Germline stem cells are critical for sexual fate decision of germ cells

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Egg or sperm? The mechanism of sexual fate decision in germ cells has been a long-standing issue in biology. A recent analysis identified *foxl3* as a gene that determines the sexual fate decision of germ cells in the teleost fish, medaka. *foxl3*/Foxl3 acts in female germline stem cells to repress commitment into male fate (spermatogenesis), indicating that the presence of mitotic germ cells in the female is critical for continuous sexual fate decision of germ cells in medaka gonads. Interestingly, *foxl3* is found in most vertebrate genomes except for mammals. This provides the interesting possibility that the sexual fate of germ cells in mammals is determined in a different way compared to *foxl*3-possessing vertebrates. Considering the fact that germline stem cells are the cells where *foxl3* begins to express and sexual fate decision initiates and mammalian ovary does not have typical germline stem cells, the mechanism in mammals may have been co-evolved with germline stem cell loss in mammalian ovary.

Keywords:
fish; germ cells; mammals; sex; stem cells

Introduction

Recent studies have shown that vertebrates employ various genes for sex determination [1]. *Sry*, located on the mammalian Y chromosome, was the first sex determination gene identified in vertebrates [2, 3]. Since then, several critical factors have been identified such as Sox9, Fgf9, and Dmrt1 [4]. However, contrary to the initial prediction that *Sry* is conserved among vertebrates, many animals do not possess an *Sry* homolog. Ten years after the discovery of *Sry*, *DMY*/*Dmrt1bY* was identified as the sex determination gene on the sex chromosome in the teleost fish, medaka [5, 6]. Since this discovery, other sex determination genes have also been identified in various vertebrates.

Regardless of these variations of the sex determination genes, the first cell type to display sexual discrimination during embryogenesis appears to be conserved among all vertebrates. All sex determination genes examined thus far are expressed in the somatic (supporting) cells that directly surround the germ cells in the gonad [3–12]. Therefore, it is reasonable to speculate that the sexual fate of germ cells (in other words, the fate decision of germ cells to develop eggs or sperms) is triggered by the sex of the surrounding somatic cells during a normal sex determination process. Thus, the precise timing and mechanism of germ cell sexual fate determination by somatic cells needs to be assessed.

The precise molecular mechanism underlying germ cell sexual fate decision is yet to be determined. However, a few studies on the cellular level have provided clues as to the mechanism. In a mouse ex vivo culture study, germ cells isolated from male gonad at 12.5 dpc (days post-coitum) maintained the male characteristics even when cultured in the presence of only female somatic cells, suggesting that the fate decision of germ cells to male occurs by around 12.5 dpc, 2 days after the onset of *Sry* expression in the supporting cells. XX germ cells do not exhibit any sign of meiosis at 12.5 dpc, but they do at 13.5 dpc in a culture condition where male gonadal primordial cells were present. Therefore, 13.5 dpc was determined as the time when the decision to female is made [13, 14].

Consistent with the results of ex vivo culture experiments, several factors – including fgf9 and retinoic acid (RA) – have been shown to be involved in the early entry into or the repression of meiosis in mouse. Fgf9, genetically located downstream of *Sry*, constitutes a major component of positive feedback for establishing male determination [15]. This factor directly acts on germ cells to repress promotion of meiosis through Fgf2 receptor and Nanos2. RA is recognized as a factor for promotion of meiosis since disruption of RA signaling in gonads represses meiotic process. The two independent factors mutually act to regulate meiosis [16, 17]. Given that an early entry into meiosis is

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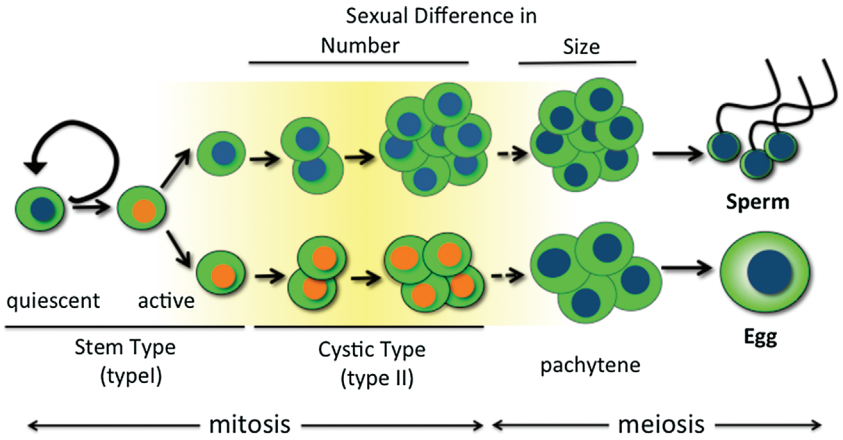

Figure 1. A schematic representation of gametogenesis and *foxl3*/Foxl3 expression. Germ cell numbers increase through mitotic divisions. *foxl3*/Foxl3 is initially detected in the germ cells of the developing female and male gonads. *foxl3*/Foxl3 expression (pink) diminishes in the male gonad while it continues in the mitotically active type of germline stem cells that subsequently undergo cystic divisions. The expression is subsequently lost before germ cells enter meiosis. The cystic division of male and female differs in terms of the number of divisions. The size of germ cells is distinguishable after the pachytene stage and female germ cells are larger than male germ cells. However, prior to the pachytene stage, the sex is difficult to distinguish merely by the size of the germ cells. The yellow-shaded area indicates the expected sexual fate decision period in germ cells.

associated with the mechanism of female determination in germ cells, these factors can be categorized as factors affecting sexual fate decision of germ cells.

Studies using the teleost fish provided further insights into the role of germ cell type for the sexual fate decision. Regardless of donor trout sex, germ cells that were transplanted into larva assumed the sexual fate of the recipient gonad [18, 19]. We also transplanted GFP-labelled germ cells isolated from adult testis into the coelomic cavity of larva and found that GFP- germ cells developed into oocytes (unpublished results). These results suggest that adult ovaries and testes contain sexually indifferent or unfixed germline stem cells. In fact, as described below, a clonal analysis demonstrated the presence of germline stem cells in the medaka ovary [20]. The germline stem cells in the medaka ovary continuously generate oocytes throughout the reproductive term, indicating the presence of germ cells with the ability to self-renew to maintain the stem cell population and to produce cells that can differentiate into eggs. The germ cells with these characters are developmentally uncommitted to gametogenesis and are likely to be sexually indeterminate (e.g., [21, 22]).

Early gametogenesis – from germline stem cells to entry of meiosis

Germ cells residing in the primordial gonad are often referred to as gonocytes or gonial cells. As primordial germ cells (PGCs) enter the primordial gonad, these cells acquire the capacity as gonial cells to develop into either eggs or sperms. Gonial cells in the mature testis were shown to include the germline stem cell population that is essential for continuous sperm production [23]. Several non-mammalian vertebrates, especially those that produce numerous eggs, are shown to possess germline stem cells or germ cells with stem cell markers [24]. In these vertebrates, gametogenesis from germline stem cell progenitors exhibits a conserved pattern of cellular division (Fig. 1).

In many organisms, progenitor cells undergo successive and synchronous mitoses, followed by entry into the first events of meiosis, the meiotic prophase I [25–28]. These successive mitoses are characterized by an incomplete cytokinesis, so that daughter cells remain connected through a cytoplasmic bridge. The interconnected germ cells are packaged as a cyst and surrounded by somatic cells. Following several rounds of mitosis, the cells enter meiosis to become eggs in the ovary or sperms in the testis [28, 29]. Interestingly, the number of successive division rounds differs between oogenesis and spermatogenesis in medaka [20, 30]. This suggests that gametogenesis-committed germ cells are sexually determined by the end of the cystic division stage.

Unlike the vertebrates mentioned above, mouse exhibits a different pattern of oogenesis in that the ovary does not retain typical germline stem cells [24] (compare Fig. 2A and B). The PGCs in mouse develop into gonocytes that are competent for gametogenesis [31]. However, this developmental status appears to be transient in female because all germ cells will eventually enter oogenesis during the fetal period. After the commitment into oocyte development, the pattern of germ cell division proceeds in a similar way as seen in non-mammalian vertebrates: the cystic division generates aggregates of germ cells that are partially interconnected to each other via intercellular bridges, and the germ cell aggregates subsequently break apart to form primordial follicles. This occurs only during a fetal period and, therefore, cystic germ cells are also absent in the adult ovary [25, 32, 33]. On the contrary, in mouse testis, gonocytes develop into spermatogonial cell populations including germline stem cells, which maintain the homeostasis of spermatogenesis [23].

The presence of germline stem cells only in male mice is atypical for vertebrates. Actually some mammals have been suggested to possess germ cells that undergo mitosis in the adult ovary [34]. The teleost and amphibian ovaries were shown to possess germline stem cells and/or mitotic germ cells [35]. Germline stem cells may exist in a prototype for the cellular gametogenesis in adult gonads.

Expected timing of sexual fate decision of germ cells

As described above, germline stem cells are very likely sexually indifferent or unfixed even in adult gonads in the teleost while the typical germline stem cells are present only in male mouse but not in female mouse. Then the question

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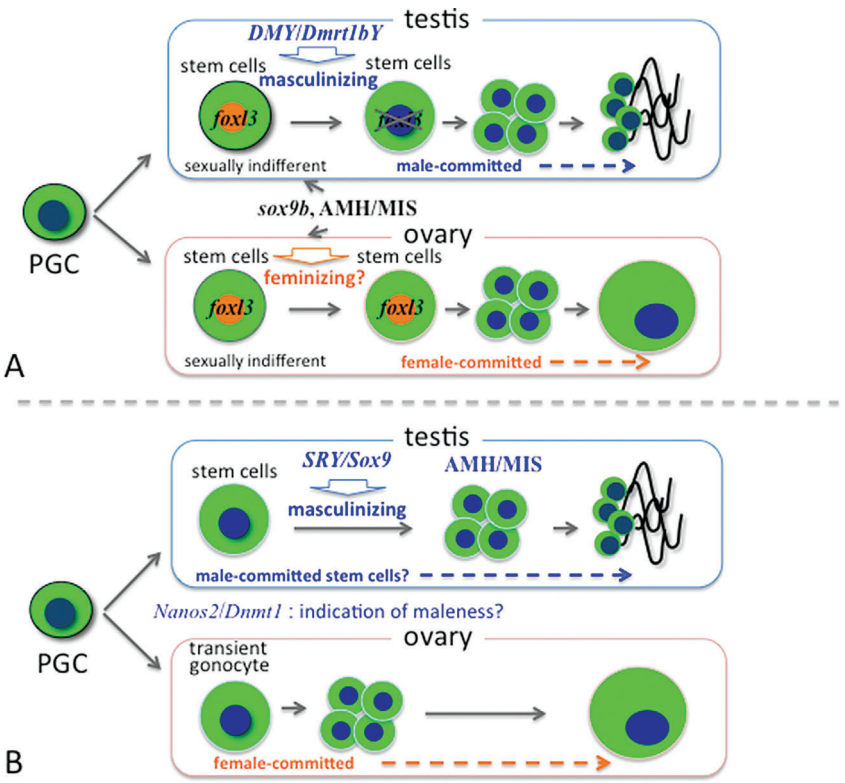

Figure 2. The sexual fate decision of germ cells and the status of germline stem cells. The mechanism of sexual fate decision in germ cells may be linked to both the oogenesis process during the development and the germline stem cell status in the mature ovary. The loss of germline stem cells may allow for the neofunctionalization of *sox9/amh,* which contributes to the masculinization of the gonad in mouse. **A:** During the ovarian and testicular development of medaka, sexually indifferent and/or unfixed germline stem cells are established. The testis and the ovary determine the sexual fate of the progeny of mitotically quiescent germline stem cells. Downregulation of *foxl3*/Foxl3 is critical for the germ cell commitment to spermatogenesis. **B:** During mouse ovarian development, all germ cells enter oogenesis, which may be mediated by the transient status of the gonocytes. Adult ovaries preserve germ cells as follicles and do not possess the typical germline stem cells. Thus, the function of *foxl3*/Foxl3 may be dispensable in mouse. Unlike in medaka, germline stem cells in mouse testis might be committed to male.

arises regarding the timing of sexual fate decision of germ cells.

In medaka, prior to the pachytene stage of meiosis, female and male germline stem cells, and mitotically dividing cystic germ cells are morphologically indistinguishable. However, female germ cells are getting larger than male germ cells by the pachytene stage. This suggests that the fate decision to eggs or sperms is made prior to this stage (Fig. 1) and occurs in the period from the timing of commitment of germline stem cells to gametogenesis and the early pachytene stage.

In mouse, sexually distinct germ cell characters were demonstrated using several meiosis mutants [36]. During meiosis, checkpoints at early meiotic prophase I, such as the double strand break and the synaptonemal complex (SC) formation, interrupt the progression of meiosis. The stage of interruption differs in female and male germ cells as early as the timing of zygotene. These observations suggest that germ cells possess sexually distinct characters before they enter zygote stage.

These observations suggest that the sexual fate decision is timed similarly in both mouse and medaka (after commitment of gonocytes/germline stem cells but before early stage of meiosis). An important point here in mouse studies is that the entry of meiosis during a fetal period has been regarded as a sign of female germ cell differentiation because male germ cells do not enter meiosis until a neonatal period. Therefore, the studies trying to address sexual fate decision have also been centered on the meiosis that occurs during female ovary development in the fetus.

Factors regulating early gametogenesis in the mouse

In this context, several germ cell-specific factors have been identified. One important factor is stra8. *stra8* is an essential gene upregulated in germ cells responding to retinoic acid (RA) that is an exogenous factor promoting meiosis. The repression of meiosis in male fetus is shown to correlate with downregulation of *stra8* by male-specific factor of fgf9 [37]. Nanos2 is another factor involving the repression of meiosis in germ cells. Dysfunctional *nanos2* in germ cells causes the precocious expression of meiotic genes during testicular development [38]. Both factors appear to prevent the precocious entry of male germ cells into meiosis. The polycomb repressive complex 1 (PRC1) may also contribute to the distinct sexual state of germ cells because premature expression of *Stra8* is only observed in female germ cells of mutant gonads [39]. These mechanisms are consistent with the expected timing of the sexual fate decision.

It is important to note that these studies are based on the assumption that an event of the early meiosis and an event of feminization are nearly equivalent in germ cells. Nonetheless, an analysis of *stra8* mutant seems to speak against this assumption. In the *stra8* mutant, a very small number of germ cells can develop into oocyte-like cells without undergoing the meiosis process. The mutant oocyte-like cells have the capacity to be fertilized in vitro [40]. This analysis suggested that a yet to be identified molecule intrinsically participates in the sexual decision of the germ cells toward female (oogenesis), but not in the promotion of meiosis that occurs in the ovary. Thus, early meiotic entry during ovarian development may be linked to the mechanism of femaleness in germ cells (therefore it can be used to indicate femaleness), but may not be equivalent to the sexual fate decision of germ cells to female. It would be possible that a

change of epigenetic status in male germ cells can be regarded as a process of stem cell establishment but not maleness of germ cells [38, 39].

Discovery of *foxl3* as a switch gene for sexual fate decision of germ cells

The mechanism of sexual fate decision may be present and act in a cell-autonomous manner in germ cells. This event may take place between the germline stem cell stage and the pachytene stage. As an alternative mechanism, the sexual fate decision is not innate in the germ cells. In this scenario, the sexual fate decision of germ cells is mechanistically identical to the germ cell development toward eggs or sperms. Germ cells are unable to reach the end of their sexual path without receiving an instructive signal(s) from the surrounding somatic cells. In this case, oogenesis and spermatogenesis are controlled by the somatic cells in a stepwise fashion.

A recent finding on *foxl3* function demonstrated that the germ cells indeed possess an intrinsic mechanism [41]. Foxl3 contains a fork-head domain and is expressed in germ cells in medaka. During a very early stage of the gonadal formation, *foxl3*/Foxl3 is transiently expressed in both female and male germ cells as early as the onset of sexual differentiation of the gonads (stage 35). However, *foxl3*/Foxl3 expression diminishes in the male, but is maintained in a subpopulation of stem type germ cells (type I germ cells) and in all cystic-type germ cells (type II germ cells) in the female (Fig. 1). Interestingly, mitotically quiescent stem type germ cells do not express Foxl3 in females, indicating that the commitment to gametogenesis is associated with the activation of *foxl3*/Foxl3 expression. As early events of meiosis (meiotic prophase I) take place in the germ cells, *foxl3*/Foxl3 expression diminishes in the female germ cells. This expression pattern is also consistent with the expected timing of the sexual fate decision described above.

The loss of *foxl3* expression leads to a remarkable phenotype in the female. Female homozygous *foxl3* mutants develop a typical ovarian structure and express ovary-specific genes. Normal ovaries display niche regions within the germinal epithelium layer, termed the germinal cradle, where germline stem cells are maintained, and oogenesis proceeds until the germ cells reach the early diplotene stage [20, 41]. Germ cells with a defective *foxl3* gene develop sperms in the germinal epithelium of the developing ovary at the larval stage. As a result, the germinal epithelium expands with numerous sperms. The sperm cannot go out from the germinal epithelium because the mutant ovary does not develop a ductal system, such as an efferent duct in testis. But artificial insemination has shown that the isolated sperm from the mutant ovary are fertile. In turn, the fertilized eggs develop to term, sexually maturing as adult females with fertile eggs, producing off-spring. This phenotype clearly demonstrates the presence of an intrinsic mechanism for the sexual fate decision in germ cells.

Duration and mechanism of the sexual fate decision in germ cells

The mutant phenotype described above provides insights into the duration and mechanism of the sexual fate decision of germ cells. Here, it is noteworthy to mention that *foxl3*/Foxl3 expression is not detected in mitotically quiescent germ cells. It is well established that stem cell populations are not homogeneous, but instead are comprised of at least two populations – one that is mostly quiescent, and one that is mitotically active [20]. As the mitotically quiescent germ cells during medaka embryonic period display no gene expression of gametogenesis and meiosis and a large cell size with less prominent DAPI (4′,6-diamidino-2-phenylindole) staining, these quiescent cells are likely the stem of stem cells or are the gonocytes that are developing into germline stem cells. The *foxl3*/Foxl3 expression is initiated in some populations of mitotically active germline stem cell type. Interestingly, the adult testis and ovary of medaka display a mosaic *nanos2* (a hallmark of germline stem cells) expression pattern in the type A spermatogonia and oogonia, including the germline stem cells, which reflect different grades of stemness characters within the gonial populations [24]. The medaka adult ovary also displays a mosaic *foxl3*/Foxl3 expression pattern in oogonia. Although, it is essential to examine if *foxl3*/Foxl3 and *nanos2* are coexpressed in the gonial populations, it is possible that the loss of stemness character is related to initiation of *foxl3*/Foxl3 expression. The mosaic expression in gonial populations implies that the initiation of sexual fate decision may be linked to the establishment (in larva) and regulation (in adult) of stem cells.

The *foxl3* mutant phenotype and expression of *foxl3*/Foxl3 also suggest the duration of the sexual fate decision during the course of gametogenesis. During a normal oogenic process, germ cells undergo three to four rounds of successive cell divisions (maximum of five rounds), resulting in between 8 and 64 premeiotic germ cells within a single cyst. Furthermore, more than nine rounds of cystic division take place successively and clonally to produce a minimum of 512 spermatocytes in wild-type male [20, 30]. However, germ cells in the mutant female divide in a fashion similar to that observed in wild-type testis. Differences in the number of cystic divisions that occur between sexes suggests that the sexual fate decision process continues during the early stage of cystic division, which is earlier than the pachytene stage predicted solely by morphology.

Because the female *foxl3* mutant exhibits mature sperm production in the developing ovary, this mutant phenotype clearly demonstrates that one major mechanism of the *foxl3*/Foxl3 sexual switch is to repress the initiation of spermatogenesis. Because of loss of the *foxl3*/Foxl3 function, derepression of spermatogenesis is initiated in the mutant around the time of hatching, which coincides with the timing of oocyte production in wild-type female. In contrast, germ cells in male *foxl3*mutant initiate spermatogenesis earlier than those in wild-type male [41].

Mature gonads are the organs that determine the sex of germ cells

The finding that *foxl3*/Foxl3 expression initially occurs in some germline stem cell populations supports the idea that

the sexual fate decision is initiated at the very beginning of the gametogenesis commitment in mitotically active germline stem cells. This finding led to an unconventional notion of the existence of a germ cell population, the sex of which is determined long after that of the organism (somatic cells) in medaka. In this case, mature ovaries and testes are not merely reproductive organs that regulate the process of gametogenesis (timing and quantity), but they also act to guide the sexual fate of germ cells by directing the expression of *foxl3*/Foxl3. To my knowledge, the view on adult testis and ovary as germ cell sex-determining organs has not been sufficiently addressed at the molecular level. However, the adult mouse ovary is different from that of medaka in that the mechanism of sexual fate decision of germ cells is dispensable since all germ cells are present as follicles and not as typical germline stem cells that are sexually indifferent and/or flexible (Fig. 2).

Rapidly evolving *foxl3*

This view may be associated with a failure to detect the *foxl3* gene in the mammalian genomes examined thus far. However, the *foxl3* gene is present in other vertebrates from teleost to marsupials [42, 43]. Thus, the evolutionary conservation of the *foxl3* gene among other vertebrates may be linked to the presence of sex determining mechanism in the adult ovary. The absence of initial germ cells that trigger *foxl3*/Foxl3 expression in the mammalian adult ovary is in agreement with the fact that the mammalian adult ovary does not possess sexually indifferent or unfixed germ cells.

It is noteworthy to mention that the *foxl3* gene shows a more rapid amino acid substitution rate than *foxl2*. *foxl2* and *foxl3* share the closest neighboring node in the phylogenetic tree. A duplication is thought to have occured before teleosts diverged [42, 43]. In mammals, *foxl2*/Foxl2 is known to be essential for development and maintenance of female somatic cells. *foxl2*/Foxl2 in both mammals and teleost is expressed in somatic cells of only female gonads but not of male gonads, suggesting that the *foxl2* of teleost and mammals share a similar role [44–49]. In contrast to the conserved role of *foxl2*/Foxl2, the rapid amino acid substitution rate of *foxl3* implies that its function changed at a relatively fast rate during vertebrate evolution. In particular, in bird and marsupial branches, a branch of *foxl3* clades is extended long in the phylogenetic tree, suggesting the possibility that the function of *foxl3*/Foxl3 is changing. As evolution proceeds toward mammalian clades, *foxl3*/Foxl3 might have lost the function of sex determination in germ cells [42, 43]. It is likely that the changes in the mechanism of germ cell sexual fate decision are linked with those in *foxl3*. The development of a novel decision mechanism may have accompanied the loss of germline stem cells. Additionally, the fact that germline stems cells in the ovary are unnecessary/not required may have promoted novel sex determination mechanisms.

Neofunctionalization of other genes with loss of germline stem cells in mammals

Actually, the development of a novel mechanism may cause the striking change of sex determination mechanism of the somatic cells. *Sox9* is a direct effector of the mammalian somatic sex determination gene, *Sry*. *Sry* is a mammalian-specific gene, while *Sox9* is conserved among vertebrates [50]. However, our recent analysis indicated that *sox9b* – an orthologue of the mammalian *Sox9* expressed in the supporting cells – is not directly involved in testicular differentiation. A study utilizing a medaka *sox9b* mutant demonstrated that the function of *sox9b* is more related to the maintenance of germ cells [51]. The mutant also indicated that *sox9b* expression is more intense in the supporting cells surrounding the germline stem cell population than in those that surround the gametogenesis-committed germ cells [24]. The function of germ cell maintenance has also been reported in the mouse adult testis [52]. It is speculated that the loss of *sox9b* function in the germ cell maintenance, in addition to the loss of germline stem cells, allows for the neofunctionalization of the mammalian *Sox9* as an effector of *Sry*.

A similar scenario may also apply to the function of the anti-Müllerian hormone (AMH/MIS). AMH/MIS is a phylogenetically ancient molecule that belongs to the bone morphogenetic protein (BMP) gene family and is regulated downstream of *Sox9* during testicular development in mammals. AMH/MIS is essential for the regression of female reproductive organs, such as the upper vagina and oviduct. However, an analysis of the mutant of a type II receptor of AMH/MIS in medaka indicated AMH/MIS involvement in the regulation of the germline stem cell numbers, but not in the formation of reproductive organs [53, 54]. This led to a hypothesis that the mammalian AMH/MIS acquired its role in the regression of female reproductive organs because the establishment of germline stem cells is dispensable during mammalian ovarian formation.

The default sexual status of germ cells – the role of *foxl3*/Foxl3

The role of *foxl3*/Foxl3 has raised a question about the default sex of germ cells. As mentioned above, immediately following PGC residence in the gonadal primordium, *foxl3*/Foxl3 is detected in both female and male. Subsequently, *foxl3*/Foxl3 expression is repressed specifically in male. This pattern of expression, in conjunction with the mutant phenotype, led us to conclude that the function of *foxl3*/Foxl3 is to repress spermatogenesis and that the repression of *foxl3*/Foxl3 results from some act by masculinized somatic cells. In other words, the gonocytes expressing *foxl3*/Foxl3 are likely in the default status.

Then, one might argue that *foxl3*/Foxl3 has a positive role in oogenesis because *foxl3*/Foxl3 is continuously expressed in the female germ cells. Interestingly, considering the absence of a *foxl3*/Foxl3 orthologue in mammals, *foxl3*/Foxl3 might not be required for the entry into oogenesis. The phenotype of the *foxl3* mutant at the adult stage may also support this notion, because few fertile oocytes appear

in a later mature stage of the sperm filled-mutant ovary [41]. This suggests that oocytes can develop in the absence of *foxl3*/Foxl3. These facts collectively imply that not becoming male germ cells leads to female germ cells where *foxl3*/Foxl3 may not contribute to becoming female germ cells. On the other hand, we do not yet know if not becoming female is equal to development to male germ cells. Forcing the expression of *foxl3*/Foxl3 in germ cells of the testis would elucidate the default sex of the germ cells.

Conclusions and outlook

Here, I have described the concept that *foxl3* has changed rapidly during the course of evolution. Nonetheless, I postulate the conservation of *foxl3*, because it is more conserved than the genes that are involved in the sexual fate decision of somatic cells (genes that are conventionally referred to as "sex determination genes"). The sex determination genes tend to change more rapidly, and are typically not conserved, even within a single class [55, 56]. However, the *foxl3* gene is found in the teleost, amphibian, reptile, bird, and marsupial genomes. Mammals are the only vertebrates that do not possess *foxl3* gene, which is consistent with mammals also not having typical germline stem cells in the ovary. Further investigation of the function of *foxl3*/Foxl3 in other classes of organism, and the presence of germline stem cells (especially in birds because germline stem cells are not yet identified and *foxl3* gene changes a lot compared to that in teleost) will provide a more comprehensive picture of how sexual fate decision is orchestrated in somatic as well as germ cells, and how diverse mechanisms are employed in different species.

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