

Review

Ovarian Germline Stem Cells in the Teleost Fish, Medaka (*Oryzias latipes*)

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Abstract

In the mammalian testis germline stem cells keep producing many sperms, while there is no direct evidence for the presence of germline stem cells in the ovary. It is widely accepted in mammals that the mature oocytes are supplied from a pool of primordial follicles in the adult ovary. In other vertebrates, such as fish, however, there has been no investigation on the mechanism underlying the high egg-producing ability. In this review, we introduce the recently identified ovarian germline stem cells and the surrounding unique structure in teleost fish, medaka (*Oryzias latipes*) [Nakamura S et al. Science. 2010; 328: 1561-1563]. We also discuss about the expression and function of *sox9* that characterizes this unique structure.

Key words: medaka, germline stem cells, *sox9*

Introduction

In mammalian ovaries, it is well known that mitotic division of germ cells completes before birth. Neo-oogenesis does not occur afterwards. Therefore the number of oocytes to be ovulated is limited to the number of primordial follicles that are generated during embryogenesis. In contrast, in many vertebrates with high fecundity, the number of oocytes is thought to be infinite. In the adult ovary of these species actually, mitotic germ cells are histologically observed as oogonia and thus the proliferation of oogonia can supply mature eggs continuously throughout the life. However, the molecular and cellular mechanisms underlying the continuous production of oocytes have not been well addressed in these species. In this review, we introduce the recently identified germline stem cells in adult medaka ovary and the possible function of *sox9* gene that expresses in the ovarian niche structure [1].

Oogenesis in the vertebrate ovary

Conceptually gametogenesis has to proceed in a chronological order of oogenesis and spermatogene-

sis, mitotic division to increase the number of gonial cells, meiosis to prepare a reductive set of chromosomes and a final maturing process to produce fertile eggs and sperms. But these distinctive steps of gametogenesis do not always proceed as successive events.

In most vertebrates, the adult testis contains all the steps of spermatogenesis and some population of spermatogonia function as germline stem cells, ensuring the production of tremendous number of sperm throughout the life. The composition of germ cells in the adult ovary, however, varies among vertebrates. In the mammalian ovary, all oogonia finish synchronous cystic divisions and enter subsequent meiosis by the date of delivery. During the development of ovary, a pool of primordial follicles is formed and some germ cells are eliminated by apoptosis [2]. All the primordial follicles are supplied from oogonia, in a short period of ovarian development around birth, through synchronous division and meiosis in cord-like structures extending from the cortical region (Figure 1).

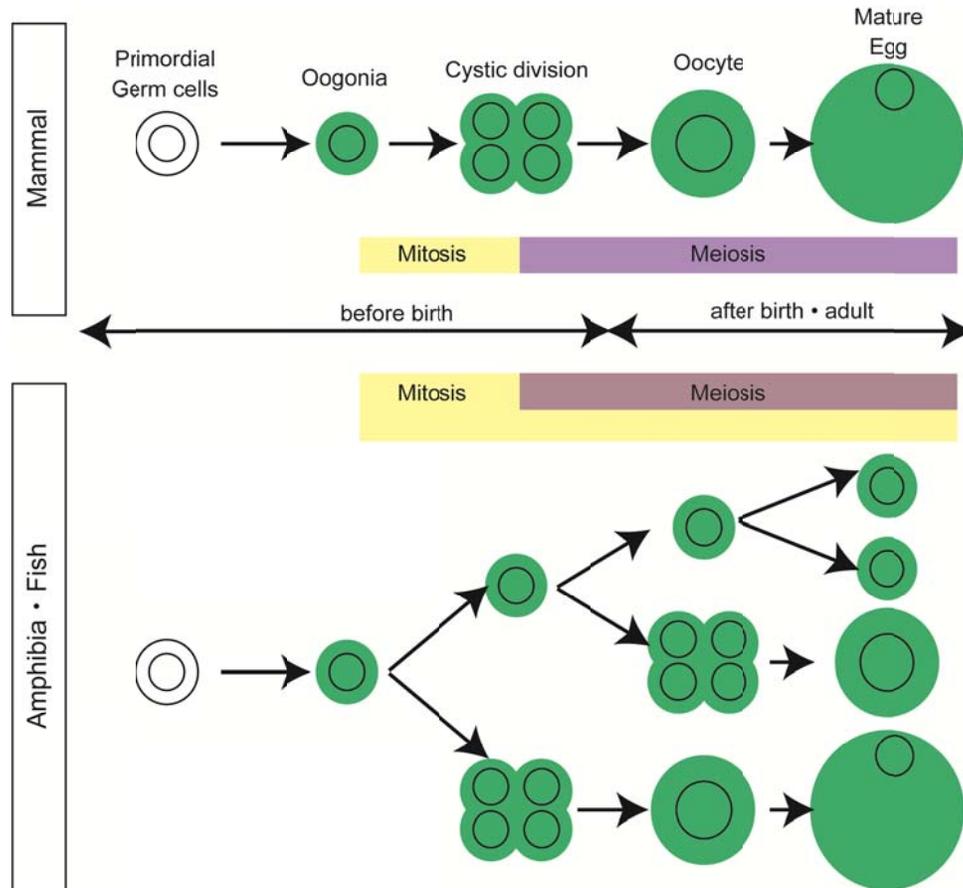


Figure 1. Oogenesis in vertebrates. In mammals, mitotic division of oogonia finish before birth and meiotic germ cells are only present in postnatal and adult ovaries. In contrast, germ cells in all the stages of oogenesis are present in adult ovaries of high fecundity species, such as amphibia and fish.

Mitotic germ cells, a good indication of oogonia, are no longer observed in ovary of many mammals after birth [2, 3].

In contrast, in the ovary of animals with high egg-producing ability, such as fish and amphibia, mitosis in oogonia is observed histologically in adult mature ovary [4, 5] (Figure 1).

Are germline stem cells present in mammalian ovary?

It has been generally accepted, as mentioned above, that mammalian ovary loses mitotic oogonia after birth. On the other hand, there has been controversial by the experts of reproduction and the stem cell biologists on the presence of germline stem cells that might produce oocytes in mature ovary. In 2004, Tilly and his colleagues showed that stem cell-like germ cells are present on the surface epithelium of ovary and neo-oogenesis actually occurs in mammalian adult ovary [6]. They also showed that these germ

cells expressed Mouse Vasa Homolog, MVH and a meiosis marker, SCP3. BrdU incorporation analysis indicated that these germ cells undergo mitosis. They also described that the numbers of follicle cells were decreased after the busulfan treatment, which abolishes only the mitotic cells. In addition, when the ovarian fragments expressing GFP were grafted to the wild-type ovary, GFP-positive oocytes differentiated in the host ovary. Next year, the same group showed that MVH-positive cells were present in the bone marrow and peripheral blood and bone marrow transplantation restored the oocyte production in the *Atm* knockout mouse, which would otherwise be incapable of making oocytes [7]. However, these result are now controversial, because the papers from Wagers' group argued that the ovulated oocytes were not derived from the bone marrow or peripheral blood in 2005 [8]. Although further experiments are required, there is no direct evidence of germline stem cells producing the functional eggs in adult mammalian ovary.

'Ovarian Cords' in medaka ovary

Many fish and amphibia have high fecundity and exhibit a variety of reproductive strategies. Salmonid fish spawns eggs once a year while goldfish spawn many times in one reproductive season within a year. Some tropical fish has no particular spawning season and spawns many times per year. The number of eggs to be spawned varies, from a few to multibillion as seen in headfish. In the fish ovaries, mitotic oogonia are morphologically observed quite often after birth [4] and therefore, unlike mammals, new eggs are thought to be produced in mature ovary. A sort of 'germline stem cells' with mitosis, if present, could be the source of many eggs. Alternatively, certain differentiated oogonia also could provide a large pool of follicles through mitosis. But the cellular and molecular mechanisms ensuring supply of eggs from oogonia have been unclear and have remained to be addressed as a question of broad interest.

We study the mechanism underlying the formation of ovary and testes using teleost fish, medaka (*Oryzias latipes*) as a model. Medaka spawns several dozens of eggs every day for about one year in artificial long-daylight conditions. Our laboratory has succeeded in visualizing several cell lineages constituting gonads by establishing the transgenic medaka [9, 10]. Additionally, we have also succeeded in applying both a cre/loxP system and a heat-inducible system to medaka. By using these techniques, we have recently identified germline stem cells and the surrounding unique structure in medaka adult ovaries [1].

Medaka ovary is composed of two major compartments; the ovarian cavity on a dorsal side of the ovary, to which the mature eggs are ovulated, and the stromal compartment on a ventral side, in which the folliculogenesis and oocyte maturation proceed. (Figure 2A).

The multilayer epithelium, called 'germinal epithelium', with the basement membrane on the ventral side, lies between the two compartments. The mature eggs are ovulated from the stromal compartment through the germinal epithelium into the ovarian cavity (Figure 2A).

We established the transgenic medaka in which, medaka homolog of mammalian *sox9*, *sox9b*-expressing cells are visualized [11]. It is known that mammalian *sox9* is essential for testis development and is not expressed in the developing ovary [12-15]. However, we have noticed that *sox9b* is ex-

pressed in the medaka ovary and found that oogonia at early stages of oogenesis are all surrounded by *sox9b*-expressing cells to form 'units'. We named the unit 'germinal cradle'. The morphological analysis revealed that the germinal cradles are connected each other by cellular processes of *sox9b*-expressing cells to form the network. We found the network buried within the germinal epithelium, between a single layer of epithelial cells and the basement membrane (Figure 2 A and B). We named the whole network 'ovarian cord'.

In the germinal cradles, germ cells can be categorized to three types by their morphology and the way germ cells are surrounded by *sox9b*-expressing cells; (1) single isolated germ cells which are completely surrounded by *sox9b*-expressing cells one by one (Gs), (2) cyst-forming germ cells which are connected by the intercellular bridge and surrounded by *sox9b*-expressing cells as a cluster (Gcys) and (3) large oocytes in the meiotic diplotene stage which are surrounded by *sox9b*-expressing cells one by one (Gdip) (Figure 2C). Although there are several types of germinal cradles by combination of the three types of germ cells, any of germinal cradles has one to five numbers of single isolated germ cells. We also found that some cyst-forming germ cells are composed of early stages of meiotic germ cells before diplotene stage.

Identification of ovarian germline stem cells

Previously, we found *nanos2* is expressed in both spermatogonial and oogonial cells [16]. Therefore we have checked the *nanos2* expression in the germinal cradle and have found that *nanos2* is exclusively expressed in the single isolated germ cells (Gs type of germ cells). We hypothesized that germline stem cells are present in the *nanos2*-expressing cells and conducted the clonal analysis using the heat-inducible cre/loxP system [1]. We have generated transgenic medaka with *hsp-cre:mCherry* and *nos2p-loxP[DsRed]-EGFP-olvas3'UTR* transgenes. With the design of these transgenes, the floxed region with DsRed is excised after heat treatment, which would lead to the transient transcription of EGFP only in a *nanos2*-expressing Gs type of germ cells. But, since the action of *olvas* 3'UTR fused to EGFP gene directs the translation of EGFP and confers EGFP transcripts with the increased stability in all types of germ cells [10, 17], the progeny of *nanos2*-expressing cells can be kept tracked by the EGFP fluorescence.

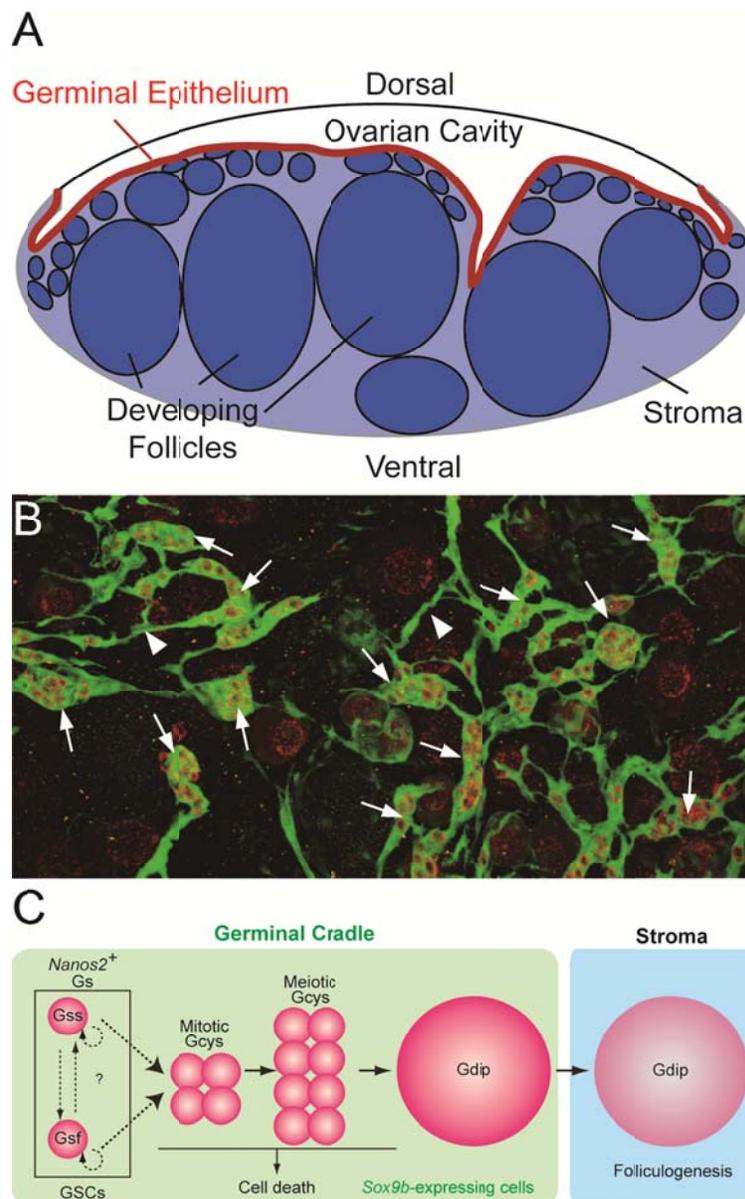


Figure 2. Ovarian cord in medaka ovary. (A) The transverse section of medaka ovary. Medaka ovary consists mainly of two compartments; the ovarian cavity to which mature oocytes are ovulated and the stroma in which folliculogenesis and oocyte maturation proceeds. Between these two compartments, there is the germinal epithelium (red) lined by the basement membrane. (B) A dorsal view of the ovarian cord in ovary of *sox9b*-EGFP transgenic medaka. *Sox9b*-expressing cells are visualized with EGFP expression immunostained with anti-GFP (green) antibody. Germ cells are stained by anti-OLVAS antibody (red). 'Germinal cradles' (arrows) composed of germ cells (red) and *sox9b*-expressing cells are connected each other by the cellular processes of *sox9b*-expressing cells (arrowheads) to form the network, 'ovarian cords'. (C) The model for early oogenesis in medaka. In the germinal cradle, three types of germ cells are present; *nanos2*-positive single isolated germ cells (Gs), cyst-forming germ cells (Gcys) and diplotene oocytes (Gdip). Fast-dividing (Gsf) and slow-dividing (Gss) germ cells exist among Gs and both or either of them function as germline stem cells (GSCs). Gs undergoes three to five rounds of synchronous mitosis and enters meiotic division (mitotic and meiotic Gcys), while some germ cells are eliminated by cell death. The remaining germ cells enter the diplotene stage of meiosis (Gdip). Oocytes in the diplotene stage undergo the folliculogenesis as they exit from the germinal cradle to the stromal compartment.

Actually EGFP was only observed in *nanos2*-expressing single isolated germ cells just after heat induction. But all the germ cells in the germinal cradles became EGFP-positive with time going. These results indicate that *nanos2*-expressing cells supply all types of germ cells in the germinal cradles.

Next we have checked if the excised DsRed transgene was transmitted to the next generation. Embryos having the excised region were obtained from the transgenic adult medaka even three months after heat-treatment, indicating that *nanos2*-expressing cells keep supplying the functional eggs. In addition, BrdU incorporation analysis has revealed two populations of the *nanos2*-expressing cells in terms of cell cycles; fast-dividing (Gsf) and slow-dividing (Gss) germ cells. These results indicate that some populations of *nanos2*-expressing germ cells contain the germline stem cells. This also demonstrates that the germline stem cell niche exists in the germinal cradle composed of *sox9b*-expressing cells.

In summary of oogenesis from germline stem cells in germinal cradles, we have shown the model in Figure 3C. Fast-dividing (Gsf) and slow-dividing (Gss) germ cells are present among *nanos2*-expressing single isolated germ cells and both or either of them

function as germline stem cells. The single isolated germ cells undergo the synchronous mitotic and meiotic division to form cyst-forming germ cells. During the cystic division, some germ cells are eliminated by cell death. Similar elimination at around birth is also reported in mammalian ovary [2]. The remaining germ cells enter the diplotene stage of meiosis as ensheathment proceeds by the *sox9b*-expressing cells one by one in the germinal cradles. Interestingly, the diplotene oocytes are very motile along or within ovarian cords and shuttle back and forth between the germinal cradles. The diplotene oocytes undergo folliculogenesis as they exit to the stromal compartment where enormous oocyte growth and a subsequent maturation event take place. The supporting cells surrounding diplotene oocytes no longer express *sox9b* but *foxl2* that is often used as a marker of female supporting cells [11].

The oogenetic process in the germinal cradle resembles that in the *Drosophila* germarium. Germarium contains germline stem cells, cyst-forming germ cells and meiotic germ cells at diplotene stage. The germ cells, as meiotic events proceed, undergo folliculogenesis with somatic supporting cells and exit from germarium (Figure 3).

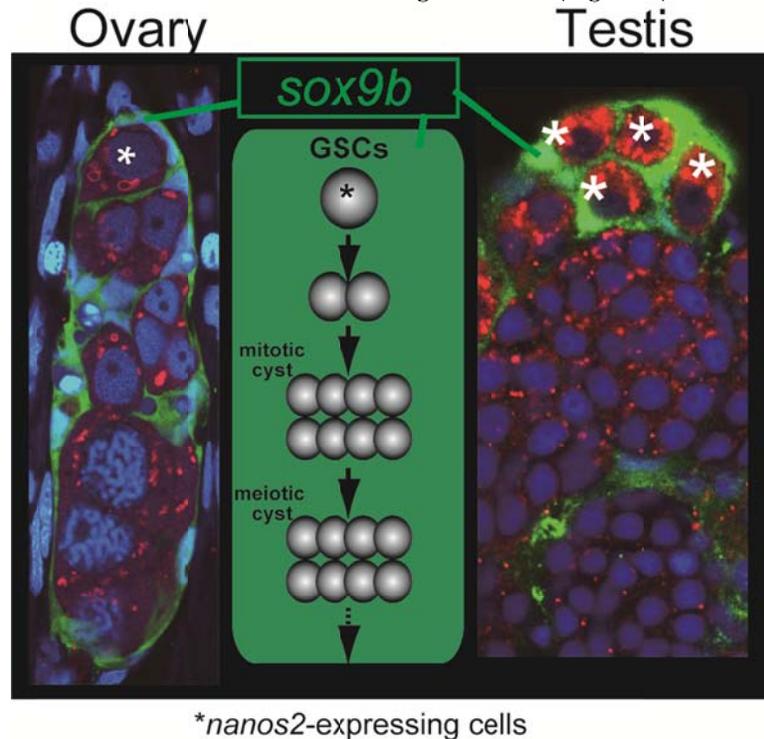


Figure 3. Common structure in ovaries and testes. Does *sox9b* have function common in regulating both male and female germline stem cells? *Nanos2*-expressing germ cells (asterisks) are surrounded by *sox9b*-expressing cells one by one in both ovaries and testes. A germinal cradle (left) and a lobule structure (right) are shown. The lobule is a typical unit in the testis. The middle depicts the process and structure common between the germinal cradle and the lobule, which reminds us of the functional unit of oogenesis, called 'germarium' in *Drosophila*.

Common structure in ovary and testis and the function of *sox9*

The structure of germinal cradles and the oogenic events occurring in germinal cradles recall spermatogenesis in medaka. Spermatogonia express *nanos2* and spermatogenesis proceeds in certain units called lobules that are surrounded by *sox9b*-expressing cells [16] (Figure 3). This is also reminiscent of mouse spermatogenesis. In mouse testes, *nanos2*-expressing cells contain the germline stem cells. Spermatogenesis occurs in the tubular structure expressing *sox9* [18]. Our previous analysis revealed that *sox9b*-expressing cells are established before gonadal primordium is present in embryos [19]. These cells form gonadal primordium with another somatic cell expressing *ftz-f1* and primordial germ cells. Then, *sox9b*-expressing cells in the primordium differentiate into either male or female supporting cells (which are subsequently called Sertoli cells in the testis and granulosa cells in the ovary, respectively) [11]. Considering that the sex of medaka can be artificially reversed after sex determination [20] and that female and male supporting cells share a common origin expressing *sox9b*, it might be interesting to assume that germline stem cells and surrounding *sox9b*-expressing cells in germinal cradles be sexually indifferent or interchangeable, or maintain sexual bipotentiality.

Another interesting question raised from our results is the function of *sox9* gene in the gonads. Medaka has two orthologues of mammalian *sox9* gene, *sox9a* and *sox9b*, but medaka gonads express only *sox9b*. Therefore *sox9b* can be regarded as a functional orthologue of mammalian *sox9* in medaka gonads [21]. Mammalian *sox9* is known to have an essential role for Sertoli cell differentiation while *sox9b* is expressed in both male and female supporting cells in medaka. In lower vertebrates like medaka, it is intriguing to know that *sox9b* has some contribution to male determination.

Recent research in stem cell biology points out another interesting function of a family of the SOX transcription factors. *Sox9* in other tissues and other members of the *sox* family have been implicated in maintaining cells in a stem cell-like state and inhibiting cell differentiation. For examples, in the central nervous system, *Sox1*, *Sox2*, and *Sox3* mark neural stem/progenitor cells and prevent their exit from the cell cycle and the induction of neurogenesis [22, 23]. *Sox9* plays a similar role in stem/progenitor cells of the hair bulge, intestinal epithelium, pancreas and neural crest [24-27]. In addition, it has been reported

that conditional *sox9* knockout testes failed to maintain germ cell [28]. Functional analysis of *sox9b* in medaka ovaries may lead to further understanding of some function on germ cells and reproduction.

Conclusions

We reviewed medaka ovarian germline stem cells and the surrounding unique structure called germinal cradle. Germinal cradles in medaka provide a good model to analyze the oogenesis and the regulation of germline stem cells in vertebrate ovaries. Further studies are required to identify the cells that function as niche in germinal cradles and to reveal the molecular mechanisms regulating germline stem cells.

The germinal cradles also give a deep insight on a variety of sex differentiation process in the gonads of many vertebrates. It is important to examine if similar structures and cells are present in other animals, especially for understanding the relation of a variety of reproductive strategies that many animal show to the cellular and molecular mechanisms that underlie the strategies. Actually, *sox9* expression in both ovaries and testes is reported in rice eels and frogs [29, 30], which may indicate that the structure in and the processes of oogenesis shown in medaka are universal among vertebrates.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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