博士論文番号：0681021

# Approaches to understanding the mechanism of mammalian folliculogenesis
# ～Analysis of gap junctions in ovarian follicles, and the establishment of an *in vitro* system for obtaining follicles from mouse embryonic stem cells～
# 哺乳類卵胞形成機構の解析：卵胞発育におけるギャップ結合の解析とマウス胚性幹細胞からの卵胞誘導系の構築

新田　麻衣
奈良先端科学技術大学院大学
バイオサイエンス研究科　細胞増殖学講座
（竹家　達夫　教授）

平成 22 年 01 月 25 日提出

# *Introduction*

## Exordium

In mammals, cell to cell interactions are critical not only to proper functioning and homeostasis in a variety of somatic cells but also to the formation and maturation of germ cells. For example, cardiac muscle cells beat in a coordinated manner by communicating with each other via gap junctions, and the cadherin family has been shown to aid in cell sorting to distinguish the many cell types and in turn allow same types of cells to adhere with each other, which is vital for embryogenesis including the neural tube's formation. Germ cells form according to bone morphogenetic protein 4 (BMP4) signaling from adjacent cells and its migration to the genital ridge dependent on signaling molecules such as integrins, cadherins, c-kit and Kit ligand (KL). Furthermore, germ cell-specific events such as meiosis are also coordinated by cell to cell communication, as the disruption of gap junctional communication between oocyte and surrounding somatic cells induces the progression of meiosis at an inappropriate stage.

## Germ cell formation and specification

Germ cells are first formed as primordial germ cell (PGC) precursor cells in the proximal region of the epiblast from embryonic day 6.25 to 6.5 before gastrulation when they receive

BMP4/8b signals from adjacent extraembryonic ectoderm [1], [2], [3], [4], [5]. Recent studies have revealed that commitment to the germ cell fate in about 40 cells in the proximal epiblast occurs when they are subjected to signaling from BMP4 and BMP8b in adjacent somatic cells at around E7.25 [6], [7], [8], [9], [10],[11]. These signaling moleculest germ cell-specific gene expression in the PGC precursor cells such as *fragilis*, *Blimp1*(also known as *Prdm1*), and *PGC7*/*stella* (also known as *Dppa3*), and repress genes like the *Hox* family, and so commitment to the PGC is accomplished, while somatic cells start to express *Hoxb1* [12], [9], [13], [14], [15], [16]. *Prdm14* also has some role in the establishment of PGC fate since a deficiency of *Prdm14* results in the loss of germ cells and impaired epigenetic reprogramming [17]. *Blimp-1* expression is restricted to the posterial region of the proximal epiblast. Consequently, there might be some signals that repress *Blimp-1* expression in the anterior region of the proximal epiblast. *Wnt3* is expressed in the anterior region of the visceral epiblasts and embryos lacking *Wnt3* exhibit defects in axial realignment, and *Wnt3* expression in the epiblast is required for generation of the primitive streak and mesoderm [18]. Cell-cell interaction by E-cadherin within the PGC precursor cells contributes to the PGC specification because the cells that diminish the expression of E-cadherin migrate and differentiate into allantois and the cells that continued to express E-cadherin gain the PGC differentiating fate [19]. PGCs can be detected as a small cluster of approximately 40 cells positive for alkaline

phosphatase (ALP) staining at around E7.25 located in the extraembryonic mesoderm posterior to the primitive streak [2], [20]. PGCs are known to have the potential to become oocytes whether they have an XX chromosome or XY chromosome at this point, and so XY PGCs have been shown to follow the female fate when they migrate into ectopic tissue [21], [22].

**Germ cell migration, colonization, proliferation and survival**

PGCs migrate through the base of the allantois, the developing hindgut endoderm, eventually enter the genital ridge[23], [24], [25], [26], [27], [28], [29] which they colonize, and undergo compaction through either adhesion molecules such as beta1-Integrin and E-cadherin, or by other molecules like Stromal cell derived factor 1 (SDF1) and its receptor CXCR4. Their fate is determined by the expression of particular genes, *Sry*, *Sox9*, *Pod1*, *SF1*, *WT1*, *M33*, *Foxl2*, *fgfr2*, *Wnt4*, and *DAX-1,* defects in any of which result in sex reversal and impaired gonadogenesis [30], [31], [32], [33], [34], [35], [36], [37], [38]. *SDF1* and *CXCR4* knockout embryos had fewer PGCs migrating into the genital ridge, which most PGCs remaining in the hindgut endoderm or mesentry [39]. In humans, PGCs change from round to spindle-shaped structure during their migration allowing them to make the amoeboid-like movements to reach the genital ridge [40]. The migratory pathway to the future gonad is guided by signaling from

the neighboring somatic cells; somatic cells produce substrates such as fragilis and Kit ligand, and PGCs migrate by nteracting with these cells by expressing E-cadherin, c-kit receptor or fragilis3, at around E8.5 to E11.5 [41], [42], [43]. Integrins that interact with the extracellular matrix (ECM) also seem to participate in the guidance of PGCs because *Integrin beta 1*-deficient PGCs fail to colonize the genital ridge, which is also impaired when gap junctional communication between PGCs themselves or PGCs and somatic cells is disrupted [44], [45]. Glycoproteins such as fibronectin, a component of the extracellular matrix, may also create a migratory pathway[46]. During their migration and after enteringthe genital ridge, PGCs proliferate until around E12 to E13 in both male and female embryo [47]. Some growth factors have also been implicated in the proliferation and survival of PGCs [48]. White stem cell factor (SCF) is required not only for PGC survival as well as leukemia inhibitory factor (LIF), FGF and IL4, SCF by preventing PGC apoptosis, but also for the PGC migration, while PGC proliferation seems to be regulated by cAMP [49], [50], [51], [52], [53], [54], [55]. *Insulin like growth factor* (*IGF-I*) is also required for germ cell apoptosis and phosphatidylinositol-3'-kinase signaling seems to participate in cytokine (such as SCF, LIF, IGF-I)-mediated germ cell survival [56]. Nanos, which encodes an RNA-binding protein, form a family of 3 and show different expression profiles; nanos 2 expressed only in male germ cells lead to the complete loss of spermatogonia when disrupted, while nanos 3 expressed in

migrating PGCs shows a complete loss of germ cells in both sexes when ablated [57]. Reduced numbers of germ cells can be observed by *Zinc-finger protein Zfx* mutation in both sexes and this mutation also results in a small body size [58]. A *Peptidyl-prolyl isomerase*, *Pin1*, helps control the timing of proliferation since *Pin 1*-deficient PGCs show a prolonged cell cycle which results in tless cell division and fewer PGCs causing profound fertility defects [47]. Other genes involved in the proliferation of PGCs are *ZFX* and *ZFY*, on the mammalian X and Y chromosomes. Mutantations in this gene result in the decreased numbers of PGCs in both sexes prior to gonadal sex diferentiation.

**Formation of cysts, Meiotic arrest, and breakdown of cysts**

Mitosis stops after PGCs enter the female gonad at around E13.5 and starts meiosis after they differentiate into oogonia [59], [60] and suddenly become meiotically arrested at the diplotene stage of meiotic prophase 1 [61], [62], [63], [64], [65], [28] while they form the germline cyst [66], [67], [68], [69] where they are connected to each other tightly by intercellular bridges. Meiosis has recently been shown not to be synchronized and it seems to take about 7 days for all oogonia to enter meiosis and be arrested at prophase 1. Shortly after birth, germ cells undergo apoptosis accompanied by the breakdown of cyst [69], [70], [71]. Some have proposed germ cell death as a process of selection to eliminate contain chromosomal

abnormalities or defects in mitochondrial-genomes [72]. Caspase-2 participates in programmed cell death because disruption of *caspase-2* resulted in the prevention of programmed germ cell death caused by the disruption of *caspase-11* [73]. It has been believed that although the male gonad retains germline stem cells, the female gonad stops producing germ cells after birth [74]. Moreover, a recent study showed that germline stem cells exist in the postnatal ovary [75].

**Formation of primordial follicles**

With the germ cell loss, only approximately 20% of gonocytes survive in rodents and 10% in humans. The gonocytes start to be surrounded by a layer of squamous somatic cells of the gonad, the future ovary, and are now termed oocytes. The newly formed structure is called a primordial follicle [76]. However, not all germ cells undergo the maturation process and their numbers greatly decrease when meiosis is initiated, by approximately 80% in rodents, 90% in humans and 95% in cows. Some genes such as *member of B-cell lymphoma leukemia 2* (*Bcl2*) and *Caspase* have been shown to be involved in this apoptosis[77], [73]. Factor in the germ line alpha (figl $\alpha$) is crucial to the formation of the primordial follicle because *figl* $\alpha$ KO females are infertile despite the presence of a normal number of meiotically competent germ cells in the embryonic ovary [78]. Dazla contributes to the formation of primordial follicles

because disruption of this gene reduces the number of germ cells embryonically and causes a complete absence of follicles in the adult ovary [79].

**From primordial follicles to primary follicles**

These primordial follicles turn into primary follicles when the surrounding somatic cells differentiate into cuboidal granulosa cells after a few days to more than a year. During this process, the diameter of the oocyte hardly changes although this is a maturation step [80]. Many factors are involved in the transition to primary follicles. In humans, retinoblastoma protein and myc oncogene expression in the oocyte nucleolus has been associated with the production of a cell proliferation inhibitor that may prevent granulosa cell proliferation [81], [82]. Member of the transforming growth factor beta (TGF-beta) superfamily like activins [83], growth differentiation factor 9 (GDF-9) and BMP-15 [84], [85] the Smad signaling pathway [86] and nerve growth factor (NGF) have also been implicated in the regulation of primordial follicles, and KL [87], fibroblast growth factor-2 (FGF-2) and LIF influence the transition to primary follicles. Tyrosine kinase (Trk) receptor works as a receptor for NGF and *NGF* knockout mice have a defect in cyst breakdown that results in a reduction in the number of follicles in the ovary [88] , a phenotype which resembles the *TrkB* knockout that might be caused by the reduced survival rate of oocytes [89]. The transition to primary follicles

proceeds independently of the direct action of a gonadotropic hormone like follicle stimulating hormone (FSH) and cell autonomously. Epithelial growth factor (EGF) has been demonstrated to support the formation of primary follicles in experiments *in vitro* with cows, goats, and neonatal mice [90]. *Mouse homolog of DMC1*, a yeast meiosis-specific homologous recombination gene, also seems to have a role in the formation of primordial follicles since *Dmc1*-knockout mice are infertile because of a defect in chromosome synapsis and devoid of follicles in the ovary due to the elimination of oocytes before they form the follicles [91], [92]. Ataxia telangiectasia mutated (ATM) and its human homolog, which function in DNA repair, also have a role in the generation of primordial follicles since *ATM*-deficient mice show increased apoptosis in oocytes and are devoid of follicles with abnormal chromosome synapsis and fragmentation [93], [94], [95].

Primary follicles develop into secondary and preantral follicles

The primary follicles continue maturing via a process called folliculogenesis, which includes the growth of the oocyte itself and the proliferation of the surrounding granulosa cells, resulting in the formation of two or more layers of granulosa cells and eventually the formation of the antrum. This step is consisted of two steps: the formation of preantral follicles and the formation of antral follicles. The surrounding granulosa cells become more proliferative and the theca layer develops atround the granulosa cells through the basal lamina

from interstitial stroma cells. The follicles reach a diameter of approximately 200 $\mu$m in rodents and humans.

Preantral follicles form through cell autonomous growth, which is coordinated by many factors such as protooncogene c-kit, c-myc, cytokines and growth factors like Macrophage colony-stimulating factor (M-CSF), IGF, BMP-15, activins, TGF-beta, GDF-9, KL, FGF-2, NGF and LIF. MCSF is a glycoprotein that belongs to a family of hematopoietic growth factors known to regulate the production of blood cells, from bone marrow progenitors to mature circulating elements. M-CSF is involved in folliculogenesis and promotes ovulation by influencing ovarian macrophages because the ovarian intrabursal administration of an anti-M-CSF antibody significantly reduced the number of ovulated oocytes in treated rat ovaries and M-CSF increased the number of ovarian macrophages in growing follicles [96]. At the primordial follicle stage, KL produced by the granulosa cells stimulates oocyte growth via the c-kit receptor tyrosine kinase present on the oocyte membrane [97], [98]. The interaction of the granulosa cell-derived kit ligand with the oocyte and theca cell-derived c-Kit is important for multiple aspects of development, including the establishment of primordial germ cells within the ovary, the activation of primordial follicles, oocyte survival and growth, granulosa cell proliferation, theca cell recruitment and the maintenance of meiotic arrest [99], [100]. Activins belong to the TGF-beta family and are formed by the homo- or

heterodimerization of activin beta subunits. Activins have various roles in cell proliferation, differentiation, apoptosis, metabolism, homeostasis, immune response, wound repair, and endocrine function, although they function differently from inhibins. GDF9 is produced by oocytes. *Gdf9*-knockout female mice showed a block in follicular development beyond the primary stage so GDF9 must be essential for the follicular growth to the preantral follicle [101]. In *Gdf9*-knockout mice, oocytes become abnormally large, while their surrounding granulosa cells don't proliferate but instead show increased KL and inhibin-alpha expression. These transgenic mice are infertile since development beyond the primary follicle stage is blocked. NGF is required for the survival and differentiation of neurons within both the central and peripheral nervous systems. Mice lacking *NGF* have markedly reduced populations of primary and secondary follicles in the presence of normal serum gonadotropin levels, and an increased number of oocytes that failed to be incorporated into a follicular structure, because of the reduction in somatic cell proliferation. So NGF might be required for the growth of primordial ovarian follicles, a process known to occur independently of pituitary gonadotropins [88] EGF also contributes to the formation of primary follicles [90].

During this step, oocyte the increases in volume as the amounts of RNA and protein, and the numbers of ribosomes, mitochondria and other cell organelles increase. The oocyte at this stage, in which meiotic arrest is maintained, also starts to secrete a glycoprotein membrane,

the zona pellucida (ZP), which forms a protective coat around the oocyte consisting of three glycoproteins, ZP1, ZP2 and ZP3, the mRNA expression of which requires the transcription factor Figl $\alpha$ ; all of these genes participate infolliculogenesis since the knockout of *Zp1* and *Zp2* results in the formation of loose and thin zona pellucidae and *Zp3*-knockout mice do not develop zona pellucidae and are sterile [102], [103]. The abnormal zona matrix doesn't affect the initial folliculogenesis, but there is a significant decrease in the number of antral follicles. Few eggs are detected in the oviduct after stimulation with gonadotropins, and no two-cell embryos are recovered after mating. Although zona-free oocytes matured and fertilized *in vitro* can progress to the blastocyst stage, the developmental potential of blastocysts derived from either *Zp2*- or *Zp3*-null eggs appears compromised and, after transfer to foster mothers, live births have not been observed. Foxl2, which participates in the formation of gonads, has also been shown to have a role in folliculogenesis at the primary stage since *Foxl2* knockout ovaries contain no primary follicles because of impaired granulosa cell differentiation. Actually, a *Foxl2* deficiency in the early embryo still results in a proper meiotic prophase, but the genetic program for determining the somatic testis is activated in the mouse XX gonads. In the *Foxl2* knockout ovary, *Sox9*, which is necessary for the differentiation into the testis and has the ability to induce ovary-to testis sex reversal, is expressed. So it is likely that Foxl2 represses the male gene pathway at several stages of female gonadal differentiation. A lack of

the *Newborn ovarian oocyte-specific homeobox*, *Nobox*, gene product, an oocyte-specific factor, accelerates the postnatal loss of oocytes and abolishes the transition from primordial to growing follicles in mice and in turn results in the downregulation of oocyte-specific genes like *Oct4* and *Gdf9*, whereas ubiquitous genes such as *Bmp4*, *Kit*, and *Bax* remain unaffected. [104]. Gap junctional communication becomes essential for maturation of the follicles. It allows signal transduction between the oocyte and granulosa cells, which coordinate the survival and growth of both cells and meiotic arrest in the oocyte. Gap junctional communication in ovarian follicular development is described below. Disruption of *connexin 43* (*Cx43*) results in neonatal lethality because of a severe heart abnormality. When *Cx43* knockout ovaries were transplanted under kidney capsules, folliculogenesis was arrested before the follicles became multilaminar [105]. Connexin37 (Cx37) appears to be the only connexin contributed by oocytes to the gap junctions, coupling them with granulosa cells, and loss of this connexin interferes with the development of antral follicles [106].

Formation of antral follicles

Follilcles begin to form a fluid-filled cavity and then the antrum with increased follicular vasculation and permeability of the blood vessels. The antrum fluid is an important source of regulatory substances derived from blood or secretions of the follicular cells like gonadotropins, steroids, growth factors, enzymes, proteoglycans and lipoproteins. FSH,

Luteneizing hormone (LH), activin and KL seem to influence the formation of the antrum. Oocytes reach a diameter of approximately 120 $\mu$m in early antral follicles of cows and humans, while in rodents the maximum diameter of 70 $\mu$m has already been reached at the end of the antral follicle stage and oocytes then acquire competence to resume meiosis. In cows, goats and pigs, full meiotic competence is acquired in follicles of about 3 mm. The competence of an oocyte to resume and complete meiosis and, after fertilization, to develop into a blastocyst is markedly increased during follicular dominance and particularly after luteolysis through a process called oocyte capacitation, whereby the nuclear envelope becomes undulated and the nucleolar remnant displays a vacuole of increasing size. Theca cells increasingly express enzymes involved in steroid biosynthesis, while granulosa cells lack aromatase expression. Antral follicles reaching a size of approximately 0.2 mm in mice become dependent on gonadotropin stimulation and are recruited for further growth after a transient increase in FSH [107]. The number of recruited Antral follicles varies between species, for example, from 5 to 10 in cows, from 1 to 4 in horses and over 50 in pigs. Antral follicle size at recruitment corresponds with the size at which the P450 side chain-cleavage enzyme and P450 aromatase first become detectable in the granulosa cells [108]. The presence of aromatase allows the recruited antral follicles to produce the potent anti-apoptotic or pro-survival hormone estradiol from thecal androgen precursors, androgen synthesis being

stimulated by LH. Estrogen production is stimulated by FSH. The selection of antral follicles takes place among falling FSH levels with high pulsatile secretion of LH. These latter follicles secrete large amounts of estradiol and inhibin and have the ability to ovulate. The remaining follicles of the cohort become subordinate and enter atresia. The activin-binding protein follistatin is produced by recruited antral follicles in increasing amounts during their growth [109]. In rodents, induction of LHRs in granulosa cells is dependent on FSH and high intrafollicular estradiol concentrations. Antral follicles show increased *Fshr* expression and appear to develop LHRs in granulosa cells, and thus are more sensitive to FSH and become responsive to LH. Under the influence of LH, they then rapidly increase in size and become larger than the other follicles. Then the oocyte is surrounded by closely associated granulosa cells, which are known as cumulus cells, forming a compact cumulus cell–oocyte complex. The oocytes express Cx37 which is essential for optimal communication between oocytes and granulosa cells and so they promote the proliferation and differentiation of granulosa cells through the secretion of paracrine factors. Communication between oocyte and surrounding follicle cells is crucial because removal of the oocyte results in premature luteinization and elevated progesterone production by the granulosa cells, or the resumption of the meiosis in early primary or preantral follicles. Shortly after the LH surge, junctions such as gap junctions between oocyte and cumulus cells are disrupted and after the cumulus cell expansion occurs,

the oocyte ovulates. An oocyte that has been arrested at MII resumes the meiotic cycle when an activation stimulus is provided by sperm penetration. Despite species differences, the basic molecular mechanisms governing the process of oocyte maturation in response to the LH surge share several regulatory pathways such as the alteration of protein phosphorylation, and cyclic adenosine monophosphate (cAMP) and calcium levels [110], [111], [112], [113], [114].

**Germ cell-specific events**

During the formation and maturation of germ cells, many germ cell-specific events such as meiosis, a process to divide chromosomes equally among the next generation with chromosomal arrangement by chromosomal recombination [115], [116], [117], [59], genomic imprinting [118], [119], [120], [121] and the reprograming of genetic imformation occur [122], [120], [123], [124], [125].

Meiosis

An oocyte which has entered the gental ridge starts meiosis and is restricted at prophase 1. During folliculogenesis, this arrest is maintaind until FSH stimulation occurs which induces the formation of antrals within the follicles. FSH allows the oocyte to go through meiosis and meiotic arrest again occurs at meiotic prophase 2. A fertilization signal allows the ovulated oocyte to complete meiosis. KL together with LIF and IGF-1 promoted meiosis in mouse fetal

ovaries cultured *in vitro* [126]. Many other factors have been implicated in the control of meiosis during folliculogenesis, as shown below.

The homologous chromosomes in meiotic cells undergo pairing (called synapsis) and recombination during which physical links, chiasmata, are established between them. Synapsis involves a meiosis-specific protein complex, the synaptonemal complex, which comprises two axial lateral elements (AEs) and a central element. The two AEs, which colocalize with the sister chromatids of each homolog, become connected along their entire length by fine fibers called transversal filaments at the pachytene stage of meiotic prophase I. The AE is composed of discrete protein filaments, organized by the cohesin complex or by two meiosis-specific proteins, synaptonemal complex protein (Sycp) 2 and Sycp3. The disruption of *Sycp3* results in defects in synapsis during meiosis, germ cell apoptosis in males, and embryonic loss in females due to aneuploidy [127], [115].

The level of cAMP in the oocyte plays a critical role in meiotic maturation. The concentration of cAMP is a crucial determinant of the meiotic arrest in the oocyte during folliculogenesis, which is coordinated by the gap junctional communication between the oocyte and granulosa cells. A high level of cAMP in the oocyte maintains meiotic arrest perhaps through activation of a protein kinase A (PKA) pathway[111] since the intercellular cAMP level is elevated when the oocyte is denuded, a phenotype which can be rescued by incubation with cAMP analogs or

a phosphodiesterase (PDE) (which metabolizes cAMP) inhibitor [128], [110]. In addition to the PKA pathway, protein kinase C (PKC) seems to be involved in the meiotic regulation of oocyte maturation. After the LH-surge, cAMP levels increase and G protein activation occurs, which then activates phospholipase C. As a result, levels of IP3 and $Ca^{2+}$ increase and cAMP/PKA levels decrease in granulosa/cumulus cells and the oocyte modifies and activates various checkpoint proteins, which may bring about germinal vesicle breakdown and further processes characteristic of the resumption of meiosis. Gap junctions also function in the transmission of cAMP to neighboring cells through pores and so participate in the meiotic arrest, consistent with the finding that when oocytes are denuded, the gap junctions between oocytes and granulose cells are abolished.

The orphan Gs-linked receptor G protein coupled receptor 3 (GPR3) is located in the oocyte and functions in the meiotic arrest. Oocytes from *Gpr3* knockout mice resume meiosis within antral follicles, whose phenotype can be reversed by the injection of *Gpr3* RNA into the oocytes [129].

Dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast) (*DMC1*) is a meiosis-specific gene first discovered in yeast that encodes a protein with homology to RecA and may be a component of recombination nodules. Yeast *dmc1* mutants are defective in crossing over and synaptonemal complex (SC) formation, and arrest in late

prophase of meiosis I. Homozygous mutant males and females for this gene are sterile with an arrest of gametogenesis in the first meiotic prophase. Chromosomes in mutant spermatocytes fail to undergo synapsis, despite the formation of axial elements that are the precursor to the SC [130].

Mitogen activated protein kinase (MAPK) is also involved in oocyte maturation [131]. In mammalian oocytes, two isoforms of MAPK are present, ERK1 (p44) and ERK2 (p42). MAPK kinase is activated by MEK, a specific MAP-kinase, which is regulated by Mos, a germ cell-specific kinase that is exclusively produced in oocytes [132]. During oocyte maturation, MAPK activity is required for the maintenance of MPF activity, formation of spindles and maintenance of MII arrest in late staged follicles [133], [134]. Of course, the knockout of c-mos results in meiotic abnormalities [135].

Resumption of meiosis requires the activation of cyclin-dependent kinase-1 (CDK1, p34cdc2), a component of maturation-promoting factor (MPF) (Cyclin B-cdc2 kinase), which is responsible for oocyte maturation since its activation occurs together with germinal vesicle breakdown (GVBD). *Cdc25b*-deficient mice are viable, and *Cdc25b* knockout female mice are sterile because of a permanent meiotic arrest resulting from the inability to activate MPF, and thus are unable to undergo GVBD, and *Cdc25b* knockout oocytes remaine arrested at prophase with low MPF activity. When *Cdc25b* mRNA was microinjected into *Cdc25b* knockout

oocytes, activation of MPF and resumption of meiosis occured [136]. Active MPF phosphorylate proteins that form the nuclear envelope and proteins involved in chromatin condensation and cytoskeletal reorganization. The fully-grown oocytes contain a pre-formed Cyclin B-Cdc2 kinase, which is maintained in an inactive form by inhibitory phosphorylation of Thr14 and Tyr15 of Cdc2. The dephosphorylation and thus activation of Cdc2 kinase requires Cdc25 phosphatase and recent findings in mice showed that this phosphatase is required for the resumption of meiosis during oocyte maturation.

Genomic imprinting

Every type of the cells has two copies of each chromosome, one from the father and one from the mother. In general, the two chromosomes work to express certain genes specific for each of the cell types, although sometimes they have a law of dominance, which will give unique properties to each cell. However, some genes have been found to be expressed in a parent-of-origin-specific manner, and this genetic phenomenon is called genomic imprinting. Genes are either expressed only from the allele inherited from the mother (for example, H19 referred to MEG), or from the allele inherited from the father (for example, IGF2 referred to PEG) [137]. In somatic cells, the imprinting is maintained throughout life, while in germ cells, it stops as germ cells form for the next generation during embryogenesis and then reoccurs in the embryonic period and finishes after birth. Imprinting is still maintained in the PGCs at

embryonic day 7.5 [138], [139], but its elimination starts around embryonic day 10.5 to 11.5 right after the migration of PGCs finished, and during embryonic day 12.5 to 13.5, the process is accomplished in both male and female germ cells [140]. In male germ cells, imprinting newly occurs after embryonic day 15.5 and is completed before birth so that only PEG works in both chromosomes, and in female germ cells, imprinting is re-established after birth so that MEG works in both chromosomes, so the maturated germ cells gain a PEG-expressing chromosome in males, and MEG-expressing chromosome in females [141]. Aberrant expression of several imprinted genes has been linked to the development of human diseases, including Beckwith–Wiedemann, Prader–Willi and Angelman syndromes. Then, what exactly is the identity of the imprinting? There are two major mechanisms involved in imprinting; DNA methylation [119] and histone modifications. DNA methylation also has a role in gene silencing, for example, in postmigratory PGCs resulting in germ cell differentiation; germ cell-specific genes such as *Mvh*, *Dazl*, and *Sycp3* are demethylated in germ cells and methylated in somatic cells, which seems to be coordinated by Dnmt1 [142]. Therefore, DNA methylation is both a heritable and reversible epigenetic modification that influences gene expression and chromatin condensation via the binding of factors such as methyl CpG-binding proteins that subsequently recruit other gene-silencing factors including histone deacetylases. As indicated, methylation differs between males and females and is maintained in somatic

cells, so the Dnmts that participate in these processes must be different. The *De novo* and maintenance methylating enzymes which participate in this imprinting are DNA methyltransferases (DNMTs), which add a methyl group to the 5-position of the cytosine within CpG dinucleotides [143]. Dnmt3L is important for the establishment of maternal methylation imprints in the female germ line. Disruption of *Dnmt3L* causes azoospermia in male and embryonic death in females with demethylation of maternal methylation sequences in oocytes [144]. Mice deficient in the DNA methyltransferase *Dnmt1*, *Dnmt1o* or *Dnmt3L* display both loss of allele-specific methylation and expression of imprinted genes implying at the very least a role for methylation in imprint maintenance [144], [145], [146], [147]. Dnmt3a or Dnmt3b works as a *de novo* methylation enzyme and the knockout of either of these genes results in death in the embryonic stage or one month after birth. Germ cell-specific knockout of *Dnmt3a* is embryonic lethal in females because of a failure to establish maternal imprinting, and failure in the formation of sperm and in the establishment of paternal imprinting, while no significant defects are found in Dnmt3b conditional knockout mice. Furthermore, the timing of the DNA methylation of each gene in both female and male germ cells also differs, which coordinates with the growth and maturation of oocytes [148], [141], [149], [150], [151], [152], [153], [154]. However, when oocytes are cultured *in vitro*, they seem to show a defect in the establishment of imprinting [137], [155], and sex reversal germ cells showed that the

methylation pattern of imprinting genes are influenced mainly by the chromosomes they possess so XX male germ cells less are methylated than XY male germ cells [156]. Epigenetic reprogramming of DNA methylation not only re-establishes the parent-specific imprinting but also restores totipotency within the germ cells.

Histone modifications and epigenetic Reprogramming

Histone modifications include phosphorylation, acetylation, ubiquitination, methylation and small ubiquitin-like modifier SUMOlation. At first, PGCs/precursor cells starting to express *Fragilis* [157] and in turn *Blimp1* [158] at E6.5 show the same Histone 3 lysine 9 di-methylation (H3K9me2) and H2K27me3 pattern as surrounding extraembryonic ectodermal or extraembryonic mesodermal cells. However, *stella*/*PGC7*, *Blimp1*and *fragilis*-positive PGCs that start to migrate to the genital ridge with E-cadherin [159] at around E7.75 begin to show a decrease in H3K9me2 and have low H3K9me2 levels at E8.75. On the other hand, H3K27me3 starts to gain modifications in *stella*-positive cells at around E8.25 gradually, and until E9.5, 90% of *stella*-positive PGCs show increased H3K27me3 levels [160], [161], [16], [162]. As for DNA methylation, genome-wide methylation decreased within a small population of PGCs at E8.0 and almost all DNA was demethylated within most of the PGCs at E9.5. As for the histone arginine methylation, Blipm1 has been shown to associate with an arginine-specific histone methyltransferase, Prmt5 [163]. The oocyte can reprogramme an

adult somatic nucleus into an embryonic state after somatic cell nuclear transfer, so that a new organism can be developed from that cell [164], [165]. Recently, Act has been implicated in promoting the efficiency of nuclear reprogramming after cell fusion, signaling of which also contributes to the maintenance of pluripotency of ES cells without LIF and the efficiency with which embryonic germ (EG) cells are derived from PGCs [166].

X chromosomal inactivation (XCI)

XCI is a process by which one of the two copies of the X chromosome in female mammals is inactivated [167]. The inactive X chromosome is silenced by packaging into transcriptionally inactivated heterochromatin. The choice of which X chromosome will be inactivated occurs randomly and once a certain X chromosome is inactivated at the ICM in the blastocyst stage, it will remain inactive throughout the lifetime of the cell although some suggest that reactivation starts in ICM since ES cells derived from ICM have the ability to reactivate the X chromosome. It has been shown that when a cell has more than two X chromosomes, only one X chromosome is active and all the remaining X chromosomes become inactivated. So there is a hypothesis that a blocking factor binds to X chromosome-specific sequences at the X inactivation center (XIC) and controls the silencing of the X chromosome by preventing its inactivation. The XIC contains two non-translated RNA genes, *X-inactive specific transcript* (*Xist*) and *Tsix*. The inactive X chromosome is coated by *Xist* RNA, whereas the active X

chromosome is not, and the *Xist* gene is the only gene which is expressed from the inactive X chromosome but not from the active one. The promoter regions of the genes on the inactive X chromosome are highly methylated. CpG islands of house-keeping genes are highly methylated on the inactive X chromosome and are low methylated in the active X chromosome. Random X chromosome inactivation occurs in implanted epiblasts, and once established, remains in the somatic cells, while reactivation occurs in oocytes that contain two X chromosomes. In males the maternal X chromosome is inactivated and forms the XY-body with the Y chromosome at meiosis prophase while the paternal X chromosome is inactivated completely in extraembryonic tissues [168]. From E8.5 to E9.5, PGCs are a mixture of cells; PGCs expressing *Xist* as well as somatic cells, PGCs with a low level of *Xist* signaling, and PGCs with no *Xist* expression, which indicate the start of reactivation of the X chromosome around these stages although the timing of the reactivation differs from cell to cell and so some PGCs show *Xist* at E14.5 [169], [170]. Re-inactivation of the X chromosome occurs randomly in female germ cells after the erasure of genomic imprinting.

**Gap junctions (connexin)**

Connexins and Gap junctions

Gap junctions are composed of connexin family members, each of which has four highly

conserved transmembrane domains, a cytoplasmic N-terminal end and two extracellular loops, and one cytoplasmic loop and carboxyl terminal ends with a specific sequence for each connexin. Roles for each domain have been reported; the N-terminal in voltage gating polarity, extracellular loops 1 (E1) and 2 (E2) in connexon-connexon interaction, E1 in voltage gating and E2 in the regulation of heterotypic channel formation, the cytoplasmic loop in pH gating, and the C terminal in channel gating by phosphorylation. To date, 20 members of the connexin family in the mouse genome and 21 members in the human genome have been reported [171], [172]. Connexins can be divided into subgroups (alpha, beta, ganma) on the basis of sequence identity and the length of the cytoplasmic loop.

Six connexins form a hemi-channel (connexon) in the plasma membrane and dock to another hemi-channel in the plasma membrane of an adjacent cell, forming hydrophilic pores across the membrane, which allows the passage of nutrients, metabolites, ions, water, electrical impulses and small signaling molecules such as second messengers like cAMP, $Ca^{2+}$ and inositol phosphates up to approximately 1kDa, although recent study has suggested that these hemichannels can display activity in a single plasma membrane, introducing large ionic conductances and dramatically changing the permeability of individual cells [173], and aggregate tightly to form the cluster at the plasma membrane.

Each connexin has a tissue-specific distribution and many cell types express more than one

isotype. For example, mouse neurons express Cx36, Cx45 and Cx57, whereas mouse hepatocytes express Cx26 and Cx32 [174]. Even in the same tissue, the expression pattern of each connexin shows cell type or developmental stage-specificity. Furthermore, the potential diversity of types of channel is further magnified by the existence of heterotypic as well as heteromeric channels where certain sets of connexins can form compatible connexons. Although gap junctions have a similar structure, channel pore size, regulation, permeability and gating characteristics depend on the connexin isoform and post-translational modifications [175].

Molecules that control gap junctions

Gap junctional communication can be modulated by connexin-associated proteins. Cadherin-mediated cell–cell adhesion is required for the formation of gap junctions. Upregulation of E-cadherin-dependent cell–cell contacts increases gap junctions, and anti-N-cadherin antibodies prevent the formation of both adherens junctions and gap junctions. E-Cadherin and -catenin colocalize with newly formed gap junctions, while N-cadherin has been shown to co-localize with Cx43 in cardiac myocytes as well as β-catenin following Wnt expression. Another catenin, p120ctn, has been found to co-localize not only with cadherins, but also with Cx43.

Zona occludens-1 (ZO-1), v-Src and c-Src are shown to interact with connexins and to

participate in the phospholylation of connexins. The viral Src oncoprotein shuts down gap junctions as well as its normal cellular counterpart c-Src, and Cx43 has been shown to be the substrate for v-Src [176]. Mutation of the putative v-Src phosphorylation site results in a lack of gap junction closure by v-Src in Xenopus oocytes. Tyrosine phosphorylation of the Cx43 C-terminal tail might trigger the closure, similar to Src action. So the inhibition of tyrosine phosphatases by pervanadate results in Cx43 tyrosine phosphorylation and closure of gap junctions. So which molecules interact with and modulate the phosphorylation of connexins? Gap junctions might be controlled by hormones, extracellular signaling molecules such as neurotransmitters, growth factors, or cytokines. Gating of the gap junctions is regulated by the phospholylation of the C-terminal of the connesins. Inhibition of tyrosine phosphatase by pervanadate results in Cx43 phosphorylation and the closure of gap junctions as mentioned above. Serin/threonine kinases participate in Cx43 phosphorylation. cAMP-dependent protein kinase (PKA), protein kinase C (PKC), Mitogen-activated protein kinase (MAPK) and tyrosine kinase seem to be involved in this regulation. PKA has been shown to upregulate Cx43 assembly. MAPK mediated Cx43 serine phosphorylation, however, its effect on gap junctional communication is unclear. First, MAPK and PKC have been shown to increase Cx43 phosphorylation and as a result, cause a rapid temporary inhibition of gap junctions on platelet-derived growth factor (PDGF) treatment [177]. However, another study shows that

MAPK-mediated Cx43 phosphorylation induced by PDGF does not result in inhibition of GJC [178]. Cdc2, which is indispensable for cell-cycle progression-mediated connexin phosphorylation might reflect plasma membrane deprivation of gap junctions during mitosis because Cx43 was phosphorylated at Ser255 in a Cdc2-dependent manner. Cx43 channel activity might be controlled by FSH. When granulosa cells receive signals from FSH receptors, pore closed gap junctions might go through the intercellular trafficking to the plasma membrane, and after they reach the proper position, phosphorylation of the C-term of Cx43 might induce gate opening. Cx43 has been shown to directly bind to alpha- and beta-tubulin and microtubules at the cell-periphery to co-localize with Cx43-based gap junctions (tubulin) or to caveolins.

Gap junctions' contributions

Gap junctions play pivotal roles in a wide range of physiological processes, for example in regulating events in the development of various organs such as bone-marrow [179], the brain [180] and the heart [181]. They play important roles in cell differentiation, growth and proliferation [182], electrical activation of the heart [183] and neuronal signaling[184]. They also participate in hormone secretion and the functions of both endocrine and exocrine glands, auditory function, wound healing [185] and even in immune functions such as tissue inflammation and tissue repair [186]. Connexins have also been shown to act as tumor

suppressors, although their mechanisms of action remain unclear. Generally, tumor cells display reduced gap junctional coupling [187]. In embryogenesis, they contribute to cell migration during morphogenesis such as for the cardiac neural crest. Gap junctional communication also has roles in both male and female reproduction. In males, gap junctions participate in germ cell proliferation and differentiation as well as in modulating the initiation and maintenance of smooth muscle tone in different structures [188]. In females, direct cell–cell communication via gap junctions seems to play important roles in oocyte maturation [189], in corpus luteum formation, in the preparation of the uterus for embryo implantation [190] and in the regulation of trophoblast invasion and placental functions.

Gap junctions and oogenesis/folliculogenesis

Gap junctional communication plays an important role both in folliculogenesis itself and in the control of germ cell-specific events [191]. During folliculogenesis, the granulosa cells become coupled and also form gap junctions with oocytes by passing through the developing ZP. Gap junctional communication is known to have a crucial role in the folliculogenesis. At present, seven connexin genes, *Cx26*, *Cx30.3*, *Cx32*, *Cx37*, *Cx43*, *Cx45* and *Cx60*, are known to be expressed in the ovary. In detail, porcine ovaries express *Cx26*, *Cx30.3*, *Cx32*, *Cx43* and *Cx60*, and the expression of *Cx43* in granulosa cells of primary follicles increases as the follicles grow. *Cx26*, *Cx30.3*, *Cx32* and *Cx43* are detected at the mRNA level in internal and

surrounding compartments of large antral follicles. Cx30.3 and Cx43 are expressed in the theca internal as well as in the granulosa cell compartment.

In cows, follicles express Cx26, Cx32, Cx37 and Cx43. The expression of Cx26 is detected in oocytes of follicles at early stages in development and in the granulosa and/or thecal cell layers of antral follicles. Cx37 is also reported to exist in both the oocyte and the granulosa cells and its levels decrease significantly at antrum formation [192].

The major connexins in the mouse ovary are Cx26, Cx30.3, Cx32, Cx37, Cx40, Cx43, Cx45 and the Cx60 homologue Cx57. They show a stage and cell -pecific expression pattern. Cx32 and Cx43 have been detected in gap junctions joining the cumulus/granulosa cells of fully grown mouse follicles [193]. Cx43 is detected in mouse embryos as early as day 11.5 of development and localizes to the somatic cells of the undifferentiated gonad, while later during embryogenesis, it is expressed between the somatic cells and germ cells; Cx43 comes to be expressed in pre-granulosa cells in mouse primordial follicles as early as postnatal day1. Cx37 is expressed from the primary follicle stage within oocyte and at the interface between granulosa cells and oocytes, existing exclusively at the oocyte surface and not on granulosa cells. So Cx37 and Cx43 are the connexins that take part in the formation of gap junctions between the oocyte and the cumulus cells, and Cx43 is restricted to the cumulus/granulosa cells. Mice that lack *Cx43* die soon after birth as a result of cardiac malfunction [194]. When

ovaries of these KO mice are planted under the kidney capsules of WT mice, they show an arrest of folliculogenesis beyond the primary follicle stage, and oocyte growth is retarded. Furthermore, when oocytes are recovered from these grafts, they fail to undergo meiotic maturation and cannot be fertilized [105], [45]. Mice lacking *Cx37* are viable and ovarian folliculogenesis proceeds apparently normally until the late preantral stage, and so the mice do not ovulate which could originally be induced by gonadotrophin stimulation [106] and oocytes are meiotically incompetent [195]. Cx45 is present in a minor compartment in granulosa cells of adult mice and rats, and colocalizes with Cx43 at some gap junctions, and also at the gap junctional plaques between cumulus cells. Gap junctions and germ cell-specific events are closely related. cAMP is transferred from granulosa cells to the oocyte and a proper concentration of cAMP is the determinant of oocyte meiotic arrest. So when gap junctions are disrupted, abnormal meiotic resumption is observed.

**Models**

Experimental models to elucidate the establishment of germ cells or germ cell-specific events have been established.

Knockout method

Generating knockout mice is the major experimental method to examine the role of a

particular gene. However, although this method enabled us to reveal the importance or role of the gene, we could not do any cellular or molecular analysis by this *in vivo* method, so that roles and functions were still largely unknown. Furthermore, the total number of germ cells in the early stage of development is around 50 cells, which also makes the analysis of germ cells difficult. Analysis at the cellular and molecular levels *in vitro* should provide information not only for understanding the development of mammalian germ cells but also for clinical purposes. To achieve this, the establishment of an appropriate *in vitro* experimental system that can follow the whole gametogenesis would be preferable.

*In vitro* culture of PGCs and follicles, Xenografting

Some investigations have been conducted into the *in vitro* culture of PGCs [196], [197] and the addition of factors like LIF [51], steel factor [198], [199], mast cell growth factor (MGF) [49], sodium pyruvate [200] and Insulin-like growth factor (IGF) [201], or the cultivation of germ cells on feeder cells [202] prolongs germ cell survival and aids proliferation. In mice, follicles begin folliculogenesis shortly after birth, and within 10–12 days they reach the secondary stage, and then develop to the large antral stage by 18–24 days after birth, so follicular growth takes about 16 days and in humans about 85 days from the primary follicle onwards. Some groups have succeeded in developing immature follicles from mouse ovary to matured follicles *in vitro*; some used alginate-extracellular matrix gel while others used

collagen gel, or some cultured follicles on membrane inserts, while others cultured one follicle in one drop of medium [203], [204], [90], [205], [206], [207], [208], [209], [210], [211], [212], [213], [214], [215], [216], [217], [218], [219]. These *in vitro* maturated oocytes/follicles have also been shown to be able to be fertilized *in vitro* [220]. However, one group reported that *in vitro* follicular growth affects oocyte imprinting in mice [155]. Analyses of DNA methylation status revealed that after *in vitro* development, there was a loss of methylation at the Igf2R locus in six of seven independent experiments and Mest/Peg1 locus (one of seven), and a gain of methylation at the H19 locus (one of seven). There are other ways to culture germ cells, which coordinate with the grafting into the kidney capsules of SCID mice or reaggregation with ovarian cells [221], [222], [223], [224], [225], [226], [227]

ES cell-derived germ cells

Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocysts, have been shown to have totipotency *in vivo* [228], [229]. ESCs were also known to differentiate into some types of somatic cells derived from all three germ layers *in vitro*. In the last seven years, several landmark investigations have demonstrated the possibility that ESCs could be differentiated into PGCs [230], [231], [232], [233], [234], [235], [236], male germ cells [237], [238], [239], female germ cells such as oocytes or follicles [240], [241], [242], [243], [244], [245], or both female and male germ cells [246], [247], and it is also reported that pancreatic

stem cells could generate oocyte-like cells *in vitro* [248] or oocyte-like cells from fetal porcine skin [249]. In 2009, *Dazl* was shown to be critical not only to the maintenance of pluripotency but also to germ cell differentiation from both human and mouse ESCs [250], [251], [252], [253]. Lin28 has a role in both germ cell development and germ cell malignancy [254]. One group showed that germ cells can be generated from mouse pluripotent somatic cell hybrids [255]. To date, however, no group has reported the successful generation of mature oocytes starting from ESCs, although one group recently reported that ESC-derived sperm could produce offspring in mice [256]. This technique, once established, will provide a new tool for investigating the mechanism of gametogenesis not only for understanding the establishment of germ cells but also for clinical purposes [257], [258], [259], [260], [261], [262].

## ***Objective***

Several genes like *Blimp1* have been identified as critical not only for the gametogenesis itself but also for these germ cell-specific events by generating knockout or transgenic mice. However, although this gene targeting analysis enables us to reveal the importance and role of a gene, we cannot do any cellular of molecular analysis with this *in vivo* method. Furthermore, the total number of germ cells in the early stage of development is around 50, which also makes the analysis of germ cells difficult. Analysis at the cellular and molecular levels should provide information not only for understanding the development of mammalian germ cells but also for clinical purposes. To achieve this, the establishment of an appropriate *in vitro* experimental system that can follow the whole gametogenesis would be preferable.

In our experiments, we tried two *in vitro* experimental approaches to understand the mechanism of germ cell development.

**Objectives and Approaches:**

In our experiments, we tried two *in vitro* experimental approaches indicated below to understand the mechanism of germ cell development.

***1. Elucidation of the role of gap junctions in folliculogenesis***

Although the molecular diversity in connexins combined with the complexity of expression

profiles seem to complicate our understanding of the physiological role of each GJC, the identification of connexin genes expressed in certain circumstances seems to be the basis for understanding the physiological significance of the respective gap junctions. Although the spatial- and stage-specific expression of multiple connexin genes in follicle cells has been reported, the species of connexin gene expressed in oocytes appeared to be very limited. Since the property of gap junctions as intercellular channels could be affected by the composition/combination of connexin [263], and also the expression of each connexin gene seems to be regulated by various conditions and factors such as gonadotropins [264], [265], identification of connexin genes in oocytes of other mammals could be informative for understanding the regulatory mechanism of mammalian oogenesis. Cx37 is reported to be well expressed in several mammalian oocytes including mouse oocytes as mentioned above, and experiments using genetically modified mice revealed its essential role in oogenesis. To examine whether this unique property of Cx37 prevails in other mammals, we extended our analysis to porcine oocytes. Using porcine oocytes, we searched for novel gap junctional protein, connexins, specifically expressed in oocytes, because while the spatial- and stage-specific expression of multiple connexin genes has been reported in GCs, the subset of connexin genes expressed in oocytes appeared to be very limited; Connexin 37 (*Gja4*/CX37) is predominantly expressed in several mammalian oocytes.

***2. Establishment of an in vitro model system for obtaining follicles from mouse ESCs***

ESCs have been demonstrated to have the ability to differentiate into PGC and, subsequently, gametes. Since we can knock down a particular gene in the ESCs, we would be able to do molecular and functional analyses spatiotemporally of gametes *in vitro* starting with ESCs. To date, however, no group has reported the successful generation of mature oocytes from ESCs. We therefore tried to construct an experimental system enabling us to obtain the mature oocytes from mouse ESCs that could be fertilized *in vitro*.

## ***Materials and Methods***

### *1. Elucidation of the role of gap junctions in folliculogenesis*

#### *Ethics*

Mice were obtained and maintained in accordance to the NAIST Animal Ethics Committee.

#### *Animals and tissues*

Ovaries obtained from 4- to 12-month-old pigs at a local slaughterhouse were transported to the laboratory in Hank's balanced salt solution (GIBCO/Invitrogen, NY), supplemented with 50 U/ml penicillin (Meiji Seika, Tokyo), 50 μg/ml streptomycin sulfate (Meiji Seika, Tokyo), and 500 ng/ml Fungizone (GIBCO/Invitrogen) at 37°C. Ovaries removed from Balb/c mice were kept frozen until sectioning.

#### *Preparation of porcine oocytes and construction of cDNA library*

Porcine ovaries were removed and placed in NCSU23 medium. Individual follicles at the antral stage were incised to release cumulus-oocyte complexes. Removal of cumulus cells was accomplished by gentle pipetting. The purity of recovered oocytes was monitored by microscopy. Total RNA was extracted using ISOGEN reagent (NIPPON GENE, Tokyo,

Japan), and genomic DNA was removed by digestion with a deoxyribonuclease-free ribonuclease, RQ1 (Promega, WI). The cDNA library was constructed using the SMART PCR cDNA synthesis kit (TAKARA BIO, Shiga, Japan). Presence of clones in the cDNA library encoding *GDF9*, *MOS*, and *CYP19* was examined by PCR using primers specific for the respective genes (Table 1). PCR was performed for 40 cycles of denaturation at 98°C, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The expression of *CYP19* in the ovary was confirmed by RT-PCR from total RNA isolated from porcine ovaries. Total RNA from human placenta was obtained from Clontech/TAKARA BIO (CLN 636527; Kyoto, Japan).

***PCR cloning with degenerate oligonucleotides***

The porcine oocyte-specific cDNA library was screened using two pairs of degenerate oligonucleotides (primers F1, F2, R1, and R2; F indicates forward primers, while R indicates reverse primers) corresponding to the regions conserved among the connexin family members (Itahana et al., 1996; Itahana et al., 1998). Primer F1 [5'-ttcccc(a/g)t(c/g)tc(c/t)cac(a/g)t(c/g)cg-3'] corresponds to the amino-terminal region. Primer F2 [5'-cagcc(a/c/g/t)gg(c/t)tg(c/t)(a/g)a(c/g)aa(c/t)gtctg-3'] is complimentary to a conserved amino acid sequence, VCYD, within the first extracellular domain. Primer R1

[5'-tgaaga(c/t)(a/c/g)(g/t)t(c/t)ttctc(g/t)gtggg-3'] matches a conserved sequence, VDCF, in the second extracellular domain, while R2 [5'-gtggg(c/t)ct(a/g)ga(a/g)a(c/t)a(a/t)a(a/g)caagt-3'] corresponds to the C-terminus of the same domain. To minimize degeneracy, not all of the possible sequence variations revealed in the alignment were represented in each probe. RT-PCR mixtures (30 μl total in volume) contained 0.2 μg of RT product, 0.2 μM of the F and R primers, and 200 μM deoxy-NTP with Taq Extended Buffer (Stratagene, CA). AmpliTaq (2U; Perkin-Elmer, CT) and Taq Extender PCR Additive (2U; Stratagene) were added after reactions reached 94°C to minimize undesired priming during the initial cycle. Thirty cycles at 94°C for 30 sec, 51°C for 2 min, and 72°C for 3 min were followed by a final extension step for 15 min at 72°C. Reaction products were separated and visualized by agarose gel electrophoresis. Products were subcloned into the pT7Blue-T cloning vector (Merck, Darmstadt, Germany) and subjected to a DNA sequence analysis using an ABI3100 sequencer (Perkin-Elmer, MA).

***Amplification of porcine GJA4, GJC1, and GJA10***

Porcine *GJA4*, *GJC1*, and *GJA10* were amplified using specific primers for each gene (Table 1) from either a porcine genomic library (Clontech/TAKARA BIO) or our porcine cDNA library. For *GJA4*, a set of *GJA4*-A primers were designed based on EST information

(GenBank ID: DB812024 and AK234742), producing a product of the expected size of 262 bp. For *GJC1*, the *GJC1*-A primer set was used, producing the expected size product of 254 bp. For *GJA10*, the *GJA10*-A primer set produced a product of the expected size of 378 bp. PCR was performed for 30 cycles of denaturation at 98°C for 30 sec, annealing for 20 sec at 54°C, 58°C, and 56°C for *GJA4*, *GJC1*, *and GJA10*, respectively, and extension at 72°C for 30 sec.

***Amplification of GJA4 and GJC1 mRNA expressed in oocytes***

Total RNA was isolated from purified oocytes in antral follicles as described above. cDNA was synthesized from total RNA with a Superscript III first-strand synthesis kit for RT-PCR (Invitrogen). The primers used to detect *GJA4*, *GJC1* and the housekeeping gene *Gapdh* are listed in Table1: *GJA4*-C and *GJC1*-C. PCR was performed for 45 cycles with denaturation at 95°C for 10 sec, annealing for 10 sec at 60°C and extension at 72°C for 8 sec.

***Screening and cloning of porcine GJC1***

The porcine oocyte-specific cDNA library was screened using standard techniques. A cDNA fragment with a sequence homologous to that of mouse *Gjc1* was obtained using the degenerated primers F and R as described above. These fragments were labeled with [$\gamma$-$^{32}$P]

deoxy-CTP (NEN, MA) using random hexamers and the Klenow fragments (TAKARA BIO) and used as the probe to screen for porcine *GJC1*.

***Sectioning and DAPI staining***

Ovarian sections from pigs or mice were prepared for *in situ* hybridization and immunohistochemistry as described previously [266], [267]. The removed ovaries were covered in OCT embedding compound (Miles, IN) and snap-frozen in liquid nitrogen. Cryosections (10 μm thick) were prepared using a Cryostat HM500-OM (Micron, Germany), collected on silane-coated slides, and stored at -80°C until staining. 4',6-diamino-2-phenylindole (DAPI)-staining was performed using a cell-permeable fluorescent DNA binding probe (Sigma-Aldrich Inc., MO).

***In situ hybridization and probes***

Procedures for *in situ* hybridization using the sections prepared as described above were carried out essentially as reported previously [266]. For the preparation of each probe, DNA regions containing unique sequences of the respective porcine or mouse connexin genes were amplified using each B set of primers (Table 1) and subcloned into the RNA transcription vector pBlue-script II SK (Stratagene) or pT7Blue T-vector (Novagen, WI). Complementary

antisense or sense RNA probes were prepared by *in vitro* transcription of the linearized constructs using SP6 or T7 RNA polymerase in the presence of digoxigenin-UTP (Roche Diagnostics, Basel). After RNA polymerization, DNA templates were hydrolyzed with RQ1 DNase. Sections were stained with a digoxigenin detection kit (Boehringer Mannheim, IN).

***Immunofluorescence staining and antibodies***

Frozen sections were rehydrated in PBS to remove OCT compound and blocked in PBS containing 5% skim milk. Incubation with anti-CX60, anti-CX37, or anti-CX45 antibodies was performed overnight at 4°C, followed by three washes in PBS. Bound antibodies were visualized with secondary antibodies conjugated to either Alexa fluor 488 or Texas-red fluorescent dyes (Molecular Probes). The Anti-CX37 antibody was obtained from Alpha Diagnostics (Cx37B12-A: rabbit anti-rat Cx37, TX). The anti-CX60 monoclonal antibody was prepared using the predicted CX60 C-terminal sequence ($^{514}$VCAEREGEKEREVNL$^{528}$) [267]. The Anti-CX45 polyclonal antibodies were purchased from Lifespan Biosciences (LS-C14572: mouse anti-human Cx45, WA) and from Millipore (AB1745: rabbit anti-human Cx45, MA).

***Gap junction-mediated intercellular communication***

Full length coding sequences of porcine CX43, CX45, and CX60 were cloned from porcine genomic DNA by nested PCR using the corresponding primer sets (1 & 2) in Table 1. The sequences franking the coding region of CX43 (GenBank ID: AY382593) and CX60 (Itahana et al., 1998) were deduced from HTGS draft sequence of the porcine genome (GenBank ID: CU914424 and CU468058, respectively). Amplified DNA fragments were inserted into XhoI-MluI site of pCX4puro or pCX4bsr whose original XhoI sites were destroyed and new ones were introduced instead at the multiple cloning sites. The constructed expression plasmids were transiently transfected into HeLa cells with lipofectamine LTX (Invitrogen, CA). Transfected cells were plated at a density of 3 x 105 cells per well in 24-well plates and donor cells were labeled with 0.5 μM calcein AM, 10 μM 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, OR) in serum-free medium for 40 min at 37 ºC. Dye-coupling activity was assessed as described previously (Asklund et al., 2003) with a slight modification in that labeled cells were co-cultured for four hours at a ratio of 1:50 (donor: recipient).

***2. Establishment of an in vitro system for obtaining follicles from mouse ESCs***

***Mouse ESCs and cell culture***

Three ESC lines, RW-4 (129/SvJ male mouse), E14TG2aIV (129/SvJ male mouse), C57BL/6 (B6J male mouse), were used for the experiments. ESCs were maintained in Dulbecco's modified Eagle's medium (D-MEM) (high glucose) (Nikken) supplemented with 20% ESC-qualified fetal (FBS) (Invitrogen), 100x nucleoside (SIGMA), 100x NEAA (Invitrogen), b-mercaptoethanol, and LIF (Chemicon), on mitomicine C-treated STO cells.

***Generation of Oct4-GFP-IRES-neo-expressing ESCs***

*Oct4* enhancer and promoter regions were isolated from a mouse (129/SvJ) lambda fix Ⅱ genomic library (Stratagene) by colony hybridization using XL1-Blue (P2+) SP (Stratagene), and *Oct4* fragments that encode 14.2Kb of the enhancer and promoter region and the *Oct4* encoding regions were isolated. Predicted PE enhancer region were removed by mutagenesis (Quick Change Site-Directed Mutagenesis Kit) (Staratagene). A neo fragment from pMC1Meo3804 (Stratagene) and IRES-hrGFP fragment from pIRES-hrGFP-1a (Stratagene) were introduced just after the initiating codon of *Oct4* instead of *Oct4* gene; neo-IREShrGFP (9.8Kb). This fragment was introduced into pBLC and a single-strand break was madeit was before electropolated into RW-4 ESCs at 270v, 1.6～1.7ms. After the electropolation, ESCs were maintained in the ES medium shown above for two days, and then cultured in ES medium containing G418. At 8days after cultivation, eight G418resistant colonies (clones A to

H) were cloned.

***Differentiation of ESCs***

ESCs were dissociated by trypsine-EDTA (0.25% trypsine / 0.02% EDTA) and resuspended in ES-medium. They were cultured standing on 0.1% gelatin-coated dishes for 15 to 20 min to remove the STO feeder cells. After centrifuging, cells were resuspended in ES-medium without LIF, whose FBS concentration was 10% or 15% (derivation medium). Cells were cultured at several concentrations, $0.5 \times 10^4$, $1 \times 10^4$, $2 \times 10^4$ and $1 \times 10^6$ cells/$cm^2$ without STO feeder cells for 3 days (37℃, 5% CO2). For elucidation of the effect of BMPs and LIF, BMP-producing cells and STO cell culture medium were added to the derivation medium.

***Establishment of BMP producing cells***

BMP genes were cloned using primers shown below from mouse testis at age 3.5 by RT-PCR and the fragments were cloned into the retroviral vectors pCX4puro and pCX4bsr. NHI3T3 was used for the infection of the virus.

*BMP4* sense 5'-CCGGGATCCATGATTCCTGGTAACCGAATGCTG-3'

*BMP4* antisense 5'-CCGGGATCCTCAGCGGCATCCACACCCCTC-3'

*BMP8b* sense 5'-CCGGGATCCCGAGATGGCTGCGCGTCCG-3'

*BMP8b* antisense 5'-CCGGGATCCTCAGTGGCAGCCACAGGCC-3'

***Morphological and histological analyses***

C57BL6 mice were used as the control for further experiments. Nine day to one month-old ovaries were isolated. Follicles were then mechanically dissected from the ovaries using 26-gauge needles. For histological experiments, PAS staining was performed over the control and ESC-derived follicle like structure (ESDFLSs). ESDFLSs were fixed with buan (a mixture of picric acid saturated solution (Nakarai) 75ml、formalin (Wako) 15ml ： acetic acid (Wako) 5ml) and embedded in paraffin. Serial sections were made and PAS staining was done.

***Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis***

Total RNA was extracted using Trizol reagent (invitrogen). RNA was reverse transcribed into cDNA by using Reverse Transcriptase, SuperScript (invitrogen). Then RT-PCR amplifications were performed with PrimeSTAR Max DNA Polymerase (TAKARA). The primers used are listed in Table 3.

The reaction profiles was as follows: 1 cycle for 98°C for 5 min, 35 cycles of denaturation at 98°C for 10 sec, annealing for 10 sec at 55°C or 60°, and extension at 72°C for 5 sec.

***Immunohistochemistry***

Follicles fixed in 4%PFA were embedded in paraffin and serial sections were used for immunostaining. Sections were washed with PBS for 20 min and blocked for 30 min with 5% skim milk-containing PBS. Then sections were incubated with anti-connexin43 (SIGMA) overnight at 4℃. After three washes with PBS, sections were incubated with appropriate FITC-conjugated secondary antibodies (Molecular probes), and embedded in VECTASHIELD (mounting medium for fluorescence with DAPI) (Vector Laborateries).

***Culture of follicles***

Alpha MEM (invitrogen) supplemented with 5% FBS (Invitrogen), 100x ITS (Invitrogen), and 200mIU/μl FSH was prepared and 20 drops (20μl) were placed on 60mm well. These drops were covered with liquid paraffin (Nacalai tesque, INC) and incubated for 4 hours at 37℃. One follicle was then added to each of the drops. Half of the medium was changed every other day.

***Transplantation of ovaries and follicles under kidney capsules***

C.B-17/Icr-scid/scidJc1 mice were anesthetized and (1) fetal ovary from C57BL6, (2) follicles from newborn ovary from C57BL6 which were embedded in 0.2% collagen gel and (3)

ESDFLSs derived from cloneG embedded in 0.2% collagen gel, were transplanted under the kidney capsules of the SCID mice. Two weeks later, transplants were harvested and PAS staining was performed with the serial sections.

***Isolation of oocyte***

Ovaries and oocytes were obtained from C57BL/6 females. Ovaries of 5-day-old mice were washed with DPBS (-) and secondary follicles were isolated using 26G needle mechanically. Follicles were transferred into drops of α-MEM supplemented with 5% FBS and 300μg/ml of hyaluronidase and incubated at 37℃ for 5 min. After the hyaluronidase treatment, follicles were transferred to drops of α-MEM supplemented with 5% FBS and granulosa cells were removed by pipetting the oocytes with MICROPET (Clay Adams), whose diameter slightly shorter than that of oocytes. Denuded oocytes were transferred into a new drop of α-MEM supplemented with 5% FBS 4 times and the granulosa cells were removed.

***Analysis of Genomic imprinting status in ESDFLSs***

2% Low melting temperature agarose was prepared by melting Low melting temperature agarose (FMC BioProducts) into 1xPBS at 80℃. 8μl of 2% Low melting temperature agarose was mixed with 2μl of 1xPBS containing 20 cells at 37℃, then dropped into cold mineral oil

(SIGMA) to make 10μl of 1.6% Low melting temperature agarose beads. Beads were then left in 800μl of Lysis solution (10mM Tris-HCl, 10mM EDTA, 1% SDS, and 20μg/μl of Proteinase K) at 50℃ over night. 1ml of PMSF (40μg/ml in TE (pH7)) was added to the beads taken out from the Lysis solution and after incubation at 37℃ for 45 minute, the PMSF was removed. After repeating the PMSF treatment twice, 1ml of TE (pH9) was added to the beads and incubated at 37℃ for 15 minutes, and then removed. After 6 repeats of the TE treatment, 100μl of 1xM buffer was added and incubated for 15 minutes at 37℃, removed, performed again, and then incubated in 100μl of restriction solution (12U HindⅢ (TaKaRa)) at 37℃ overnight. On removal of the restriction solution, 0.3M NaOH treatment at 37℃ was performed for 15 minutes, repeated twice. After treatment with 500μl of 0.1M NaOH at 3℃ for 15 minutes, cells and beads were left for 15 minutes at 80℃, and then re-seeded by dropping into cold mineral oil. Beads were added to 1 ml of 5M Bisulfite solution (2.5M sodium meta bisulfate (SIGMA), 125mM hydroquinone(SIGMA), pH5), shielded from light, left at 50℃ for 7 hours. The bifulfite solution was removed and 6 rounds of TE (pH8) treatment (1ml) and removal at 37℃ for 15 minutes were done followed by 3 rounds of NaOH treatment (0.2ml) at 37℃ for 15 minutes, finally neutralized with 0.2ml of 1M HCl and all the solutions were removed. TE (pH8.0) treatments were done for 15 minutes at 37 ℃ three times and the TE was removed. Three D.W. washes were done and then the agarose

beads were used for Nested PCR. Primers used are listed below.

Snrpn-DMR sense (F1) 5'- TATGTAATATGATATAGTTTAGAAATTAG-3'

Snrpn-DMR sense (F2) 5'- AATTTGTGTGATGTTTGTAATTATTTGG-3'

Snrpn-DMR antisense (R2) 5'- ATAAAATACACTTTCACTACTAAAATCC-3'

Snrpn-DMR antisense (R1) 5'- AATAAACCCAAATCTAAAATATTTTAATC-3'

H19-DMR sense (F1) 5'- GAGTATTTAGGAGGTATAAGAATT-3'

H19-DMR sense (F2) 5'- GTAAGGAGATTATGTTTATTTTTGG-3'

H19-DMR antisense (R2) 5'-CCTCATTAATCCCATAACTAT-3'

H19-DMR antisense (R1) 5'-ATCAAAAACTAACATAAACCCCT-3'

The first round of PCR was performed using 10μl of agarose beads and LaTaq (TaKaRa) following the manufacture's protocol with following modifications. Agarose beads (10.0μl) was mixed with 10×LA buffer (9.6μl), 2.5mM dNTP (8.0μl,9 2.5mM MgCl2 (9.6μl), LA Taq (TaKaRa) (0.5μl), Snrpn-DMR (F1) primer (4.0 $\mu$ l), H19-DMR (F1) primer (4.0μl), Snrpn-DMR (R1) primer (4.0μl) and H19-DMR (R1) primer (4.0μl), and D.W. was added to make the total volume up to 96μl (one cycle at 94 °C for 2 min, 40 cycles at 94 °C for 30 sec, at 50 °C for 30 sec and at 68 °C for 1 min; and, finally, one cycle at 72 °C for 7 min). As for the appropriate polymerase, rTaq polymerase (TaKaRa), EX Taq polymerase (TaKaRa), LA Taq polymerase (TaKaRa) or Prime Star HS DNA polymerase (TaKaRa) was examined. First

round PCR products were purified using the Wizard® SV Gal and PCR Clean-UP System (promega) and used for a second PCR (one cycle at 94 °C for 2 min, 40 cycles at 94 °C for 30 sec, at 50 °C for 30 sec and at 68 °C for 1 min; and, finally, one cycle at 72 °C for 7 min). 1.00μl of first round PCR products were mixed with 3.6μl of 10xLA buffer, 3μl of 2.5mM dNTP, 3.6μl of 2.5mM of MgCl2, 0.15μl of LA Taq and 1.5μl of each forward and reverse primers and D.W. was added to make the total volume up to 36μl. Second round products were purified using the Wizard® SV Gal and PCR Clean-UP System and cloned into the TA cloning vector using a DynaExpress TA PCR Cloning Kit (pTAC-1) Large (BioDynamics Laboratory Inc.). Colony PCR and blue-white selection were done to check positive colonies, plasmids were purified using the Wizard®Plus SV Minipreps DNA Purification System, and the products were sequenced by standard methods to check the state of methylation.

## ***Results***

### *1. Elucidation of the role of gap junctions in folliculogenesis*

#### *Construction of a porcine oocyte cDNA library*

We focused on connexin genes specifically expressed in porcine oocytes. Since the sequence of the porcine genome was unavailable when we began this study, we first constructed an oocyte-specific cDNA library to identify which connexin genes are expressed in these cells. We collected about 3,000 oocyte/cumulus cell complexes from porcine antral follicles, and then mechanically denuded the oocytes (Fig. 1A). We isolated total RNA from the oocytes and synthesized cDNA by reverse transcription (Fig. 1B). Products longer than 5 kb were observed, generating a profile comparable to that obtained from human placental total RNA. Using this RNA sample, we created a porcine oocyte cDNA library containing 1.6 x $10^6$ independent clones. The library was validated by confirming the presence of clones for genes known to be expressed in mammalian oocytes, such as *GDF9* and *MOS*, and the exclusion of clones for somatic cell-specific markers, such as *CYP19* [268] (Fig. 1C).

#### *Identification of GJC1 and GJA10 clones in the cDNA library*

Next, we searched for connexin genes expressed in our porcine oocyte cDNA library. Each connexin contains highly conserved domains as well as divergent domains that confer each

member with unique properties. Two sets of degenerate oligonucleotide primers specific for domains common to the connexin family were prepared; primer sets F1/F2 and R1/R2 correspond to extracellular domains I and II, respectively. Using this method, we previously identified multiple connexin genes expressed in porcine follicle cells [266]. PCR amplification using these primers and the oocyte cDNA library as a template produced distinct bands by agarose gel electrophoresis (Fig. 2A). F2/R2 generated two bands, F2/R1 and F1/R2 produced a single major band, and F1/R1 did not amplify any detectable product (data not shown).

PCR products were subcloned into the pT7Blue-T vector and sequenced. The products obtained from F2/R2 reactions generated two distinct bands encoding a portion of *GJA10* (upper band), which encodes the CX60 protein [267] and a homolog of mouse *Gjc1* (lower band) [269], which encodes the CX45 protein[269]. In contrast, the F2/R1 reaction generated a mixture of fragments encoding *GJA10* and *GJC1*, while F1/R2 amplified only the *GJC1* fragment. The clones traced to either *GJC1* or *GJA10* are shown in Fig. 2B. As porcine *GJC1* has not been previously reported at either the DNA or amino acid sequence level, we screened a porcine genomic library for porcine *GJC1* and cloned the entire coding region (DDBJ: AB470925).

Notably, we did not detect clones of *GJA4*, which encodes CX37, despite previous studies reporting strong expression of CX37 in several mammalian species [175]. Since the

pan-connexin primers we used might not have been efficient at amplifying porcine *GJA4*, we designed specific primers for porcine *GJA4*, *GJA4*-A (Table 1; [270]), and attempted to amplify this transcript from the cDNA library, using the porcine genomic library as a control. We obtained a product of the expected size from the genomic library (Fig. 3A) that showed the predicted AccI restriction digest pattern. However, no *GJA4* product was obtained from the cDNA library, suggesting that *GJA4* is not expressed at detectable levels in porcine oocytes. A faint band could be obtained using the cDNA library as a template, but this product differed in size from the expected band and could not be digested with AccI (Fig. 3A), suggesting it was a nonspecific product. In contrast, when *GJC1* and *GJA10* were PCR-amplified, both the cDNA and genomic libraries gave products of appropriate sizes with the expected restriction digest patterns (Fig. 3A), suggesting the absence of *GJA4* and the presence of *GJC1* and *GJA10* in our cDNA library.

To further confirm our findings, we directly analyzed the expression of *GJA4* mRNA in porcine oocytes by RT-PCR using *GJC1* as a positive control. *GJC1* appeared to be expressed at a significant level in both the cDNA library and oocytes, while *GJA4* was barely detected, confirming the above observations (Fig. 3B).

***Expression of GJC1/CX45 and GJA10/CX60 in porcine oocytes***

Given the identification of *GJC1* and *GJA10* clones in our oocyte cDNA library, we examined their expression and that of *GJA4* directly in porcine follicles using slices from porcine ovaries. First, the expression of these mRNAs in porcine oocytes was examined by *in situ* hybridization (Fig. 4A). We detected specific signals for both *GJC1* and *GJA10* in oocytes at three different stages from preantral through antral follicles. Little signal, however, was obtained with the *GJA4* probe, supporting the results of our cDNA screening. *GJA10* signal was also detected in cumulus and mural granulosa cells in antral follicles, as was previously reported [267].

The expression of CX45 and CX60 was further confirmed by immunohistochemistry. CX45 signal was clearly detected in oocytes, but not in surrounding cells, including cumulus cells (Fig. 4B). To examine CX60 expression, we prepared a monoclonal antibody against CX60 recognizing the extreme C-terminal sequence. CX60 signal was detected at the boundaries between oocytes and cumulus cells, with faint signal also found in cumulus granulosa cells in antral follicles (Fig. 4B). Since the expression of *GJA4* was undetectable at the mRNA level using multiple methods, we did not examine CX37 expression at the protein level. (Bars in figures represent 100μm)

***Comparative analysis of connexin genes expressed in porcine and mouse oocytes***

Since the expression of CX37 in mammalian oocytes is well-established, whereas the expression of CX45 in oocytes has never been reported, we compared the expression of CX37 and CX45 in mouse and porcine oocytes by immunohistochemistry. The anti-CX45 antibody used was raised against a 14 amino acid residue portion of human CX45, which shares 92% sequence similarity with the mouse and porcine homologs. The expression of CX37 in mouse oocytes was easily observed, whereas little CX45/*Gjc1* expression could be detected at either the protein or mRNA level in mouse oocytes (Fig. 5A, B). These results are consistent with previous reports [271], [191].

***Gap junction-mediated intercellular communication***

As the GJC activity of CX60 was unknown, we assessed it by a fluorescent dye-transfer experiment. When examined using a homotypic system of CX60 and a heterotypic system between CX60 and CX43 (Itahana et al., 1996; Kidder and Mhawi, 2002), dye-coupling was verified in both systems (Fig. 5). However, CX45/CX60 heterotypic channels did not show dye transfer activity (data not shown). Since the expression of GJA4 was undetectable at the mRNA level using multiple methods, we did not examine CX37 expression at the protein level.

***2. Establishment of an in vitro system for obtaining follicles from mouse ESCs***

***Construction of an Oct4-GFP-expressing ESC line***

Hübner and his co-workers have shown that by culturing ESCs without LIF or MEF with certain cell numbers allows ESCs to differentiate into primary follicles that contain oocytes *in vitro*. They introduced an *Oct4*-GFP reporter gene into ESCs so that they could follow the differentiation of ESCs to germ line cells because *Oct4* genes are expressed only in ESCs or germ cells. We established an ESC line that expressed ΔPE *Oct4*-neo-IRES-hr-GFP (*Oct4*-GFP) stably to follow their experiments and neomycin selection was done to obtain 8 clones (Clone A to G).

***Derivation of follicles from mouseESCs***

We next examined three ESC lines and one *Oct4*-GFP expressing ES cell clone; Clone G, to see whether they can differentiate into oocytes or follicles. With a certain cell number and culture conditions, two ESC lines from 129 Sv/J and Clone G differentiated into follicle-like structures (FLSs) (Table2). Furthermore, during the cloning of *Oct4*-GFP-expressing ESCs, we obtained ESC clones that derive FLSs much more efficiently than parental ESCs. While intact ESCs produce follicles very occasionally, clone G showed the dozens of efficiency in

derivation of FLSs much more than parental ESCs. There were two types of follicles on the day follicles appeared (termed as day 3 ESDFLSs because these FLSs appeared 3 days after the induction of germ cells from ESCs in our system.), those that adhered to the dish, and the others floated in the medium.

***Morphological and histological analyses***

ESDFLSs had almost the same morphology as control follicles of mouse ovaries under the microscope (Fig. 6A and B). When serial sections of ESDFLSs were viewed with PAS staining, they showed large oocyte-like cells in the center, and several layers of granulose-like cells, basal lamina, and theca-like cells in the outermost layer, which were the fundamental structures seen in the follicles derived from intact mouse ovaries (Fig. 6C and D). PGC that had entered the ovary first differentiated into inmature oocytes that were surrounded by one layer of somatic cells called granulosa cells during embryogenesis, which were called primary follicles. Then they began folliclogenesis to generate the mature oocyte: the growth of the oocyte itself and the increase in the number of layers of granulosa cells (pre-antral follicle), and, in the end, the formation of antrum (antral follicles). The derived oocyte was surrounded by one layer or a few layers of granulose cells, which means that our ESDFLSs were primary follicles. In the derivation dish, other types of cells were observed, such as epithelial-like cells,

fibroblast-like cells, and sometimes cardiac muscle-like cells (Fig. 6E). Besides the follicle-like structure, oocyte-like cells were observed too (Fig. 6F, white arrow), which contain a polar body-like structure (Fig. 6F, pink arrow)

***BMP4/8b and STO cell supernatant supports the derivation of follicles from mouse ESCs***

It has been reported that BMP4 and 8b were essential for the derivation of primordial germ cells *in vivo* and *in vitro*. It was also reported that cytokine leukemia inhibitory factor (LIF) promoted the growth of mouse primordial germ cells (PGCs) *in vitro* [199]. STO cells have been shown to produce LIF, and LIF has some effect on the survival and proliferation of germ cells *in vitro* as well as steel factor. So we generated BMP4, BMP8b, and BMP4/8b (both BMP4 and BMP8b)-expressing cell lines (Fig. 7A). Their supernatants were collected and added to the medium at various concentrations in our *in vitro* follicle-deriving system. We found that BMP4/8b supports the derivation of FLSs from mouse ESCs in our *in vitro* system (Fig. 7B), while STO cell cultured medium had little effect on the efficiency of the germ cell derivation (data not shown).

***Characterization of derived follicles***

We next examined whether these ESDFLSs expressed germ cell-specific genes in a RT-PCR

analysis and observed the expression of several marker genes such as *Figla*, *Gdf9*, *fragilis*, *Mv*h, *Dazl*, *stella*, *Gpr3*, *Zp3*, *Gpr3, Dmc1, Sycp2* and *Sycp3* (Fig. 8A). *Figlα* is essential for the very first step of primordial follicle formation, which expression results in the transcription of *Zps* that are the components of oocyte surrounding zaona, so the expression of this gene might have allowed the formation of follicles from ESCs. The expression of *Gdf9* from oocyte works for the early staged hormone independent cell autonomous folliculogenesis and decreases during in late staged ovarian growth. *Mvh* and *stella* are detected in developing oocytes. The expression of *Mvh* decreases as the follicles mature, while, although *stella* expression diminishes once after PGCs enter meiosis at E13.5, its expression reappears within immature oocytes of new born ovaries that last throughout the oocyte maturation. All of these genes essential for the early staged follicles were expressed in ESDFLSs. *Fragilis* and *Dazl* are also crucial for germ cell maintenance. The expression of *Gpr3, Dmc1, Sycp2* and *Sycp3*, four meiosos controlling gene indicated that the oocyte-like cell within ESDFLSs has entered meiosis and was meiotically arrested at prophase. However, several abnormal gene expression patterns were obserbed over ESDFLSs. *Nobox* and *Meisetz* (data not shown) were not detected in ESDFLSs. *Nobox* disruption results in the defect in folliculogenesi. C-kit is the tyrosine kinase receptor for Kit ligand (also termed as SCF or steel factor) and their interaction works not only for the PGC migration, proliferation, survival but also for the folliculogenesis,

although c-kit expression starts to decrease gradually 7 days after birth while Kit ligand exist on oocytes throughout the folliculogenesis. The decrease of *c-kit* expression observed in control ovary was not observed in ESDFLSs. Furthermore, *Oct4* that must be repressed by the disruption of *Nobox* was still expressed in the ESDFLSs.

Immunostaining for Cx43 suggested that ESDFLSs form gap junctions composed of Cx43 between the granulosa cells (Fig. 8B).

***In vitro cultivation of follicles***

A system for maturing the primary follicles *in vitro* has already been established and these follicles develop into antral follicles. Recently, Fukunaga and his coworkers showed that *in vitro* maturated follicles could ovulate when stimulated by FSH and LH, and that the oocyte could be fertilized *in vitro*. So we examined whether day 3 floating ESDFLSs could go through the same maturation process. On culturing for 14 days, control follicles showed complete folliculogenesis and formed the antrum (Fig. 9A-C), while day 3 ESDFLSs didn't show any folliculogenesis, and rather the oocyte-gulanulosa cell complex was degraded in this one drop – one follicle cultivation system. Some of the day 3 ESDFLSs showed parthenogenetic development and differentiated into somatic cells a few days after derivation from ESCs. So we cultured all day3 ESDFLSs in our derivation medium for a few days and

excluded such non-follicle cells. When we cultured the remaining non-abnormal ESDFLSs at the proper density / number with selection several times, although no antrum formation was observed, they did go through some maturation, including the growth of the oocyte itself and an increase of granulosa cells (Fig. 9D-F). When we cultured the day 3 follicles, some of them started to adhere while others continued to float next day (day 4 ESDFLSs). Little folliculogenesis was observed from day4 adherent ESDFLSs because of the growth of other somatic cells to an dish that resulted in the arrest of cell proliferation due to contact inhibition (Fig. 9G). If we cultured day 3 ESDFLSs at lower concentrations, almost all ESDFLSs, in turn, went through abnormal differentiation. So we next picked up the floating day4 or later ESDFLSs and cultured them in the derivation medium, and they adhered to the dish and showed the folliculogenesis indicated before (Fig. 9H and I). Although the folliculogenesis from primary follicles to pre-antral follicles occured almost cell autonomously, the folliculogenesis to form the antrum was coordinated by hormones like follicle-stimulating hormone (FSH). So we checked whether our follicles expressed the *Fshr* gene. As expected, *Fshr* was not detected in ESDFLSs, which might be the cause of the arrest of folliculogenesis at the pre-antral stage (Fig. 8A).

***Transplantation of follicles under kidney capsules***

It has been reported that transplanting the fetal ovary under the kidney capsules of female severe combined immunodeficient (SCID) mice induces folliculogenesis within the ovary. So we next checked the ability of derived follicles to develop *in vivo*. We transplanted the fetal ovary as a control and the derived follicles embedded in collagen gel under the kidney capsules of SCID mice. Two weeks later, transplants were examined. The transplanted fetal ovary showed folliculogenesis and formation of the antrum. However, the ESDFLSs formed a teratoma, which included cells of all three germ layers, like gastrointestinal cells, blood cells (of bone marrow), muscle cells, Cardiac muscle cells, and Pancreatic cells or glandula mammaria (Fig. 10).

***Requirements of the arranged bisulfate method using agarose gel***

To check the state of methylation in oocytes, which cannot be obtained in large numbers, we tried to utilize bisulfate genomic sequencing using a low melting agarose gel, which allows a small number of cells to be examined [272]. We first examined whether this system works using our experimental materials; we checked for an appropriate polymerase that can amplify AT rich sequence from small scale of DNA, and appropriate time for bisulfate treatment because this treatment is known to induce DNA damage [273], and minimum number of cells required for the proper reaction, as for Snrpn and H19. Snrpn contains 16 CpGs within its

DMR [274] and they are demethylated completely in the paternal allele and methylated completely in the maternal allele in somatic cells [275], a methylation pattern maintained also in ES cells [276]. In male germ cells, after the demethylation of all CpGs in PGCs, CpGs of Snrpn remain to be demethylated while they become methylated gradually 10 days after birth in females [137]. H19 contains 15 CpGs in its DMR [137] and they are methylated completely in the paternal allele and are demethylated completely in maternal allele in somatic cells.

rTaq, EX Taq, LA Taq, and Prime Star were examined using 20 or 100 splenic somatic cells. LA Taq was most effective in terms of both cell number and DNA (Fig. 11A). So we next examined the appropriate time for bisulfite treatment because this treatment causes DNA damage time dependently [273]. Using 100 somatic cells, reaction time was examined for 4, 7, 10 and 13 hours and Snrpn genomic imprinting was checked. 7 hours was found to be the proper period of time for the further experiments (Fig. 11B). Finally, we searched for the minimum number of cells required for the proper bisulfate sequencing reaction and examined 5, 10 and 20 cells for Snrpn and H19 PCR amplification. Twenty cells were indicated as the minimam number required for the further experiments (Fig. 11C).

***Analysis of Genomic imprinting status in the oocytes from mouse ovary, granulose cells and oocytes from ESDFLSs***

Twenty oocytes, 20 granulose cells and 20 oocytes of ESDFLSs were examined for genomic imprinting. We investigated CpG regions of Snrpn and H19, and found that oocytes from mice showed complete demethylation while granulosa cells showed a mixed methylation pattern (Fig. 12A and B). However, oocytes of ESDFLSs showed that the CpG regions of Snrpn were demethylated completely and on the other hand CpGs of H19 were methylated completely, which resemble the genomic imprinting in male germ cells (Fig. 12C).

## ***Discussion***

### *1. Elucidation of the role of gap junctions in folliculogenesis*

The mammalian cumulus-oocyte complex (COC) is a model example of a multicellular structure that expresses multiple connexins and relies upon GJCs for proper development. The expression of several connexins, including CX26, CX30.3, CX32, CX37, CX43, and CX57/CX60, has been reported in ovarian follicles of different species, including the mouse, rat, pig, sheep, and cow [266], [277], [278]. The genes involved in folliculogenesis and/or oogenesis have been extensively analyzed using rodent systems [191], [175]. For example, CX43 and CX37 in the mouse localize to the zona pellucida [279], [280] and play critical roles in ovarian function [281], [282], [283]. The absence of CX43 in granulosa cells or CX37 in oocytes is sufficient to inhibit the development of both oocytes and follicles [175]. Namely, the oocytes of mice lacking CX37 do not reach their normal size and are meiotically incompetent, inducing premature luteinization of granulosa cells. However, previous work showed that CX43 can substitute for CX37 in oocytes [284], indicating that CX37 may not be uniquely required to link oocytes and granulosa cells. These results suggest that analyzing the expression pattern of each connexin and its regulatory mechanism is informative for understanding the physiological role of the gap junctions they form.

To understand the properties of gap junctions in the ovarian follicles of non-rodent mammals, we systematically searched for connexin genes expressed in porcine follicle cells in our

previous studies [266], [267]. Here, we searched for connexin genes expressed in porcine oocytes and identified *GJC1* and *GJA10*. Expression of these genes in oocytes was confirmed by RT-PCR and also by *in situ* hybridization, and immunofluorescence on sectioned porcine ovaries. *GJC1* and *GJA10* mRNA was detected in oocytes at at least three different stages: preantral, and early-antral, and late-antral. Immunofluorescence labeling showed gap junction-like CX60 signal at the interface between oocytes and cumulus cells and also diffuse staining in cumulus cells, consistent with *in situ* hybridization results here and previous studies [267]. CX45 signal, on the other hand, was observed not only at the surface, but also in the ooplasm. Interestingly, a similar pattern of localization was reported for CX37 in bovine oocytes [192]. It has been suggested that the profile may reflect the status of CX37 in storage or assembly [106]. These results suggest that CX45 and CX60 may possess different properties and/or play distinct roles in oogenesis/folliculogenesis, despite their similar expression profiles. Further studies are needed to clarify these points. We focused here on the analysis of connexin genes expressed during folliculogenesis in the porcine ovary. Obviously, many different approaches can be used to understand the process of folliculogenesis. For example, Whitworth et al. carried out an expressed sequence tag project to identify genes expressed in pig reproductive tissues and embryos, and a clone corresponding to GJC1 was identified as being expressed in germinal vesicle oocytes by Whitworth et al. in 2005.

CX37 has been shown to be expressed in the oocytes of many other species, such as the mouse, rat, sheep, and cow [192], [278], [285], and plays an essential role in mouse oogenesis [45], [106], [281], [284]. However, we did not detect the expression of CX37*GJA4* in porcine oocytes at either the mRNA or protein level under conditions that revealed *GJC1*/CX45 and *GJA10*/CX60 expression. Nonetheless, since the level of CX37 expression in bovine and sheep ovaries is known to change during folliculogenesis and oogenesis [192], [285], it is possible that CX37 is expressed at a specific stage other than the ones we examined in porcine ovaries, or is present at undetectable levels in porcine oocytes.

What then is the significance of CX45 and CX60, and not CX37, acting as the major connexins in porcine oocytes? It is possible that this is related to differences between the pig and other species, such as the mouse, in the process of folliculogenesis/oogenesis. For example, it is well-known that the components as well as the size of oocytes are not identical between these two species. Meiotic resumption in response to a luteinizing hormone (LH) surge takes a longer time in the pig before ovulation. However, it is necessary to identify the gap junctions formed between oocytes and cumulus cells to understand the significance of the expression of these genes in porcine oocytes. We did not observe the expression of CX45 in follicle cells in this study, whereas we previously observed the expression of CX30.3/*GJB4* and CX43/*GJA1* in addition to CX60/*GJA10* in cumulus cells, suggesting that one of these

connexins contributes to intercellular communication in the porcine oocyte/cumulus cell complex. CX45, however, is unable to form heterochannels with CX30.3 [263], while CX45 and CX43 have been reported to be compatible and to form functional channels, although the gating activity of heterotypic channels was rectified [286]. On the other hand, both homotypic channels of CX60 and heterotypic channels consisting of CX60 and CX43 in HeLa cells appeared to be capable of forming functional GJC. Regarding this, mice lacking CX57, a mouse ortholog of porcine CX60, did not exhibit any changes in oogenesis/folliculogenesis [191], [287], questioning the functional role of CX60 homotypic channels in porcine follicles, if CX60 and CX57 are equivalent in their physicochemical properties. Regardless, precise analyses of the regulatory mechanisms of *GJA4*, *GJC1* and *GJA10* expression and characterization of gap junction channels containing CX45 and CX60 are needed to better understand the physiological significance of these findings.

***2. Establishment of an in vitro system for obtaining follicles from mouseESCs***

PGCs are known to have the potential to become oocytes whether they have an XX chromosome or XY chromosome, and XY PGCs have been shown to follow the female fate when they migrate into ectopic tissue [21], [22], which correlates with the fact that one XY ESCs produced both male and female germ cells [246], [247], and that only the SRY gene that

started to be expressed at E10.5 to 11 and not the existence of the Y chromosome seems to trigger male differentiation and actually XY germ cells have been shown to differentiate into oocytes [288]. Sry expression seems to trigger the differentiation of the Sertoli cell lineage in the testis and oppose the differentiation into follicle cells, which results in the other cell fate determination. The hormones secreted by the gonad also in turn have some effect on its sex determination. Lim1 [289], [290], Sf1 [291], [292], Wt1 [293], [294], Lhx9 and Emx-2 mutant mice show the lack gonads, and GATA4 also seems to be involved in gonad differentiation [295]. After sex determination has occurred, other genes suggested below start to be expressed and participate in sex determination, mutation of which results in male-to-female sex reversal to different degrees. *M33*, *a mouse homologue of polycomb*, mutation causes half of the early stage leatha before weaning and resting pups showed the impaired gonad development of different degrees of male-to female sex reversal, which phenotype started to be observed at the time of the Sry expression [296]. *Fgf9* mutant results in a complete male-to female sex reversal [297], which correlate with the fact that *Fgf2 receptor* that act as fgf9 receptor deficient mice show the sex-reversal [298], and lack of *Pod1* also results in male-to female sex reversal [299]. A male to female sex reversal model has been established, named as B6.Y $^{TIR}$, B6.Y$^{POS}$ or B6.Y$^{DOM}$, and in these mouse models the Y chromosome of some local varieties of *Mus musculus domesticus* is placed onto the C57BL/6J (B6) inbred genetic background and

they develop only ovaries or ovotestes. Treatment with gonadotropins on these mice results in the ovulation of small numbers of eggs, which can be fertilized but cannot develop beyond the 2-cell stage. Further investigation has showed that although the XY oocyte goes through follicle formation, it has little ability to attach to cumulus cells and so XY females contain fewer late preantral follicles [300].

As for now, we have succeeded in establishing an *in vitro* system for generating follicles at the preantral stage (pre-late stage). We have established the high efficient GC deriving ESC line clone G starting from 129/SvJ XY ESC line. Two given ESC line and Clone G showed the potential to produce female germ cells under a certain conditions, Clone G showed almost the same morphology as parental ESCs when cultured on the feeder cells and follow the embryoid body (EB) formation by hanging drop method, although abnormal EB formation were observed for some degree. Clone G induced the follicle like structure or oocyte-like cells 3 days after derivation in our system, which require the shortest time for deriving germ cells from ESCs within the previously reported system. This indicates that clone G has committed to GC fate somehow, although clone G still possess the ability to differentiate into some somatic cells like beating cardiac muscle cells (data not shown), fibroblast cells or epithelial cells when differentiation was induced. Some of the germ cell specific genes have been identified to be expressed in these follicles, which suggest that they are likely to go through

some germ cell specific events.

However, many defects were also found as for the morphology, gene expression, the progression of folliculogenesis and establishment of genomic imprinting in ESDFLSs. Serial section of ESDFLSs revealed that although ESDFLSs possessed the fundamental follicle structure, the cell alignment and thus cell-cell interaction seemed abnormal. Cell-cell communication between GCs and the surrounding somatic cells work critically for the proper oogenesis and GC specific events. Disruption of cell-cell communication causes many defects such as the progression of meiosis at inappropriate stage when oocyte was denuded from surrounding follicle cells and gap junction between these cells were abolished since gap junctional communication transfer cAMP that controls the arrest of meiosis. Furthermore, *Cx43* deficient mice show the arrest of folliculogenesis and reduced oocyte growth. Although Cx43 seemed to exist, no gap junctional plaque was obserbed in ESDFLSs and some showed cytoplasmic expression, which indicates that gap junction might not have established properly within ESDFLSs, and this defect might participate in the arrest of folliculogenesis.

ESDFLSs showed the expression of genes that are essential in early staged follicles and that will decrease in further maturation steps such as *Mvh* and *Gdf9*, which indicated that the ESDFs were at early staged follicles, which correlate with their morphology. However, ESDFs showed abnormal gene expression pattern; expression of *c-kit* that must be repressed 7 days

after birth during folliculogenesis, and loss of *Nobox* expression that is essential for the early folliculogenesis. Furthermore, despite the repression of *Nobox*, *Oct4* expression was maintained in ESDFLSs. Transplantation of ESDFs into the kidney cupsul of SCID mice resulted in the formation of teratoma, which consisted of all three germ layers, like gastrointestinal cells, blood cells, cardiac muscle cells, and pancreatic cells or glandula mammaria while transplanted fetal ovary showed complete folliculogenesis and formed the antrum (Fig. 10), Since intact oocytes express *Oct4* and have no effect in teratoma formation, resulting pluripotency of ESDFLSs caused by *Oct4* expression probably not only in oocytes but also in surrounding follicle cells ectopically might have caused teratoma formation. Further immunostaining experiments should reveal the abnormal existence of GC specific proteins within ESDFLSs.

Day 3 ESDFLSs didn't undergo proper folliculogenesisi with the proper medium for ovarian follicles. Furthermore, day3 ESDFLSs seemed to require the selection and elimination of follicles that would go through abnormal development of differentiation, which resemble the PGC/oocyte apoptosis and oocyte selection in the ovary. Cultivation of the selected follicles with the derivation medium allowed the growth of the oocyte itself and the proliferation of the granulose cells. However, the follicles stopped their folliculogenesis before they entered the antral follicle stage, in other words, the late stage. We found that the *Fshr* was not expressed in

the derived follicles, though its expression is essential for the folliculogenesis from pre-late stage to late stage.

PGCs follow their sex determination according to the sex of the environmental around them whether they have an XX chromosome or XY chromosome. Therefore XY PGCs differentiate into oocytes and enter meiosis in a fetal ovary while XX PGCs develop into spermatogonia that enter mitotic arrest in a fetal testis, which correlate with the fact that XY ESCs produced both male and female GCs and ESDFs were derived from out XY ESC line. On the other hand, although XX GCs before E13.5 do not enter meiosis when cultured with fetal testis, E13.5 XX GCs enter meiosis properly even cultured with fetal testis, which indicate once sex commitment occurs, sexual environment no longer affect the sex determination. In our experiments, follicles appear from cells of XY karyotype. So combinational cultivation with female cells may help to obtain perfect female GCs or complete folliculogeneiss, although recent study showed that ESC derived oocytes seemed to have a developmental defect even when cultured with fetal gonadal somatic cells [245], which indicates.co-culturing with *Fshr* expressing cell might not be efficient enough to rescue them. Further inquests of conditions that resemble in vivo environment are preferable. We assume that derivation or cultivation of Clone G or ESDFLSs under 5% CO2, 5% O2, 90% N2 condition might show some effect on derivation of germ cells of on germ cell survival. We also suppose that addition of several

factors such as RA, nerve growth factor (NGF), bFGF, stem cell factor (SCF), forskolin, Glia cell-line derived neurotrophic factor (GDNF), TNF-alpha, leptin, activin, EGF, Insulin-Transferrin-Selenium (ITS), fetuin, sodium pyruvate, calcium lactate, stromal cell-derived factor 1 (SDF1) and 5-Azacytidine in order to activate some genes expression that are essential for egg derivation and maturation such as *FSH receptor* at the start of derivation or during the FLSs cultivation. The medium itself must be examined to find suitable one for germ cell derivation and culture, such as MEM alpha, Waymouth's MB 752/1 medium, Medium 199, DMEM/F-12, mWM medium that is used for cultivation of 2 cell to blastocyst, TYH medium that is used for sperm and unfertilized egg cultivation or high glucose DMEM. Co culture with gonadal cells, ovarian cells, or epithelial cells, which ever use them as feeder cells or aggrigate tem with ES cells, must be essential for normal and healthy germ cell development.

Recent study has shown that ESCs are likely to reverse into ESC state even after the differentiation into germ-like cells occurred and PGCs can differentiate back into pluripotent stem cells when exposed to a certain factors, and generally GCs and ESCs show the similar gene expression pattern as is also shown in our experiments. Actually, some day 3 ESDFs underwent abnormal differentiation and so follicle selection was dispensable for the further folliculogenesis experiments, which resemble the oocytes apoptosis and oocyte selection exist

in the ovary to exclude the abnormal ones.

Recently male to female sex reversal mouse model revealed several defect in XY oocytes. Although they developed ovaries and produced oocytes and follicles normaly, XY ovary contain fewer late preantral and no preovulately follicles since XY oocyte has low ability to attach to cumulus cells, which correlate with the observation in the arrest of folliculogenesis in our XY ESDFLSs. XY oocytes can be fertilized but failed to develop beyond the 2-cell stage maybe due to the defective oocyte cytoplasm. It has been reported that the presence of the Y chromosome impairs the progression of the second meiotic division and X chromosome activation / inactivation might affect the progression of meiosis because the paternal X chromosome is inactivated, while the Y chromosome remains activatied state and continues transcribing some genes on Y chromosomes [301]. Impaired pairing of sex chromosome has been observed in XY oocytes in second meiosis while first meiosis segregate X and Y chromosome separately as normally as other chromosomes. These reports indicate that sex chromosome has crucial effect in establishing the oocyte maturation.

Furthermore, we analyzed the erasure and reestablishment of genomic imprinting in the derived oocyte, which resulted in the male genomic imprinting pattern in the oocyte. Some reported that the chromosome one cell possessed affected the methylation state of imprinting genes, while others reported that oocytes in the testis show the female imprinting pattern

[302]. Furthermore, overexpression of Sry gene in XX GCs resulted in ablation of establishing the genomic imprinting and male impring pattern was established for some degree [303]. As is indicated before, several genes have been reported to be transcribed by y chromosome in XY female GCs. This indicates that he transcribes from Y chromosome might have affected ESDFLSs' oocyte to gain the male imprinting properties, so using *Sry* deficient ESCs might help overcome impairment in imprinting establishment in XY ESC-derived GCs. *In vitro* cultivation itself seems to have an effect on establishing abnormal imprinting pattern [304, 305]. However, recent study implicated that sex chromosome has no effect on establishment of imprinting. Since we didn't check out the state of imprinting in Clone G, we're not sure whether the establishment of imprinting, that is, the erasure and reestablishment of methylation, did occur in our derived oocyte. Surrounding XY cells such as granulose-like cells, epithelial like cells or fibroblast like cells under the ESDFLSs might have affected the imprinting state in ESDFLSs' oocyte.

We suppose these defects of the germ cell development in the derived oocyte were due to the imcomplete cell to cell communication between oocytes and GCs within the derived follicles. So we suppose the arrangement of condition itself to derive the follicle must help back up the incompleteness of the derivation of germ cells. And of course the use of XX ESCs or EG cells rather than XY ESCs would easily help solve these defects observed in ESDFLSs.

***Figures***

Figure 1

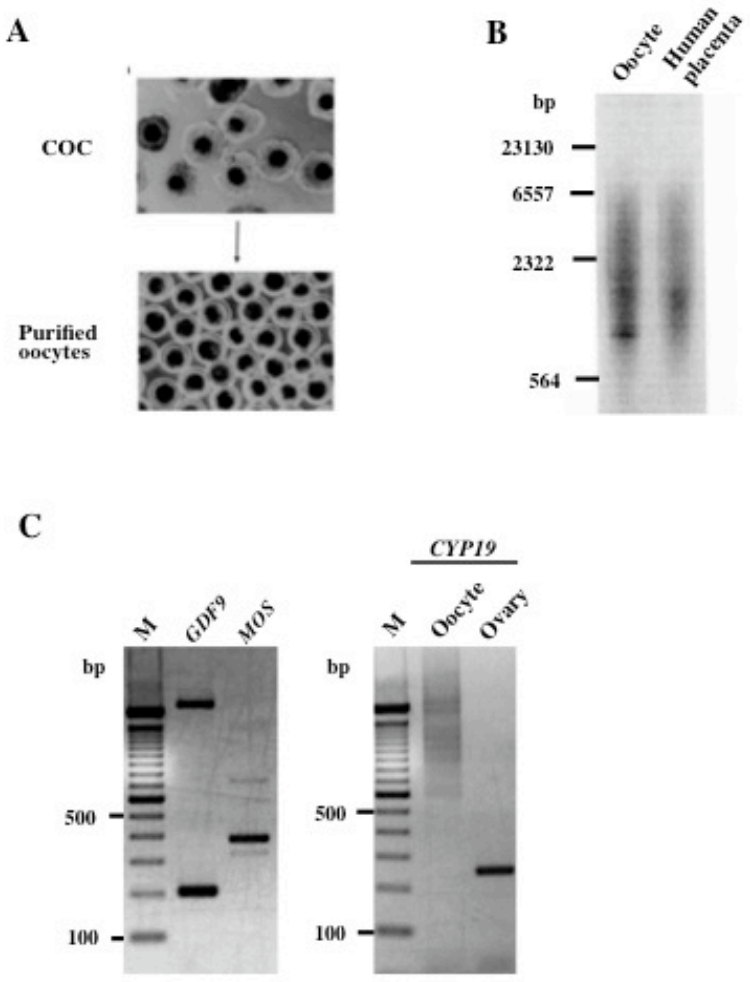
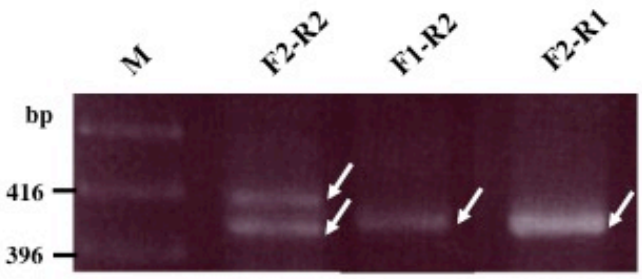



**Figure 1.** Construction of a porcine oocyte cDNA library.
A) Purification of porcine oocytes. Isolated cumulus oocyte complexes (COC) from antral follicles and denuded and purified oocytes are shown. B) Quality of total RNA isolated from the purified oocytes. cDNA was synthesized using RNA prepared from oocytes and control human placental RNA, and the products were subjected to agarose gel electrophoresis. The templates used for cDNA synthesis are indicated at the top. C) Validation of the cDNA library. Our porcine oocyte cDNA library was validated by pulling out clones encoding oocyte-specific genes, such as *GDF9* and *MOS*, by PCR with gene-specific primers (Table 1) (*left panel*). In addition, the exclusion of clones encoding genes specific to somatic cells, such as *CYP19*, was confirmed (*right panel*). The expression of *CYP19* in somatic cells was examined by RT-PCR from total ovary RNA. M: size markers.

**Figure 2**

**A**




**B**

| | Number of clones analyzed | | |
|---|---|---|---|
| **Primers:** | **F2/R2** | **F1/R2** | **F2/R1** |
| ***GJC1*** | **5** | **19** | **2** |
| ***GJA10*** | **5** | **0** | **6** |

**Figure 2.** Screening of connexin genes expressed in the oocyte cDNA library.
A) Agarose gel electrophoresis of PCR products amplified with degenerate primers. The products obtained using the combinations of degenerate primers were subjected to agarose gel electrophoresis with size markers as a reference. F1 and R1 did not produce any products and are not included here. The arrows indicate bands that were recovered and subjected to DNA sequence analysis. M: size markers. B) DNA sequence analysis and annotation of the PCR products. PCR products obtained on agarose gels were extracted and subjected to DNA sequence analysis. The number of clones analyzed and those encoding either *GJC1* or *GJA10* are shown.

**Figure 3**

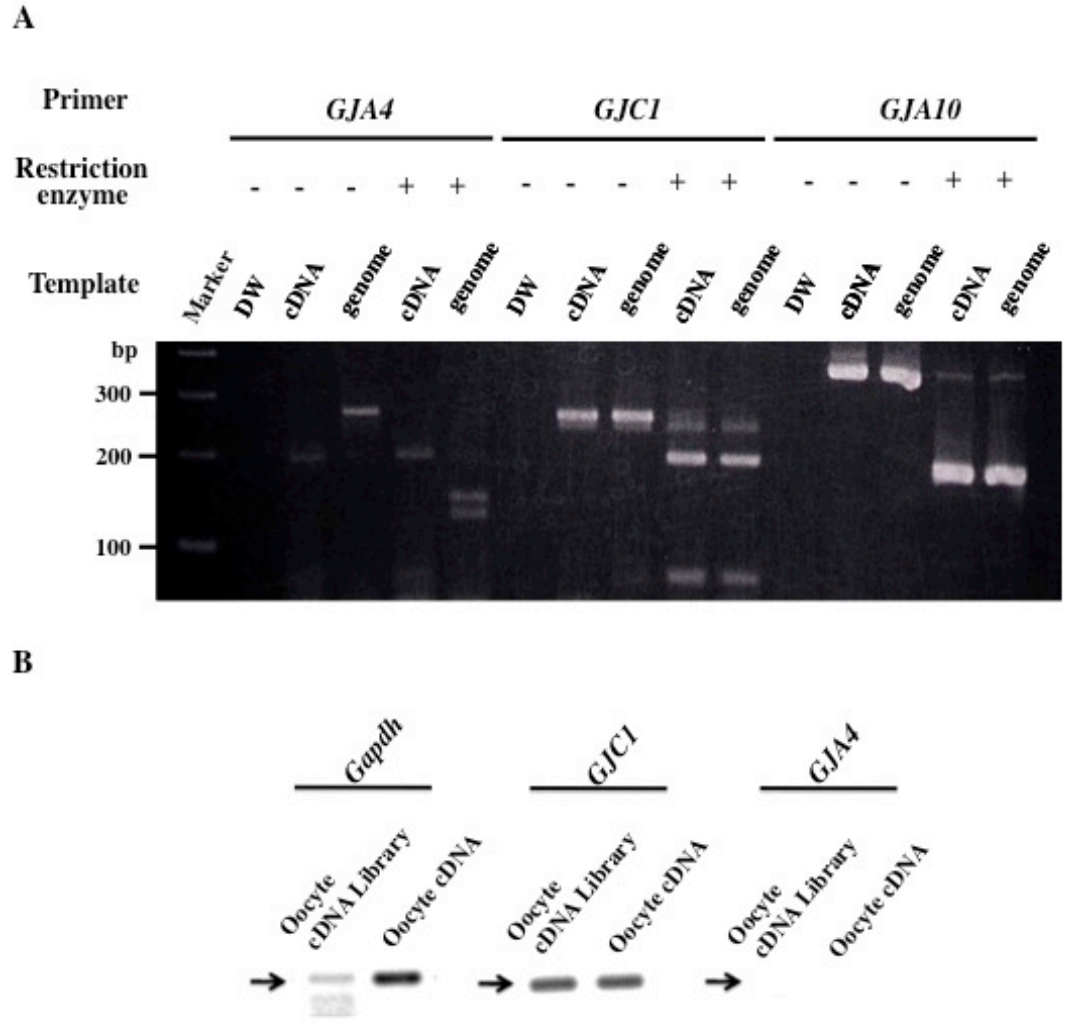



**Figure 3.** Expression analysis of *GJA4*, *GJC1*, and *GJA10*.
A) Detection of *GJA4*, *GJC1*, and *GJA10* in porcine genomic and oocyte cDNA libraries. Primers specific for *GJA4*, *GJC1*, and *GJA10*, respectively, were prepared (A sets in Table 1) and used for PCR with the porcine and oocyte cDNA libraries as templates. The products were treated with or without a restriction enzyme unique to each connexin gene and separated on agarose gels; AccI and SacI served as the restriction enzymes for *GJA4* and *GJC1* and for *GJA10*, respectively. DW: distilled water. B) Expression analysis of *GJC1,* and *GJA4* mRNA in porcine oocytes. RT-PCR was conducted using RNA from porcine oocytes in antral follicles with specific primer pairs listed in Table 1 (C-set). PCR using the oocyte cDNA library with the same primers was also carried out for the control. *Gapdh* was used as an internal marker. Arrows indicate the expected mobility of each PCR product.

**Figure 4**

**A**

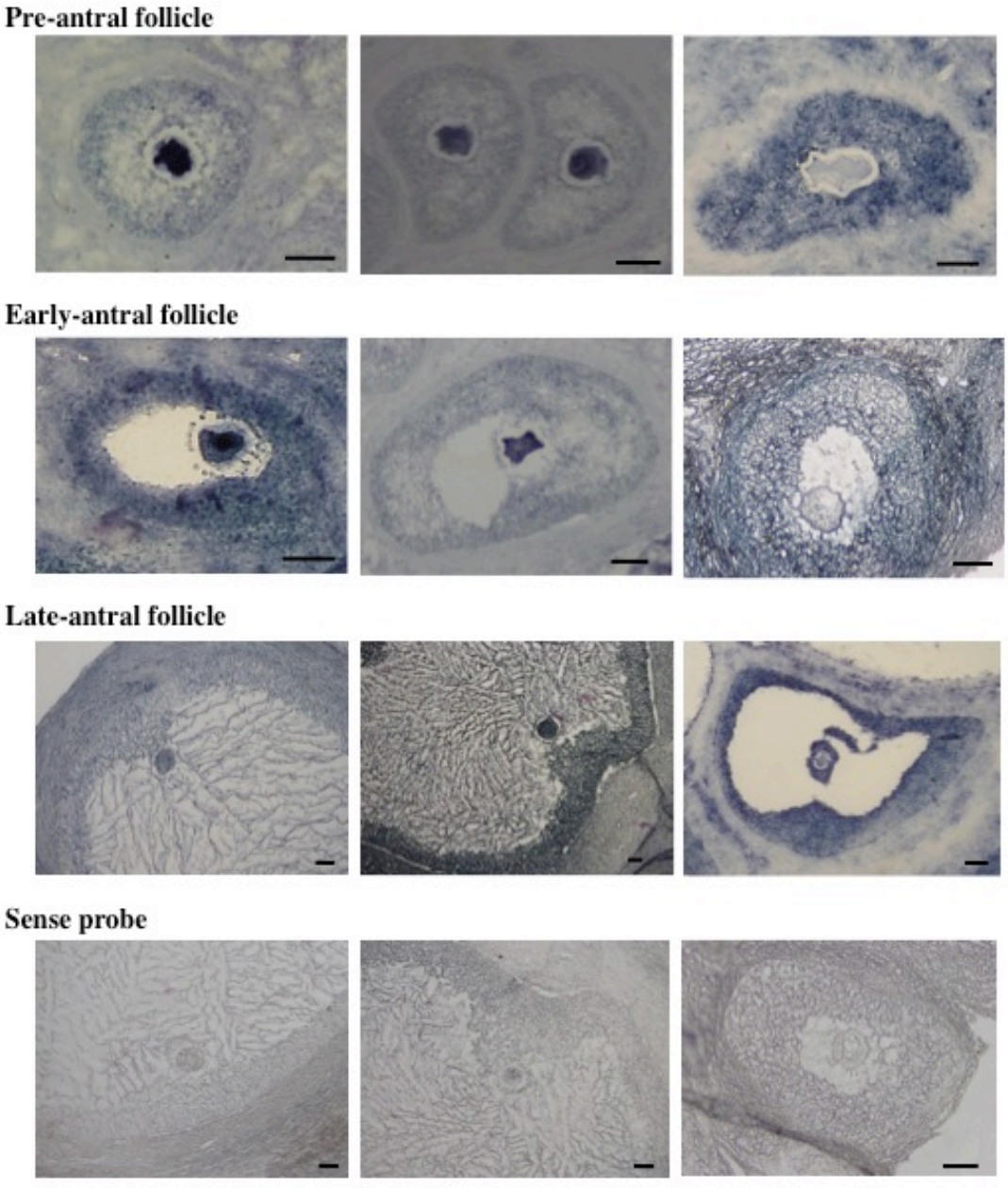

GJC1
GJA10
GJA4
Pre-antral follicle
Early-antral follicle
Late-antral follicle
Sense probe

**Figure 4**

**B**

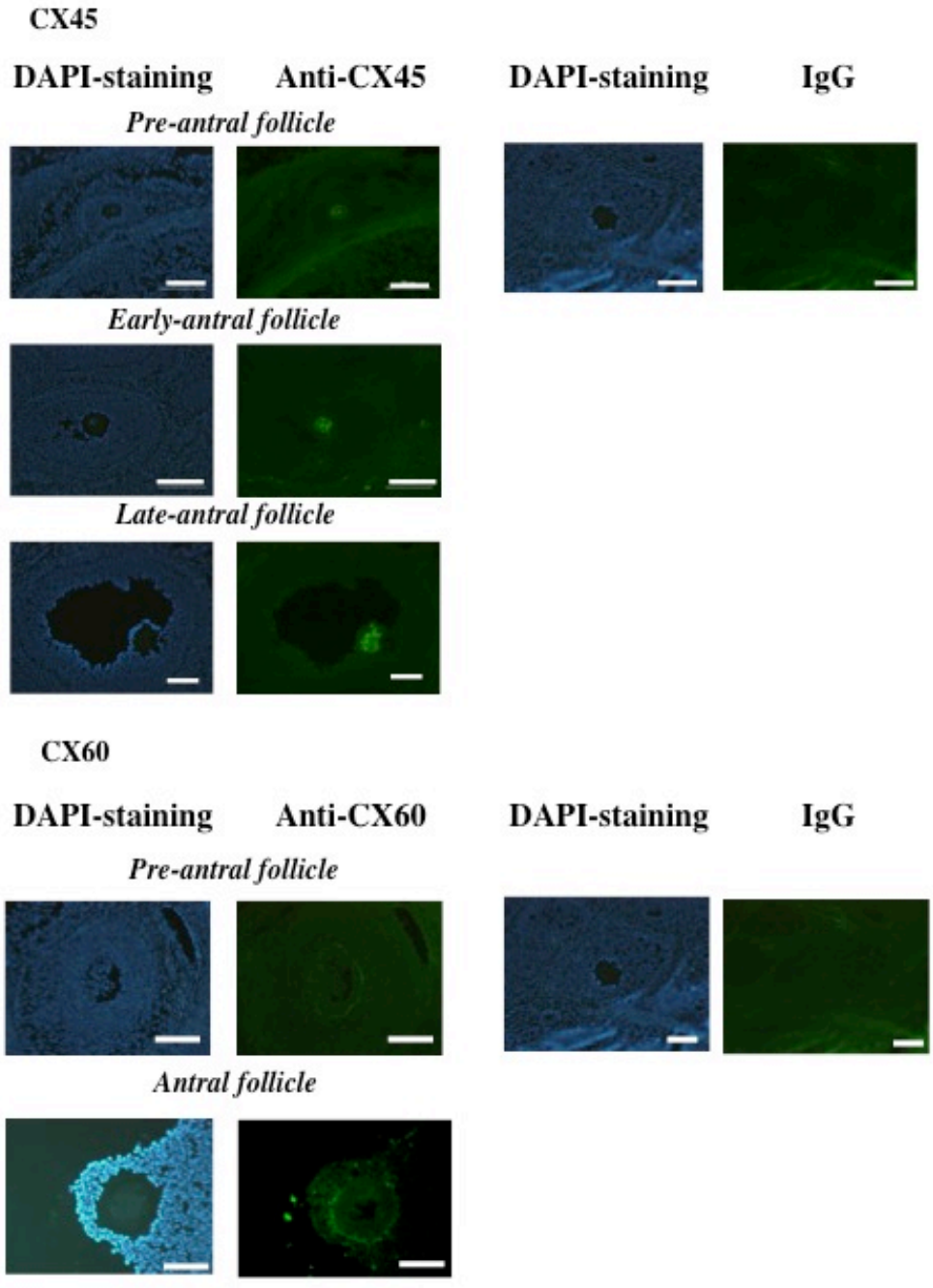

CX45
DAPI-staining
Anti-CX45
DAPI-staining
IgG
Pre-antral follicle
Early-antral follicle
Late-antral follicle
CX60
DAPI-staining
Anti-CX60
DAPI-staining
IgG
Pre-antral follicle
Antral follicle

**Figure 4.** Expression of CX45/*GJC1* and CX60/*GJA10* in porcine oocytes.
A) *In situ* hybridization using porcine ovarian sections. The expression of *GJC1*, *GJA10,* and *GJA4* mRNA was examined with either digoxigenin-labeled antisense or sense probes specific for each gene. Representative data with follicles from three different stages are shown. Bars, 100 μm.
B) Immunofluorescence for CX45 and CX60 in porcine follicles. Sections containing follicles were prepared and stained with either DAPI or the indicated antibodies, as described in the Materials and Methods. Bars, 200 μm.

**Figure 5**

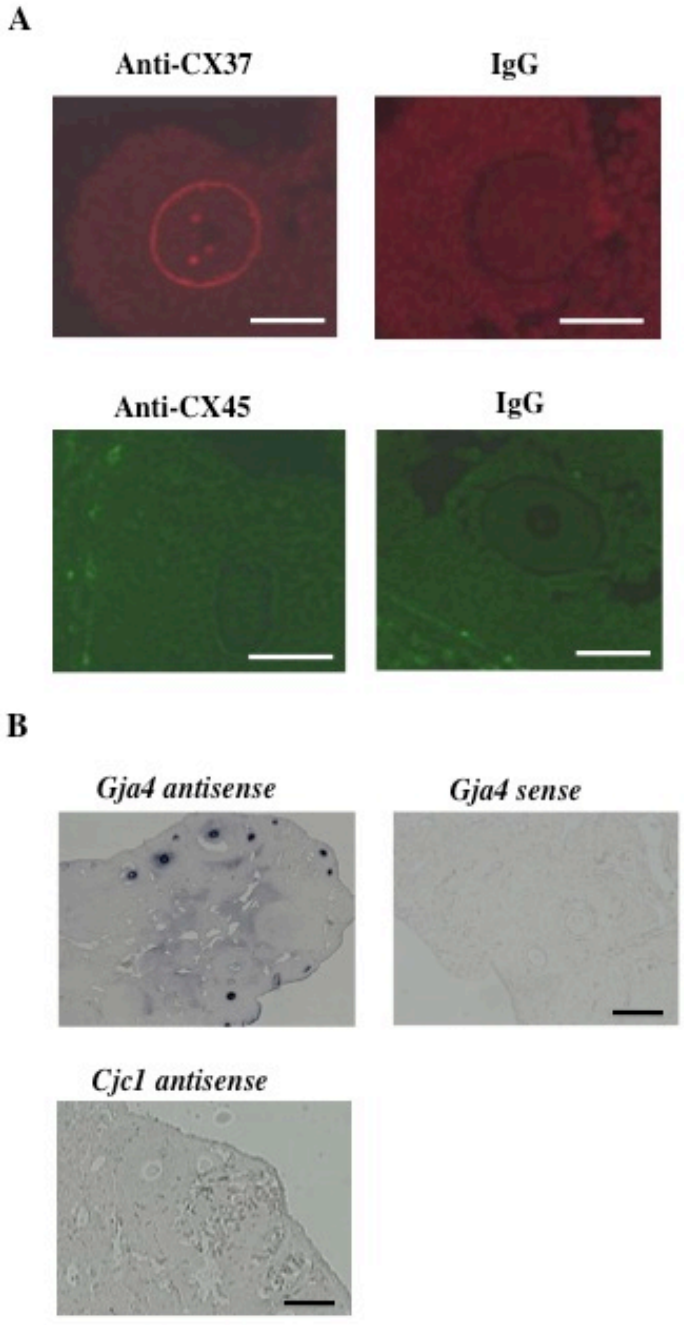



**Figure 5.** Expression of Cx37/*Gja4* and Cx45/*Gjc1* in mouse ovarian follicles.

A) Sections were prepared from mouse ovaries and stained with antibodies against either CX37 or CX45, as described in the Materials and Methods. IgG was used as a negative control. Bars, 20 μm. B) *In situ* hybridization using sections prepared as described in A) was performed with the respective antisense or sense probes. Bars, 100μm (*Gjc1*); 25 μm (*Gja4* sense).

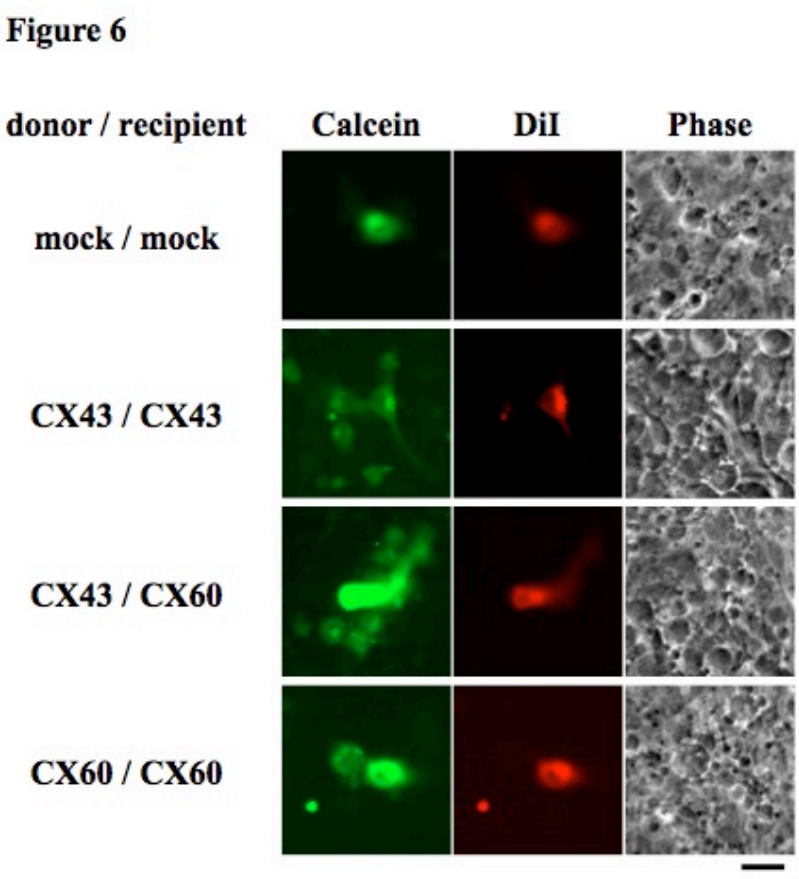



**Figure 6**. Fluorescent dye transfer experiments. HeLa cells transfected the porcine CX43 or CX60 expression vector were loaded with calcein-AM and DiI. The donor cells were settled onto the unstained recipient cell monolayers. Dye transfer was monitored under the fluorescent microscope at 4 h after the contact between donor and recipient cells. Mock transfected cells were also used as a negative control. Bar, 20μm.

**Table 1 Gene-specific primer sets**

| Gene | Forward | Reverse |
|---|---|---|
| Porcine | | |
| *GDF9* | 5’ -ctactgtaaaggggactctc- 3’ | 5’ -gttgcgatcatgtcttcgtattc- 3’ |
| *MOS* | 5’ -caaaatcagcgactttggttg- 3’ | 5’ -cagcccagttcagcttgtag- 3’ |
| *ZP3* | 5’ -ggaagggcctgctgttatc- 3’ | 5’ -gggaagcagacacggggctcacaag- 3’ |
| *CYP19* | 5’ -atgctgaacccaatgaacatcagc- 3’ | 5’ -cctatccagaccctcatgaattc- 3’ |
| *GJA4*-A | 5’ -acttcgagtgtaacactgcc- 3’ | 5’ -gagatcttggccatctgtcg- 3’ |
| *GJC1*-A | 5’ -cacggcgaagcagacaagaaggc- 3’ | 5’ -ctcaaacatggtccttgccagc- 3’ |
| *GJA10*-A | 5’ -ggcattgaggatgaaagggg- 3’ | 5’ -gaagccattgttgtacctagcc- 3’ |
| *GJA4*-B | 5’ -gagcccgtgtttgtgtgc- 3’ | 5’ -ggcccttacacgtactgt- 3’ |
| *GJC1*-B | 5’ -cgcggatccccaacagcatccctgaagat- 3’ | 5’ -cggggtaccctgctccccctggctataa- 3’ |
| *GJA10*-B | 5’ -cggggtaccgctagaattcagcactcagg- 3’ | 5’ –gctctagacctctctttccttctctcc- 3’ |
| *GJA4* -C | 5’ –ctcgtccagtgagcagaa- 3’ | 5’ –ctatacatactgcttctag- 3’ |
| *GJC1* -C | 5’ –gtccccgggaaaaaaaagccaaag- 3’ | 5’ –ttaaatccagacggaggtcttccc- 3’ |
| *SsCX43_1* | 5’ –aggtagtgtccgggcaacat– 3’ | 5’ –gattggctctagatctccagg- 3’ |
| *SsCX43_2* | 5’ –ctgaccgctcgagacatgggtgactggagt- 3’ | 5’ –ctgaccgacgcgtctctagatctccaggtc- 3’ |
| *SsCX45_1* | 5’ –cagggcaaaccagttccgtca- 3’ | 5’ –gcatccctgaagataaccagagcc- 3’ |
| *SsCX45_2* | 5’ –ctgaccgctcgagccatgagttggagcttc- 3’ | 5’ –ctgaccgacgcgtttaaatccagacggaggtc- 3’ |
| *SsCX60_1* | 5’ –agttgtctcttaccatggggg- 3’ | 5’ –cacttttgatggcagaggtgg- 3’ |
| *SsCX60_2* | 5’ –ctgaccgctcgagccatgggggattggaat- 3’ | 5’ –ctgaccgacgcgtgatggcagaggtggtat- 3’ |
| Control gene | | |
| *Gapdh* | 5’ –catgttccagtatgactccactc- 3’ | 5’ –ggcctcaccccatttgatgt- 3’ |

| Mouse | | |
|---|---|---|
| *Gja4* | 5’ -cccgggcccgatctggggctaatgtca- 3’ | 5’ -cgcggatcccaagacatcaaccagttccc- 3’ |
| *Gjc1* | 5’ –cccgagctcgtaactacagcagtccaatg- 3’ | 5’ -cggggtaccgtagcaaatcaggggatgg- 3’ |

**Table 2**

| | | $0.5 \times 10^4$ cells/cm$^2$ | $1 \times 10^4$ cells/cm$^2$ | $2 \times 10^4$ cells/cm$^2$ | $14 \times 10^4$ cells/cm$^2$ |
|---|---|---|---|---|---|
| RW-4 | 10%FCS | — | — | — | — |
| | 15%FCS | — | ○ | — | — |
| B6 | 10%FCS | — | — | — | — |
| | 15%FCS | — | — | — | — |
| E14 | 10%FCS | — | — | — | — |
| | 15%FCS | — | ○ | — | — |
| Clone | 10%FCS | — | — | — | — |
| | 15%FCS | — | ◎ | — | — |

**TABLE 2**. The conditions for obtaining the follicles from mouse ESCs
Two mouse ESC lines showed the ability to generate germ cells under certain conditions.
RW-4 : RW-4 mouse ESC line from 129Sv/J, B6 : C57BL/6 mouse ESC line, E14 : E14TG2a Ⅳ mouse ESC line from 129Sv/J

**Figure 7**

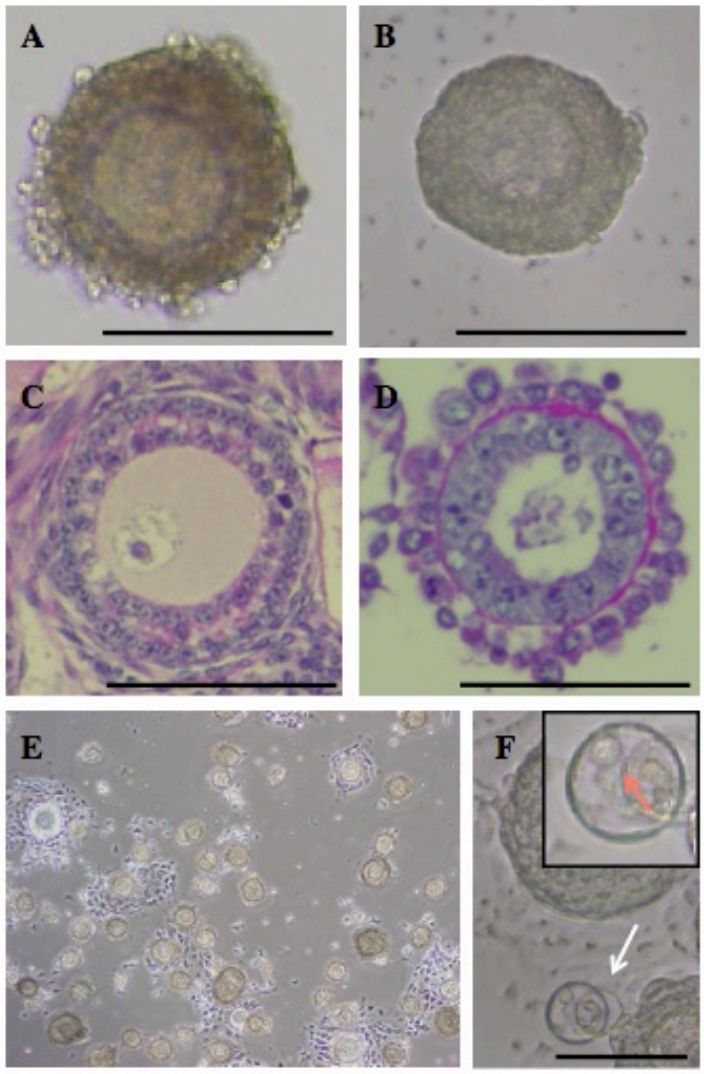



**Fig 7.** Morphological and histological analysis
A) and C) follicles from BL6 mouse ovary or B) and D) the ESDFLSs A) and B) under microscopy and C) and D) the PAS staining of the serial section of the follicles. E) Wide field of the derivation dish. F) oocyte-like cell (white arrow) with the polar body-like structure (pink arrow). Bars, 100μm

**Figure 8**

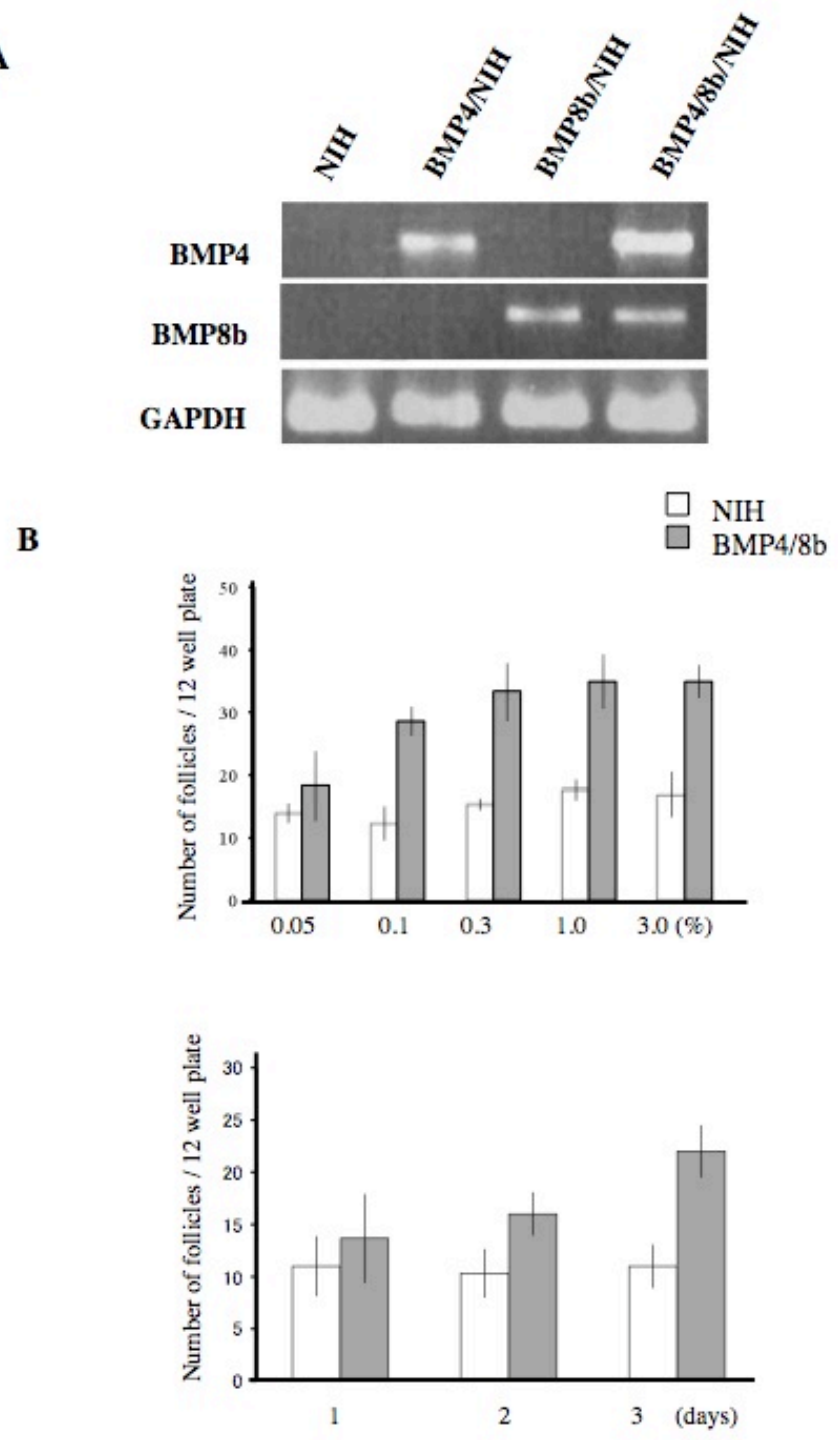



**Fig 8.** Construction of BMP-expressing cell lines and their effect on the follicular derivation. A) BMP genes were cloned and NHI3T3 was used for the introductionof each gene. B) Effects of the derivation of follicles from mouse ESCs were examined.

**Figure 9**

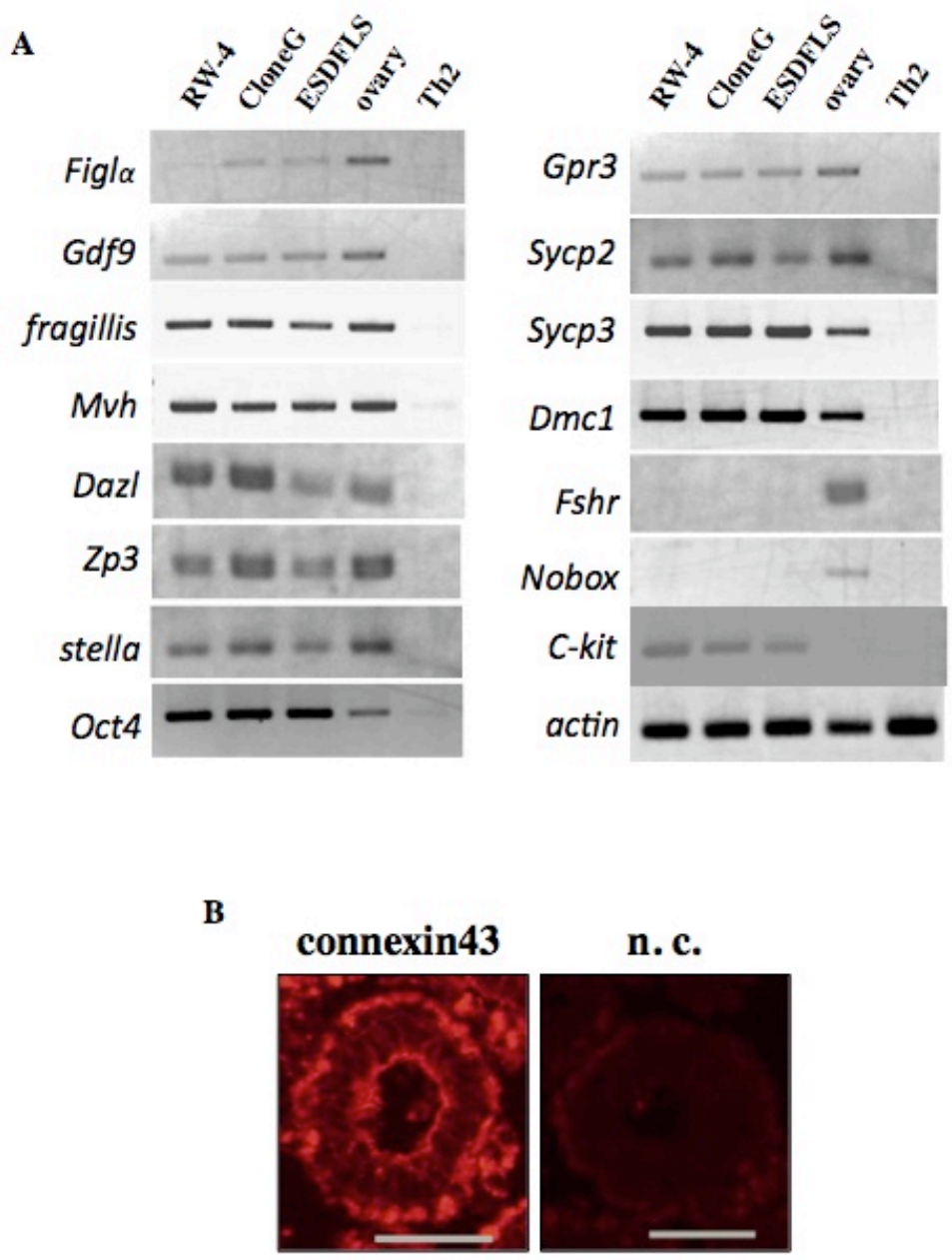



**Figure 9.** RT-PCR analysis of the germ cell specific genes

A) Expression of germ cell specific genes was examined by RT-PCR in RW-4, Clone G, ESDFLSs, ovarian cells and Th2 cells. B) Immunofluorescence staining of the mouse ovary and ESDFLSs.

**Figure 10**

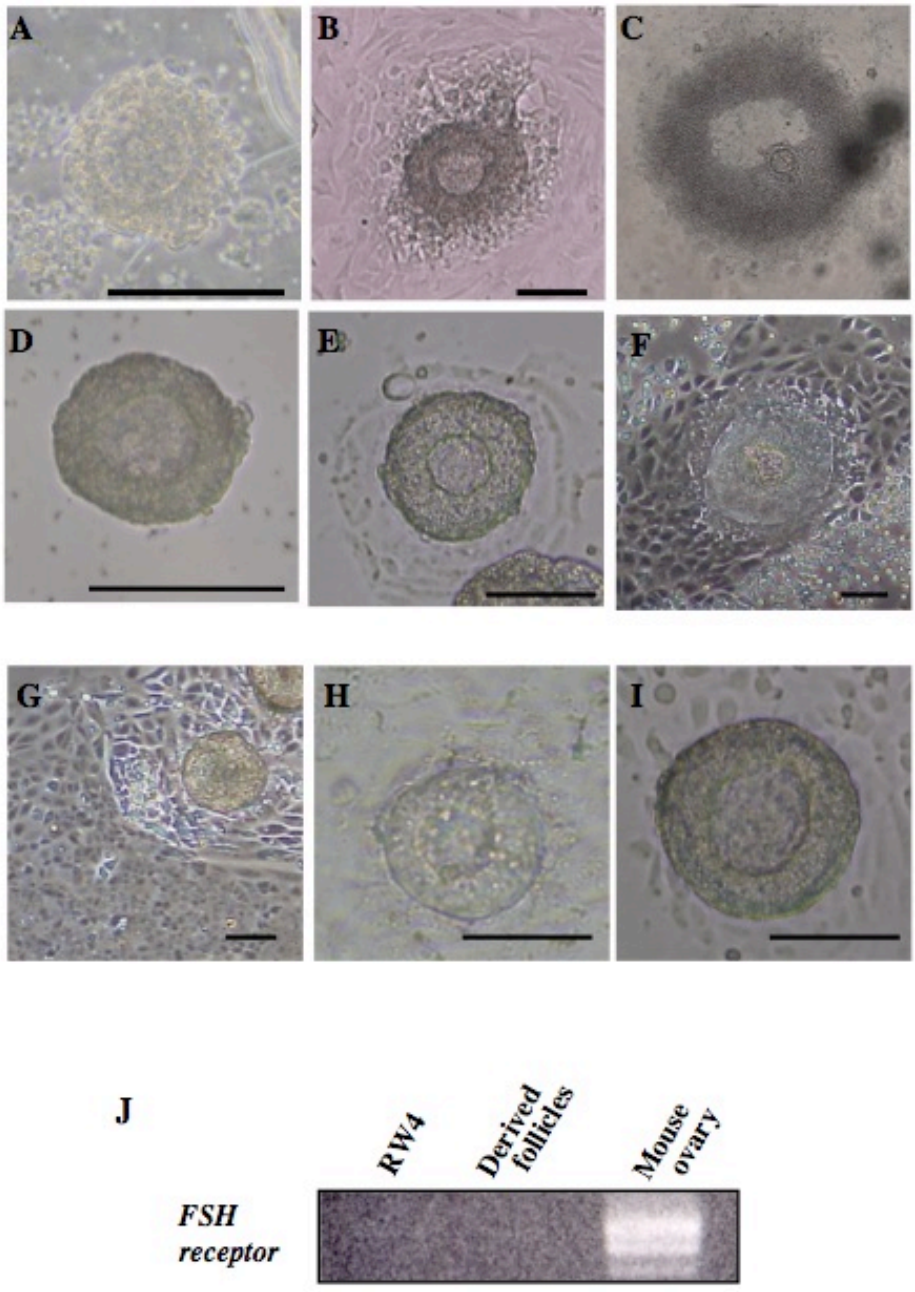



**Fig 10.** *In vitro* follliculogenesis

A)-C) *In vitro* culture of the follicles derived from C57BL6 mouse ovary and D)-F) ESDFLSs. G) Growth of somatic cells in the derivation dish. H) and I) Selected follicles that adhere.

**Figure 11**

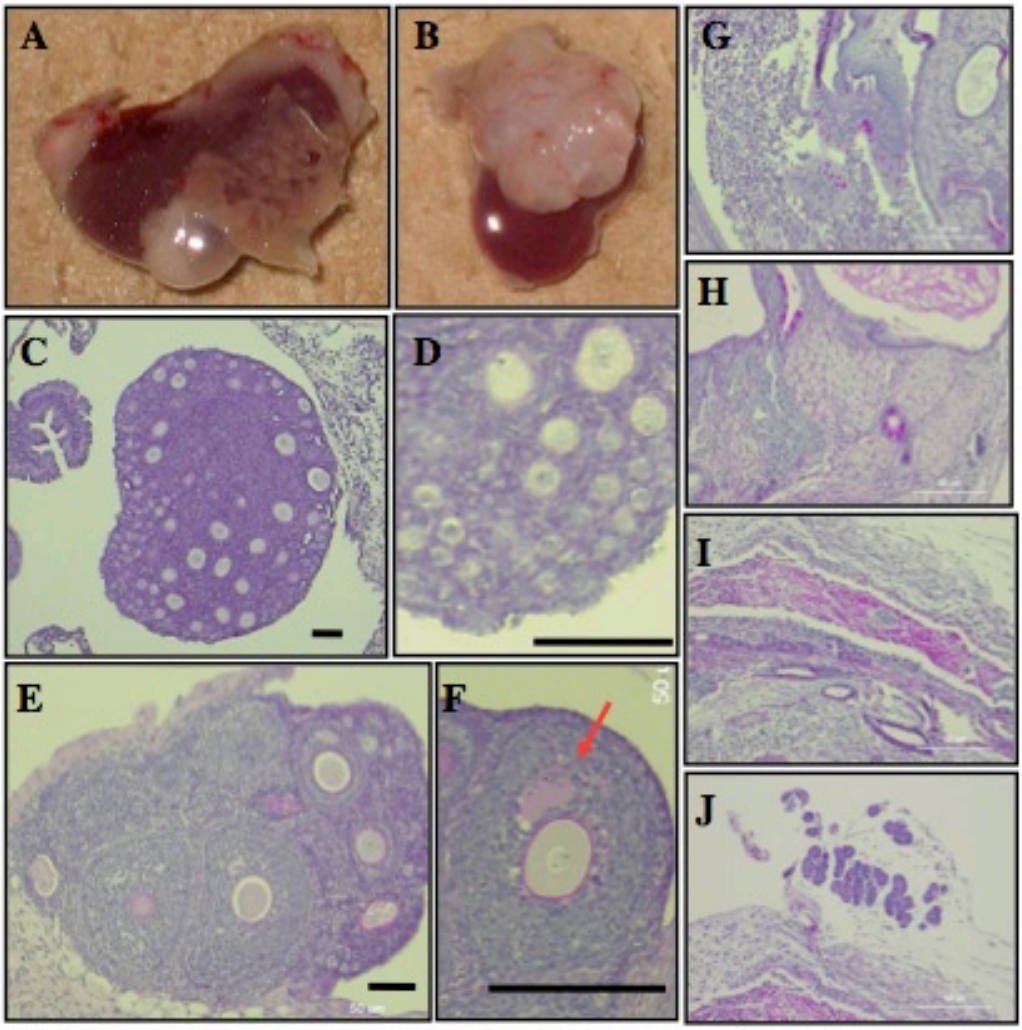
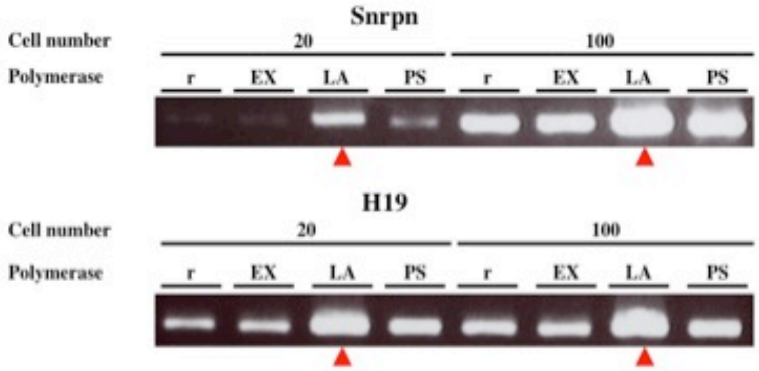



**Fig 11.** Transplantation of a ESDFLSs under the kidney capsule of a SCID mouse resulted in the formation of a teratoma

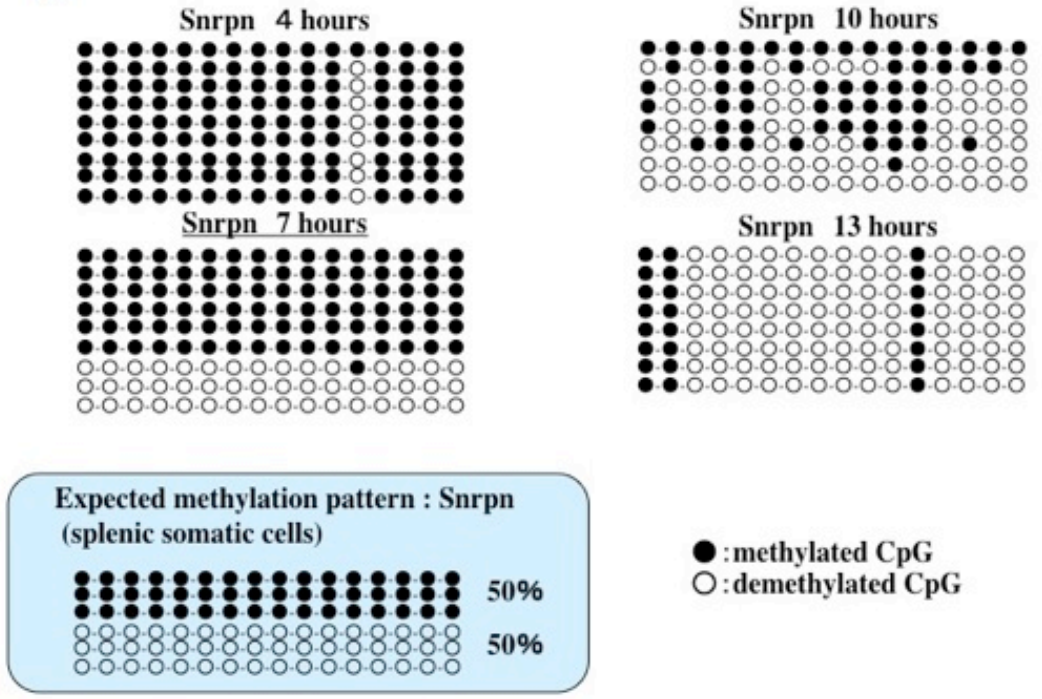
A), C)-F) When newborn mouse ovaries were grafted under the kidney capsule, folliculogenesis occurred and the antral cavity was seen in the ovary. Arrows in F) indicate the antrum. B), G)-J) ESDFLSs formed a teratoma which contained cells of various tissues.

**Figure 12**

A


Snrpn
Cell number
20
100
Polymerase
r
EX
LA
PS
r
EX
LA
PS
H19
Cell number
20
100
Polymerase
r
EX
LA
PS
r
EX
LA
PS

B


Snrpn 4 hours
Snrpn 10 hours
Snrpn 7 hours
Snrpn 13 hours
Expected methylation pattern : Snrpn
(splenic somatic cells)
50%
50%
●:methylated CpG
○:demethylated CpG

C

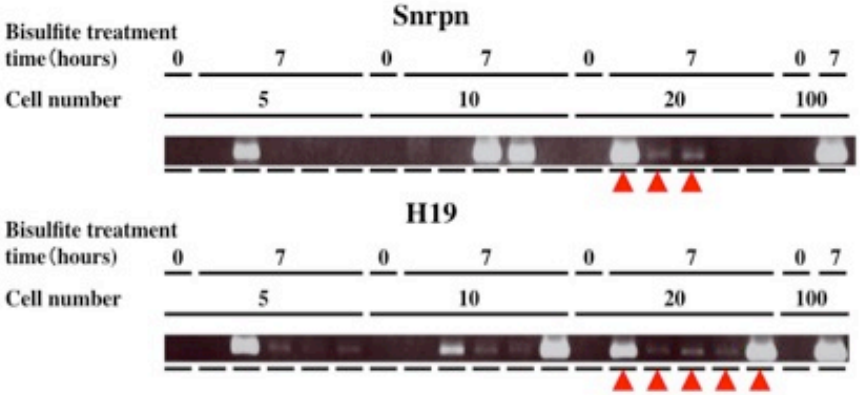






**Fig 12.**Requirements for proper genomic imprinting

Requirements for proper genomic imprinting over A) the polymerase, B) the treatment time and C) the cell number. Blue box shows the respectable pattern of genomic imprinting in B).

**Figure 13**

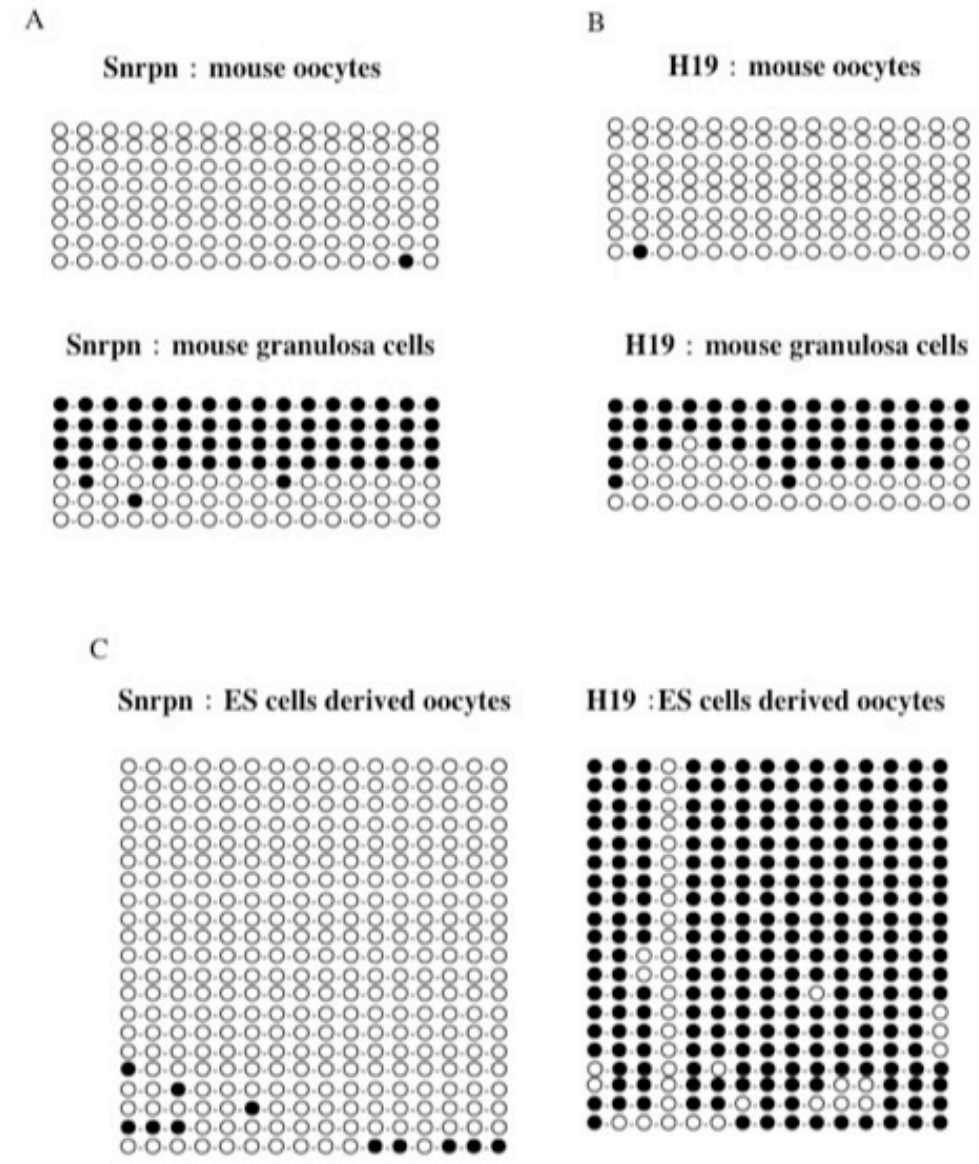



**Fig 13.** Methylation state of Snrpn and H19

Mouse oocytes showed almost complete demethylation while granulosa cells showed a mixed methylation pattern in both regions. CpGs of Snrpn were demethylated in oocytes derived from mouse ES cells while CpGs of H19 were methylated.

**Table 3 Gene-specific primer sets**

| Gene | Forward | Reverse |
|---|---|---|
| *Mvh* | 5' - TGCCTTTGCTCCGCACCAT - 3' | 5' - GGGTGAAGCACTTCAGGACC - 3' |
| *c-kit* | 5' - CAAGCTTTCGTTTCTGTGTA - 3' | 5' - CCTGCTATGTGAGAATCCAA - 3' |
| *Figl*α | 5' - ACTCCACCACGGATGACCTG - 3' | 5' - CTCGCACAGCTGGTAGGTTGG - 3' |
| *Sycp2* | 5' - GACACTGAAACCGAATGTGGA - 3' | 5' - TGTGGGTCTTGGTTGTCCTTT - 3' |
| *Sycp3* | 5' - ATGATGGAAACTCAGCAGCAAGAGA - 3' | 5' - TTGACACAATCGTGGAGAGAACAAC - 3' |
| *Gdf9* | 5' - CTGTATGTGTGACCTTGTGG - 3' | 5' - GATACAAGATGAGGGAAGGA - 3' |
| *stella* | 5' - AGCCCTGGGCCTCACAGCTT - 3' | 5' - CAGCCGTACCTGTGGAGAACAAGAG - 3' |
| *Oct4* | 5' - GGATCCTCGAACCTGGCTAA - 3' | 5' - CCACCTTCTCCAACTTCACG - 3' |
| *Fshr* | 5' - GCCTTACCTACCCCAGTCAC - 3' | 5' - TCAGCAAAGGCGAGGTTACA - 3' |
| *fragilis* | 5' - TTGCTCGGCACCATGAACCA - 3' | 5' - TGAAGCACTTCAGGACCGGA - 3' |
| *Dazl* | 5' - GCCAGCACTCAGTCTTCATC - 3' | 5' – GTTGGAGGCTGCATGTAAGT - 3' |
| *Nobox* | 5' – CCTTCAGTCACAGTTTCCGTAT - 3' | 5' – GGGAGGTTCTGGCAAGCAAT - 3' |
| *Zp3* | 5' – TTGAGCAGAAGCAGTCCAGC - 3' | 5' – CGGTTGCCTTGTGGATGGTC - 3' |
| *Gpr3* | 5' – TATCCACTCTCCAAGAACCATCTCG - 3' | 5' – GGAATTAAGCCCTGGTGGACCTAAC - 3' |
| *Dmc1* | 5' – GGACATTGCTGACCGCTTCAACGT - 3' | 5' – GGCGATCCTCAGTTCTCCTCTTCC - 3' |
| *β-actin* | 5' – GGGGTGTTGAAGGTCTCAAA - 3' | 5' – CCCTGAAGTACCCCATTGAA - 3' |

## ***Acknowledgements***

I would like to express profound gratitude to Dr. Jun Miyoshi, Dr. Eimei Sato and Prof. Tatsuo Takeya for invaluable support, encouragement, supervision and suggestions throughout this research. I feel grateful to Dr. Takuya. Ogawa for his support on my experiments and academic life. I also would like to thank Dr. Miki Okamoto for teaching me how to treat mouse embryonic stem cells, Dr. Hiroyoshi Ishizaki for teaching the transplant experiments, and Keiko Hori and Yu Ito for advices on the experiments. I thank Dr. Keiichiro. Yogo, Dr. Norihiro. Kitagawa-Ishida and Dr. Motofusa Akiyama for valuable suggestions throughout this study. I am also grateful for the cooperation of Yu Inoue, Masayo Ohash and Yasuo Kunitomo for this work. BMP4/8b experiments were done by Yu Inoue, the bisulfate genomic sequencing experiments were done by Yasuo Kunitomo, and the amplification of the novel connexin gene itself was done by Masayo Ohashi. I feel greatful to my advisor, Prof. Masashi Kawaichi, Prof. Junya Kato and Prof Kinichi Nakashima for their support and advices throughout the last 6 years. I thank all of my seniors and juniors for their support and advices. I would like to thank all of my friends for their indispensable supports. I feel most grateful to Dr. Masanori Imamura for his indispensable support, research advices, and love. Finally, I would like to express my appreciation to my family, my mother Midori Nitta, my father Nobuhiro Nitta and my aunt Matsuko Chiba, for their love and support throughout my life.

## ***References***


1. Spiegelman, M. and D. Bennett, *A light- and electron-microscopic study of primordial germ cells in the early mouse embryo*. J Embryol Exp Morphol, 1973. **30**(1): p. 97-118.
2. Ginsburg, M., M.H. Snow, and A. McLaren, *Primordial germ cells in the mouse embryo during gastrulation*. Development, 1990. **110**(2): p. 521-8.
3. Lawson, K.A., J.J. Meneses, and R.A. Pedersen, *Clonal analysis of epiblast fate during germ layer formation in the mouse embryo*. Development, 1991. **113**(3): p. 891-911.
4. Lawson, K.A. and W.J. Hage, *Clonal analysis of the origin of primordial germ cells in the mouse*. Ciba Found Symp, 1994. **182**: p. 68-84; discussion 84-91.
5. Tam, P.P. and S.X. Zhou, *The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo*. Dev Biol, 1996. **178**(1): p. 124-32.
6. Lawson, K.A., et al., *Bmp4 is required for the generation of primordial germ cells in the mouse embryo*. Genes Dev, 1999. **13**(4): p. 424-36.
7. Ying, Y., et al., *Requirement of Bmp8b for the generation of primordial germ cells in the mouse*. Mol Endocrinol, 2000. **14**(7): p. 1053-63.
8. Ying, Y., X. Qi, and G.Q. Zhao, *Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways*. Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7858-62.
9. Hayashi, K., et al., *SMAD1 signaling is critical for initial commitment of germ cell lineage from mouse epiblast*. Mech Dev, 2002. **118**(1-2): p. 99-109.
10. Saitou, M., et al., *Specification of germ cell fate in mice*. Philos Trans R Soc Lond B Biol Sci, 2003. **358**(1436): p. 1363-70.
11. Ohinata, Y., et al., *A signaling principle for the specification of the germ cell lineage in mice*. Cell, 2009. **137**(3): p. 571-84.
12. Sato, M., et al., *Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells*. Mech Dev, 2002. **113**(1): p. 91-4.
13. Saitou, M., S.C. Barton, and M.A. Surani, *A molecular programme for the specification of germ cell fate in mice*. Nature, 2002. **418**(6895): p. 293-300.
14. Ohinata, Y., et al., *Blimp1 is a critical determinant of the germ cell lineage in mice*. Nature, 2005. **436**(7048): p. 207-13.
15. Yabuta, Y., et al., *Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling*. Biol Reprod, 2006. **75**(5): p. 705-16.
16. Hayashi, K., S.M. de Sousa Lopes, and M.A. Surani, *Germ cell specification in mice*. Science, 2007. **316**(5823): p. 394-6.
17. Yamaji, M., et al., *Critical function of Prdm14 for the establishment of the germ cell lineage in mice*. Nat

Genet, 2008. **40**(8): p. 1016-22.

18. Barrow, J.R., et al., *Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis*. Dev Biol, 2007. **312**(1): p. 312-20.

19. Okamura, D., et al., *Cadherin-mediated cell interaction regulates germ cell determination in mice*. Development, 2003. **130**(26): p. 6423-30.

20. Chiquoine, A.D., *The identification, origin, and migration of the primordial germ cells in the mouse embryo*. Anat Rec, 1954. **118**(2): p. 135-46.

21. McLaren, A., *Oocytes in the testis*. Nature, 1980. **283**(5748): p. 688-9.

22. McLaren, A., *Germ cells and germ cell sex*. Philos Trans R Soc Lond B Biol Sci, 1995. **350**(1333): p. 229-33.

23. Tam, P.P. and M.H. Snow, *Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos*. J Embryol Exp Morphol, 1981. **64**: p. 133-47.

24. Anderson, R., et al., *The onset of germ cell migration in the mouse embryo*. Mech Dev, 2000. **91**(1-2): p. 61-8.

25. Bendel-Stenzel, M.R., et al., *The role of cadherins during primordial germ cell migration and early gonad formation in the mouse*. Mech Dev, 2000. **91**(1-2): p. 143-52.

26. Molyneaux, K.A., et al., *Time-lapse analysis of living mouse germ cell migration*. Dev Biol, 2001. **240**(2): p. 488-98.

27. Mahakali Zama, A., F.P. Hudson, 3rd, and M.A. Bedell, *Analysis of hypomorphic KitlSl mutants suggests different requirements for KITL in proliferation and migration of mouse primordial germ cells*. Biol Reprod, 2005. **73**(4): p. 639-47.

28. Peters, H., *Migration of gonocytes into the mammalian gonad and their differentiation*. Philos Trans R Soc Lond B Biol Sci, 1970. **259**(828): p. 91-101.

29. Blandau, R.J., B.J. White, and R.E. Rumery, *Observations on the Movements of the Living Primordial Germ Cells in the Mouse*. Fertil Steril, 1963. **14**: p. 482-9.

30. Koopman, P., et al., *Expression of a candidate sex-determining gene during mouse testis differentiation*. Nature, 1990. **348**(6300): p. 450-2.

31. Katoh-Fukui, Y., et al., *Male-to-female sex reversal in M33 mutant mice*. Nature, 1998. **393**(6686): p. 688-92.

32. Vainio, S., et al., *Female development in mammals is regulated by Wnt-4 signalling*. Nature, 1999. **397**(6718): p. 405-9.

33. Ara, T., et al., *Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1)*. Proc Natl Acad Sci U S A, 2003. **100**(9): p. 5319-23.

34. Yao, H.H., L. DiNapoli, and B. Capel, *Cellular mechanisms of sex determination in the red-eared slider turtle, Trachemys scripta*. Mech Dev, 2004. **121**(11): p. 1393-401.

35. Cui, S., et al., *Disrupted gonadogenesis and male-to-female sex reversal in Pod1 knockout mice*.

Development, 2004. **131**(16): p. 4095-105.

36. Fleming, A. and E. Vilain, *The endless quest for sex determination genes*. Clin Genet, 2005. **67**(1): p. 15-25.

37. Ottolenghi, C., et al., *Foxl2 is required for commitment to ovary differentiation*. Hum Mol Genet, 2005. **14**(14): p. 2053-62.

38. Bagheri-Fam, S., et al., *Loss of Fgfr2 leads to partial XY sex reversal*. Dev Biol, 2008. **314**(1): p. 71-83.

39. Molyneaux, K.A., et al., *The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival*. Development, 2003. **130**(18): p. 4279-86.

40. Motta, P.M., S. Makabe, and S.A. Nottola, *The ultrastructure of human reproduction. I. The natural history of the female germ cell: origin, migration and differentiation inside the developing ovary*. Hum Reprod Update, 1997. **3**(3): p. 281-95.

41. Fleischman, R.A., *From white spots to stem cells: the role of the Kit receptor in mammalian development*. Trends Genet, 1993. **9**(8): p. 285-90.

42. McCoshen, J.A. and D.J. McCallion, *A study of the primordial germ cells during their migratory phase in Steel mutant mice*. Experientia, 1975. **31**(5): p. 589-90.

43. Tanaka, S.S., et al., *IFITM/Mil/fragilis family proteins IFITM1 and IFITM3 play distinct roles in mouse primordial germ cell homing and repulsion*. Dev Cell, 2005. **9**(6): p. 745-56.

44. Anderson, R., et al., *Mouse primordial germ cells lacking beta1 integrins enter the germline but fail to migrate normally to the gonads*. Development, 1999. **126**(8): p. 1655-64.

45. Juneja, S.C., et al., *Defects in the germ line and gonads of mice lacking connexin43*. Biol Reprod, 1999. **60**(5): p. 1263-70.

46. Fujimoto, T., K. Yoshinaga, and I. Kono, *Distribution of fibronectin on the migratory pathway of primordial germ cells in mice*. Anat Rec, 1985. **211**(3): p. 271-8.

47. Atchison, F.W., B. Capel, and A.R. Means, *Pin1 regulates the timing of mammalian primordial germ cell proliferation*. Development, 2003. **130**(15): p. 3579-86.

48. De Felici, M., et al., *Experimental approaches to the study of primordial germ cell lineage and proliferation*. Hum Reprod Update, 2004. **10**(3): p. 197-206.

49. Dolci, S., et al., *Requirement for mast cell growth factor for primordial germ cell survival in culture*. Nature, 1991. **352**(6338): p. 809-11.

50. Dolci, S., M. Pesce, and M. De Felici, *Combined action of stem cell factor, leukemia inhibitory factor, and cAMP on in vitro proliferation of mouse primordial germ cells*. Mol Reprod Dev, 1993. **35**(2): p. 134-9.

51. Resnick, J.L., et al., *Long-term proliferation of mouse primordial germ cells in culture*. Nature, 1992. **359**(6395): p. 550-1.

52. Cooke, J.E., J. Heasman, and C.C. Wylie, *The role of interleukin-4 in the regulation of mouse primordial germ cell numbers*. Dev Biol, 1996. **174**(1): p. 14-21.

53. Resnick, J.L., et al., *Role of fibroblast growth factors and their receptors in mouse primordial germ cell growth*. Biol Reprod, 1998. **59**(5): p. 1224-9.

54. Pesce, M., et al., *Stem cell factor and leukemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis)*. Development, 1993. **118**(4): p. 1089-94.

55. De Felici, M. and M. Pesce, *Growth factors in mouse primordial germ cell migration and proliferation*. Prog Growth Factor Res, 1994. **5**(2): p. 135-43.

56. Morita, Y., et al., *Requirement for phosphatidylinositol-3'-kinase in cytokine-mediated germ cell survival during fetal oogenesis in the mouse*. Endocrinology, 1999. **140**(2): p. 941-9.

57. Tsuda, M., et al., *Conserved role of nanos proteins in germ cell development*. Science, 2003. **301**(5637): p. 1239-41.

58. Luoh, S.W., et al., *Zfx mutation results in small animal size and reduced germ cell number in male and female mice*. Development, 1997. **124**(11): p. 2275-84.

59. McLaren, A. and D. Southee, *Entry of mouse embryonic germ cells into meiosis*. Dev Biol, 1997. **187**(1): p. 107-13.

60. Koubova, J., et al., *Retinoic acid regulates sex-specific timing of meiotic initiation in mice*. Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2474-9.

61. Mehlmann, L.M., T.L. Jones, and L.A. Jaffe, *Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte*. Science, 2002. **297**(5585): p. 1343-5.

62. Hilscher, B., et al., *Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis*. Cell Tissue Res, 1974. **154**(4): p. 443-70.

63. Sorensen, R.A. and P.M. Wassarman, *Relationship between growth and meiotic maturation of the mouse oocyte*. Dev Biol, 1976. **50**(2): p. 531-6.

64. Motta, P.M., S.A. Nottola, and S. Makabe, *Natural history of the female germ cell from its origin to full maturation through prenatal ovarian development*. Eur J Obstet Gynecol Reprod Biol, 1997. **75**(1): p. 5-10.

65. Menke, D.B., J. Koubova, and D.C. Page, *Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave*. Dev Biol, 2003. **262**(2): p. 303-12.

66. Ruby, J.R., R.F. Dyer, and R.G. Skalko, *The occurrence of intercellular bridges during oogenesis in the mouse*. J Morphol, 1969. **127**(3): p. 307-39.

67. Pepling, M.E. and A.C. Spradling, *Female mouse germ cells form synchronously dividing cysts*. Development, 1998. **125**(17): p. 3323-8.

68. Pepling, M.E., M. de Cuevas, and A.C. Spradling, *Germline cysts: a conserved phase of germ cell development?* Trends Cell Biol, 1999. **9**(7): p. 257-62.

69. Pepling, M.E. and A.C. Spradling, *Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles*. Dev Biol, 2001. **234**(2): p. 339-51.

70. Jefferson, W., et al., *Neonatal genistein treatment alters ovarian differentiation in the mouse: inhibition of oocyte nest breakdown and increased oocyte survival*. Biol Reprod, 2006. **74**(1): p. 161-8.

71. Tingen, C., A. Kim, and T.K. Woodruff, *The primordial pool of follicles and nest breakdown in mammalian ovaries*. Mol Hum Reprod, 2009. **15**(12): p. 795-803.

72. Baker, T.G. and L.L. Franchi, *The fine structure of oogonia and oocytes in the rhesus monkey (Macaca mulatta)*. Z Zellforsch Mikrosk Anat, 1972. **126**(1): p. 53-74.

73. Morita, Y., et al., *Caspase-2 deficiency prevents programmed germ cell death resulting from cytokine insufficiency but not meiotic defects caused by loss of ataxia telangiectasia-mutated (Atm) gene function*. Cell Death Differ, 2001. **8**(6): p. 614-20.

74. Borum, K., *Oogenesis in the mouse. A study of the origin of the mature ova*. Exp Cell Res, 1967. **45**(1): p. 39-47.

75. Johnson, J., et al., *Germline stem cells and follicular renewal in the postnatal mammalian ovary*. Nature, 2004. **428**(6979): p. 145-50.

76. Pepling, M.E., *From primordial germ cell to primordial follicle: mammalian female germ cell development*. Genesis, 2006. **44**(12): p. 622-32.

77. Flaws, J.A., et al., *Effect of bcl-2 on the primordial follicle endowment in the mouse ovary*. Biol Reprod, 2001. **64**(4): p. 1153-9.

78. Soyal, S.M., A. Amleh, and J. Dean, *FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation*. Development, 2000. **127**(21): p. 4645-54.

79. McNeilly, J.R., et al., *Loss of oocytes in Dazl knockout mice results in maintained ovarian steroidogenic function but altered gonadotropin secretion in adult animals*. Endocrinology, 2000. **141**(11): p. 4284-94.

80. Salha, O., N. Abusheikha, and V. Sharma, *Dynamics of human follicular growth and in-vitro oocyte maturation*. Hum Reprod Update, 1998. **4**(6): p. 816-32.

81. Bukovsky, A., et al., *Quantitative evaluation of the cell cycle-related retinoblastoma protein and localization of Thy-1 differentiation protein and macrophages during follicular development and atresia, and in human corpora lutea*. Biol Reprod, 1995. **52**(4): p. 776-92.

82. Li, S., et al., *Stage-limited expression of myc oncoprotein in the human ovary during follicular growth, regression and atresia*. Endocr J, 1994. **41**(1): p. 83-92.

83. Smitz, J., et al., *Effects of recombinant activin A on in vitro culture of mouse preantral follicles*. Mol Reprod Dev, 1998. **50**(3): p. 294-304.

84. McGrath, S.A., A.F. Esquela, and S.J. Lee, *Oocyte-specific expression of growth/differentiation factor-9*. Mol Endocrinol, 1995. **9**(1): p. 131-6.

85. Knight, P.G. and C. Glister, *TGF-beta superfamily members and ovarian follicle development*. Reproduction, 2006. **132**(2): p. 191-206.

86. Kaivo-oja, N., et al., *Smad signalling in the ovary*. Reprod Biol Endocrinol, 2006. **4**: p. 21.

87. Thomas, F.H. and B.C. Vanderhyden, *Oocyte-granulosa cell interactions during mouse follicular*

*development: regulation of kit ligand expression and its role in oocyte growth*. Reprod Biol Endocrinol, 2006. **4**: p. 19.

88. Dissen, G.A., et al., *Nerve growth factor is required for early follicular development in the mammalian ovary*. Endocrinology, 2001. **142**(5): p. 2078-86.

89. Spears, N., et al., *The role of neurotrophin receptors in female germ-cell survival in mouse and human*. Development, 2003. **130**(22): p. 5481-91.

90. Eppig, J.J. and M.J. O'Brien, *Development in vitro of mouse oocytes from primordial follicles*. Biol Reprod, 1996. **54**(1): p. 197-207.

91. Bannister, L.A. and J.C. Schimenti, *Homologous recombinational repair proteins in mouse meiosis*. Cytogenet Genome Res, 2004. **107**(3-4): p. 191-200.

92. Di Giacomo, M., et al., *Distinct DNA-damage-dependent and -independent responses drive the loss of oocytes in recombination-defective mouse mutants*. Proc Natl Acad Sci U S A, 2005. **102**(3): p. 737-42.

93. Barlow, C., et al., *Atm-deficient mice: a paradigm of ataxia telangiectasia*. Cell, 1996. **86**(1): p. 159-71.

94. Barlow, C., et al., *Atm deficiency results in severe meiotic disruption as early as leptonema of prophase I*. Development, 1998. **125**(20): p. 4007-17.

95. Xu, Y., et al., *Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma*. Genes Dev, 1996. **10**(19): p. 2411-22.

96. Nishimura, K., et al., *Effects of macrophage colony-stimulating factor on folliculogenesis in gonadotrophin-primed immature rats*. J Reprod Fertil, 1995. **104**(2): p. 325-30.

97. Motro, B. and A. Bernstein, *Dynamic changes in ovarian c-kit and Steel expression during the estrous reproductive cycle*. Dev Dyn, 1993. **197**(1): p. 69-79.

98. Tisdall, D.J., et al., *Stem cell factor and c-kit gene expression and protein localization in the sheep ovary during fetal development*. J Reprod Fertil, 1999. **116**(2): p. 277-91.

99. Hutt, K.J., E.A. McLaughlin, and M.K. Holland, *Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis*. Mol Hum Reprod, 2006. **12**(2): p. 61-9.

100. Clark, D.E., et al., *Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries*. J Reprod Fertil, 1996. **106**(2): p. 329-35.

101. Dong, J., et al., *Growth differentiation factor-9 is required during early ovarian folliculogenesis*. Nature, 1996. **383**(6600): p. 531-5.

102. Rankin, T., et al., *Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss*. Development, 1999. **126**(17): p. 3847-55.

103. Rankin, T.L., et al., *Defective zonae pellucidae in Zp2-null mice disrupt folliculogenesis, fertility and development*. Development, 2001. **128**(7): p. 1119-26.

104. Rajkovic, A., et al., *NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression*. Science, 2004. **305**(5687): p. 1157-9.

105. Ackert, C.L., et al., *Intercellular communication via connexin43 gap junctions is required for ovarian*

*folliculogenesis in the mouse*. Dev Biol, 2001. **233**(2): p. 258-70.

106. Simon, A.M., et al., *Female infertility in mice lacking connexin 37*. Nature, 1997. **385**(6616): p. 525-9.

107. Driancourt, M.A., *Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction*. Theriogenology, 2001. **55**(6): p. 1211-39.

108. Webb, R., et al., *Molecular mechanisms regulating follicular recruitment and selection*. J Reprod Fertil Suppl, 1999. **54**: p. 33-48.

109. Findlay, J.K., et al., *Recruitment and development of the follicle; the roles of the transforming growth factor-beta superfamily*. Mol Cell Endocrinol, 2002. **191**(1): p. 35-43.

110. Conti, M., et al., *Role of cyclic nucleotide phosphodiesterases in resumption of meiosis*. Mol Cell Endocrinol, 1998. **145**(1-2): p. 9-14.

111. Bornslaeger, E.A., P. Mattei, and R.M. Schultz, *Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation*. Dev Biol, 1986. **114**(2): p. 453-62.

112. Wiersma, A., et al., *Phosphodiesterase 3 inhibitors suppress oocyte maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents*. J Clin Invest, 1998. **102**(3): p. 532-7.

113. Homa, S.T., *Calcium and meiotic maturation of the mammalian oocyte*. Mol Reprod Dev, 1995. **40**(1): p. 122-34.

114. Inoue, M., et al., *Activation of mitogen-activated protein kinase during meiotic maturation in porcine oocytes*. Zygote, 1995. **3**(3): p. 265-71.

115. Yuan, L., et al., *Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3*. Science, 2002. **296**(5570): p. 1115-8.

116. Chuma, S. and N. Nakatsuji, *Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling*. Dev Biol, 2001. **229**(2): p. 468-79.

117. Dolci, S. and M. De Felici, *A study of meiosis in chimeric mouse fetal gonads*. Development, 1990. **109**(1): p. 37-40.

118. Zemel, S., M.S. Bartolomei, and S.M. Tilghman, *Physical linkage of two mammalian imprinted genes, H19 and insulin-like growth factor 2*. Nat Genet, 1992. **2**(1): p. 61-5.

119. Li, E., C. Beard, and R. Jaenisch, *Role for DNA methylation in genomic imprinting*. Nature, 1993. **366**(6453): p. 362-5.

120. McGrath, J. and D. Solter, *Completion of mouse embryogenesis requires both the maternal and paternal genomes*. Cell, 1984. **37**(1): p. 179-83.

121. Rougier, N., et al., *Chromosome methylation patterns during mammalian preimplantation development*. Genes Dev, 1998. **12**(14): p. 2108-13.

122. Kanka, J., *Nuclear transplantation: reprogramming of transplanted nuclei*. Reprod Nutr Dev, 1999. **39**(5-6): p. 545-54.

123. Morgan, H.D., et al., *Epigenetic reprogramming in mammals*. Hum Mol Genet, 2005. **14 Spec No 1**: p. R47-58.

124. Reik, W., *Stability and flexibility of epigenetic gene regulation in mammalian development*. Nature, 2007. **447**(7143): p. 425-32.

125. Hajkova, P., et al., *Chromatin dynamics during epigenetic reprogramming in the mouse germ line*. Nature, 2008. **452**(7189): p. 877-81.

126. Lyrakou, S., M.A. Hulten, and G.M. Hartshorne, *Growth factors promote meiosis in mouse fetal ovaries in vitro*. Mol Hum Reprod, 2002. **8**(10): p. 906-11.

127. Yuan, L., et al., *The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility*. Mol Cell, 2000. **5**(1): p. 73-83.

128. Conti, M., et al., *Role of cyclic nucleotide signaling in oocyte maturation*. Mol Cell Endocrinol, 2002. **187**(1-2): p. 153-9.

129. Mehlmann, L.M., et al., *The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes*. Science, 2004. **306**(5703): p. 1947-50.

130. Pittman, D.L., et al., *Meiotic prophase arrest with failure of chromosome synapsis in mice deficient for Dmc1, a germline-specific RecA homolog*. Mol Cell, 1998. **1**(5): p. 697-705.

131. Gordo, A.C., et al., *Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes*. Mol Reprod Dev, 2001. **59**(1): p. 106-14.

132. Sagata, N., *What does Mos do in oocytes and somatic cells?* Bioessays, 1997. **19**(1): p. 13-21.

133. Colledge, W.H., et al., *Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs*. Nature, 1994. **370**(6484): p. 65-8.

134. Hashimoto, N., et al., *Parthenogenetic activation of oocytes in c-mos-deficient mice*. Nature, 1994. **370**(6484): p. 68-71.

135. Araki, K., et al., *Meiotic abnormalities of c-mos knockout mouse oocytes: activation after first meiosis or entrance into third meiotic metaphase*. Biol Reprod, 1996. **55**(6): p. 1315-24.

136. Lincoln, A.J., et al., *Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation*. Nat Genet, 2002. **30**(4): p. 446-9.

137. Lucifero, D., et al., *Methylation dynamics of imprinted genes in mouse germ cells*. Genomics, 2002. **79**(4): p. 530-8.

138. Szabo, P.E., et al., *Allele-specific expression of imprinted genes in mouse migratory primordial germ cells*. Mech Dev, 2002. **115**(1-2): p. 157-60.

139. Yamazaki, Y., et al., *Reprogramming of primordial germ cells begins before migration into the genital ridge, making these cells inadequate donors for reproductive cloning*. Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12207-12.

140. Lee, J., et al., *Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells*. Development, 2002. **129**(8): p. 1807-17.

141. Sanford, J.P., et al., *Differences in DNA methylation during oogenesis and spermatogenesis and their*

*persistence during early embryogenesis in the mouse*. Genes Dev, 1987. **1**(10): p. 1039-46.

142. Maatouk, D.M., et al., *DNA methylation is a primary mechanism for silencing postmigratory primordial germ cell genes in both germ cell and somatic cell lineages*. Development, 2006. **133**(17): p. 3411-8.

143. Bestor, T.H., *The DNA methyltransferases of mammals*. Hum Mol Genet, 2000. **9**(16): p. 2395-402.

144. Bourc'his, D., et al., *Dnmt3L and the establishment of maternal genomic imprints*. Science, 2001. **294**(5551): p. 2536-9.

145. Hata, K., et al., *Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice*. Development, 2002. **129**(8): p. 1983-93.

146. Li, E., T.H. Bestor, and R. Jaenisch, *Targeted mutation of the DNA methyltransferase gene results in embryonic lethality*. Cell, 1992. **69**(6): p. 915-26.

147. Howell, C.Y., et al., *Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene*. Cell, 2001. **104**(6): p. 829-38.

148. Kafri, T., et al., *Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line*. Genes Dev, 1992. **6**(5): p. 705-14.

149. Obata, Y. and T. Kono, *Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth*. J Biol Chem, 2002. **277**(7): p. 5285-9.

150. Lucifero, D., et al., *Gene-specific timing and epigenetic memory in oocyte imprinting*. Hum Mol Genet, 2004. **13**(8): p. 839-49.

151. Hiura, H., et al., *Oocyte growth-dependent progression of maternal imprinting in mice*. Genes Cells, 2006. **11**(4): p. 353-61.

152. La Salle, S., et al., *Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells*. Dev Biol, 2004. **268**(2): p. 403-15.

153. Song, Z., et al., *Maternal imprinting during mouse oocyte growth in vivo and in vitro*. Biochem Biophys Res Commun, 2009. **387**(4): p. 800-5.

154. Hajkova, P., et al., *Epigenetic reprogramming in mouse primordial germ cells*. Mech Dev, 2002. **117**(1-2): p. 15-23.

155. Kerjean, A., et al., *In vitro follicular growth affects oocyte imprinting establishment in mice*. Eur J Hum Genet, 2003. **11**(7): p. 493-6.

156. Durcova-Hills, G., et al., *Influence of sex chromosome constitution on the genomic imprinting of germ cells*. Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11184-8.

157. Lange, U.C., et al., *The fragilis interferon-inducible gene family of transmembrane proteins is associated with germ cell specification in mice*. BMC Dev Biol, 2003. **3**: p. 1.

158. Saitou, M., et al., *Blimp1 and the emergence of the germ line during development in the mouse*. Cell Cycle, 2005. **4**(12): p. 1736-40.

159. Di Carlo, A. and M. De Felici, *A role for E-cadherin in mouse primordial germ cell development*. Dev Biol, 2000. **226**(2): p. 209-19.

160. Seki, Y., et al., *Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice*. Dev Biol, 2005. **278**(2): p. 440-58.

161. Seki, Y., et al., *Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice*. Development, 2007. **134**(14): p. 2627-38.

162. Kurimoto, K., et al., *Specification of the germ cell lineage in mice: a process orchestrated by the PR-domain proteins, Blimp1 and Prdm14*. Cell Cycle, 2008. **7**(22): p. 3514-8.

163. Ancelin, K., et al., *Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells*. Nat Cell Biol, 2006. **8**(6): p. 623-30.

164. Surani, M.A., *Reprogramming of genome function through epigenetic inheritance*. Nature, 2001. **414**(6859): p. 122-8.

165. Arney, K.L., et al., *Epigenetic reprogramming of the genome--from the germ line to the embryo and back again*. Int J Dev Biol, 2001. **45**(3): p. 533-40.

166. Nakamura, T., et al., *Effects of Akt signaling on nuclear reprogramming*. Genes Cells, 2008. **13**(12): p. 1269-77.

167. Gardner, R.L., et al., *Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo*. J Embryol Exp Morphol, 1985. **88**: p. 349-63.

168. Takagi, N. and M. Sasaki, *Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse*. Nature, 1975. **256**(5519): p. 640-2.

169. Sugimoto, M. and K. Abe, *X chromosome reactivation initiates in nascent primordial germ cells in mice*. PLoS Genet, 2007. **3**(7): p. e116.

170. Chuva de Sousa Lopes, S.M., et al., *X chromosome activity in mouse XX primordial germ cells*. PLoS Genet, 2008. **4**(2): p. e30.

171. Willecke, K., et al., *Structural and functional diversity of connexin genes in the mouse and human genome*. Biol Chem, 2002. **383**(5): p. 725-37.

172. Sohl, G. and K. Willecke, *An update on connexin genes and their nomenclature in mouse and man*. Cell Commun Adhes, 2003. **10**(4-6): p. 173-80.

173. Stout, C., D.A. Goodenough, and D.L. Paul, *Connexins: functions without junctions*. Curr Opin Cell Biol, 2004. **16**(5): p. 507-12.

174. Kojima, T., et al., *Cx32 but not Cx26 is associated with tight junctions in primary cultures of rat hepatocytes*. Exp Cell Res, 2001. **263**(2): p. 193-201.

175. Gershon, E., V. Plaks, and N. Dekel, *Gap junctions in the ovary: expression, localization and function*. Mol Cell Endocrinol, 2008. **282**(1-2): p. 18-25.

176. Loewenstein, W.R., *Junctional intercellular communication and the control of growth*. Biochim Biophys Acta, 1979. **560**(1): p. 1-65.

177. Pelletier, D.B. and A.L. Boynton, *Dissociation of PDGF receptor tyrosine kinase activity from*

*PDGF-mediated inhibition of gap junctional communication*. J Cell Physiol, 1994. **158**(3): p. 427-34.

178. Hossain, M.Z., et al., *Disruption of gap junctional communication by the platelet-derived growth factor is mediated via multiple signaling pathways*. J Biol Chem, 1999. **274**(15): p. 10489-96.

179. Montecino-Rodriguez, E., H. Leathers, and K. Dorshkind, *Expression of connexin 43 (Cx43) is critical for normal hematopoiesis*. Blood, 2000. **96**(3): p. 917-24.

180. Sutor, B. and T. Hagerty, *Involvement of gap junctions in the development of the neocortex*. Biochim Biophys Acta, 2005. **1719**(1-2): p. 59-68.

181. Liu, S., et al., *Distinct cardiac malformations caused by absence of connexin 43 in the neural crest and in the non-crest neural tube*. Development, 2006. **133**(10): p. 2063-73.

182. Lo, C.W., *Genes, gene knockouts, and mutations in the analysis of gap junctions*. Dev Genet, 1999. **24**(1-2): p. 1-4.

183. Morley, G.E., et al., *Characterization of conduction in the ventricles of normal and heterozygous Cx43 knockout mice using optical mapping*. J Cardiovasc Electrophysiol, 1999. **10**(10): p. 1361-75.

184. Nagy, J.I., F.E. Dudek, and J.E. Rash, *Update on connexins and gap junctions in neurons and glia in the mammalian nervous system*. Brain Res Brain Res Rev, 2004. **47**(1-3): p. 191-215.

185. Ehrlich, H.P. and T. Diez, *Role for gap junctional intercellular communications in wound repair.* Wound Repair Regen, 2003. **11**(6): p. 481-9.

186. Chanson, M., et al., *Gap junctional communication in tissue inflammation and repair.* Biochim Biophys Acta, 2005. **1711**(2): p. 197-207.

187. Hotz-Wagenblatt, A. and D. Shalloway, *Gap junctional communication and neoplastic transformation*. Crit Rev Oncog, 1993. **4**(5): p. 541-58.

188. Pointis, G., et al., *Gap junctional communication in the male reproductive system*. Biochim Biophys Acta, 2005. **1719**(1-2): p. 102-16.

189. Sela-Abramovich, S., et al., *Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation*. Endocrinology, 2006. **147**(5): p. 2280-6.

190. Grummer, R., et al., *Different regulatory pathways of endometrial connexin expression: preimplantation hormonal-mediated pathway versus embryo implantation-initiated pathway.* Biol Reprod, 2004. **71**(1): p. 273-81.

191. Kidder, G.M. and A.A. Mhawi, *Gap junctions and ovarian folliculogenesis*. Reproduction, 2002. **123**(5): p. 613-20.

192. Nuttinck, F., et al., *Comparative immunohistochemical distribution of connexin 37 and connexin 43 throughout folliculogenesis in the bovine ovary.* Mol Reprod Dev, 2000. **57**(1): p. 60-6.

193. Valdimarsson, G., P.A. De Sousa, and G.M. Kidder, *Coexpression of gap junction proteins in the cumulus-oocyte complex*. Mol Reprod Dev, 1993. **36**(1): p. 7-15.

194. Reaume, A.G., et al., *Cardiac malformation in neonatal mice lacking connexin43*. Science, 1995. **267**(5205): p. 1831-4.

195. Carabatsos, M.J., et al., *Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence*. Dev Biol, 2000. **226**(2): p. 167-79.

196. Labosky, P.A. and B.L. Hogan, *Mouse primordial germ cells. Isolation and in vitro culture*. Methods Mol Biol, 1999. **97**: p. 201-12.

197. De Felici, M., *Regulation of primordial germ cell development in the mouse*. Int J Dev Biol, 2000. **44**(6): p. 575-80.

198. Godin, I., et al., *Effects of the steel gene product on mouse primordial germ cells in culture*. Nature, 1991. **352**(6338): p. 807-9.

199. Matsui, Y., et al., *Effect of Steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture*. Nature, 1991. **353**(6346): p. 750-2.

200. Geshi, M., et al., *Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells*. Biol Reprod, 2000. **63**(6): p. 1730-4.

201. Demeestere, I., et al., *Effect of insulin-like growth factor-I during preantral follicular culture on steroidogenesis, in vitro oocyte maturation, and embryo development in mice*. Biol Reprod, 2004. **70**(6): p. 1664-9.

202. Donovan, P.J., et al., *Studies on the migration of mouse germ cells*. J Cell Sci Suppl, 1987. **8**: p. 359-67.

203. Shen, W., et al., *In vitro development of mouse fetal germ cells into mature oocytes*. Reproduction, 2007. **134**(2): p. 223-31.

204. O'Brien, M.J., J.K. Pendola, and J.J. Eppig, *A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence*. Biol Reprod, 2003. **68**(5): p. 1682-6.

205. Eppig, J.J. and A.C. Schroeder, *Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro*. Biol Reprod, 1989. **41**(2): p. 268-76.

206. Hartshorne, G.M., *In vitro culture of ovarian follicles*. Rev Reprod, 1997. **2**(2): p. 94-104.

207. Ohtake, H., et al., *A novel in vitro experimental model for ovarian endometriosis: the three-dimensional culture of human ovarian surface epithelial cells in collagen gels*. Fertil Steril, 1999. **71**(1): p. 50-5.

208. Hardy, K., et al., *In vitro maturation of oocytes*. Br Med Bull, 2000. **56**(3): p. 588-602.

209. Gosden, R.G., et al., *Current perspective on primordial follicle cryopreservation and culture for reproductive medicine*. Hum Reprod Update, 2002. **8**(2): p. 105-10.

210. Klinger, F.G. and M. De Felici, *In vitro development of growing oocytes from fetal mouse oocytes: stage-specific regulation by stem cell factor and granulosa cells*. Dev Biol, 2002. **244**(1): p. 85-95.

211. Okazaki, T., et al., *LH reduces proliferative activity of cumulus cells and accelerates GVBD of porcine oocytes*. Mol Cell Endocrinol, 2003. **209**(1-2): p. 43-50.

212. Adriaens, I., R. Cortvrindt, and J. Smitz, *Differential FSH exposure in preantral follicle culture has*

*marked effects on folliculogenesis and oocyte developmental competence*. Hum Reprod, 2004. **19**(2): p. 398-408.

213. Lenie, S., et al., *A reproducible two-step culture system for isolated primary mouse ovarian follicles as single functional units*. Biol Reprod, 2004. **71**(5): p. 1730-8.

214. Adam, A.A., et al., *In vitro culture of mouse preantral follicles using membrane inserts and developmental competence of in vitro ovulated oocytes*. J Reprod Dev, 2004. **50**(5): p. 579-86.

215. Miyano, T., *JSAR Outstanding Research Award. In vitro growth of mammalian oocytes*. J Reprod Dev, 2005. **51**(2): p. 169-76.

216. Combelles, C.M., et al., *In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system*. Hum Reprod, 2005. **20**(5): p. 1349-58.

217. Xu, M., et al., *Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development*. Biol Reprod, 2006. **75**(6): p. 916-23.

218. Kreeger, P.K., et al., *The in vitro regulation of ovarian follicle development using alginate-extracellular matrix gels*. Biomaterials, 2006. **27**(5): p. 714-23.

219. Abir, R., et al., *In vitro maturation of human primordial ovarian follicles: clinical significance, progress in mammals, and methods for growth evaluation*. Histol Histopathol, 2006. **21**(8): p. 887-98.

220. Shen, W., et al., *Live offspring produced by mouse oocytes derived from premeiotic fetal germ cells*. Biol Reprod, 2006. **75**(4): p. 615-23.

221. Eppig, J.J., K. Wigglesworth, and F.L. Pendola, *The mammalian oocyte orchestrates the rate of ovarian follicular development*. Proc Natl Acad Sci U S A, 2002. **99**(5): p. 2890-4.

222. Eppig, J.J. and K. Wigglesworth, *Development of mouse and rat oocytes in chimeric reaggregated ovaries after interspecific exchange of somatic and germ cell components*. Biol Reprod, 2000. **63**(4): p. 1014-23.

223. Eppig, J.J., K. Wigglesworth, and Y. Hirao, *Metaphase I arrest and spontaneous parthenogenetic activation of strain LTXBO oocytes: chimeric reaggregated ovaries establish primary lesion in oocytes*. Dev Biol, 2000. **224**(1): p. 60-8.

224. Senbon, S., et al., *Xenografting of bovine secondary follicles into ovariectomized female severe combined immunodeficient mice*. J Reprod Dev, 2004. **50**(4): p. 439-44.

225. Senbon, S., et al., *Fertilization and development of bovine oocytes grown in female SCID mice*. Zygote, 2005. **13**(4): p. 309-15.

226. Kagawa, N., et al., *Growth and maturation of follicles and oocytes following xenotransplantation of porcine ovarian tissues and in vitro maturation*. J Reprod Dev, 2005. **51**(6): p. 741-8.

227. Liu, J., et al., *Maturation of mouse primordial follicles by combination of grafting and in vitro culture*. Biol Reprod, 2000. **62**(5): p. 1218-23.

228. Evans, M.J. and M.H. Kaufman, *Establishment in culture of pluripotential cells from mouse embryos*. Nature, 1981. **292**(5819): p. 154-6.

229. Martin, G.R., *Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells*. Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7634-8.

230. Toyooka, Y., et al., *Embryonic stem cells can form germ cells in vitro*. Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11457-62.

231. Clark, A.T., et al., *Spontaneous differentiation of germ cells from human embryonic stem cells in vitro*. Hum Mol Genet, 2004. **13**(7): p. 727-39.

232. Kee, K., et al., *Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells*. Stem Cells Dev, 2006. **15**(6): p. 831-7.

233. Teramura, T., et al., *Primate embryonic stem cells proceed to early gametogenesis in vitro*. Cloning Stem Cells, 2007. **9**(2): p. 144-56.

234. Aflatoonian, B., et al., *In vitro post-meiotic germ cell development from human embryonic stem cells*. Hum Reprod, 2009. **24**(12): p. 3150-9.

235. Eguizabal, C., et al., *Generation of primordial germ cells from pluripotent stem cells*. Differentiation, 2009. **78**(2-3): p. 116-23.

236. Young, J.C., V.L. Dias, and K.L. Loveland, *Defining the Window of Germline Genesis In Vitro from Murine Embryonic Stem Cells*. Biol Reprod, 2009.

237. Geijsen, N., et al., *Derivation of embryonic germ cells and male gametes from embryonic stem cells*. Nature, 2004. **427**(6970): p. 148-54.

238. Lacham-Kaplan, O., *In vivo and in vitro differentiation of male germ cells in the mouse*. Reproduction, 2004. **128**(2): p. 147-52.

239. Mizuno, K., et al., *Genes associated with the formation of germ cells from embryonic stem cells in cultures containing different glucose concentrations*. Mol Reprod Dev, 2006. **73**(4): p. 437-45.

240. Hubner, K., et al., *Derivation of oocytes from mouse embryonic stem cells*. Science, 2003. **300**(5623): p. 1251-6.

241. Lacham-Kaplan, O., H. Chy, and A. Trounson, *Testicular cell conditioned medium supports differentiation of embryonic stem cells into ovarian structures containing oocytes*. Stem Cells, 2006. **24**(2): p. 266-73.

242. Novak, I., et al., *Mouse embryonic stem cells form follicle-like ovarian structures but do not progress through meiosis*. Stem Cells, 2006. **24**(8): p. 1931-6.

243. Qing, T., et al., *Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells*. Differentiation, 2007. **75**(10): p. 902-11.

244. Salvador, L.M., et al., *The promoter of the oocyte-specific gene, Gdf9, is active in population of cultured mouse embryonic stem cells with an oocyte-like phenotype*. Methods, 2008. **45**(2): p. 172-81.

245. Nicholas, C.R., et al., *Transplantation directs oocyte maturation from embryonic stem cells and provides a therapeutic strategy for female infertility*. Hum Mol Genet, 2009. **18**(22): p. 4376-89.

246. Kehler, J., et al., *Generating oocytes and sperm from embryonic stem cells*. Semin Reprod Med, 2005.

**23**(3): p. 222-33.

247. Kerkis, A., et al., *In vitro differentiation of male mouse embryonic stem cells into both presumptive sperm cells and oocytes*. Cloning Stem Cells, 2007. **9**(4): p. 535-48.

248. Danner, S., et al., *Derivation of oocyte-like cells from a clonal pancreatic stem cell line*. Mol Hum Reprod, 2007. **13**(1): p. 11-20.

249. Dyce, P.W., L. Wen, and J. Li, *In vitro germline potential of stem cells derived from fetal porcine skin*. Nat Cell Biol, 2006. **8**(4): p. 384-90.

250. Haston, K.M., J.Y. Tung, and R.A. Reijo Pera, *Dazl functions in maintenance of pluripotency and genetic and epigenetic programs of differentiation in mouse primordial germ cells in vivo and in vitro*. PLoS One, 2009. **4**(5): p. e5654.

251. Yu, Z., et al., *Dazl promotes germ cell differentiation from embryonic stem cells*. J Mol Cell Biol, 2009. **1**(2): p. 93-103.

252. Kee, K., et al., *Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation*. Nature, 2009. **462**(7270): p. 222-5.

253. Kerr, C.L. and L. Cheng, *The Dazzle in Germ Cell Differentiation*. J Mol Cell Biol, 2009.

254. West, J.A., et al., *A role for Lin28 in primordial germ-cell development and germ-cell malignancy.* Nature, 2009. **460**(7257): p. 909-13.

255. Lavagnolli, T.M., et al., *Presumptive germ cells derived from mouse pluripotent somatic cell hybrids*. Differentiation, 2009. **78**(2-3): p. 124-30.

256. Nayernia, K., et al., *In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice*. Dev Cell, 2006. **11**(1): p. 125-32.

257. Baughman, J.M. and N. Geijsen, *In vitro generation of germ cells: new techniques to solve current issues*. Ann N Y Acad Sci, 2005. **1061**: p. 33-40.

258. Wu, M.Y. and S.N. Chow, *Derivation of germ cells from mouse embryonic stem cells*. J Formos Med Assoc, 2005. **104**(10): p. 697-706.

259. West, J.A. and G.Q. Daley, *In vitro gametogenesis from embryonic stem cells*. Curr Opin Cell Biol, 2004. **16**(6): p. 688-92.

260. Aflatoonian, B. and H. Moore, *Germ cells from mouse and human embryonic stem cells*. Reproduction, 2006. **132**(5): p. 699-707.

261. Whittaker, P., *Stem cells to gametes: how far should we go?* Hum Fertil (Camb), 2007. **10**(1): p. 1-5.

262. Nicholas, C.R., et al., *Instructing an embryonic stem cell-derived oocyte fate: lessons from endogenous oogenesis*. Endocr Rev, 2009. **30**(3): p. 264-83.

263. Cottrell, G.T. and J.M. Burt, *Functional consequences of heterogeneous gap junction channel formation and its influence in health and disease*. Biochim Biophys Acta, 2005. **1711**(2): p. 126-41.

264. Dekel, N., *Cellular, biochemical and molecular mechanisms regulating oocyte maturation*. Mol Cell Endocrinol, 2005. **234**(1-2): p. 19-25.

265. Grazul-Bilska, A.T., L.P. Reynolds, and D.A. Redmer, *Gap junctions in the ovaries*. Biol Reprod, 1997. **57**(5): p. 947-57.

266. Itahana, K., Y. Morikazu, and T. Takeya, *Differential expression of four connexin genes, Cx-26, Cx-30.3, Cx-32, and Cx-43, in the porcine ovarian follicle*. Endocrinology, 1996. **137**(11): p. 5036-44.

267. Itahana, K., et al., *Isolation and characterization of a novel connexin gene, Cx-60, in porcine ovarian follicles*. Endocrinology, 1998. **139**(1): p. 320-9.

268. Gray, S.A., M.A. Mannan, and P.J. O'Shaughnessy, *Development of cytochrome P450 17 alpha-hydroxylase (P450c17) mRNA and enzyme activity in neonatal ovaries of normal and hypogonadal (hpg) mice*. J Mol Endocrinol, 1996. **17**(1): p. 55-60.

269. Baldridge, D., et al., *Sequence and structure of the mouse connexin45 gene*. Biosci Rep, 2001. **21**(5): p. 683-9.

270. Grapes, L., Y. Zhang, and M.F. Rothschild, *Rapid communication: physical and linkage mapping of the porcine connexin 37 (CX37) gene*. J Anim Sci, 2002. **80**(5): p. 1375-6.

271. Wright, C.S., et al., *Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation*. Reproduction, 2001. **121**(1): p. 77-88.

272. Olek, A., J. Oswald, and J. Walter, *A modified and improved method for bisulphite based cytosine methylation analysis*. Nucleic Acids Res, 1996. **24**(24): p. 5064-6.

273. Tanaka, K. and A. Okamoto, *Degradation of DNA by bisulfite treatment*. Bioorg Med Chem Lett, 2007. **17**(7): p. 1912-5.

274. Lucifero, D., et al., *Coordinate regulation of DNA methyltransferase expression during oogenesis*. BMC Dev Biol, 2007. **7**: p. 36.

275. Lucifero, D., et al., *Bovine SNRPN methylation imprint in oocytes and day 17 in vitro-produced and somatic cell nuclear transfer embryos*. Biol Reprod, 2006. **75**(4): p. 531-8.

276. Mitalipova, M.M., et al., *Preserving the genetic integrity of human embryonic stem cells*. Nat Biotechnol, 2005. **23**(1): p. 19-20.

277. Wright, C.S., et al., *Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation*. Reproduction, 2001. **121**(1): p. 77-88.

278. Granot, I., et al., *Connexin43 in rat oocytes: developmental modulation of its phosphorylation*. Biol Reprod, 2002. **66**(3): p. 568-73.

279. Teilmann, S.C., *Differential expression and localisation of connexin-37 and connexin-43 in follicles of different stages in the 4-week-old mouse ovary*. Mol Cell Endocrinol, 2005. **234**(1-2): p. 27-35.

280. Simon, A.M., H. Chen, and C.L. Jackson, *Cx37 and Cx43 localize to zona pellucida in mouse ovarian follicles*. Cell Commun Adhes, 2006. **13**(1-2): p. 61-77.

281. Veitch, G.I., et al., *Selective assembly of connexin37 into heterocellular gap junctions at the oocyte/granulosa cell interface*. J Cell Sci, 2004. **117**(Pt 13): p. 2699-707.

289.

282. Gittens, J.E. and G.M. Kidder, *Differential contributions of connexin37 and connexin43 to oogenesis revealed in chimeric reaggregated mouse ovaries*. J Cell Sci, 2005. **118**(Pt 21): p. 5071-8.

283. Tong, D., et al., *Patch-clamp study reveals that the importance of connexin43-mediated gap junctional communication for ovarian folliculogenesis is strain specific in the mouse*. Am J Physiol Cell Physiol, 2006. **290**(1): p. C290-7.

284. Li, T.Y., et al., *Rescue of oogenesis in Cx37-null mutant mice by oocyte-specific replacement with Cx43*. J Cell Sci, 2007. **120**(Pt 23): p. 4117-25.

285. Borowczyk, E., et al., *Gap junctional connexin 37 is expressed in sheep ovaries*. Endocrine, 2006. **30**(2): p. 223-30.

286. Elenes, S., et al., *Heterotypic docking of Cx43 and Cx45 connexons blocks fast voltage gating of Cx43*. Biophys J, 2001. **81**(3): p. 1406-18.

287. Hombach, S., et al., *Functional expression of connexin57 in horizontal cells of the mouse retina*. Eur J Neurosci, 2004. **19**(10): p. 2633-40.

288. Evans, E.P., C.E. Ford, and M.F. Lyon, *Direct evidence of the capacity of the XY germ cell in the mouse to become an oocyte*. Nature, 1977. **267**(5610): p. 430-1.

289. Shawlot, W. and R.R. Behringer, *Requirement for Lim1 in head-organizer function*. Nature, 1995. **374**(6521): p. 425-30.

290. Dong, W.F., et al., *Cloning, expression, and chromosomal localization to 11p12-13 of a human LIM/HOMEOBOX gene, hLim-1*. DNA Cell Biol, 1997. **16**(6): p. 671-8.

291. Luo, X., Y. Ikeda, and K.L. Parker, *A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation*. Cell, 1994. **77**(4): p. 481-90.

292. Achermann, J.C., J.J. Meeks, and J.L. Jameson, *Phenotypic spectrum of mutations in DAX-1 and SF-1*. Mol Cell Endocrinol, 2001. **185**(1-2): p. 17-25.

293. Kreidberg, J.A., et al., *WT-1 is required for early kidney development*. Cell, 1993. **74**(4): p. 679-91.

294. Hammes, A., et al., *Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation*. Cell, 2001. **106**(3): p. 319-29.

295. Molkentin, J.D., et al., *Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis*. Genes Dev, 1997. **11**(8): p. 1061-72.

296. Katoh-Fukui, Y., et al., *Male-to-female sex reversal in M33 mutant mice*. Nature, 1998. **393**(6686): p. 688-92.

297. Colvin, J.S., et al., *Male-to-female sex reversal in mice lacking fibroblast growth factor 9*. Cell, 2001. **104**(6): p. 875-89.

298. Bagheri-Fam, S., et al., *Loss of Fgfr2 leads to partial XY sex reversal*. Dev Biol, 2008. **314**(1): p. 71-83.

299. Cui, S., et al., *Disrupted gonadogenesis and male-to-female sex reversal in Pod1 knockout mice*. Development, 2004. **131**(16): p. 4095-105.

300. Vanderhyden, B.C., et al., *Interactions between the oocyte and cumulus cells in the ovary of the B6.Y(TIR) sex-reversed female mouse*. Biol Reprod, 1997. **57**(3): p. 641-6.

301. Alton, M., et al., *The behavior of the X- and Y-chromosomes in the oocyte during meiotic prophase in the B6.Y(TIR)sex-reversed mouse ovary*. Reproduction, 2008. **135**(2): p. 241-52.

302. Isotani, A., et al., *Genomic imprinting of XX spermatogonia and XX oocytes recovered from XX<-->XY chimeric testes*. Proc Natl Acad Sci U S A, 2005. **102**(11): p. 4039-44.

303. Durcova-Hills, G., et al., *Influence of sex chromosome constitution on the genomic imprinting of germ cells*. Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11184-8.

304. Liang, L., et al., *Dynamic readjustment of parental methylation patterns of the 5'-flank of the mouse H19 gene during in vitro organogenesis*. Int J Dev Biol, 2000. **44**(7): p. 785-90.

305. Borghol, N., et al., *Epigenetic status of the H19 locus in human oocytes following in vitro maturation*. Genomics, 2006. **87**(3): p. 417-26.