

Mechanism of mammalian germ cell development: toward assisted new reproductive technologies

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Received: 8 October 2011 / Accepted: 22 November 2011 / Published online: 23 December 2011
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Abstract Approximately 10% of Japanese couples who want to have children experience infertility, and the majority of these individuals have either abnormal egg cell or abnormal sperm development. In addition, currently, there is a low birth rate in many parts of the developed world; therefore, it is critical to elucidate the mechanisms of germ cell development. Thus, the study of assisted reproductive technologies has scientific significance, and is a key to solving infertility problems. This review summarizes the development of mouse germ cells, particularly focusing on the role of Nanos genes, which are essential for the development of mouse germ cells and have been identified as genes that are involved in human infertility. We also briefly introduce a study in which this information is applied to assisted reproductive technologies.

Keywords ART · Germ cells · Infertility · iPS · Mouse

Introduction

All the cells in the body are generated from one fertilized egg. There are approximately 60 trillion of these cells, comprising hundreds of different types. Cells are roughly classified into either somatic or germ lines. The cells from the somatic line make up the organs and tissues of the body, while the germ line, including egg cells and sperm, is involved in the transfer of genetic information down the generations. In order to acquire and maintain their characteristics, the germ cells are controlled by genes that are specific to them, such as those involved in the processes of germ cell specification, sexual differentiation (oocyte or sperm), gametogenesis (meiosis), and reacquisition of pluripotency (Fig. 1).

In animals, the mechanism underlying the development of germ cells is divided into two modes. The first is preformation, in which cells in organisms such as nematodes, flies, and frogs take up cytoplasmic components called germplasm into the fertilized ovum at the time of cell division, and subsequently develop into germ cells [1]. The other mode is epigenesis, in which cells are isolated from the somatic line by signals from the surrounding tissues and turn into germ cells [2]. This occurs in mammals such as humans and mice [3]. Mouse germ cells are generated in an extraembryonic tissue during early development and migrate to the future gonads during cell proliferation. These germ cells are called primordial germ cells, and they have potency to differentiate into either sperm or egg cells. However, when they reach the gonads, sex determination of germ cells occurs in accordance with the sex of the somatic cells surrounding them; germ cells that colonize the female gonads enter meiosis immediately, whereas germ cells in the male gonads undergo cell cycle arrest and never enter meiosis at the embryonic stage [4]. This review

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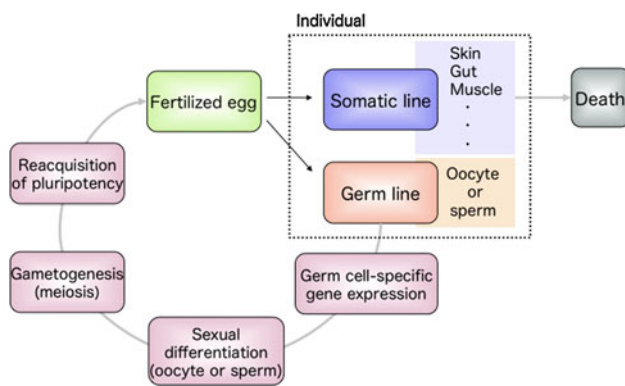


Fig. 1 Life cycle of germ cells. Germ cells are special cells that transmit genetic information to the next generation. As such, their developmental process is strictly regulated to acquire and maintain specific potency. This process is roughly divided into four steps: germ cell-specific gene expression, sexual differentiation, gametogenesis, and reacquisition of pluripotency

explains the mechanisms of mammalian embryonic germ cell development by using mouse germ cells as an example, and briefly introduces a study that applies information obtained by analysis of the mechanisms of germ cell development to assisted reproductive technologies.

Specification of primordial germ cells

The inhibition of somatic gene expression plays a key role in the development of mouse primordial germ cells because they are isolated from the somatic line [5]. In mouse germ cells, some epiblast cells receive bone morphogenic protein (BMP) signals from extraembryonic ectoderm and visceral endoderm approximately 6 days after fertilization [3, 6, 7], which leads to the production of extraembryonic mesoderm. Subsequently, a few of these cells that are located in the prospective posterior proximal site of the embryo express *Blimp1/Prdm1*, and they are then completely isolated from the somatic line, a process called specification to primordial germ cells (PGCs). *Blimp1* is a transcription factor responsible for repressing the somatic program in PGCs while allowing the establishment of germ cell characteristics in these cells. In *Blimp1*-expressing cells, mesodermal genes including *T*, *Fgf8*, and *Snail* become repressed, whereas pluripotency-associated genes such as *Sox2* and *Nanos* are up-regulated [8].

On the other hand, another transcription factor, *Prdm14*, is also specifically expressed in PGCs at almost the same time as *Blimp1*, and is involved in reacquisition of pluripotency [9]. In *Prdm14*-null mouse, although somatic gene expression in the PGCs remains suppressed owing to the expression of *Blimp1*, one of the essential genes for reacquisition of pluripotency, *Sox2*, is severely down-regulated.

In addition, the histone H3K9me2 signal of these cells is prevented from decreasing by the up-regulation of the histone methyltransferase GLP, which suggests that the epigenetic state of the chromatin structure cannot be normally reprogrammed. Therefore, both *Blimp1* and *Prdm14* are responsible for the development of primordial germ cells.

Migration to the future gonads

After specification, the germ cells become transcriptionally silent at 8.5 days post-coitum (8.5 dpc) and are subjected to extensive reprogramming of their genomes, which involves histone modifications such as the erasure of H3K9me2 and up-regulation of H3K27me3, as well as decreased DNA methylation [10]. Although the germ cells are known to migrate actively toward the future gonads after 8.5 dpc, little is known about the molecular mechanisms that underlie this migration or that protect the identity of germ cells during their migration among somatic cells. Gene knockout studies, however, have revealed that some factors are essential for the survival of germ cells during migration, and these include *Nanos3*.

Nanos homologues in human and mouse

Although the Nanos protein was first identified in fruit flies as a gene responsible for posterior pattern formation [11, 12], subsequent studies have shown that Nanos is a conserved protein implicated in germ cell development in many species [13–17]. The protein contains two CCHC-type zinc-finger motifs, which play an essential role in the development of germ cells. There are three *Nanos* homologues in mouse and human, among which mouse *Nanos2* and *Nanos3* have been shown to be essential for germ cell development by knockout analyses [17]. In addition, human *Nanos3* has also been identified as a gene responsible for human infertility [18, 19], which suggests that this gene has common functions among mammals.

Function of mouse *Nanos3*

In mouse, the initial expression of *Nanos3* is detected at around E7.5 in the putative PGCs (Fig. 2) [20]. The expression is subsequently maintained during the PGC migration stage. In the *Nanos3*-knockout mouse, few germ cells are detectable in the E12.5 genital ridge [17], indicating that the specification and derivation of PGCs is unaltered, but that these cells are not subsequently maintained during migration. In *Nanos3*-null cells, an increased

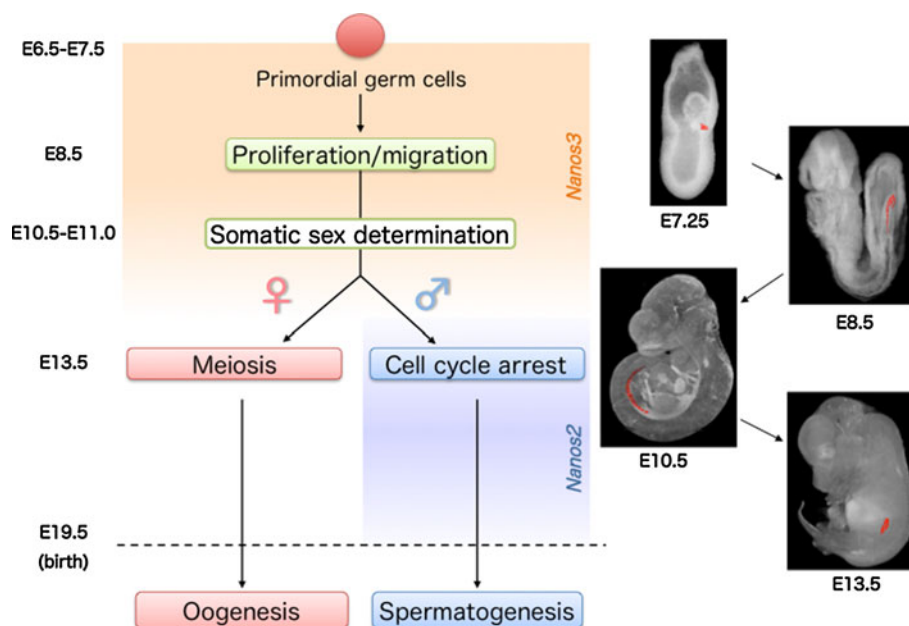


Fig. 2 An overview of mouse germ cell development and the expression profiles of *Nanos2* and *Nanos3* In mouse, primordial germ cells (PGCs) are segregated from somatic cells at around 6.5–7.25 dpc and then begin to migrate toward the future gonad. After colonization of the gonadal somatic environment, they undergo sexual differentiation in accordance with the sex of the somatic cells;

germ cells colonizing female gonad enter meiosis immediately whereas germ cells in male gonad undergo cell cycle arrest at G1/G0 and never enter meiosis at the embryonic stage. Subsequently, *Nanos3* expression is first detected at 7.5 dpc and continues in migrating PGCs, whereas *Nanos2* is expressed only in germ cells colonizing male gonads

number of apoptotic cells can be detected without any change in the proliferation rate. In addition, lineage-tracing experiments using the *Nanos3*-cre mouse revealed that no abnormal differentiation into somatic cells occurred, even when apoptotic cell death was suppressed by the elimination of the *Bax* gene in the *Nanos3*-null background. In the absence of *Bax*, however, only a portion of the *Nanos3*-null PGCs escape from apoptosis and enter the normal differentiation pathway; most still undergo apoptotic cell death. This result indicates that *Nanos3* is required to prevent PGCs from undergoing apoptosis via both *Bax*-dependent and *Bax*-independent mechanisms during migration [20].

Sexual differentiation of germ cells in the mouse gonads

Germ cells are sexually bipotential at the migrating stage; their sex-specific differentiation begins after the colonization of the gonads. Female germ cells enter meiosis whereas male germ cells undergo cell cycle arrest at G1/G0 and never enter meiosis during the embryonic stage of development. There is a long-standing controversy with regard to whether the induction of meiosis in female or the inhibition of meiosis in male germ cells is the principal mechanism underlying their sex determination. Notably, a number of reports over the past 5 years have indicated that

both pathways are valid. Retinoic acid (RA) signaling is implicated as the initial trigger for feminization [21, 22]. RA molecules derived from the mesonephros induce meiotic initiation of germ cells in female embryonic gonads via induction of the RA-responsive gene *Stra8*, which is required for premeiotic replication [23]. On the other hand, at least two somatic factors are required for masculinization of germ cells in male embryonic gonads. *CYP26B1*, an RA-metabolizing enzyme, is expressed in the Sertoli cells and protects germ cells from exposure to RA, resulting in the suppression of meiosis [21, 22]. In addition, somatically derived fibroblast growth factor 9 (FGF9) promotes the expression of male-type genes via its receptors on the surfaces of germ cells [24].

Nanos2 and sexual differentiation of germ cells

Nanos2 is one of the male-type genes directly induced by FGF9, so its mRNA is detected only in the germ cells that have colonized the male embryonic gonads at around E13.0. This expression is maintained during embryogenesis. Adult male *Nanos2*-knockout mice show complete loss of male germ cells as they undergo apoptotic cell death from as early as E15.5 [17]. However, it was recently reported that *Nanos2* is involved in the sexual

differentiation of germ cells in addition to its role in suppressing apoptosis [20].

In the RA signaling pathway, an RA-responsive gene, *Stra8*, has been shown to be required for premeiotic DNA replication. Gene knockout studies clearly show that, in female embryos lacking *Stra8* gene function, the germ cells fail to enter meiosis [23]. Moreover, in the absence of CYP26B1, the up-regulation of *Stra8* is observed in male germ cells, which induces entry into meiosis. A similar phenotype has also been described in *Nanos2*-null male germ cells in which *Stra8* expression was found to be up-regulated and meiosis-initiated (Fig. 3a, b). However, the timing of the relevant events is different in these two knockout mice. In *Cyp26b1*-null testes, *Stra8* induction occurs at E13.5, which is similar to the stage at which it is induced in embryonic ovaries [22], but this does not occur until E14.5 in *Nanos2*-null testes. The difference is due to the period of *Cyp26b1* expression, which is higher at E12.5–E13.5 but is gradually reduced after E13.5, whereas high levels of RA are maintained in the male embryonic mesonephros [21]. *Nanos2* begins to be expressed at the point at which *Cyp26b1* is down-regulated. Once *Nanos2* is induced after E13.5, a male germ-cell-specific program is initiated and the germ cells then acquire a masculine fate even though RA is present.

This idea is further supported by the results of ectopic expression of *Nanos2* in the female germ cells, which suppresses *Stra8* expression (Fig. 3c) and eventually leads to the up-regulation of male-specific genes such as DNMT3L (Fig. 3d–f). Hence, *Nanos2* appears to have a male-promoting function rather than a permissive one.

Toward new assisted reproductive technologies

Study of the production of a gamete from a cultured cell has been performed since embryonic stem (ES) cells and embryonic germ (EG) cells were established. Interest in the application of the results of these studies to clinical settings has grown since an article on the production of oocyte-like cells from mouse ES cells was published in 2003 [25]. Subsequently, other researchers reported that sperm could also be produced in the same manner [26], and functionality of these sperm was confirmed [27]. However, efficiency of the methods used in these papers is very low, so that many scientists have made efforts to establish an improved method. Recently, a group at Kyoto University has announced that they have successfully produced sperm cells from both ES cells and induced pluripotent stem cells (iPS cells) [28]. The group identified *Blimp1* and *Prdm14* as genes that play important roles in the development of primordial germ cells and, in 2009, they found that primordial germ cells that were implanted into a testis after birth normally differentiated into sperm [29]. In this study, they first produced both ES cells and iPS cells that contained transgenes for blue and green fluorescent protein in the gene region of *Blimp1* and *Prdm14*, respectively. These cells were then incubated with several kinds of secreted protein and selected with the fluorescent proteins as indicators for expression of *Blimp1* and *Prdm14*. In this process, they finally obtained cells expressing both blue and green fluorescent proteins. The transcriptomes of these cells are similar to those of primordial germ cells. When these cells were transplanted into mouse adult testes, normal meiosis

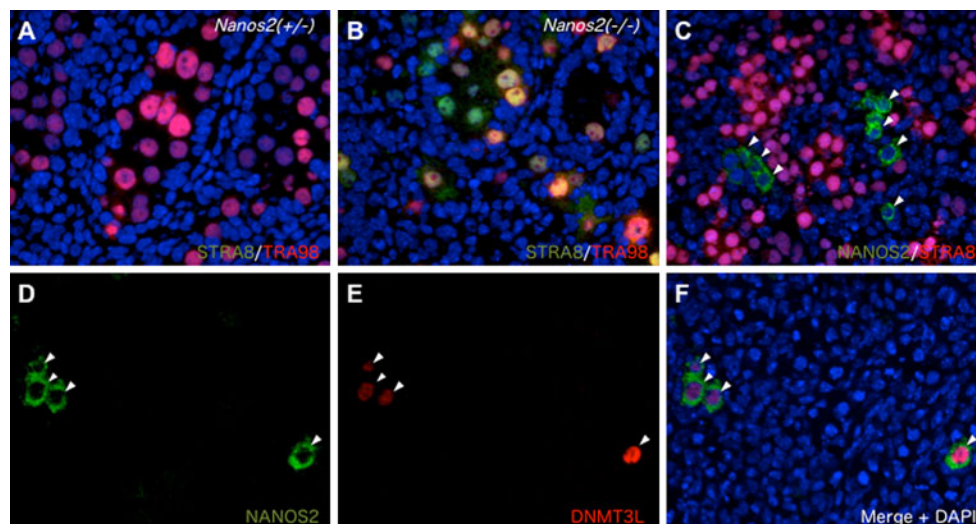


Fig. 3 *Nanos2* suppresses meiosis and promotes male-type differentiation. Sections from *Nanos2*(+/+) (a) and *Nanos2*(-/-) (b) male gonad at 15.5 dpc are immunostained with anti-STRA8 (green) and TRA98 (red) antibodies. c–f Sections from 3× FLAG *Nanos2*-

expressing female gonad at 16.5 dpc are immunostained with anti-FLAG (green) (c, d, f) and anti-STRA8 (red) (c) or anti-DNMT3L (red) (e, f) antibodies

progressed, which resulted in the differentiation into sperm. In addition, the male mice sired litters of normal pups. Thus, this study succeeded in producing functional sperm from artificial cells, such as ES cells and iPS cells. When ES cells and iPS cells themselves are implanted into a mouse testis, a teratoma is always produced. Therefore, the study results demonstrate the successful induction of differentiation into primordial germ cells.

Among the life-cycle of germ cells illustrated in Fig. 1, production of PGC-like cells from ES or iPS cells corresponds to the first step, “gene expression specific to germ line”. The final step of differentiation into sperm was achieved by transplantation into a testis of a host mouse. Although this method might theoretically be able to be applied to human clinical application, it is desirable that all steps be performed in vitro because a human host is unnecessary if functional sperm can be produced in a culture dish. In addition, the derivation of sperm from ES or iPS cells in vitro provides an invaluable assay for the basic information of germ cell development, and such information itself will contribute to improving the efficiency of in vitro production of sperm. Therefore, future studies should focus on the production of functional sperm in a culture dish. In addition, studies for production of egg cells in the same manner can be initiated. In order to perform these studies, it is essential to collect information on genes that play important roles in each of the steps indicated Fig. 1. Since *Nanos2* plays a key role in the sexual differentiation of germ cells, it may be possible to enhance the efficiency of sperm or egg cell formation in vitro by inhibiting or promoting the expression of this gene.

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