). In addition, the early parthenogenetic cells are pluripotent and contribute to the formation of germ line chimera (Stevens, 1978). The ovulated parthenotes undergo apparently normal development up to the expanded blastocyst stage, but after implantation they are absorbed, since their genomes are incorrectly imprinted (Kono *et al.*, 1996). However, when the parthenogenic four- and eight-cell embryos were aggregated with normal embryos, the parthenogenic cells populated the germ cell line in chimera, indicating that they are pluripotent (Stevens, 1978). From these observations, oocytes seem to restore pluripotency after accumulation of maternal molecules, even if they do not complete meiosis and do not undergo fertilization, suggesting that some maternal factors are critical for expressing pluripotency (Fig. 2). The roles of maternal factors in early development are more obvious in *Drosophila*. In *Drosophila*, each oogonium divides four times to produce a clone of 16 cells (cystocytes) connected by cytoplasmic connections. Only 1 of the 16 cystocytes becomes an oocyte, and all others become nurse cells. The nurse cells actively synthesize RNA and protein which are transported into the oocyte through the cytoplasmic connection. Some of the maternal RNAs and proteins are then localized or form gradients in oocytes, which is critical for the construction of the anterior-posterior axis of the embryo or for germ cell establishment. Thus, in the case of *Drosophila*, maternal molecules are essential not only for the restoration of pluripotency of oocytes but also for the formation of the basic body plan of embryos in oocytes.

Oct-3/4 has been identified as the only gene that is specifically expressed in pluripotential embryonic cells and the germ cell line in mice (Scholer, 1991). Oct-3/4 encodes a transcription factor and is involved in the maintenance of the undifferentiation status of embryonal carcinoma cells (Shimazaki *et al.*, 1993). The expression pattern and its function in embryonal carcinoma cells suggest that it plays an important role to protect pluripotential cells from undergoing somatic cell differentiation in early embryos. This gene may also contribute to the realization of the fate of PGCs. This and other yet unknown molecules expressed in this cell lineage, together with maternal molecules, may be essential for restoration of the pluripotency of oocytes.

Conclusion

The mammalian germ cell line is derived from pluripotential cell populations in embryos at the egg cylinder stage. Although the mechanisms of germ cell specification in mammals are unknown, it is supposed that cell communication among the pluripotential cells may induce germ cell determinants in particular cells in the proximal epiblast. After the allocation, PGCs might remain plastic with regard to their developmental fate and might be protected from deviation from this fate by environmental factors and/or intrinsic mechanisms. Fetal germ cells seem to be destined only for gametes after entry into the G0 phase or meiotic prophase, which entry may also be regulated by cell-autonomous and/or instructive mechanisms. Even after their developmental fate becomes rigid, a large portion of germ cells still suffer programmed cell death before finally differentiating to gametes. Their cell death may be necessary either for selecting healthy germ cells capable of giving rise to the next generation or for optimizing the environment for their maturation. Maturating germ cells can differentiate only to gametes, but pluripotency may be restored in oocytes by maternal as well as germ line specific regulators. As described here, the saga of the germ line is compelling, but the molecular mechanisms directing the fates of germ cells remain to be determined.

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