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Multioocyte follicles in adult mammalian ovaries

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Abstract

The majority of follicles contain only a single oocyte. However, it has been shown that more than two oocytes can exist within the same follicle. These follicles have been well documented during fetal development, but their presence in adult ovaries is an intriguing physiologic phenomenon. Apoptotic pathway members, locally produced factors, circulating hormones and steroid factors may be involved in their persistence. The origin and fate of these structures, as well as their contribution to ovulation and fertility in adult females, are discussed in this review.

Keywords: mammal, oocyte, ovary, multioocyte follicle.

Introduction

Follicle assembly and development has been well documented for many mammalian species (Byskov and Lintern-Moore, 1973; Hirshfield, 1991; Pepling and Spradling, 2001; Sawyer *et al*., 2002; Tingen *et al*., 2009). The timing of specific developmental changes is different among mammals, but the overall pattern appears to be reasonably well conserved. As the primordial germ cells arrive at the genital ridge, they differentiate into oogonia and develop in clusters or nests. These structures have been highly conserved during evolution (Pepling *et al*., 1999), although they are not strictly analogous to the cysts in *Drosophila* ovaries because mammalian germ cells never transform into nurse cells (de Cuevas *et al*., 1997). As oogonia undergo mitosis, they interact with somatic cells in the ovary, giving rise to germline cysts. Inside the cysts, oogonia are connected by intercellular bridges (Gondos *et al*., 1971; Gondos, 1987). Premeiotic female germ cells from insects, rabbits, mice, rats, hamsters and humans all show intercellular cytoplasmic bridges characteristic of cysts (Pepling *et al*., 1999). In ruminants, the plasma membranes of oogonia and pregranulosa cells are in close apposition to one another, and along their surfaces, focal points of physical attachment (desmosomes) were observed (Sawyer *et al*., 2002). When nest formation is complete, a few single mitotic germ cells and many germline cysts are surrounded by epithelial pregranulosa cells, becoming organized into ovigerous or ovarian cords (Fig. 1), which remain until primordial follicles begin to form (Odor and Blandau, 1969; Byskov, 1986; Hirshfield, 1991; Pepling and Spradling, 2001; Guigon and Magre, 2006; Luis-Díaz *et al*., 2008; Tingen *et al*., 2009). Ovarian cords are described as a tube-like structure, containing germ cells, with an external wall of pregranulosa cells bounded by a basal lamina (Juengel *et al*., 2002; Fig. 1).

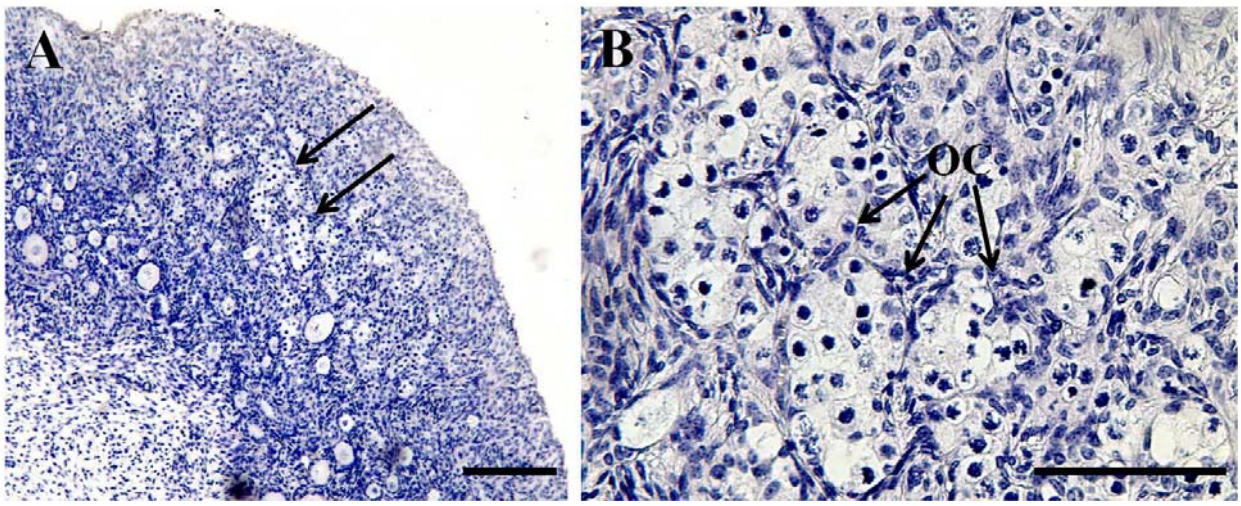

Figure 1. Histological section of ovigerous cords in an ovary from a *Bos taurus* fetus. Ovigerous cords (OCs) are indicated by arrows. Sections were stained with periodic acid Schiff (PAS) and hematoxylin. Scale bars: 50 µm. Original magnifications 100X (A) and 400X (B).

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After several mitoses, oogonia enter meiosis, becoming oocytes, which are arrested in the diplotene stage of meiotic prophase I (Pepling, 2006). The formation of primordial follicles occurs when the oocytes are individualized, as the nest germline undergoes programmed breakdown (Bristol-Gould *et al*., 2006). Nest breakdown involves the degeneration of many germ cell nuclei and the invasion of the pre-granulosa cells into the germ cell nests (Pepling and Spradling, 2001). This way, the majority of ovarian follicles enclose only one single oocyte, although there are reports of two or more oocytes within a single follicle in the ovaries of several mammalian species (Hartman, 1926; Mainland, 1928; Gondos *et al*., 1971; Lucci *et al*., 1999; Smitz and Cortvrindt, 2002; Balciuniene *et al*., 2006; Payan-Carreira and Pires, 2008; Yang and Fortune, 2008; Carrijo Jr., 2009; Stankiewicz *et al*., 2009; Silva-Santos *et al*., 2011).

This review focuses on the presence of these structures, called multioocyte follicles, in the ovaries of mammalian females, which are similar to the organization of oocytes in germline cysts.

Multioocyte follicles

Multioocyte follicles are follicles in which two or more oocytes are contained within a single follicle without a separating basement membrane between them (Tingen *et al*., 2009). These structures have already been described in several mammalian species: bats (Guthrie and Jeffers, 1938), mice (Iguchi *et al*., 1990; Balciuniene *et al*., 2006), rabbits (Al-Mufti *et al*., 1988), cats (Dederer, 1934; Miclãus *et al*., 2007), dogs (Telfer and Gosden, 1987; McDougall *et al*., 1997; Payan-Carreira and Pires, 2008; Reynaud *et al*., 2009), pigs (Greenwald and Moor, 1989; Stankiewicz *et al*., 2009), sheep (Hadek, 1958), goats (Lucci *et al*., 1999), cattle (Nuttinck *et al*., 1993; Lucci *et al*., 2002; Ireland *et al*., 2008; Yang and Fortune, 2008; Silva-Santos *et al*., 2011) and humans (Gondos *et al*., 1971; Smitz and Cortvrindt, 2002). Although these follicles have frequently been regarded as pathological entities, Telfer and Gosden (1987) argued that the development of multioocyte follicles is neither a spurious nor a pathological phenomenon, but rather a natural polymorphism arising from a spectrum of possible numerical combinations of oocytes and pregranulosa cells.

Characteristics of multioocyte follicles

Morphology

The number of oocytes inside the multioocyte follicle varies among species: 2 to 24 oocytes in rabbits (Al-Mufti *et al*., 1988), 2 to 9 in goats (Lucci *et al*., 1999), 2 to 17 in dogs (McDougall *et al*., 1997) and 2 to 9 in cattle (Silva-Santos *et al*., 2011). When the oocytes inside a multioocyte follicle are at different developmental stages, it seems that the granulosa cells and the more developed oocytes are at the same stage of development (Hartman, 1926).

Multioocyte follicles can be 2 to 3 times larger in volume compared to follicles with a single oocyte at the same developmental stage, due to the greater volumes of the oocytes and the greater number of granulosa cells (Telfer and Gosden, 1987). Oocytes inside the multioocyte follicle can be aligned or not, giving the follicle an elongated or rounded shape (Fig. 2).

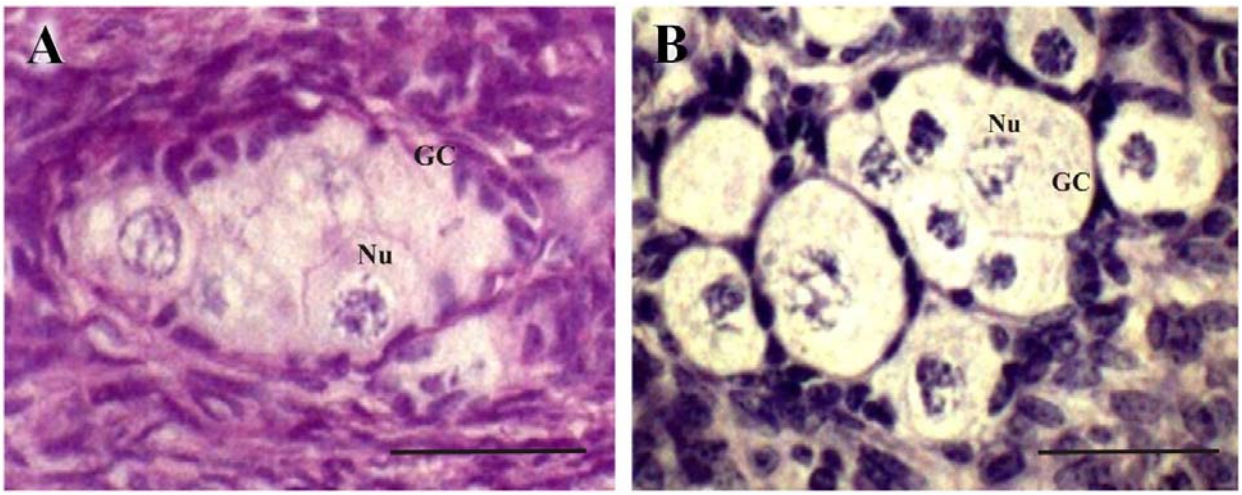

Figure 2. Histological sections of an elongated multioocyte follicle in the ovary of a Nelore heifer (A) and a rounded multioocyte follicle in an Aberdeen Angus (B) heifer. Presumptive nuclei of oocytes (Nu) enclosed within a follicle-like cell with a single layer of granulosa cells (GCs). Sections were stained with PAS and hematoxylin. Scale bars: 50 µm. Adapted from Silva-Santos *et al*. (2011).

Frequency

The frequency of multioocyte follicles in the ovaries is species- and individual-dependent (Table 1). For the opossum, mouse, pig and goat, multioocyte follicles are more common in the ovaries of younger, rather than in older, females (Hartman, 1926; Davis and Hall, 1950; Lucci *et al*., 1999; Stankiewicz *et al*., 2009). For dogs, there is disagreement on this topic. Some authors have shown that they are less common in older dogs than in animals at the beginning of their reproductive lives (Telfer and Gosden, 1987; Payan-Carreira and Pires, 2008), but other authors have reported no difference in the numbers of multioocyte follicles between young and adult females (McDougall *et al*., 1997). Multioocyte follicles represented 7% (before LH peak) and 4% (after LH peak) of the follicular population (Reynaud *et al*., 2009). Regarding breed, these structures were observed more frequently in mongrels (52.3%) than in purebred animals (25.5%; Payan-Carrera and Pires, 2008). In cattle, the number of multioocyte follicles gradually decreased throughout gestation in 80 to 244-day-old fetuses (Yang and Fortune, 2008), but their frequency was constant in fetuses, heifers and cows (Silva-Santos *et al*., 2011; Table 1). For humans, the frequency of these follicles did not change among different age groups (Gougeon, 1981), but decreased over time (Dandekar *et al*., 1988). Moreover, the frequency of their appearance was unaffected by gonadotropic hormones, oral contraceptives, pregnancy, or the day of the menstrual cycle (Gougeon, 1981).

Table 1. Frequency of multioocyte follicles in four mammalian species.

Species	Category	Frequency % (observed/total)	Reference
Goat	Prepubertal	100.0 (6/6)	Lucci *et al*., 1999
	Non-pregnant	50.0 (3/6)	
	Pregnant	17.0 (1/6)	
Bitch	Prepubertal	68.4 (13/19)	Payan-Carreira and Pires, 2008
	Mature	36.6 (48/131)	
Pig	Gilts	6.4 (49/819)	Stankiewicz *et al*., 2009
	Sows	1.4 (9/659)	
Bovine	Fetus	40.0 (8/20)	Silva-Santos *et al*., 2011
	Heifer	37.5 (9/24)	
	Cow	45.0 (9/20)	

Although there have been some reports of low frequency of multioocyte follicles, it seems that these follicles are not an extremely rare occurrence (Kennedy, 1924; Nuttinck *et al*., 1993; Lucci *et al*., 1999; Payan-Carreira and Pires, 2008). The prevalence of follicles containing more than a single oocyte is 40.7% in dogs (Payan-Carreira and Pires, 2008), 41% in cattle (Silva-Santos *et al*., 2011) and 85% in humans (Gougeon, 1981). In mice, the incidence of multioocyte follicles was reduced by gonadotropin treatment (Iguchi *et al*., 1990) and was enhanced by overexpression of inhibin-α (McMullen *et al*., 2001), knockout of the *Dmrt4* gene (Balciuniene *et al*., 2006) and treatment with estrogen (Iguchi *et al*., 1990).

Follicle developmental stage

Multioocyte follicles have already been reported during all phases of folliculogenesis, from primordial to preovulatory stages (Al-Mufti *et al*., 1988; Miclãus *et al*., 2007; Ireland *et al*., 2008; Payan-Carreira and Pires, 2008; Stankiewicz *et al*., 2009; Fig. 3). In young ovaries, the frequency was constant in the early stages of follicular growth but decreased in their largest preantral stage (Telfer and Gosden, 1987). The high variation among heifers regarding the number of antral follicles was strongly and positively correlated with the number of multioocyte follicles (Ireland *et al*., 2008).

Characteristics of oocytes from multioocyte follicles

When multioocyte follicles contain two oocytes, they seem to be at the same developmental stage because the thickness of the zona pellucida and the oocyte diameter is similar between the oocytes, but this relationship was not observed in follicles containing more than three oocytes (Payan-Carreira and Pires, 2008). Despite this observation, the meiotic status of the pronucleus must be taken into account.

Some authors have suggested that multioocyte follicles may ovulate (Bysted *et al*., 2001; Reynaud *et al*., 2009). Other authors have suggested that the smallest of the oocytes in a multioocyte follicle might be arrested in development and will probably degenerate, so that all might disappear except one or that all might undergo atresia and never reach the stage of extrusion of the first polar body (Kennedy, 1924). In cases where the differences among cells are more pronounced, some of those cells may undergo degeneration (Payan-Carreira and Pires, 2008). It is

possible that the fate of atypical follicles is the common destiny of the great majority of all follicles of the mammalian ovary, i.e., atresia, but occasionally such a follicle may reach maturity, even though the evidence in favor of this end is not complete (Hartman, 1926). It has been speculated that multioocyte follicles containing two oocytes would explain the higher rate of twinning in giant pandas if the oocytes were ovulated simultaneously (Zhang *et al*., 2001), but they could not be a significant cause of the birth of dizygotic twins because the probability that such follicles will ovulate is very low (Gougeon, 1981).

The higher concentration of estradiol-17β and the lower concentration of progesterone in multioocyte follicles from porcine ovaries may suggest that the presence of a greater number of oocytes in an ovarian follicle can lead to increased secretion of oocyte factors and an increase in these factors' influence on steroidogenesis (Stankiewicz *et al*., 2009).

Oocytes from multioocyte follicles have a significantly decreased fertilization capacity *in vitro* (Graham and Bradley Carolyn, 1971; Iguchi *et al*., 1991). The number of mature oocytes from these follicles is significantly lower than those from unioocyte follicles. Similarly, the total number of embryos that develop to the two or more cell stage, as well as to the morula and blastocyst stages after *in vitro* fertilization, is lower. In this way, beyond the differences in size between them, oocytes from multioocyte follicles differ in maturity and quality from unioocyte follicles (Stankiewicz *et al*., 2009). However, embryos obtained from these oocytes are able to successfully develop *in vitro* until the blastocyst stage. Despite reports of lower fertilization rates of oocytes from multioocyte follicles compared to unioocyte ones in both porcine and murine ovaries (Iguchi *et al*., 1991; Stankiewicz *et al*., 2009), the highest numbers of multioocyte follicles were observed in two mouse lines after long-term selection for high fecundity compared to the control (Alm *et al*., 2010).

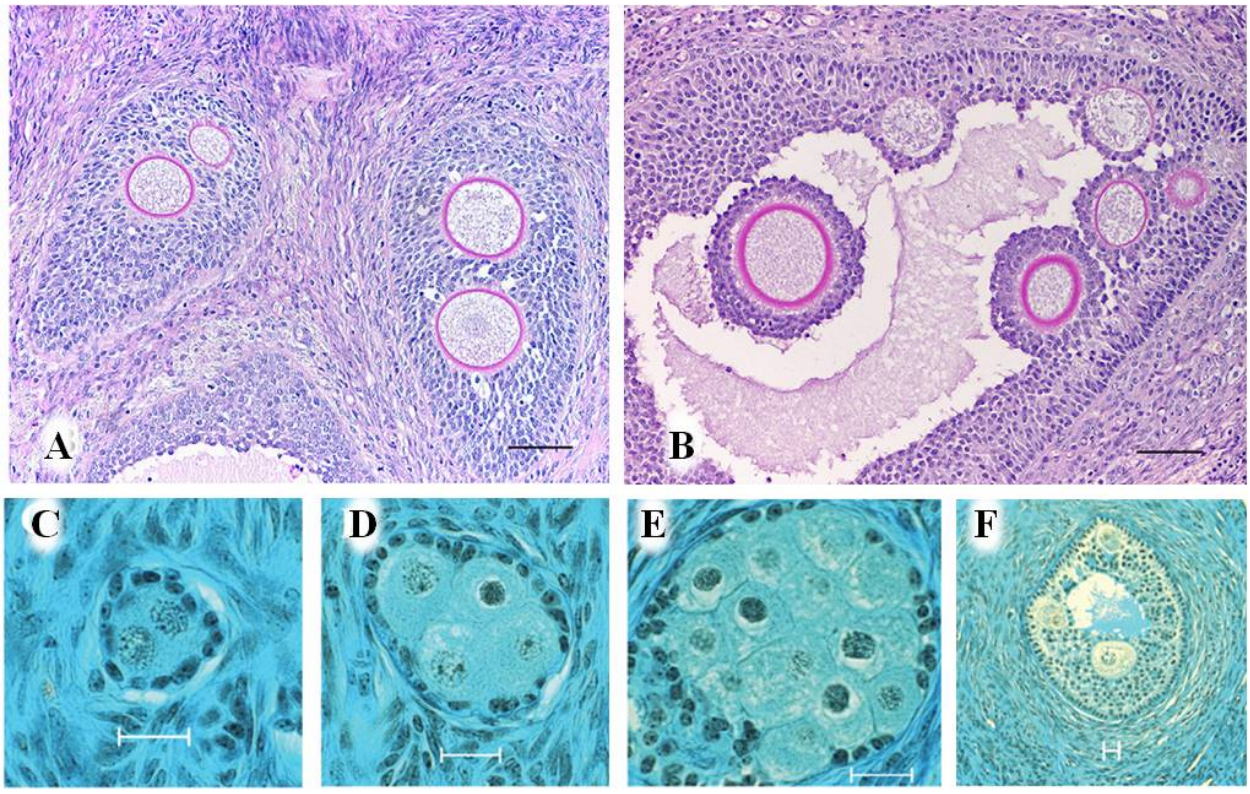

Figure 3. Preantral and antral multioocyte follicles in mature bitch (A-B) and *Bos taurus* heifer (C-F) ovaries. Multioocyte follicle at the secondary stage of development with two oocytes in each follicle (A), at the antral stage with six oocytes (B), at the primary stage with two (C), four (D) and 13 oocytes (E), and at the antral stage with three oocytes (F). Sections A-B were stained with PAS and hematoxylin. Scale bars: 70 µm (Adapted from Payan-Carreira and Pires, 2008). Sections C-F were stained with hematoxylin and picric methyl blue. Scale bars: 20 µm (Adapted from Ireland et al., 2008).

Multioocyte follicle organization

Studies on multioocyte follicles have been available in the literature since the early 19th century, but their origin has still not been established. It has been proposed that they may be derived from the division of a single cell body with two or more nuclei formed by amitotic division (Kennedy, 1924), from the encasement

of multiple oocytes in primordial follicles during folliculogenesis due to their more rapid developmental rate compared to the differentiation of the surrounding somatic cells (Kennedy, 1924; Telfer and Gosden, 1987; Balciuniene *et al*., 2006; Bristol-Gould *et al*., 2006), or from the failure of germ cell breakdown during the early stages of folliculogenesis (Zeilmaker *et al*., 1983; Safran *et al*., 1998; Lucci *et al*., 1999; Bristol-Gould *et al*., 2006; Tingen *et al*., 2009). The last hypothesis seems most likely because it phylogenetically evokes folliculogenesis in *Drosophila* (Reynaud *et al*., 2010). The *Drosophila* oocyte develops within a cyst of 16 interconnected germ cells (cystocytes), which are formed by a process of incomplete cell division to produce a germline cluster (King and Aggarwal, 1965; King, 1970; Miya *et al*., 1970; Mandelbaum, 1980; Spradling, 1993; Büning, 1994). Following each division, daughter cystocytes remain connected by cytoplasmic bridges, called *ring canals*. Later, only one of the 16 cystocytes in each cyst differentiates as an oocyte, and the other 15 become nurse cells that provide an assortment of RNA and proteins to be transported into the future oocyte (Büning, 1994; de Cuevas *et al*., 1997). Despite differences in the timing of breakdown during oogenesis in different organisms, germ cell breakdown occurs by apoptosis in both vertebrates and invertebrates (Foley and Cooley, 1998). Therefore, the molecular mechanisms controlling *Drosophila* cyst breakdown are likely to be relevant to the understanding of mouse cyst breakdown. Furthermore, the finding that cysts are evolutionarily conserved during early stages of oogenesis, but not in later stages, suggests that there is a separate function for these structures prior to follicle formation (Pepling and Spradling, 2001).

It is still unknown how some oocytes are selected to survive, while the others are designated as nurse cells destined to die. *Drosophila* mutants that disrupt cyst formation have demonstrated that the cysts are necessary for gamete development and fertility (Pepling *et al*., 1999). Some authors have suggested that the existence of multioocyte follicles provides evidence that correct germline breakdown selectively rids the ovary of defective oocytes (Iguchi *et al*., 1990, 1991; Hahn *et al*., 2005; Kipp *et al*., 2007). In mice, a 30% lower fertilization rate obtained with oocytes from multioocyte follicles, compared to unioocyte follicles, demonstrates the link between correct breakdown and oocyte quality (Iguchi *et al*., 1991; Tingen *et al*., 2009). Payan-Carreira and Pires (2008) suggested that multioocyte follicles' persistence in adult ovaries may indicate the inability of these follicles to ovulate, which in turn could be responsible for a decrease in the number of those follicles observed over time. However, the presence of multioocyte follicles of ovulatory diameter suggests that they probably do ovulate (Reynaud *et al*., 2009). Furthermore, it was already reported that more oocytes/embryos were collected by flushings than corpora lutea counted, which suggests that multioocyte follicles may ovulate (Bysted *et al*., 2001; Reynaud *et al*., 2005).

Molecular modifiers of nest breakdown

Germ cell breakdown can be affected by factors produced by the oocytes or somatic cells themselves that are not necessarily part of the apoptotic loss of the germ cell nuclei (Fig. 4). Synaptonemal complex protein-1 (SCP1) is expressed only in oocytes and drops precipitously within 24 h after birth, when germ cell breakdown is known to begin in the mouse (Tingen *et al*., 2009). Knock down of SCP1 leads to premature arrival of oocytes in the diplotene stage of meiosis and germ cell breakdown, causing acceleration of primordial follicle assembly, which suggests a link between cell cycle stage and primordial follicle development (Paredes *et al*., 2005). Thus, meiotic progression is not merely concomitant with germ cell breakdown, but causative of it (Tingen *et al*., 2009). The onset of germ cell breakdown is also affected by other proteins, including Foxl2, Nobox and members of the Notch signaling pathway and transforming growth factor (TGFβ) superfamily. Mutations in Foxl2, a transcription factor gene normally expressed in pregranulosa cells, are associated with premature ovarian failure (Crisponi *et al*., 2001; Uda *et al*., 2004). Nobox (newborn ovary homeobox-encoding gene) is expressed in germ cell nests and in oocytes of primordial and growing follicles (Suzumori *et al*., 2002). Lack of Nobox does not merely delay the development of germ cells nests, but accelerates postnatal oocyte loss and abolishes the transition from primordial to growing follicles in mice (Rajkovic *et al*., 2004). Components of the Notch signaling pathways are expressed in the mouse by germ cells in the nest and surrounding pregranulosa cells when bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) are mutated together (Yan *et al*., 2001). Both proteins, members of the TGFβ superfamily, are oocyte-secreted factors expressed early in ovarian differentiation (Elvin *et al*., 2000).

Mice lacking BMP15 or GDF9 have more multioocyte follicles as well as other defects of ovarian differentiation compared to the wild type (Yan *et al*., 2001), which suggests their influence in nest breakdown. Yet another TGFβ family member, activin, has also been implicated in early oocyte differentiation. Activin-deficient females have abnormal ovarian development and develop multioocyte follicles (McMullen *et al*., 2001; Bristol-Gould *et al*., 2005). Ovarian activin knockout mice demonstrate decreasing fertility with sequential deletion of the β-subunits, culminating in sterility when all intraovarian activins are removed (Pangas *et al*., 2007). Notch signaling can be stimulated or inhibited by the Lunatic fringe (Lfng) gene, a member of the fringe family of proteins expressed in granulosa

and theca cells of developing follicles (Bruckner *et al*., 2000; Hicks *et al*., 2000; Hahn *et al*., 2005). Lfng mutant mice are infertile and also have multioocyte follicles (Hahn *et al*., 2005). Interestingly, in Drosophila, fringe mutants also have follicles with more than one oocyte (Grammont and Irvine, 2001).

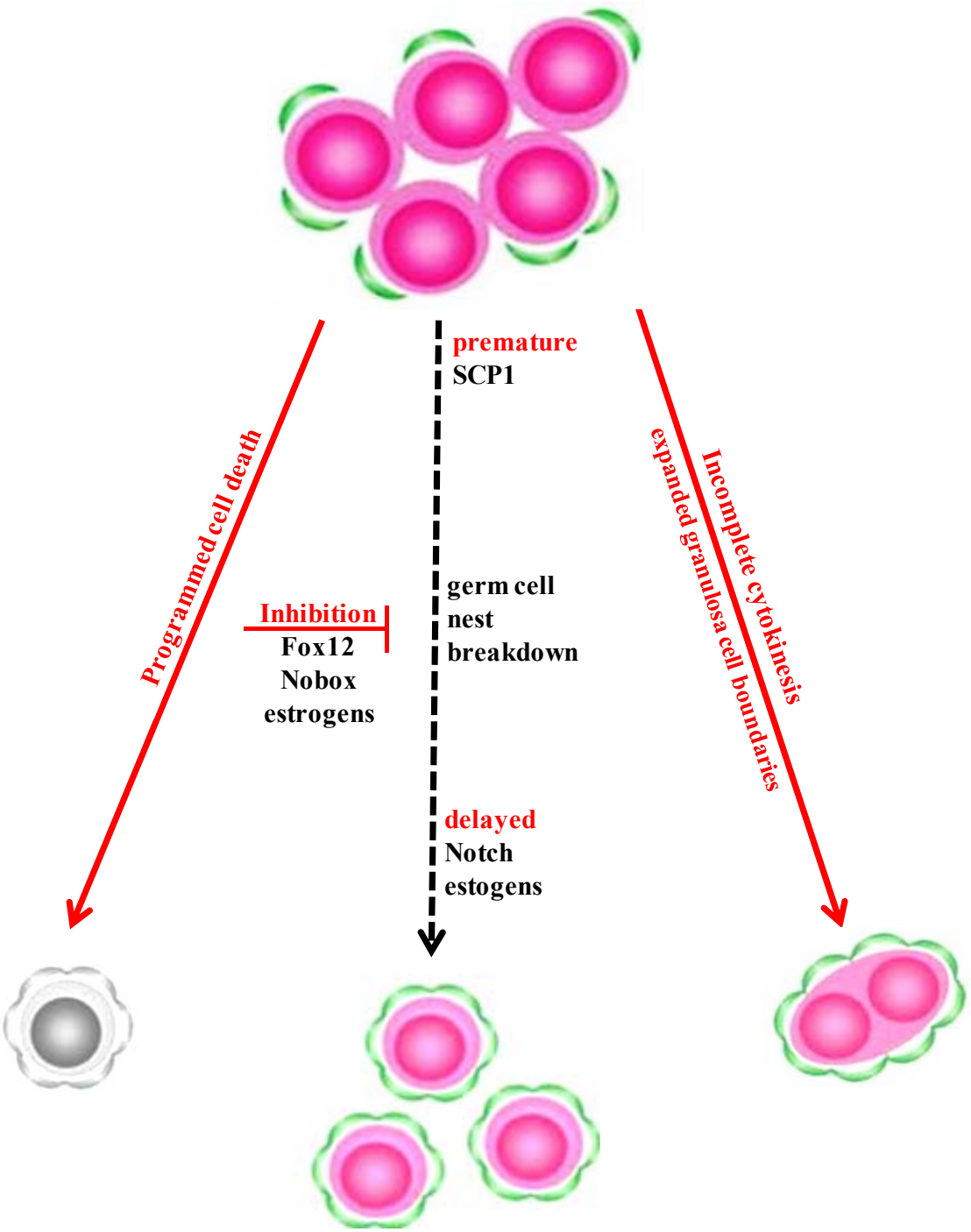

Figure 4. Schematic diagram of the process of multioocyte formation. Adapted from Tingen *et al*. (2009).

Circulating hormone and steroid factors are also involved in nest breakdown and primordial follicle assembly. Estradiol, progesterone and other estrogenic compounds like genistein, a phytoestrogen, and diethylstilbestrol (DES), a synthetic estrogen, can inhibit nest breakdown and induce multioocyte follicles (Iguchi *et al*., 1988, 1990; Jefferson *et al*., 2002; Suzuki *et al*., 2002; Kezele and Skinner, 2003; Chen *et al*., 2007; Kipp *et al*., 2007). Neonatal expose to genistein and DES leads to the inhibition or delay of nest breakdown in mouse ovaries (Jefferson *et al*., 2006; Chen *et al*., 2007). Genistein delays cyst breakdown and

reduces oocyte death through suppression of apoptosis, supporting the idea that multioocyte follicles are nests that did not break down (Jefferson *et al*., 2006). Induction of multioocyte follicles by DES is thought to be similar to that by genistein exposure; namely, an inhibition of oocyte death (Jefferson *et al*., 2006). Both genistein and DES signaling in multioocyte induction are mediated by receptor beta (ESR2), which is localized in granulosa cells (Sar and Welsch, 1999; Jefferson *et al*., 2002; Kirigaya *et al*., 2009). Estrogens influence on oocyte quality is unclear, but a lower fertilization rate was reported for oocytes from multioocyte follicles compared to oocytes from unioocyte follicles in control and DES-exposed mice (Iguchi *et al*., 1990; 1991). It seems that oocytes are kept in nests by the high levels of estrogens and progesterone present in the maternal environment of pregnancy, since progesterone's actions were independent of the estrogen receptor and addition of either exogenous estrogen or progesterone inhibited premature breakdown in ovaries removed at 16.5 days post coitum (Chen *et al*., 2007). Before birth, the high maternal hormone levels inhibit nest breakdown and oocyte apoptosis (Jefferson *et al*., 2006). The decrease of estrogen and progesterone levels at birth stimulates nest breakdown and follicle assembly in both mice and large animals (Zachos *et al*., 2004; Chen *et al*., 2007), but it is still not known why nests need to break apart at birth. Possibly, if this process occurred earlier, the oocytes would not yet be in the right stage of meiosis (Chen *et al*., 2007).

As stated before, the drop in estrogen and progesterone levels at birth is the primary signal to initiate nest breakdown and follicle assembly. However, it is possible that this mechanism is different in humans, since follicle formation occurs at about 4.5 months of gestation, not at birth as occurs in rodents (Gillman, 1948; Witschi, 1948; Gondos *et al*., 1971). We can also hypothesize that even though total progesterone and estrogen levels are high late in human pregnancy, the amounts that fetal tissues are exposed to are reduced at this time, which is supported by a report of reduced progesterone levels in monkey fetal tissues during late pregnancy (Thau *et al*., 1976). In ruminants, follicle formation also occurs asynchronously over a fairly long period of time (van Wagenen and Simpson, 1965; Russe, 1983; McNatty *et al*., 1995); at about 75-100 days of gestation in sheep (McNatty *et al*., 1995; Sawyer *et al*., 2002) and 74-130 days of gestation in cattle (Erickson, 1966; Russe, 1983; Dominguez *et al*., 1988; Yang and Fortune, 2008). Decreased progesterone and estradiol levels were observed between Days 83 and 140 of bovine gestation, during follicle formation and before the appearance of primary follicles (Yang and Fortune, 2008). Conversely, ovarian progesterone and estradiol levels increased 3-to 4-fold from Days 55 to 75 of ovine pregnancy (Quirke *et al*., 2001), but they did not measure steroids after Day 75.

Conclusions

Multioocyte follicles are an intriguing physiologic phenomenon. In insects, germline cysts are essential to the development of a single oocyte through the presence of nurse cells, but their significance in mammalian ovaries remains unclear. Although they are typically described in early folliculogenesis, there is a lack of information about them. It was shown that apoptotic pathway members, locally produced factors, circulating hormone and steroid factors can affect germ cell breakdown and primordial follicle assembly, thereby inducing multioocyte follicles. Oocytes from multioocyte follicles, after fertilization, are capable of developing *in vitro* until the blastocyst stage, but would they be able to reach ovulation and release a viable oocyte *in vivo,* or does their presence indicate the inability of these follicles to ovulate? New approaches regarding the function of multioocyte follicles and their correlation with fertility would improve our knowledge of this subject.

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