IN VITRO MATURATION, FERTILIZATION, AND PREIMPLANTATION DEVELOPMENT OF OOCYTES ISOLATED FROM B6.Y DOM SEX-REVERSED AND XO FEMALE MICE

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ABSTRACT

When the Y chromosome of a *Mus. musculus domesticus* mouse strain is placed onto the C57BL/6J(B6) background, half of the XY (B6.Y^{Dom}) progeny develop as females, which ovulate very few eggs and fail to develop zygotes beyond the two-cell stage. In the present study, oocyte-cumulus complexes were isolated from ovaries of juvenile B6.Y^{Dom} females, as well as XX and XO females for comparison, and matured and fertilized *in vitro*. The results indicate that (1) *In vitro* procedure yielded far more mature oocytes than through ovulation; (2) The oocytes underwent apparently normal nuclear maturation, but failed to develop after fertilization; and (3) Addition of FSH during oocyte maturation increased the percentage of fertilization and the first cell cleavage, but not further development. The oocytes from XO ovaries were comparable with those from XX rather than XY ovaries, suggesting that the presence of the Y chromosome is responsible for the infertility of the B6.Y^{Dom} female.

ABSTRACT

Quand le chromosome Y de la lignée murine *Mus. musculus domesticus* est introduit dans le contingent génétique C57BL/6J(B6), la moitié de la progéniture XY (B6.Y^{Dom}) se développe en femelle, qui ovule peu d'ovocytes et échoue à tout dévelopement d'embryon après deux cellules. Dans cette étude, des complexes ovocyte-cumulus sont isolés à partir d'ovaires de femelles juvéniles B6.Y^{Dom} mais aussi XX et XO pour permettre la comparaison. Ils sont ensuite maturés et fécondés *in vitro*. Les résultats indiquent que (1) la technique *in vitro* permet d'obtenir plus d'ovocytes matures que l'ovulation; (2) ces ovocytes montrent une maturation nucléaire apparemment normale mais restent néanmoins incompétents à tout developpement après fécondation; et (3) l'addition de FSH durant la maturation ovocytaire augmente le pourcentage de fécondation et de premier clivage mais pas le developement ultérieur. Les ovocytes isolés des ovaires XO se comparent plus aux ovocytes XX qu'XY, suggérant que la présence du chromosome Y est responsable de l'infertilité de la femelle B6.Y^{Dom}.

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INTRODUCTION

A) INTRODUCTION

It has been generally accepted that the presence of the Y chromosome determines the development of testes which in turn determines the male phenotype. On the other hand, the absence of the Y chromosome results in the development of ovaries and the female phenotype. The B6.Y^{Dom} mouse is an exception to this rule since female gonadal and phenotypic sex develops with XY chromosomes. The oocytes isolated from B6.Y^{Dom} ovaries can be fertilized and undergo an apparently normal development up to the two-cell stage, but almost all embryos die before the blastocyst stage. In contrast to the XY (B6.Y^{Dom}) infertile female, the XO female is fertile, suggesting that the absence of the second X chromosome does not necessarily lead to infertility. Previous study has demonstrated some endocrine abnormalities in the XY ovary which may disturb the development of competence in oocytes. The objective of my study is to understand (1) how the oocytes from the XY female perform during maturation and zygotic development *in vitro*; (2) how gonadotropins, when added during oocyte maturation, influence these events; and (3) how the sex chromosomal composition affects the fertility by comparison between XO and XY oocytes.

B) GONADAL SEX DETERMINATION AND DIFFERENTIATION

1. Determination of Gonadal sex

In normal development, differentiation of an indifferent gonad into a testis is regulated in early embryogenesis by the Y chromosome. A regulatory gene responsible for these events has been mapped to the short arm of the human Y

chromosome and named *SRY* (or *Sry* in the mouse). The *SRY* (*Sry*) gene encodes a DNA binding protein which shares sequence homology with members of the HMG box family of transcription factors (Sinclair et al, 1990; Berta et al, 1990; Gubbay et al, 1990). The mechanism of action of *SRY* is still unknown. The expression of the *Sry* transgene in XX mice appears to be sufficient to initiate a cascade of regulatory genetic events which lead to the formation of testes-specific structures and suppression of female development. It is likely that activation of the *SRY* gene leads to the expression of Mullerian Inhibiting Substance (MIS) which reflects an early phase of Sertoli cell differentiation. However, the molecules mediating these two events remain to be identified.

2. Gonadal sex differentiation

Gonadal differentiation begins with the establishment of a sexually undifferentiated gonad, which develops as a stratification of the coelomic epithelium of the mesonephric kidney. Most cell types of the gonad are derived from the mesoderm of the urogenital ridge except for primordial germ cells which are initially located in the ectoderm and migrate into the gonad. The first sign of sexual dimorphism is the differentiation of primordial Sertoli cells and their aggregation into cords in the fetal testis. Consequently, Sertoli cells secrete MIS, which acts locally to suppress Mullerian duct development. Leydig cells differentiate in the interstitium, on the other hand, and produce testosterone which is essential for differentiation of the male urogenital tract and most of other male characteristics.

Failure of testicular differentiation results in ovarian development, which is characterized by initiation of meiosis in primordial germ cells. An increase in the

population of oogonia by proliferation is followed by a dramatic decrease by atresia at the pachytene stage. Then, remaining oocytes become arrested at the diplotene stage, also defined as the dictyate stage. Until this stage of meiosis, sex chromosomes appear to play little role, as particularly documented in the mouse (Burgoyne, 1978). (1) The second X chromosome is not essential for fertility of oocytes since the XO female is fully fertile in the mouse (Lyon and Hawker, 1973; Burgoyne and Baker, 1981). (2) The XY germ cells located outside of the testis cords often enter the meiotic prophase (McLaren, 1983). (3) The ectopic germ cells that have migrated into the adrenal gland differentiate into oocytes in the normal male fetus (Upadhyay and Zamboni, 1982; Zamboni and Upadhyay, 1983). Sex chromosomes may become essential for the oocyte to progress to the second meiotic metaphase because non-disjunction of pairing chromosomes often results in degeneration of oocytes.

3. Sex reversal in the B6.Y^{Dom} gonad

When the Y chromosome of a *Mus. musculus domesticus* (Dom) strain is placed onto the C57BL/6J (B6) background, none of the XY progeny (B6.Y^{Dom}) develop normal testes during the fetal life, and half of its population eventually develop bilateral ovaries and female internal and external genitalia (Eicher and Washburn, 1983; Taketo-Hosotani et al, 1989). No mutation is found on the Y chromosome to explain the sex-reversal. Furthermore, the *Sry* gene has been found to be expressed in the fetal gonad at the normal onset (Lee and Taketo, 1994). Eicher and others have postulated that the primary testis-determination gene on the Y chromosome (*Tdy*), probably identical with *Sry*, needs to interact with other autosomal genes to impose testicular development, and that the *Tdy*

gene of the Dom strain cannot properly interact with recessive autosomal gene(s) of the B6 mouse strain.

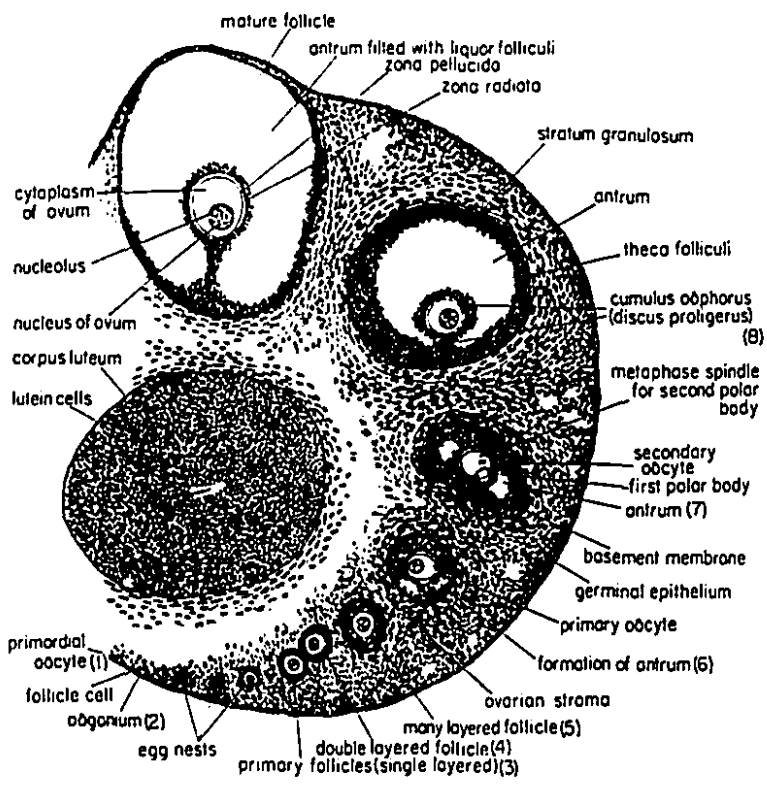
In the B6.Y^{Dom} ovotestis, seminiferous cords and ovarian cords are often enveloped by common basal lamina, indicating that both structures share the embryonic origin. Postnatally, ovarian components of the ovotestis regress to give the appearance of true but small testes. The fetal B6.Y^{Dom} ovary is morphologically indistinguishable from the XX ovary between 12 and 16 days of gestation (dg), when germs cells are distributed all over the ovary and reach the zygotene or pachytene stage of meiotic prophase (Taketo-Hosotani et al, 1989). Between 17 and 19 dg, many oocytes progress to the diplotene stage in the medullary area of the normal XX ovary. In contrast, all oocytes in the medullary region of the XY ovary degenerate while many continue to develop in the cortex region. Consequently, no follicles develop in the medullary region while folliculogenesis appears to proceed normally in the cortex region. Nonetheless, the XY ovary loses oocytes far more rapidly than the control XX female and very few follicles remain in the XY ovary by two months of age.

C) FEMALE FERTILITY

1. Ovarian development

In normal development, a few distinct steps are required to form mature follicles. First, the primordial follicle accumulates a few thousand granulosa cells to form a solid multilaminar structure (antral follicle) and then the extracellular space expands to form a cavity, called the antrum, presumably in response to gonadotropins. The granulosa cells differentiate into two sub- populations: mural

granulosa cells are attached to the basement membrane enclosing the follicle, and cumulus granulosa cells occupy the area between the oocyte and the mural granulosa cells. The illustration below schematically shows the development of the mouse ovum and ovarian follicles.



Development of the mouse ovum and ovarian follicles (Rugh, 1990)

In response to hormonal stimulation, two major events occur in the oocyte-cumulus complex (OCC). The fully grown oocyte resumes meiosis and becomes arrested at the metaphase II while the cumulus undergoes expansion (preovulatory follicles). With the luteinizing hormone (LH) surge, the metaphase II oocyte, still enclosed in the expanded cumulus, completes the first meiotic division upon ovulation and undergoes the second meiotic division after fertilization.

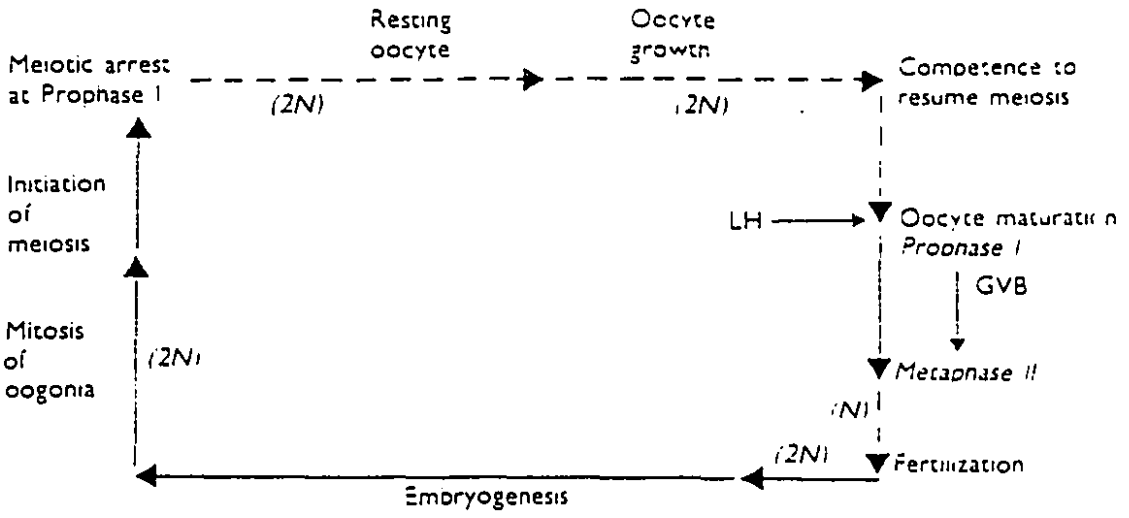


Illustration from "Ovarian Endocrinology" (Eppig , 1991)

Stop-and-Go pattern of meiosis during oocyte development. Meiosis is initiated after mitotic proliferation of oogonia in primitive ovary. Oocyte development with arrested meiosis is indicated by broken arrows. Meiosis is arrested in resting oocytes residing in primordial follicles, growing oocytes, and fully grown oocytes in non-atretic antral follicles. The preovulatory surge of LH triggers the resumption of meiosis which progresses from prophase I to metaphase II. Eggs (secondary oocytes) remain arrested in metaphase II until they are penetrated by sperm; meiosis II is then completed with the production of the second polar body. N refers to the ploidy (2N= diploid, 1N= haploid).

2. Interaction between oocytes and granulosa cells

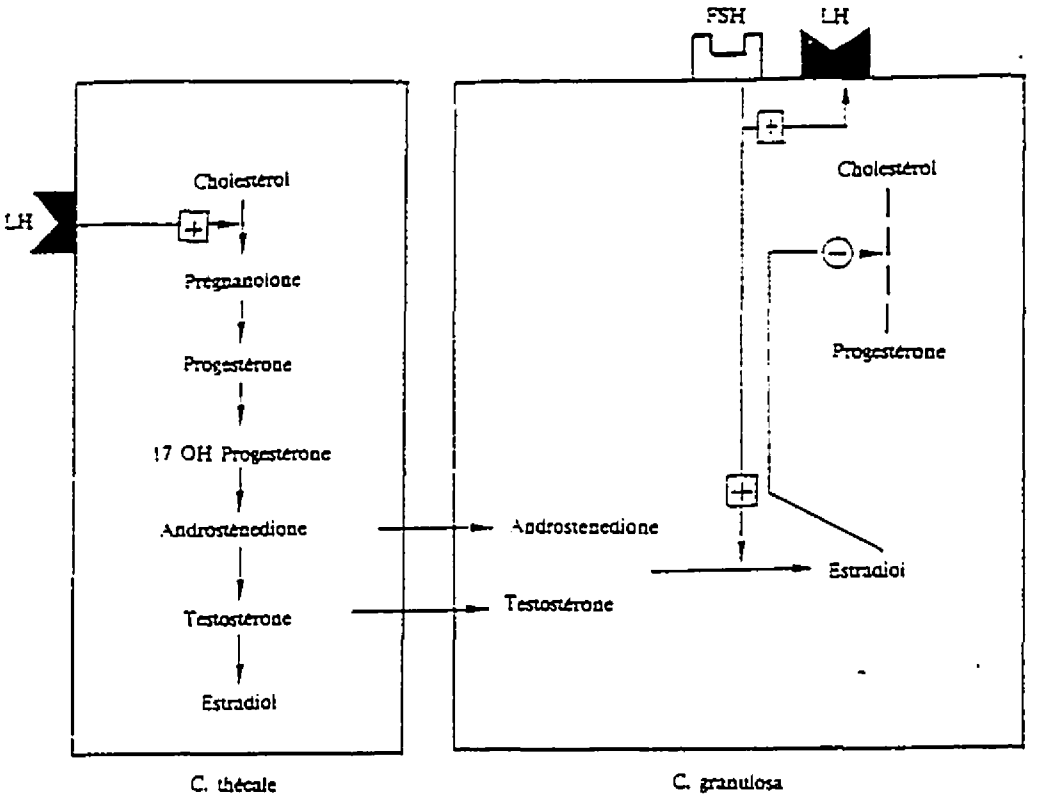
The association between oocytes and somatic granulosa cells persists throughout differentiation, growth, maturation, and fertilization of the oocyte. This association is crucial, ensuring the ultimate success of oogenesis (Schroëder and Eppig, 1984). The mural and cumulus granulosa cells of mature follicles are functionally interconnected by gap junctions. Furthermore, a threadlike extension of cumulus granulosa cells traverses the zona pellucida and makes close contact with the oocyte plasma membrane (Anderson and Albertini, 1976). A fundamental unit of the gap junction is connexon, which is a hexamer of proteins called connexins. Coexpression of mouse connexin 32 and 43 has been found in both oocytes and cumulus granulosa cells (Valdimarsson et al, 1993). Intercellular coupling mediated by these channels has been implicated in cell growth regulation (Zhu et al, 1991; 1992) and spatial patterning of cell differentiation. In addition to providing a pathway for metabolic cooperation between the oocyte and its surrounding somatic cells during follicular development, the cumulus-oocyte gap junctions transmit an as yet unidentified signal which triggers meiotic maturation (Fagbohum and Downs, 1991). Down regulation of the gap junction occurs during cumulus expansion under hormonal regulation (Chen et al, 1990).

3. Development of endocrine functions

Ovarian activity is regulated by follicle stimulating hormone (FSH) and LH synthesized by the gonadotrophes of the anterior pituitary under the influence of hypothalamic releasing factor LHRH. FSH and LH are heterodimeric glycoproteins composed of a common α subunit, and a unique β subunit. Steroid production by theca interna and interstitial cells is mainly regulated by

LH. In each estrous cycle, the theca cells surrounding the follicles that are destined to ovulate become more sensitive to LH than their cohorts, probably due to an increase in LH receptor density and induction of 17 α-hydroxylase and 17-20 lyase enzymes. Thus, production of androgens, especially testosterone, increases. On the other hand, action of FSH is restricted to granulosa cells with follicular development, resulting in the induction of aromatase activity which converts C-19 androgens to estrogens. The production of estrogens by two ovarian cell types is known as the "two-cell theory". According to a modified version of this theory, LH stimulates androgen production by theca cells. Consequently, the androgens are aromatized partially by theca cells and the rest by granulosa cells. Estrogens produced by theca cells are secreted into the circulation while estrogens synthesized by granulosa cells promote locally the follicular development. Estrogens act in an autocrine and paracrine manner in that they induce an increase in the population of granulosa cells and also the density of FSH receptors on these cells.

The granulosa cells at later stages (antral or preovulatory follicles) acquire the receptors for LH and prolactin under the influence of FSH and begin to synthesize progesterone (P) following ovulation. Prolactin appears to inhibit estrogen synthesis by both reducing the FSH-induced aromatase activity in granulosa cells and LH-induced androgen synthesis by theca cells. The shift from production of estrogens to that of progesterone is a complex process involving the interaction between FSH, LH, androgens and estrogens. The densities of FSH receptors in granulosa cells and LH receptors in theca cells increase not only in the follicles which are destined to mature into graffian follicles but also in those which eventually undergo atresia.



Action of Gonadotropins on antral follicles- Illustration from "gynécologie du praticien" (Emperaire, 1988)

4. Maturation of the oocyte

After initiation of meiosis in fetal life, oocytes become arrested at the diplotene stage of meiotic prophase I, also referred to as dictyate stage. *In vivo,* oocytes resume meiosis in response to a surge of gonadotropins (LH). The induction of oocyte maturation must be mainly mediated by the somatic component of follicles. There are currently two hypotheses to explain the mechanism by which gonadotropins induce resumption of meiosis *in vivo.* First, gonadotropin stimulation initiates the breakdown of the intercellular communication apparatus

in the follicle, thus depriving the oocyte of factors, such as cAMP, which maintain the meiotic arrest. Second, gonadotropins elicit a maturation-inducing signal by granulosa cells, overriding the inhibitory milieu of follicles (Buccione et al, 1990; Schroëder and Eppig, 1984).

As oocytes complete the growth phase, critical developmental changes occur to acquire the competence for nuclear and cytoplasmic maturation. Both the nuclear and the cytoplasmic maturation are essential for the formation of an egg having the capacity for fertilization and development to live offspring. Nuclear maturation encompasses the process of resuming meiosis at prophase I and driving the progression of meiosis to metaphase II, a stage at which meiosis normally becomes arrested again until fertilization. Cytoplasmic maturation refers to the process which prepares the egg for activation, pronuclear formation, and preimplantation development (Eppig et al, 1994).

Competence of oocytes at the germinal vesicle (GV) stage for both nuclear and cytoplasmic maturation is acquired in a stepwise manner during oocyte growth. The competence for the nuclear maturation differs as a function of the age of oocytes (Eppig et al, 1994). At 12 days postpartum (dpp), isolated oocytes are incompetent to undergo nuclear maturation. At 15 dpp, they are able to undergo GVB but almost all become arrested at the metaphase I. At 18 dpp, almost all are competent for completing nuclear maturation. Competence for cytoplasmic maturation is also acquired by oocytes in a stepwise manner. For example, mouse oocytes first acquire the competence for fertilization and development to the two-cell stage, but further growth of oocytes is required for acquiring the competence for development to the blastocyst stage (Eppig and Schroëder, 1989).

Eppig et al (1994) have shown recently that the competence of oocytes for nuclear and cytoplasmic maturation is acquired independently of each other in the developing GV-stage oocytes. Even though oocytes may be competent for completing nuclear maturation, they still can be deficient in cytoplasmic maturation. Further differentiation of these oocytes at the GV stage is required for production of the maternal factors essential for development of embryos beyond the two-cell stage. Nuclear and cytoplasmic maturation usually occur in approximate synchrony since the pronuclear formation fails unless penetration of the oocyte by sperm occurs shortly before or at the time of the exclusion of the first polar body. Nevertheless, Eppig et al (1994) have reported that oocytes incompetent for complete nuclear maturation can undergo at least some events of the cytoplasmic maturation. Previous studies comparing matured cumulus intact oocytes and matured cumulus free oocytes have shown the essential role of cumulus cells in promoting normal cytoplasmic maturation necessary for pronuclear formation and subsequent developmental capacity (Vanderhyden and Armstrong, 1989).

5. Cumulus expansion

The oocyte-cumulus complexes in preovulatory follicles undergo dramatic changes during oocyte maturation. The cumulus cells desegregate from one another and synthesize and secrete large amounts of hyaluronic acid-enriched cumulus extracellular matrix, increasing the volume of the OCC by 20 to 40-fold. This process of cumulus expansion or mucification may be required for oocyte maturation, ovulation, and fertilization.

Vanderhyden et al (1990) have demonstrated that differentiation of granulosa cells and cumulus expansion are influenced by the oocyte. The removal of the oocyte from OCC (oocytectomy) in the mouse prevents the production of hyaluronic acid and the cumulus expansion induced by FSH *in vitro* (Buccione et al, 1990). The ability of oocytectomized OCC is restored if the complexes are cultured in the medium conditioned with fully grown oocytes, showing that the oocyte secretes a cumulus expansion-enabling factor which allows cumulus cells to undergo expansion in response to FSH (Buccione et al, 1990; Salustri et al, 1990). The ability of the oocyte to secrete cumulus expansion enabling factors depends upon the stage of development of the oocyte. *In vivo*, cumulus expansion occurs as a prelude to ovulation and fertilization. *In vitro*, the oocyte becomes competent for secretion of the cumulus enabling-factor coincidentally with that for the resumption of GVB and the first meiotic division. Vanderhyden et al (1990) have also shown that the medium conditioned with fully grown oocytes can promote the proliferation of granulosa cells but cannot induce cumulus expansion despite the presence of both FSH and cumulus expansion-enabling factors. They conclude that continuous close association between oocytes and surrounding granulosa cells is necessary to promote the differentiation of granulosa cells into cumulus cells. Perhaps this association plays an important role in determining which granulosa cells to differentiate into cumulus cells or mural granulosa cells.

The mechanism of gonadotropin-mediated cumulus expansion is confounded because expansion *in vivo* and *vitro* appears to be mediated by different gonadotropic hormones. *In vivo*, cumulus expansion of antral follicles is a central component of the ovulatory response to the LH surge (Amstrong, 1991).

In vitro, it is FSH that stimulates the expansion of the cumulus oophorus of isolated OCC (Eppig, 1979). The nature of interaction between LH and FSH in relation to the process of cumulus expansion is not well understood.
Eppig et al (1980) postulated that the cumuli oophori probably do not respond directly to LH. LH seems to stimulate cumulus expansion via an indirect mechanism which allows the cumulus cells to respond to FSH-like activity present in the follicle. So the hormonal regulation of follicular development associated with ovulation appears to be a complex mechanism triggered by LH but requiring the interplay of both FSH and LH. Hen et al (1994) have shown that a synergistic action of LH and FSH is required for optimal expansion of the cumulus oophorus *in vitro.* FSH needs to first act and up-regulate the number of functional LH receptors. In turn, LH can act upon cumulus cells to stimulate the production and the secretion of the hyaluronic acid-enriched extracellular matrix. Other studies (Larsen et al, 1986) support the possibility that FSH stimulates the loss of gap junctions and other cell adhesion molecules.

D) STERILITY OF THE B6.Y^{Dom} FEMALE MOUSE

All B6.Y^{Dom} females mated with normal males fail to produce litters except for one (Eicher et al, 1982). To delineate the cause of infertility, Taketo and others studied the fertilization of XY oocytes *in vitro* (Merchant-Larios et al, 1994) and examined the endocrine profile of the B6.Y^{Dom} ovary (Villalpando et al, 1993). Their results indicated several abnormal aspects of the B6.Y^{Dom} ovary which may impair the fertility.

1. Incompetence of B6.Y^{Dom} oocytes for postfertilization development

Oocytes from the XY female are able to get fertilized both *in vivo* or *vitro*, but almost all embryos stop their development at the 1- or 2-cell stage and none reach the blastocyst stage. Merchant-Larios et al (1994) concluded that incompetence of the B6.Y^{Dom} oocyte for postfertilization development is programmed during differentiation or maturation of the oocyte in the XY ovary.

2. The defect inside the XY ovary

The XY females never show regular estrous cyclicity, and most stay at the persistent diestrous stage (Taketo-Hosotani et al, 1989). When XY females have been ovariectomized and received XX ovarian grafts, regular estrous cyclicity is initiated. In contrast, XX females which have been ovariectomized and received XY ovarian grafts do not resume their estrous cyclicity. These findings suggest that the pituary of the XY female responds to normal ovarian signals to induce regular estrous cyclicity. Therefore, the XY female is infertile due to a defect inside the XY ovary.

3. Hormonal profile of the B6.Y^{Dom} female

At a prepubertal stage (14 dpp), the XY ovary shows a higher 3β-HSD activity in the medullary region and produces more P than the XX ovary. The levels of androgens and estrogens in the XY ovary are constantly half of those in the XX ovary at 14 and 35 dpp. Villalpando et al (1993) postulated that because of the loss of oocytes prior to folliculogenesis, pregranulosa cells may differentiate into luteal-like cells, which mainly produce P, while interstitial androgenic cells fail to develop.

The most distinct difference between XX and XY ovaries was found in the response to gonadotropins. The XY ovary fails to increase as much P and T production as the XX ovary in the presence of gonadotropins. The insensitivity to gonadotropins may affect the competence of the oocyte for postfertilization development by modulating granulosa cell differentiation or function. Since testosterone synthesis is regulated mainly by LH while follicular growth and aromatase activity are regulated by FSH, the gonadotropin insensitivity of the XY ovary can be more likely attributed to a problem of hCG/LH action. Accordingly, distribution of LH receptors was examined by the binding of ^{125}I-hCG *in vivo* (Amleh et al, submitted). Distribution of hCG in theca-interstitial cells in XX and XY ovaries is almost identical with or without treatment with PMSG. This observation suggests that the lack of response to LH/hCG in the XY ovary is caused at the level of signal transduction or further downstream. Two more observations were made in the above studies: (1) In contrast to the XX ovary, hCG-binding in the B6.Y^{Dom} ovary is rare in mural granulosa cells of preovulatory follicles, suggesting abnormal differentiation of somatic cells; (2) hCG binding is more abundant in newly formed corpus luteal structures in the XY ovary if compared with the XX ovary. This finding suggests that antral follicles may undergo premature luteinisation instead of ovulation in the XY ovary.

The origin of the endocrine abnormalities in the XY ovary is perhaps the massive loss of oocytes in the medullary region before birth. This event prevents follicular formation and subsequent differentiation of interstitial steroidogenetic cells. In the XX ovary, the first generation of follicles in the medullary region undergo atresia. The medullary region is then occupied with stromal tissue and

steroidogenic cells. The role of the interstitial steroidogenic cells is not fully understood but is thought to be essential for normal endocrine functions.

E) REDUCED FERTILITY OF THE XO FEMALE MOUSE

Behavior of XO oocytes is of particular interest for comparison with XY oocytes. Similarities can be attributed to the missing second X chromosome whereas differences to the Y chromosome. Morphologic appearance of XO ovaries during the fetal life shows some similarities with XY ovaries. Both ovaries show normal oogonial proliferation and meiotic progress up to 16 dg, followed by a dramatic loss of oocytes by 19 - 20 dg (Burgoyne and Baker, 1985; Taketo-Hosotani et al., 1989). At 4 - 5 dpp, the XO ovary has only 40% as many oocytes as the XX control. This oocyte depletion is mainly due to elimination of atretic oocytes at the pachytene stage (Burgoyne and Baker 1981; 1985). It was postulated that the reduced number of oocytes is responsible for the short reproductive life of the XO female (Lyon and Hawker, 1973) as it is observed in the XY female. Contrary to the XY ovary, however, most of the degenerating oocytes appear to be located in the cortex region of the XO ovary. Miklos (1974) and Burgoyne and Baker (1985) explained that loss of oocytes is a mechanism to selectively destroy the meiotic cells carrying chromosomes which are unpaired or incompletely paired during the pachytene stage.

Despite the morphological similarities between XO and XY ovaries during the fetal life, XO females are invariably fertile whereas XY females are infertile. Partly due to the single X chromosome, reduced fertility of XO females has been nevertheless reported as their mean litter size is about 55% and their reproductive life is only one-third of normal XX females (Lyon and Hawker,

1972). A greater number of abnormal preimplantation embryos has also been reported in XO females. It is suggested that YO embryos do not develop beyond the two-cell stage while some XO embryos die at later stages (Morris, 1968). Chromosome counts on metaphase II oocytes from XO mice revealed a significant increase in the number of oocytes containing the X chromosome, suggesting a nonrandom segregation with a preferential retention of the single X chromosome into the secondary oocyte (Luthard, 1976). Whether the absence of the second X chromosome in the XY ovary contributes to infertility remains to be understood.

F) OBJECTIVES

The first objective of the present study is to find out whether fertility of XY oocytes can be improved by *in vitro* maturation and to determine the developmental age at which the XY oocytes become incompetent for embryonic development. The second objective is to examine the effect of FSH on maturation of OCC *in vitro* and later on fertilization and embryo development. The third objective is to assess the influence of the Y chromosome on fertility of the XY female by comparison with the hypofertile XO female.

MATERIALS AND METHODS

A) PREPARATION OF JUVENILE B6.Y^{DOM} FEMALES

1. Preparation of the B6.Y^{Dom} mouse

A *Mus musculus domesticus* male (Tirano, Italy) was mated with B6 females (Jackson Laboratories, Bar Harbor, Maine), and the F1 males were further backcrossed with B6 females as described previously (Nagamine et al, 1987). Each B6.Y^{Dom} male mouse (N22-28 backcross generations) of 60 to 180 dpp was caged with three B6 females (60 to 180 dpp) overnight, and the presence or absence of copulation plugs was checked the next morning. The day of delivery was defined as O dpp. At 25 dpp, the offspring were weaned and separated into female, male, and hermaphrodite groups according to the external genitalia.

2. Determination of the chromosomal sex of F1 (B6 x B6.Y^{Dom}) progeny

The chromosomal sex of rach mouse was determined by dot blot hybridization with a mouse Y chromosome-specific DNA probe according to the methods described previously (Taketo-Hosotani et al, 1989). Briefly, 4 to 5 μl of blood was aspirated from the tail of each mouse between 18 and 20 dpp and disposed onto a HYBOND-N+ nylon membrane (Amersham). Each mouse was then ear tagged and his/her phenotypic sex recorded. The membrane was baked at 120°C for 30 minutes.

The DNA samples on the membrane were denatured by incubation with 0.4 N NaOH for 5 min and then washed in 1 M TRIS-HCl (pH 7.5) for 5 min with

shaking. Subsequently, the membrane was incubated for 30 min with a prehybridization mix in a sealed plastic bag at 42°C in a water bath.

The pre-hybridization mix was composed as follow:

- Deionized formamide	50 %
- Dextran sulfate	5 %
- NaCl	1 M
- SDS	1 %

Meanwhile, the DNA probe (145SC5) labelled with ^{32}P by random priming and salmon sperm DNA were denatured by boiling for 5-10 min and chilled on ice. The probe ($1x10^5$ cpm/ml) and salmon sperm DNA (0.2 mg/ml) were then added to the membrane in the prehybridization mixture, which was further incubated overnight at 42°C in a water bath with shaking. The next day, the membrane was taken out from the bag and washed 5 times in 0.1 × SSC (0.15 M NaCl, 15 mM Na citrate). The first wash lasted for one to two hours and the others half an hour each. The membrane was then wrapped with plastic film, placed in a cassette with an X-ray film, and kept for two days at minus 70 °C until development.

B) PREPARATION OF JUVENILE XO FEMALES

The breeding pairs for XO mice were purchased from the Jackson Lab. (Bar Harbor, Maine). The X-linked recessive gene, Tabby was used as a marker to identify the XO progeny. XO females with normal coat color (+/O), when mated with Tabby males (Ta/Y), were expected to produce three types of offspring: males with normal coat colar (+/Y), females with a greasy coat color (Ta/O), and

females with stripes (Ta/+). XO females (Ta/O) were used in the present experiment and XX females (Ta/+) were used as the control.

C) MEDIA FOR OOCYTE AND EMBRYO CULTURE

- Oocyte maturation	Waymouth + FBS
- In vitro fertilization - Embryo development up to the 2-cell stage	MEM + BSA
- Embryo development after the 2-cell stage	KSOM + BSA

WAYMOUTH + FBS MEDIUM

Composition of the stock medium

Waymouth (GIBCO/BRL)	13.8 g
$NaHCO_3$	2.24 g
Streptomycin	50 mg
Penicillin G potassium salt	75 mg
Pyruvic acid sodium salt	25 mg
Double distilled water (ddH_2O)	
Total volume	1 litter

Waymouth stock medium was sterilized by filtration (0.45 µm) and gassed for 10 min vigorously with a mixture of 5% $C0_2$, 5% O_2 and 90% N_2 (referred as 5-5-90). This medium was stored at 4 °C and used within one month. For culture, 5 ml of

fetal bovine serum (FBS) was added to 100 ml of Waymouth stock medium, which was then kept at 4°C up to 2 weeks.

MEM + BSA MEDIUM

EBSS (10 X)

$CaCl_2$- 2 H_2O	0.53 g
KCl	0.8 g
$MgSO_4$-7H_2O	0.4 g
NaCl	13.6 g
NaH_2PO_4	0.25 g
Glucose	2.0 g
Phenol red	0.02 g
ddH_2O	100 ml

Aliquoted and stored at - 20°C.

MEM

EBSS (10x)	20 ml
$NaHCO_3$	0.44 g
Pyruvic acid	5.0 mg
Penicillin G	15 mg
Streptomycin	10 mg
L-Glutamine	58 mg
Essential amino acids (50x)	4.0 ml
Vitamins (100x)	2.0 ml
EDTA	0.76 mg
ddH_2O	
Total volume	100 ml

The MEM stock medium was sterilized, and gassed vigorously with the 5-5-90 mixture and stored up to one month at 4°C. For culture, the medium was supplemented with BSA at 3 mg/ml and stored up to two weeks at 4°C.

KSOM + BSA MEDIUM

Composition of the stock medium

NaCl	1.11 g
KCl	37 mg
KH_2PO_4	9.5 mg
$MgSO_4$	9.9 mg
Lactic acid	0.348 g
Pyruvic acid	4.4 mg
Glucose	7.2 mg
EDTA	0.76 mg
$NaHCO_3$	0.42 g
$CaCl_2$-$2H_2O$	0.05 g
Penicillin G	12.6 mg
Steptomycin	0.01 g
L-Glutamine	29 mg
Essential amino acid (100×)	2.0 ml
Non-essential amino acid (50×)	1.0 ml
ddH_2O	
Total volume	200 ml

The KSOM stock medium was sterilized, gassed vigorously with the 5-5-90 mixture and stored up to one month at 4°C. For culture, the medium was supplemented with BSA at 1 mg/ml and stored up to two weeks at 4°C.

D) MATURATION OF OOCYTES

1. Priming of female mice with gonadotropins

Juvenile B6.Y^{Dom} females, their XX litter mates, XO (Tab/O) females and their XX (Tab/+) litter mates at 25 dpp were used to provide oocytes in the present study. Each mouse was injected intraperitoneally with 5 IU of pregnant mare's serum

gonadotropin (PMSG) to stimulate the follicular development, and the ovary was dissected out 17 hr later. To collect at least 35 oocyte-cumulus cell complexes (OCC) in each group, 3 to 4 females of each type were sacrified.

2. Isolation of oocyte-cumulus cell complexes (OCC) for in vitro oocyte maturation

Each mouse was killed by cervical dislocation and the two ovaries collected. The ovaries were then blotted on Kimwipe and placed in a petri dish containing Waymouth+FBS medium. The OCC were liberated into the medium upon puncture of follicles using two 30-gauge needles which were attached to 1 ml tuberculin syringes. Using a micropipette, OCC were collected and washed by transferring through fresh medium in three dishes. At every transfer, preantral follicles, denuded oocytes, partially denuded oocytes and follicular debris were removed, so that in the last dish only OCC were recovered. The dishes containing OCC were placed in a modular incubator chamber (Billups-Rothenberg), which was flushed with 5-5-90 gas for 5 min, sealed with a positive pressure, and placed in an incubator at 37°C for 15-17 hr. After incubation, cumulus cells were removed from the oocyte by gently drawing OCC in and out of a Pasteur pipette a few times in Waymouth+FBS medium. This mechanical denudation allowed observation of maturation stages of oocytes.

3. Treatment of OCC with FSH during in vitro maturation

In some experiments, effect of FSH was assessed by adding FSH (SIGMA, F4520) to the maturation medium (1 μg/ml). No mechanical denudation was performed, and the intact OCC was subjected to fertilization. OCC collected from 3 or 4 females were first mixed up and then divided into two groups, one with FSH and the other whithout FSH.

E) IN VITRO FERTILIZATION

1. Collection of sperm

In each experiment, two CD1 males (3-5 months old) were killed by cervical dislocation, and one cauda epididymidis from each was placed in a petri dish containing 0.9 ml of MEM+BSA medium overlaid with paraffin oil which was prewarmed and gassed for 10 min. The cauda epididymidis was then cut into 10-15 pieces using a pair of sharp sterile scissors, and returned to an incubator for 10 min. Then, sperm number and motility were assessed under a dissecting microscope. Dense large swirls with small whirlpools of sperm on the surface of a drop of medium indicated a good quality of preparation.

2. Fertilization

The denuded oocytes (without FSH) or intact OCC (with FSH) were washed in MEM+BSA medium three times to remove any residue of serum, which was detrimental to sperm viability and fertilization. The washing was accomplished as quickly as possible, as the zona pellucidae of the ova is known "harden" in the medium without serum. 10 μl of sperm preparation was added into 0.5 ml of MEM+BSA medium overlaid with paraffin oil in a petri dish, and incubated under 5-5-90 gas for 15 min at 37°C. The ova after three washings in MEM+BSA medium were then transferred into the dishes containing sperm. The dishes were then placed in a modular incubation chamber, flushed with 5-5-90 gas, and incubated at 37°C for 4 hr.

3. Embryo culture

After fertilization, the zygotes were washed twice in MEM+BSA medium and transferred into petri dishes, each containing 2.5 ml of MEM+BSA medium. The dishes were placed in a modular incubation chamber, which was then flushed with 5-5-90 gas and incubated at 37℃. Between 24 hr and 48 hr in culture, most control zygotes reached the 2-cell stage. Then, the 2-cell stage embryos were washed by transferring into three dishes of KSOM+BSA medium and further incubated for 4 days.

F) STATISTICAL ANALYSIS

In each experiment, percentages of oocytes or zygotes which reached specific stages were calculated. The data from three experiments were used to evaluate statistic significance using Mann-Whitney U test. Difference with $p< 0.05$ was considered to be statistically significant.

RESULTS

A) IN VITRO MATURATION, FERTILIZATION, AND PREIMPLANTATION DEVELOPMENT OF OOCYTES FROM THE B6.Y^{Dom} FEMALE

All data shown in Table 1, 2 , 3 and 5 indicate the mean of 3 experiments. For the development of embryos beyond the two-cell stage and up to the blastocyst stage, the results are expressed as percentages of the two-cell stage embryos. The means and standard errors of the means are given in figures.

female genotype	n° of GV-OCC /female	maturation (%)	fertilization (%)	develop. to 2CS (%)	develop. beyond 2CS/2CS embryos (%)	develop. to blast./2CS embryos (%)
B6.Y^{Dom}	21	87	44	28	14	0
XX	33	94	74	70	64	33

Table 1: *In vitro* maturation, fertilization and preimplantation development of oocytes from B6.Y^{Dom} and control XX females. (develop. = development; 2CS = two-cell stage; blast. = blastocyst)

The number of OCC collected from each B6.Y^{Dom} female was smaller than that from XX control female (Figure 1). At the time of OCC collection, the morphologies of the oocytes at GV stage isolated from B6.Y^{Dom} and XX females were identical. The percentage of maturation of oocytes from the B6.Y^{Dom} female was comparable with the XX control (Figure 2).

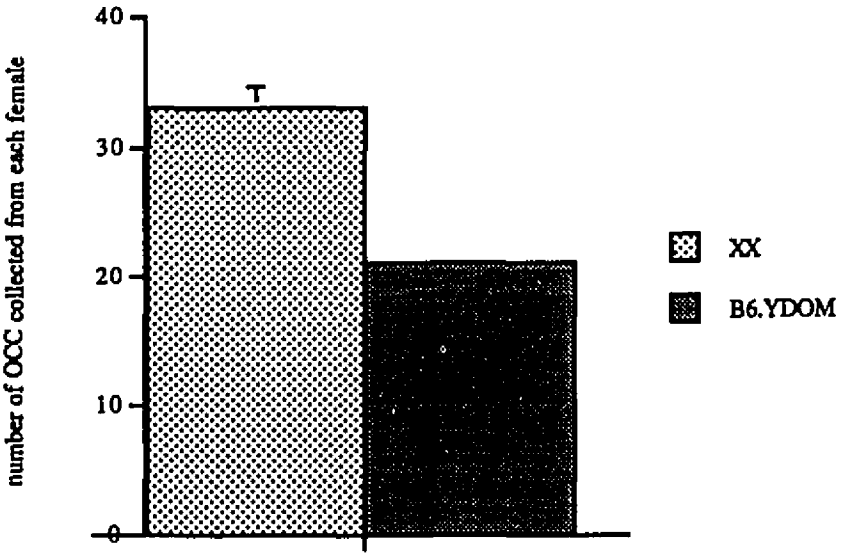


Figure 1: Number of OCC collected from B6.Y^{Dom} and control XX females

After *in vitro* maturation, OCC from B6.Y^{Dom} females and those from XX controls showed a distinct difference in the appearance of cumulus cells. XX OCC were still surrounded by cumulus cells (Appendix 1a), and, hence, to access the maturation status (GVBD or extrusion of the first polar body), denuding the oocytes mechanically by pippeting was necessary. In contrast, the majority of B6.Y^{Dom} oocytes were spontaneously denuded (Appendix 1b), and the maturation status could be assessed without mechanical force. It appears that the link between cumulus cells and oocytes in the OCC isolated from B6.Y^{Dom} females was looser than that from XX control females.

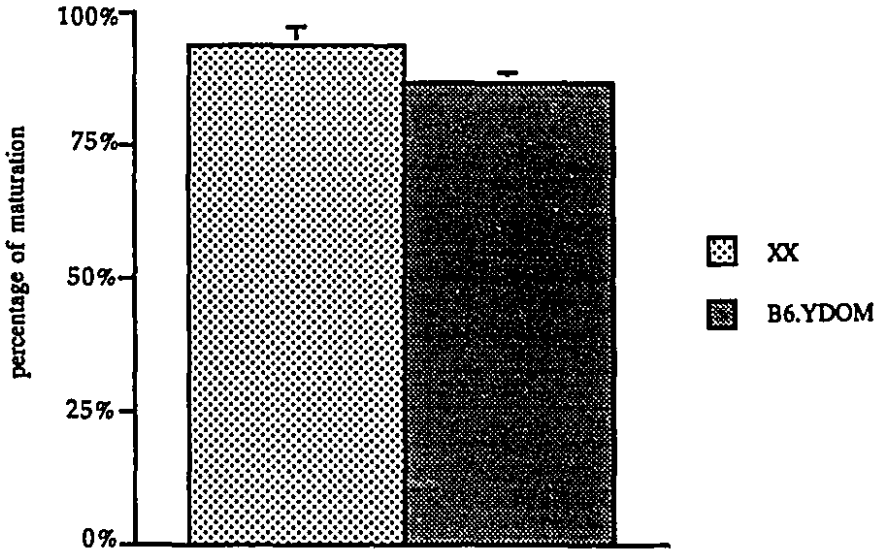


Figure 2: Maturation of oocytes from B6.Y^{Dom} versus control XX females

Further differences between B6.Y^{Dom} and control XX groups began to appear at fertilization, which was confirmed by formation of two pronuclei and development to the 2-cell stage. Smaller percentages of mature oocytes were fertilized and reached the two cell stage in the B6.Y^{Dom} group when compared with the XX control group (Figure 3).

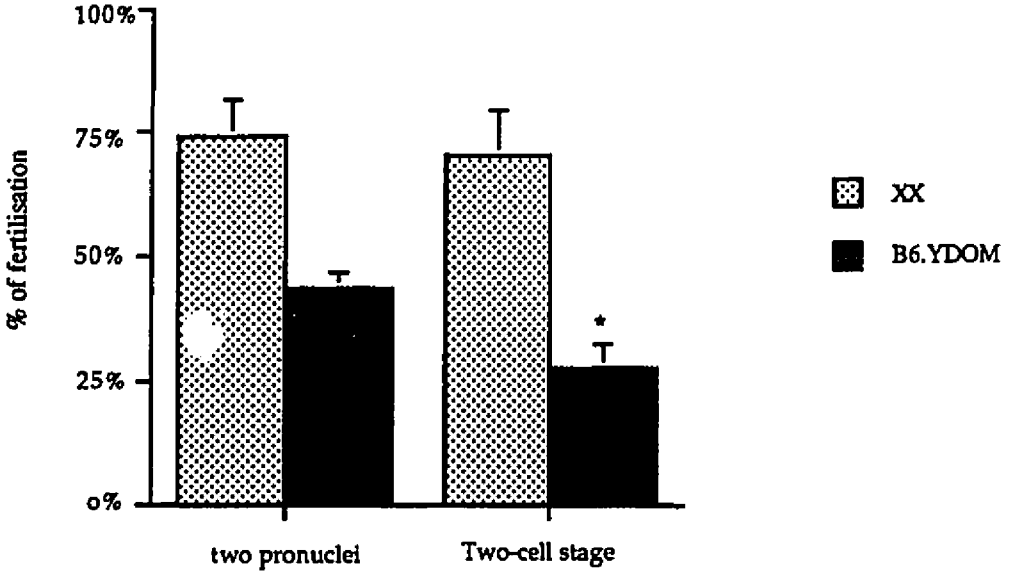


Figure 3: Fertilization of oocytes isolated from B6.Y^{Dom} and control XX females, and their progression to the two-cell stage

The majority of the two-cell stage embryos developed further and one-third reached the blastocyst stage in XX control group (Figure 4). In B6.Y^{Dom} group, much fewer embryos developed beyond the two-cell stage. Most were arrested at the four-cell stage, a few reached the eight-cell stage, and none reached the blastocyst stage.

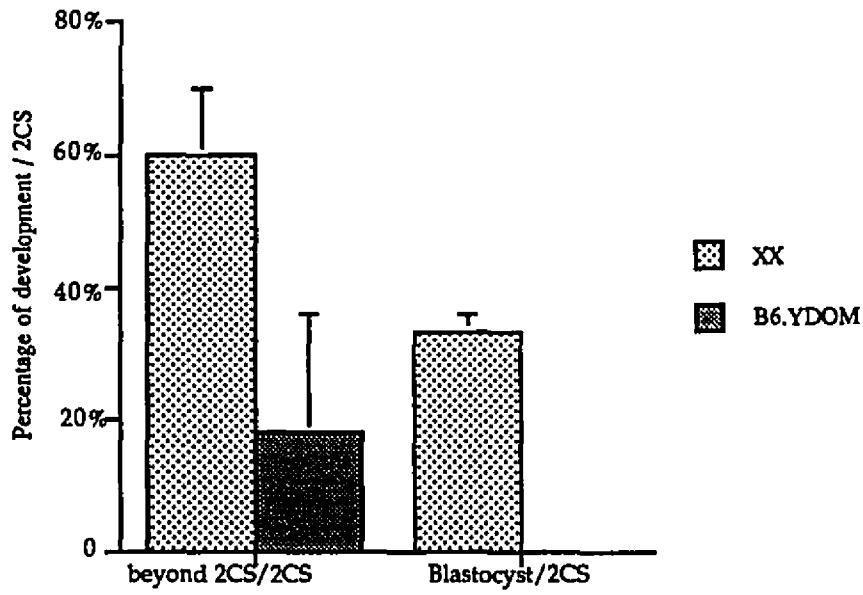


Figure 4: Development beyond the two-cell stage and to the blastocyst stage from the two-cell stage embryos collected from B6.Y^{Dom} and control XX females

B) DOSAGE OF **PMSG** FOR PRETREATMENT OF FEMALES

Priming mice with 2 instead of 5 IU of PMSG did not cause a dramatic difference in the rate of fertilization or embryo development of oocytes from B6.Y^{Dom} and XX ovaries (Table 2). It appears that 2 IU is enough to recrute antral follicles for maturation, whereas 5 IU does not cause any detrimental effect on the developmental competence of the oocyte. Since most of the published studies used 5IU of PMSG, we also chose this dosage for the rest of studies although the smaller dosage may be more physiological.

female genotype-PMSG dosage	n° of GV-OCC /female	maturation (%)	fertilization (%)	develop. to 2CS (%)	develop. beyond 2CS/2CS embryos (%)	develop. to blast/2CS embryos (%)
B6.Y^{Dom}-2IU	23	95	61	37	18	0
B6.Y^{Dom}-5IU	21	99	44	35	0	0
XX-2IU	27	86	69	77	61	30

Table 2: Dosage of PMSF for pretreatment of females and its effects on the subsequent development of oocytes in vitro

C) MATURATION, FERTILIZATION AND PREIMPLANTATION DEVELOPMENT OF OOCYTES FROM THE XO FEMALE

The numbers of OCC collected from XO and XX females were comparable (Table 3). The percentages of maturation were also not different between the two groups. No morphologic difference was observed between XO and the control groups after *in vitro* maturation. The percentage of zygotes which developed to the two-cell stage was also comparable. The rate of development beyond the two-cell stage in the XO group was lower than the XX control. Embryos developed from XO oocytes were arrested equally at four-cell, eight-cell and morula-stages and only 18% reached the blastocyst stage if compared with 35% in the XX control group (Figure 7).

female genotype	n° of GV-OCC /female	maturation (%)	develop. to 2CS (%)	develop. beyond 2CS/2CS embryos (%)	develop. to blast./2CS embryos (%)
XO(Tab/O)	30	96	68	48	18
XX(Tab/+)	27	97	69	71	35

Table 3: In vitro maturation, fertilization and embryo development from oocytes of XO versus XX females

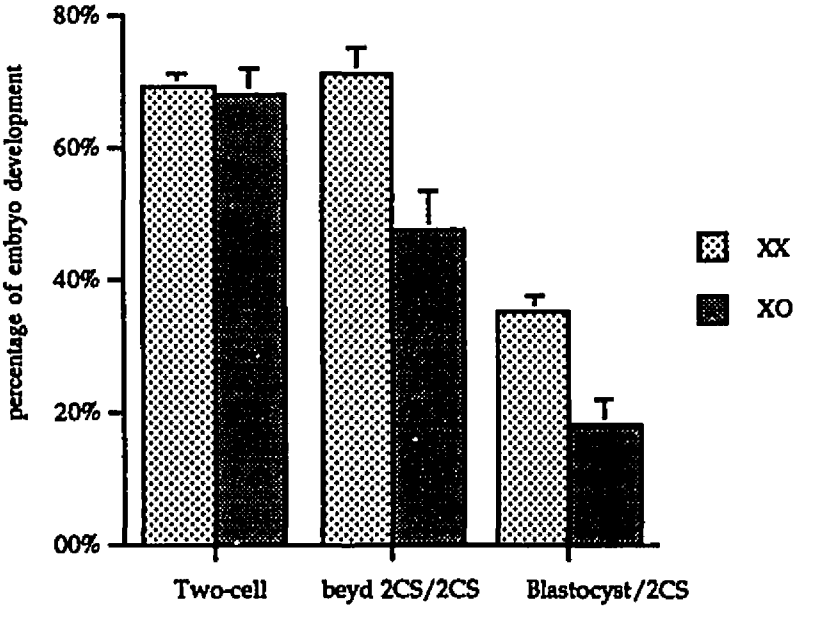


Figure 7: Fertilization and embryo development of oocytes isolated from XO versus control XX females

D) EFFECTS OF FSH ADDED DURING IN VITRO MATURATION ON FERTILIZATION AND PREIMPLANTATION DEVELOPMENT OF OOCYTES FROM THE B6.Y^{DOM} FEMALE

Table 4 presents the result of each experiment and Table 5 the mean of three experiments. In contrast to the previous set of experiment, OCC were not denuded before fertilization to avoid interference with the contact between cumulus cells and oocytes.

Exp		N° of GV-OCC (total)	Fertil (%)	develop to 2CS (%)	Develop beyond 2CS/2CS embryos (%)	Develop to blast /2CS embryos (%)
1	XX	56	75	57	55	30
	XX+FSH	70	62	54	55	33
	B6.YDOM	40	41	33	0	0
	B6.YDOM +FSH	48	65	48	0	0
2	XX	60	80	80	66	33
	XX+FSH	60	65	70	77	30
	B6.YDOM	42	41	33	0	0
	B6.YDOM +FSH	34	78	50	29	0
3	XX	60	70	60	55	33
	XX+FSH	35	80	77	60	40
	B6.YDOM	50	50	36	0	0
	B6.YDOM +FSH	48	81	50	50	0

Table 4: In vitro maturation with or without FSH, and subsequent fertilization and embryo development from oocytes of B6.Y^{Dom} and control XX females

Experiments	fertilization (%)	develop. to 2CS (%)	develop. beyond 2CS/2CS embryos (%)	develop. to blast./2CS embryos (%)
XX	75±3	65±7	59±4	31±1
XX+FSH	69±6	67±7	64±7	34±3
B6.Y^{Dom}	44±3	34±1	0	0
B6.Y^{Dom}+FSH	74*±5	49*±1	26±14	0

Table 5: Summary of the result comparing the maturation, fertilization and preimplantation development of OCC with or without FSH.

* significant difference from the value without FSH

Addition of FSH in the maturation medium of OCC from the B6.Y^{Dom} group induced a significant increase in the success rate of fertilization as indicated by the presence of two pronuclei (Table 5 and Figure 8) as well as progression to the two-cell stage (Table 5).

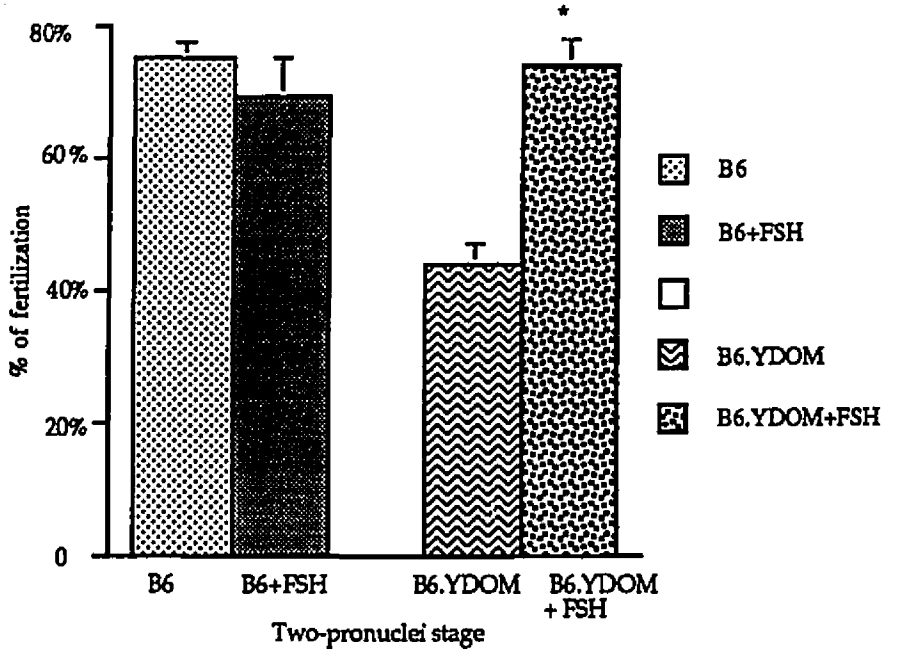


Figure 8: Fertilization of oocytes from B6.Y^{Dom} and control XX females with and without FSH treatment during maturation. * significant difference from the control (without FSH treatment).

Less spontaneous denudation was observed in OCC from B6.Y^{Dom} females when matured in the presence of FSH (Appendix 1d) than its absence (Appendix 1b). However, cumulus expansion was not as prominent as in the control XX oocytes (Appendix 1c). Addition of FSH in the maturation medium improved the development of zygotes from the B6.Y^{Dom} female beyond the two-cell stage, but none reached the blastocyst stage (Table 5).

DISCUSSION

Genetic variants often provide key information for the understanding of normal developmental process. We assume that study of the infertile B6.Y^{Dom} female mouse could give us some clue for identifying crucial steps in female fertility. Previous studies have shown that the oocytes ovulated from the B6.Y^{Dom} female can be fertilized but do not develop beyond the two-cell stage (Merchant-Larios et al, 1993). This observation was, however, restricted by the small number of oocytes, a mean of 6 oocytes ovulated by each female (Taketo-Hosotani et al, 1989). The present study using the *in vitro* maturation allows us (1) to increase significantly the recovery of oocytes which are capable for fertilization, (2) to separate oocytes from the XY sex-reversed host environment, and (3) to avoid problems associated with ovulation. Mammalian ovarian follicles in response to an ovulatory surge of LH (and FSH) undergo several dramatic and acute reactions prior to ovulation. Taking into account the well-documented endocrine abnormalities of the B6.Y^{Dom} ovary (Villalpando et al, 1992), observation of oocytes *in vitro* should simplify the analysis of the cause of infertility.

A) THE NUCLEAR MATURATION IS APPARENTLY NORMAL IN THE OOCYTES ISOLATED FROM THE B6.Y^{DOM} FEMALE.

The present study indicates that the nuclear maturation of oocytes from the B6.Y^{Dom} female appears to be comparable with those from XX control and XO females. They underwent GVB and progressed to metaphase II in a similar proportion. Furthermore, both the time to undergo GVB and that to reach the first cell cleavage were comparable. In the previous studies, by contrast, *in vivo* ovulated oocytes from the B6.Y^{Dom} female demonstrated delayed onset of the first cell cleavage (Merchant-Larios et al, 1994; Amleh et al, submitted). Abnormal

endocrine features, particularly insensivity to gonadotropins, may prevent recruitment of proper oocytes for ovulation in the XY female.

B) OOCYTES FROM THE B6.Y^{Dom} FEMALE ARE INCOMPETENT FOR POSTFERTILIZATION DEVELOPMENT.

After *in vitro* maturation, most oocytes from the B6.Y^{Dom} female got fertilized but stopped their development at the two-cell stage or earlier. A few developed to the four-cell stage but their nuclei were found to be fragmented. We conclude that the oocytes from the B6.Y^{Dom} female are incompetent for postfertilization development, independent of ovulation. The developmental incompetence may be imposed during differentiation, growth or maturation of oocytes. The death of all oocytes in the medullary region during the fetal life (Taketo-Hosotani et al, 1989) and the abnormal differentiation of cumulus cells at the late stage of follicular development (Amleh et al, submitted) may reflect the abnormality during growth or differentiation of oocytes in the B6.Y^{Dom} ovary. Although nuclear maturation appeared to progress normally in the XY oocyte, the cytoplasmic maturation may likely be defective. It is known that proper accumulation of cytoplasmic factors in oocytes is required for progression of zygotes (fertilized eggs) beyond the two-cell stage (Eppig et al, 1994). Competence for cytoplasmic and nuclear maturation is acquired independently, although in an approximate synchrony and in a stepwise manner (Eppig et al, 1994). The present results may further support the hypothesis that apparent normal nuclear maturation is not sufficient for proper cytoplasmic maturation.

C) LOSS OF CUMULUS CELLS OCCURS DURING IN VITRO MATURATION OF OOCYTES FROM THE B6.Y^{Dom} OVARY.

A crucial difference was observed during *in vitro* maturation of oocytes as almost all OCC from the B6.Y^{Dom} female were spontaneously denuded whereas such premature loss of cumulus cells was not observed among the oocytes from XO or XX females. The spontaneous denudation may reflect a problem of granulosa cell differentiation or a defect in the oocyte itself. The present findings may emphasize the importance of communication between oocytes and granulosa cells for acquisition of competence for postfertilization development. It will be informative to examine the junctions between granulosa cells or between oocytes and granulosa cells in the B6.Y^{Dom} ovary.

D) FSH REDUCES SPONTANEOUS DENUDATION OF B6.Y^{Dom} OOCYTES AND INCREASES THE PERCENTAGE OF FERTILIZATION, BUT DOES NOT IMPROVE ZYGOTIC DEVELOPMENT.

The presence of FSH in the medium during oocyte maturation increased significantly the percentage of fertilization and progression to the two-cell stage but not further development of the oocytes from the B6.Y^{Dom} female. These effects must be attributable to granulosa cells since no FSH receptors have been identified on the normal oocyte. With adjunction of FSH in the maturation medium, OCC from the B6.Y^{Dom} female appeared less denuded although they did not reach the extent of cumulus expansion that was observed with XX or XO oocytes. These observations may suggest a significant role of FSH in oocyte-cumulus interaction. On the other hand, failure of zygotic development appears to be independent of spontaneous denudation.

While the molecular and cellular bases for the effects of the cumulus upon fertilization are not known, a few hypotheses have been advanced recently. The rate of fertilization of the oocytes with expanded cumuli is significantly greater than that with compact cumulus mass (Downs et al, 1986; Vanderhyden and Armstrong, 1989). Higher fertilization and developmental potential have been ascribed to expansion of the cumulus oophorus (Ball et al, 1983) which is frequently used as a gross indication of "maturation" of oocytes (Testard et al, 1983). Furthermore, the inhibition of hyaluronic acid synthesis and consequent expansion of the cumulus extracellular matrix result in low percentage of fertilization (Chen et al, 1990). On the other hand, Downs et al (1986) reported a reduction in the percentage of fertilization following the removal of cumulus cells from OCC prior to maturation. Thus, close association between oocytes and cumulus cells is necessary for subsequent cumulus expansion. Hence, loose contact with cumulus cells may disrupt proper cytoplasmic maturation of oocytes or production of factors essential for cumulus expansion in the B6.Y^{Dom} ovary.

E) FSH TREATMENT RESULTS IN INCOMPLETE CUMULUS EXPANSION OF OCC FROM THE B6.Y^{DOM} FEMALE.

When spontaneous denudation was prevented in the presence of FSH, OCC from the B6.Y^{Dom} female underwent cumulus expansion, but not as much as those from XX or XO females. Previous studies have suggested that cumulus cells do not differentiate properly at late stages of follicular development in the B6.Y^{Dom} ovary since LH receptors cannot be identified as in the normal XX ovary (Amleh et al, submitted). Furthermore, LH receptors in theca cells are not functional in the XY ovary since the production of testosterone does not increase in response to

gonadotropins (Villalpando et al, 1992). Chen et al (1994) suggested that for optimal cumulus expansion, FSH first acts to up-regulate the number of functional LH receptors in granulosa cells. In turn, LH acts upon cumulus cells to allow the expansion. Since treatment with FSH did not result in appropriate expansion of cumulus cells, up-regulation of LH receptors in cumulus cells by FSH may also be impaired in the B6.Y^{Dom} ovary. We cannot exclude a possibility that the sensitivity to FSH is reduced in the B6.Y^{Dom} ovary although it appears to respond to FSH better than to LH (unpublished observation). It remains to be determined whether incomplete cumulus expansion plays any roie in the failure of zygotic development in the B6.Y^{Dom} female.

F) THE ABSENCE OF THE SECOND X CHROMOSOME MAY CONTRIBUTE TO BUT CANNOT FULLY EXPLAIN THE INFERTILITY OF THE B6.Y^{DOM} FEMALE.

The present study revealed more different aspects than similarities between XY and XO females. The major difference was the behavior of oocytes during the postfertilization development. The majority of the zygotes from the B6.Y^{Dom} female stopped their development at the two-cell stage or earlier whereas those from the XO female lost their population gradually at all stages during preimplantation periods and some developed normally up to the blastocyst stage. It is known that all YO embryos die before implantation while XO embryos can develop to live-borns. In addition, OCC from the XO female did not get denuded during *in vitro* maturation and were able to expand in response to FSH. Thus, the comparison between XO and XY oocytes highlights the problems associated with the Y chromosome rather than the single X chromosome in the behavior of XY oocytes during fertilization and preimplantation development.

However, very little is known of oogenesis in either B6.Y^{Dom} or XO ovary, such as meiotic recombination and transcriptional activity of sex chromosomes.

Some crucial events reported during development of ovaries in fetal life may explain later differences in the fertility between the two females. Lee and Taketo (1994) reported the expression of a Y-encoded gene, *Sry* in the B6.Y^{Dom} ovarian primordium. In addition, the XY ovary loses all oocytes in the medulary region (Taketo-Hosotani et al, 1989) whereas the XO ovary loses oocytes mainly in the cortex region (Burgoyne and Baker, 1985) during fetal life. We do not know whether these differences are due to the sex chromosomal composition of germ cells or somatic cells. At the onset of meiosis, the X chromosome of XO oocytes is known to be active whereas the transcriptional activity of X and Y chromosomes in the B6.Y^{Dom} gonad is yet to be determined.

G) FUTURE DIRECTIONS

The present study raises the following interesting points for further investigation.

(1) The difference in oocyte-cumulus interactions observed between XO and XY ovaries could be at least partly responsible for the difference in fertility. Gap junctions between oocyte-cumulus and cumulus-cumulus cells can be studied morphologically and biochemically. Furthermore, endocrine features of the XO ovary should be examined in comparison with the XY ovary.

(2) The zygotic gene activation, evident by the two-cell stage, is known to be required for further cleavage (involving reprogramming of gene expression) (Zimmermann and Schultz, 1994). It would be interesting to examine the gene expression in the B6.Y^{Dom} zygote.

CONCLUSION

An *in vitro* maturation procedure made it possible to recover a reasonable number of oocytes from the XY ovary, which would otherwise have produced very few oocytes by either spontaneous or gonadotropin-induced ovulation. Furthermore, *in vitro*, we could observe abnormal features of OCC from the XY ovary during maturation in comparison with those from XX or XO ovaries. Most prominently, oocytes from the XY ovary were often spontaneously denuded after *in vitro* maturation, and when denudation was prevented by treatment with FSH during maturation, the cumulus cells underwent only partial expansion. Although oocytes from the XY ovary underwent apparently normal nuclear maturation and fertilization *in vitro*, they failed to develop beyond the two-cell stage. Treatment with FSH increased the percentage of oocytes to undergo fertilization and the first cell cleavage, but not further development. These findings suggest that the XY oocytes are programmed to be incompetent for zygotic development at early stages of differentiation, growth or maturation. We speculate that the loose contact between oocytes and cumulus cells in the XY ovary may disrupt proper cytoplasmic maturation of oocytes which is essential for postfertilization development. Thus, fertility of oocytes may be assessed in part by the appearence of OCC as well as their response (cumulus expansion) to FSH. Since OCC from the fertile XO ovary resembled those from the XX ovary more than the XY ovary, the developmental incompetence of oocytes in the XY ovary can be attributed to the presence of the Y chromosome rather than to the absence of the second X chromosome.

BIBLIOGRAPHY

1. Abatangelo G; Cortivo R; Martelli M; Vecchia P. Cell detachment mediated by hyaluronic acid. Exp Cell Res. 1982; 137: 73-78.

2. Amleh A; Lédée N; Saeed J; Taketo T. Competence of XY oocytes from the B6.YDom sex-reversed female mouse for maturation, fertilization, and preimplantation development. unpublished.

3. Amsterdam A; Knecht M; Catt K J. Hormonal regulation of cytodifferentiation and intercellular communication in cultured granulosa cells. Proc Natl Acad Sci USA. 1981; 78: 3000-3004.

4. Amsterdam A; Koch Y; Lieberman ME; Lindner HR. Distribution of binding sites for human chorionic gonadotropin in the preovulatory follicle of the rat. J Cell Biology. 1975; 67: 894-900.

5. Amstrong. Role of hormones during oocyte maturation. Ann New York Acad Sc. 1991; 626: 137-158.

6. Anderson E; Albertini DF. Gap junction between the oocyte and companion follicle cells in the mammalian ovary. J Cell Biology. 1976; 71: 680-686.

7. Baker TG. A quantative and cytological study of germ cells in human ovaries. Proc R Soc London. 1963; 158: 417-433.

8. Ball GD; Liebfried ML; Lenz RW; Ax RL; Bavister BD; First NL. Factors affecting successful in vitro fertilization of bovine follicular oocytes. Biol Reprod. 1983; 28: 717-725.

9. Bar-ami S; Zlotkin E; Brandes JM; Itskovitz-eldor J. Failure of meiotic competence in human oocytes. Biol Reprod. 1994; 50: 1100-1107.

10. Bennet MVL; Barrio LC; Bargiello TA; Spray DC; Hertzberg E; Saez JC. Gap junctions: new tools, new answres, new questions. Neuron. 1991; 6: 305-320.

11. Berta B; Hawkins JR; Sinclair AH; Taylor A; Griffiths BL; Goodfellow PN; Fellous M. Genetic evidence equating SRY and the testis-determinating factor. Nature. 1990; 348: 448-450.

12. Bortollussi M; Marini G; Reolon ML. A histochemical study of the binding of ^{125}I-HCG to the rat throughout the estrous cycle. Cell Tissue Res. 1979; 197: 213-226.

13. Brook JD. X-chromosome segregation, maternal age and aneuploidy in the XO mouse. Genet Res Camb. 1983; 41: 85-95.

14. Buccione R; Cecconi S; Tatone C; Mangia F; Colonna R. Follicle cell regulation of mammalian oocyte growth. J Exp Zool. 1987; 242: 351-354.

15. Buccione R; Schroëder AC; Eppig JJ. Interactions between somatic cells and germ cells throughout mammalian oogenesis. Biol Reprod. 1990: 43: 543-547.

16. Buccione R; Vanderhyden BC; Caron PJ; Eppig JJ. FSH-induced expansion of the mouse cumulus oophorus *in vitro* is dependent upon a specific factor(s) secreted by the oocyte. Dev Biol. 1990; 138: 16-25.

17. Bukovsky A; Chen T T; Caudle M R. Cellular localization of luteinizing Hormone receptor Immunoreactivity in the ovaries of immature, gonadotropin-primed and normal cycling Rats. Biol Reprod. 1993; 48: 1367-1382.

18. Burgoyne PS. The role of the mammalian Y chromosome in spermatogenesis. Development. 101: 133-141 .

19. Burgoyne PS. The role of the sex chromosomes in mammalian germ cell differentiation. J Anim Bioch Biophys. 1978; 18 (2B): 317-325.

20. Burgoyne PS; Baker TG. Oocyte depletion in XO mice and their XX sibs from 12 to 200 days *post partum*. J Reprod Fert. 1981; 61: 207-212.

21. Burgoyne PS; Baker TG. Perinatal loss in XO mice and its implication for the aetiology of gonadal dysgenesis in XO women. J Reprod Fert. 1985; 75: 633-645.

22. Burgoyne PS; Mahadevaiah SK; Sucliffe MJ. Fertility in mice requires X-Y pairing and a Y-chromosomal " spermiogenesis" gene mapping to the long arm. Cell. 1992; 71: 391-398.

23. Camaioni A; Hascall VC; Yanagishita M; Salustri A. Effects of exogenous hyaluronic acid and serum on matrix organization and stability in the mouse cumulus cell-oocyte. J Biol Chem. 1993; 268: 20473-20481.

24. Capel B; Rasberry C; Dyson J; Bishop CE; Simpson E; Vivian N; Lovell-Badge R; Rastan S; Cattanach BM. Deletion of Y chromosome sequences located outside the testis determining region can cause XY female sex reversal. Nature Genet. 1993; 5: 301-307.

25. Chen L; Russell P T; Larsen W J. Functional Significance of Cumulus Expansion in the Mouse: Roles for the Preovulatory Synthesis of Hyaluronic Acid Within the Cumulus Mass. Mol Reprod Devel. 1993; 34: 87-93.

26. Chen L; Russel PT; Larsen WJ. Sequential Effects of Follicle-stimulating Hormone and Luteinizing Hormone on Mouse Cumulus Expansion In Vitro. Biol Reprod. 1994; 51: 290-295.

27. Chen L; Wert SE; Hendrix E M; Russel PT; Cannon M; Larsen WJ. Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. Mol Reprod Devel. 1990; 26: 236-247.

28. Chesnel F; Wigglesworth K; Eppig JJ. Acquisition of meitic competence by denuded mouse oocytes: participation of somatic cell product(s) and cAMP. Dev Biol. 1994; 161: 285-295.

29. Downs SM; Coleman DL; EppigJJ. Maintenance of murine oocyte meiotic arrest: Uptake and metabolism of hypoxanthine and adenosine by cumulus cell-enclosed and denuded oocytes. Dev Biol. 1986;1; 117: 174-183.

30. Downs SM; Schröder AC; Eppig JJ. Serum maintains the fertilizability of mouse oocytes matured in vitro by preventing the hardenning of the zona pellucida. Gamete Res. 1986;2; 15: 115-122.

31. Duncan M; CummingsL; Chada K. Germ cell deficient mouse as a model of premature ovarian failure. Biol Reprod. 1993; 49: 221-227.

32. Eicher EM; Washburn LL. Genetic control of primary sex determination in mice. Ann Rev Genet. 1986; 20: 327-60.

33. Eicher E; Washburn LL. Inherited sex reversal in mice: Identification of a new primary sex-determinating gene. J Exp Zool. 1983; 228: 297-304.

34. Emperaire JC. Gynécologie endocrinienne du praticien. Chapitre 2: Edition Frisson Roche; 1988.

35. Eppig JJ. FSH stimulate hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. Nature. 1979; 281: 483-484.

36. Eppig JJ. Regulation of cumulus oophorus expansion by gonadotropins in vivo and in vitro. Biol Reprod. 1980; 23: 545-552.

37. Eppig JJ. The relationship between parthenogenetic embryonic development and cumulus cell-oocyte intercellular coupling during oocyte meiotic maturation. Gamete Research. 1982; 5: 229-237.

38. Eppig JJ; Schroeder AC. 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: 268-276.

39. Eppig JJ; Schroeder AC; O'brien MJ. Developmental capacity of mouse oocytes matured in vitro: effects of gonadotropic stimulation, follicular origin and oocyte size. J Reprod Fert. 1992; 95: 119-127.

40. Eppig JJ; Schultz RM; O'Brien M; Chesnel F. Relationship between the developmental programs controlling nuclear and cytoplasmic maturation of mouse oocytes. Dev Biol. 1994; 164: 1-9.

41. Eppig JJ; Wiglesworth K. Atypical maturation of oocytes of strain I/LnJ mice. Human Reprod. 1994; 9: 1136-1142.

42. Eppig JJ; Wigglesworth K; Chesnel F. Secretion of cumulus expansion enabling factor by mouse oocytes: relationship to oocyte growth and competence to resume meiosis. Dev Biol. 1993; 158: 400-409.

43. Fagbohum CF; Downs SM. Metabolic coupling and ligand stimulated meiotic maturation in the mouse oocyte-cumulus cell complex. Biol Reprod. 1991; 45: 851-859.

44. Goodman HM. Basic medical endocrinology. Raven Press New York; 1990.

45. Gubbay J; Collignon J; Koopman P; Capel B; GoodfellowP; Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of novel family of embryonically expressed genes. Nature. 1990; 346: 245-250.

46. Haqq CM; King CY; Ukiyama E; Falsafi S; Haqq TN; Donahoe PK; Weiss MA. Molecular basis of mammalian sexual determination: Activation of Müllerian Inhibiting Substance gene expression by SRY. Science. 1994; 266: 1494-1500.

47. Hillensjo T; Magnusson C; Svensson U; Thelander H. Effect of luteinizing hormone and follicle-stimulating hormone on

progesterone synthesis by cultured rat cumulus cells. Endocrinology. 1980; 108: 1920-1924.

48. Hillier SG. Ovarian Endocrinology. Chapter 4: Blackwell Scientific Publication; 1991.

49. Houle AM; Teruko T. True hermaphodites: An experimental model in the mouse. J Urol. 1992; 148: 672-676.

50. Koopman P; Gubbay J; Vivian N; Goodfellow P. Male development of chromosomally female mice transgenic for Sry. Nature. 1991; 351: 117-121.

51. Kotsuji F; Kubo M; Tominaga T. Effect of interactions between granulosa and theca cells on meiotic arrest in bovine oocytes . J Reprod Fert. 1994; 100: 151-156

52. Kotsuji F; Tominaga T. The role of granulosa and theca cell interactions in ovarian structure and function. Microsc Res Techn. 1994; 27: 97-107.

53. Larsen WJ; Wert SE; Brunner GD. Differential modulation of rat follicle cell gap junction populations at ovulation. Dev Biol. 1987; 122:61-71 .

54. Laurincik J; Hyttel P; Vesela J. Ooplasmectomy has not influence on FSH-induced expansion of bovine cumulus investment *in vitro*. Anat Embryol. 1994; 190: 65-72.

55. Lawrence TS; Dekel N; Beers W. Binding of human chorionic gonadotropin by rat cumuli oophori and granulosa cells: A comparative study. Endocrinology. 1980; 106: 1114-1118.

56. Lee CH; Taketo T. Normal onset, but prolonged expression, of Sry gene in the B6.Y^{dom} sex-reversed mouse gonad. Dev Biol. 1994; 165: 442-452.

57. Lovel-Badge R; Robertson E. XY female mice resulting from a heritable mutation in the primary testis-determining gene, tdy. Development. 1990; 109: 635-646.

58. Luthardt FW. Cytogenetic analysis of oocytes and early preimplantation Embryos from XO mice. Dev Biol. 1976; 54: 73-81.

59. Lyon MF; Hawer SG. Reproductive lifespan in irradiatred and unirradiated chromosomally XO mice. Genet Res Camb. 1973; 21: 185-194.

60. Merchant-Larios H; Clarke HJ; Taketo T. Developmental arrest of fertilized eggs from the B6.Y^{Dom} sex-reversed female Mouse. Dev Genet. 1994; 15: 435-442.

61. Merchant-Larios H; Taketo T. Testicular differentiation in mammals under normal and experimental conditions. J Electr Microsc Techn. 1991; 19: 158-171.

62. Miklos GLG. Sex-chromosome pairing and male infertility. Cytogenet Cell . 1974; 13: 558-577.

63. Moor RM; Smith MW; Dawson RMC. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. Exp Cell Res. 1980; 126: 15-29.

64. Nagai T; Ding J; Moor RM. Effect of follicle cells and steroidogenesis on maturation and fertilization *in vitro* of pig oocytes . J Exp Zool. 1993; 266: 146-151.

65. Nagamine CM; Taketo T; Koo GC. Morphological development of the mouse gonad in *tda*-1XY sex reversal. Differentiation. 1987; 33: 214-222.

66. O'Brien M; Wiggleworth K; Eppig J. Method inToxicology. 129-141: Academic Press; 1993.

67. Peng XR; Hsueh A JW; Lapolt PS; Bjersing L. Localization of luteinizing hormone receptor messenger ribonucleic aid

expression in ovarian cell types during follile development and ovulation. Endocrinology. 1991; 129: 3200-3207.

68. Renard JP; Baldacci P; Richoux-Duranthon V; Pournin S; Babinet C. A maternal factor affecting mouse blastocyst formation. Development. 1994; 120: 797-802.

69. Richard JS; Midley AS. Protein hormone action: a key to understanding ovarian follicular and luteal cell development. Biol Reprod. 1976; 14: 82-94.

70. Salustri A; Yanagishita M; Hascall VC. Synthesis and accumululation of hyaluronic acid and proteoglycans in the mouse cumulus cell-oocyte complex during follicle-stimulating hormone-induced mucification. J Biol Chem. 1989; 23: 13840-13847.

71. Schroëder AC; Eppig JJ. The developmental capacity of mouse oocytes that matured *in vitro* is normal. Dev Biol. 1984; 102: 493-497.

72. Schultz RM; Montgomery RR; Ward-Bailey P; Eppig JJ. Regulation of oocyte maturation in the mouse: Possible roles of intercellular communication, cAMP, and testosterone. Dev Biol. 1983; 95: 294-304.

73. Shen WH; Moore CC; Ikeda Y; Parker KL; Ingraham HA. Nuclear receptor steroidogenetic factor 1 regulates the Mullerian inhibiting substance gene: a link to the sex determination cascade. Cell. 1994; 77: 651-661.

74. Sinclair Ah; Berta P; Palmer MS; Hawkins JR; GRiffiths BL; Smith MJ; Foster JW; Frischaulf AM; Lovell-Badge R; Goddfellow PN. A gene from the human sex-determinating region encodes a protein with homology to conserved DNA-binding motif. Nature. 1990; 346
: 240-244.

75. Sorenson RA; Wasserman PM. Reliationship between growth and meiotic maturation of the mouse oocyte. Dev Biol. 1976; 50: 531-536.

76. Speed RM. The possible role of the meiotic pairing anomalies in the atresia of human fetal oocytes. Hum Genet. 1988; 78: 260-266.

77. Sutovsky P; FlechonJ.E; Flechon B. Dynamic change of gap junctions and cytoskeleton during *in vitro* culture of cattle oocyte cumulus complexes. Biol Reprod. 1993; 49: 1277-1287.

78. Taketo-Hosotani T; Nishioka Y; Nagamine C.M; Villalpando I; Merchant-LariosH. Development and fertility of ovaries in the B6.Y^{Dom} sex-reversed female mouse. Development. 1989; 107: 95-105.

79. Taketo-Hosotani T; Sinclair-Thompson E. Influence of the mesonephros on the development of fetal mouse ovaries following transplantation into adult male and female mice. Dev Biol. 1987; 124: 423-430.

80. Temeles GL; Ram PT; Rothstein JL; Schultz RM. Expression patterns of novel genes during mouse preimplantation development. Mol Reprod Devel. 1994; 37: 121-129.

81. Testard J; Lassale B; Frydman R; Belaisch JC. A study of factors affecting the sucess of human fertilization in vitro. Influence of semen quality and oocyte maturity on fertilization and cleavage. Biol Reprod. 1983; 28: 425-431.

82. Textbook of endocrine physiology. 1992.
Griffin JE//Ojeda SR
Oxford University Press.

83. Thibault C; LMevasseur MC. La reproduction chez les mammifères et les hommes. Chapitre 11: Ellipse; 1991.

84. Tilly JL; Lapolt PS; Hsueh JW. Hormonal regulation of follicle-stimulating hormone receptor messenger ribonucleic acid levels in cultured rat granulosa cells. Endocrinology. 1992; 130: 1296-1302.

85. Upadhyay S; Zamboni L. Ectopic germ cells: a natural model for the study of germ cell sexual differentiation. Proc Natl Acad Sci USA. 1982; 79: 6584-6588.

86. Valdimarsson G; De sousa PA; Kidder GM. Coexpression of Gap junction proteins in the cumulus- oocyte complex. Mol Reprod Dev. 1993; 36: 7-15.

87. Valdimarsson G; De sousa PA; Kidder GM. Coexpression of gap junction proteins in the cumulus-oocyte complex. Mol Reprod Dev. 1993; 36: 7-15.

88. Vanderhyden BC. Species differences in the regulation of cumulus expansion by an oocyte-secreted factor(s). J Reprod ertil. 1993; 98: 219-227.

89. Vanderhyden BC; Armstrong DT. Role of cumulus cells and serum on the in vitro maturation, and subsequent development of rat oocytes. Biol Reprod. 1989; 40: 720-728.

90. Vanderhyden BC; Caron PJ; Buccione R; Eppig JJ. Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. Dev Biol. 1990; 140: 307-317.

91. Vanderhyden BC; Telfer EE; Eppig JJ. Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles in vitro. Biol Reprod. 1992; 46: 1196-1204.

92. Villalpando I; Nishioka Y; Taketo T. Endocrine differentiation of the XY sex-reversed mouse ovary during postnatal development. J Steroid Biochem Molec Biol. 1993; 45: 265-273.

93. Wang XN; Greenwald GS. Human chorionic gonadotropin or human recombinant follicle-stimulating hormone (FSH)-induced ovulation and subsequent fertilization and early embryo development in hypophysectomized FSH-primed mice. Endocrinology. 1993; 132: 2009-2015.

94. Wang XN; Greenwald GS. Hypophysectomy of the cyclic mouse. II Effecs of follicle-stimulating hormone (FSH) and luteinizing hormone on folliculogenesis, FSH and human chorionic gonadotropins receptors, and steroidogenesis. Biol Reprod. 1993; 48: 595-605.

95. West J. Genetic control of gamete production and function. 49-51: Academic Press, London; 1982.

96. Wiekowski M; Miranda M; Depamphilis ML. Regulation of gene expression in preimplantation moouse embryos: Effects of the zygotic clock and the first mitosis on promoter and enhancer activities. Dev Biol. 1991; 147: 403-414.

97. Wiekowski M; Miranda M; Depamphilis ML. Requirements for promoter activity in mouse oocytes and embryos distinguish paternal pronuclei from maternal and zygotic nuclei. Dev Biol. 1993; 159: 366-378.

98. Zamboni L; Upahyay S. Germ cell differentiation in mouse adrenal glands. J Exp Zool. 1983; 228: 173-193.

99. Zhu D; Caveney S; Kidder GM; Naus CCG. Transfection of C6 glioma cells with connexin 43 c DNA: analysis of expression, intercellular coupling, and cell proliferation. Proc Natl Acad Sci USA. 1991; 88: 1883-1887.

100. Zhu D; Kidder GM; Caveney S; Naus CCG. Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. Proc Natl Acad Sci USA. 1992; 89: 10218-10221.

101. Zimmerman JW; Schultz RM. Analysis of gene expression in the preimplantation mouse embryo: Use of mRNA differential display. Proc Natl Acad Sci USA. 1994; 91: 5456-5460.

Supplement

Morris T. The XO and OY chromosome constitutions in the mouse. Genet Res Camb. 1968; 12: 125-137.

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APPENDIX

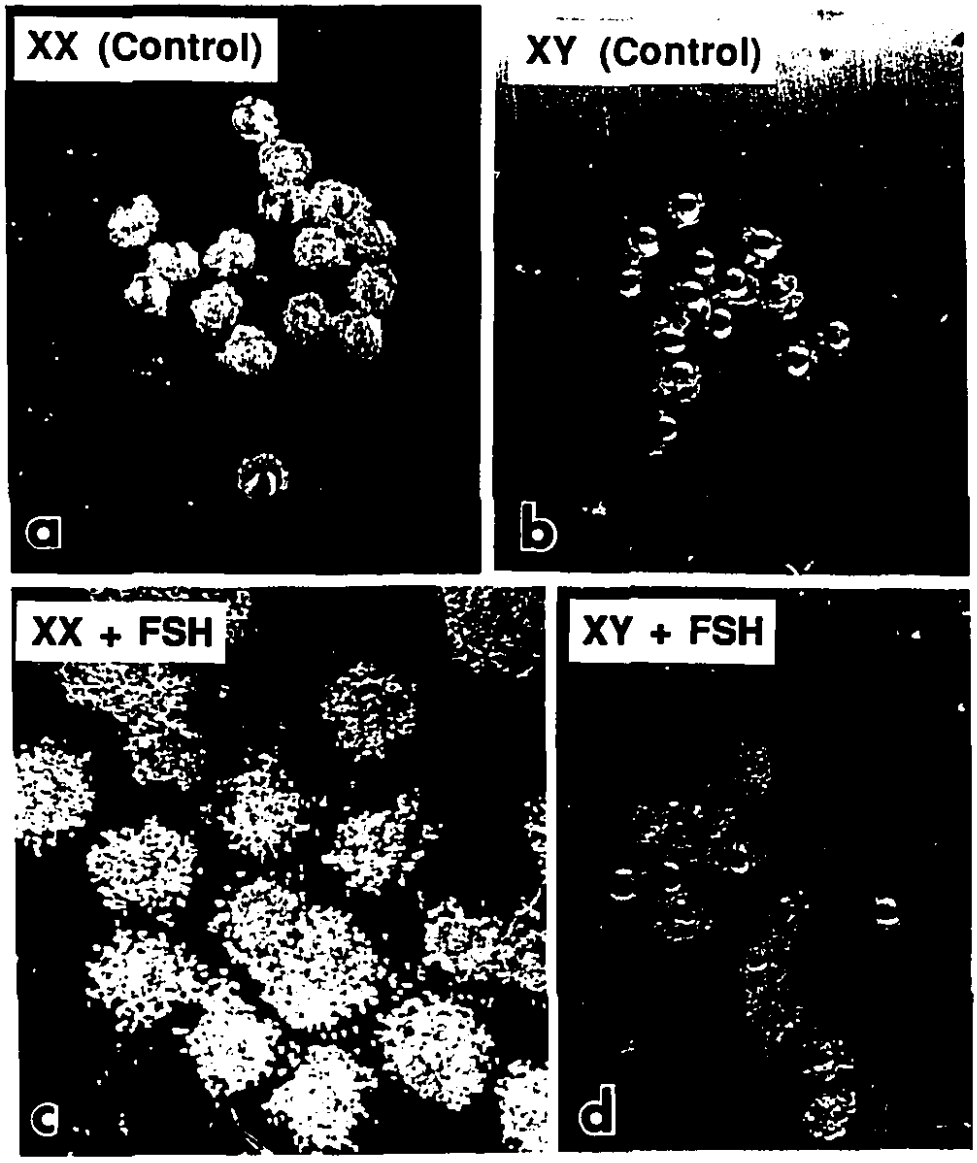
Appendix 1: Oocyte-cumulus complexes shown with transillumination under a dissecting microscope.

a. OCC from the XX ovary after incubation in the control medium. Cumulus cells are tightly attached to the oocyte.

b. OCC from the B6.Y^{Dom} ovary after incubation in the control medium. No or only a few cumulus cells are attached to the oocyte.

c. OCC from the XX ovary after incubation in the presence of FSH. Cumulus cells around the oocyte are fully expanded.

d. OCC from the B6.Y^{Dom} ovary after incubation in the presence of FSH. Cumulus cells around the oocyte are partially expanded.



Appendix 1