# Intraovarian regulation of folliculogenesis in the dog: a review

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**Running title**: Dog ovarian folliculogenesis

## Summary

Dog reproductive cycle is unique among other mammals in that females experience long and variable periods of ovarian inactivity. Neuroendocrine controls of the reproductive cycle have been thoroughly studied in the dog. However, there is little information regarding endocrine, paracrine and autocrine controls of dog ovarian folliculogenesis. Advancements in the understanding of mechanisms regulating dog ovarian follicle development will be helpful in the development of an approach to control cyclicity in this species. Furthermore, such information will likely be useful for the establishment of an in vitro follicle culture system to preserve fertility of genetically valuable disease models or endangered canids. This review highlights current knowledge on dog folliculogenesis with emphasis on endocrine, paracrine and autocrine controls of follicular development.

**Keywords**: dog, follicles, gonadotropins, growth factors

## Introduction:

Within the ovary, there is a vast reserve of intraovarian follicles. During reproductive life, poorly understood intraovarian mechanisms activate small numbers of dormant primordial follicles that subsequently develop into primary, secondary and antral stages (Gougeon, 2010). Folliculogenesis can be divided into four stages: activation of primordial follicles, growth of early stage follicles, selection of dominant follicle(s) and maturation of preovulatory follicles (Gougeon, 2010) (Fig. 1). The first two stages (activation and growth) are regulated by intraovarian factors

(McGee & Hsueh, 2000; Hsueh, Kawamura, Cheng & Fauser, 2015) whereas the final two stages are gonadotrophin-dependent. Most conventional wisdom about mechanisms regulating mammalian folliculogenesis has been derived from extensive studies in rodents (Eppig, 2001; Richard & Pangas, 2010), and some from larger species (Fortune, Cushman, Wahl & Kito 2000; van den Hurk, & Zhao, 2005). This review focuses on endocrine, paracrine and autocrine controls of folliculogenesis in the domestic dog. Because there is limited information about mechanisms regulating dog folliculogenesis, knowledge gained from mouse and larger mammal studies also will be discussed when it is relevant.

**Ovarian folliculogenesis in the dog**

Mammalian oocyte arises from primordial germ cells (PGCs) developed during the embryogenesis (Edson, Nagaraja, & Matzuk, 2009). Once formed, the PGCs proliferate and migrate to the undifferentiated or bipotential gonad at the genital ridge (Edson et al., 2009). Upon reaching the undifferentiated gonad, PGCs continue to rapidly proliferate with incomplete cytokinesis to form clusters of germ cell nest consisting of oogonia connected to each other by intercellular bridges, and the syncytia units are surrounded by pre-granulosa and stromal mesenchymal cells of the ovary (Tingen, Kim, & Woodruff, 2009). Shortly after germ cell proliferation commences, differentiation of the bipotential gonad takes place (Wear, McPike, & Watanabe, 2016). In the dog, ovarian differentiation occurs at Day 36 of gestation (Andersen & Good, 1970; Meyers-Wallen et al., 1991; Pretzer, 2008). This developmental process is characterised by inward proliferation of the surface epithelium, and together with the surrounding connective tissue forms ovarian structure (Andersen & Good, 1970). Within the developing ovary, mitosis ceases and oogonia within germ cell nests transform into oocytes by entering the first meiotic prophase and being arrested at the late diplotene stage (Wear et al., 2016).

The formation of dog primordial follicles begins with the breakdown of germ cell nests during the second or third weeks after birth (Andersen & Simpson, 1973; Peter & McNatty, 1980). To date, little is known about the regulatory process of germ cell nest breakdown in the dog. Studies in the mouse have demonstrated that activation of the neurogenic locus homolog protein 2 (NOTCH2) signaling pathway in granulosa cells is required for the breakdown of the germ cell nests (Wear et al, 2016). Previous studies also have shown that germ cell nest breakdown is associated with decreased estrogen concentration (Tingen et al., 2009; Wear et al., 2016) and the cytoplasmic-to nuclear localization of forkhead transcription factor 3 in the oocyte of neonatal mice (John, Gallardo, Shirley & Castrillon, 2008). Furthermore, members of transforming growth factor β, activin and follistatin have been shown to play roles in regulating germ cell nest breakdown and primordial follicle formation (Wang et al 2015). Specifically, culturing mouse fetal ovaries with activin increases primordial follicle pool whereas follistatin treated ovaries exhibit reduced granulosa cell proliferation and down regulation of Notch signaling pathway (Wang, Niu, Wang, Teng, Wen, Xia, Wang, 2015). During the breakdown of germ cell nests, granulosa cells invade and surround a single oocyte, eventually forming a primordial follicle (Wear et al., 2016). Within a dog ovary, there are ~100,000 primordial follicles, each of which contains a small (10-26 μm in diameter) oocyte voided of the zona pellucida with a single layer of flattened granulosa cells (Durrant, Pratt, Russ, & Bolamba, 1998; Songsasen, Fickes, Pukazhenthi, & Wildt, 2009) (Fig. 2a). To date, there is no information about the influence of dog breed on the numbers of primordial follicles within the ovarian reserve. However, the study by Durrant et al., (1998) demonstrated that a greater number of follicles can be obtained by enzyme digestion of ovarian tissue in crossbred than purebred individuals; however, there are no differences in follicle numbers among purebred dogs. Domestic dog ovaries are also noted for their high frequency of multi-oocyte

follicles (Payan-Carriera and Pires, 2008), though the mechanism of this phenomenon is not yet well understood. The activation of primordial follicles begins in 6 weeks old pups (Zlotnik, 1994). During this period, granulosa cells transition from flattened to cuboidal (Durrant et al., 1998) (Fig.2b). In primary follicles, the oocyte is nearly double in size (16-62 μm, average 31 μm) compared to the primordial stage (Songsasen et al., 2009; Zlotnik, 1994). As primary follicles develop into the secondary stage, granulosa cells rapidly proliferate and theca cell differentiate from interstitial stroma cells and surround the granulosa layers (van den Hurk & Zhao, 2005) (Fig. 2c & d). In the secondary stage follicle, the oocyte expands in size (35-88 μm, average 60.5 μm) and secretes a glycoprotein membrane, the zona pellucida which forms a protective coat around the gamete (van den Hurk & Zhao, 2005) (Fig 2c & d). Formation and accumulation of a fluid filled cavity is a characteristic of early and antral stage follicles (Fig. 2e). At this developmental stage, granulosa cells also undergo massive proliferation and the gamete continues to increase in size (Songsasen et al., 2009). During the antral phase, dog oocytes grow from ~80 μm in early antral stage to the maximum size of >120 μm when the follicles reach >0.4 mm in diameter (Reynaud et al., 2012). From 120 through 160 days after birth, primary, secondary and early antral follicles can be observed within the dog ovarian cortex (Andersen & Simpson, 1973; Durrant et al., 1998). Advanced antral follicles (>2 mm diameter) can be found in bitches as young as 6 months of age and shortly before pro-oestrus (England & Allen, 1989; Wildt, Levinson, & Seager, 1977). At the final stage of follicular growth, dog preovulatory follicle can reach 5-7 mm in diameter (Reynaud et al., 2012). A previous study shows that large dogs have a higher number of preovulatory follicles than small dogs, and these follicular stage is larger in the former than the latter (Reynaud, Chastat-Maillard, Batard, Thoumire, & Monget, 2010). However, unlike other

mammals, dog preovulatory follicles contain an immature oocyte(s) that require additional 48-72 hours in the oviduct to complete maturation (Chastant-Maillard et al., 2011).

**Neuroendocrine controls of dog ovarian cycle**

Dog ovarian cycle is unique among other mammalian species (Concannon, 2011). Specifically, dog reproductive cycle is characterised by an extended period of pro-oestrus and then oestrus (~1 week each) followed by dioestrus, a luteal phase averaging 2 months in duration irrespective of pregnancy. Dioestrus is succeeded by anoestrus, an extended (2-10 months) interval of ovarian quiescence (Concannon, 2011). To date, little is known about factors regulating the interval of ovarian cycle in the dog. It has been suggested that dog (and wild canids) ovarian cycle interval is part of an endogenous circannual cycle that is influenced by age, genetics and pheromones (Concannon, 2012).

Like other mammals, the ovarian cycle in the dog is regulated by the hypothalamic-pituitary-gonadal axis (Concannon, 2009; Concannon, Castracane, Temple, & Montanez, 1999; Wildt, Panko, Chakraborty, & Seager, 1979)(Fig. 3). Prior to pro-oestrus, there is an increase in gonadotropin releasing hormone (GnRH) pulses from the hypothalamus which, in turn, stimulate follicle stimulating hormone (FSH) and luteinizing hormone (LH) release from the anterior pituitary (Concannon, 2009). The increase in pituitary hormone pulses initiates small follicle growth and stimulates gonadal steroidogenesis (estradiol and progesterone) (England, Russo, & Freeman, 2009) that results in the selection of dominant follicles. Continuing rise in estradiol during pro-oestrus triggers an LH surge that is followed by ovulation ~60 hours later (Concannon, 2011). Currently, the mechanisms underlying the transition from anoestrus to pro-oestrus have not been fully elucidated. It has been shown that there is a rise GnRH pulse frequency during the late

anoestrus (de Gier, Beijerink, Kooistra, & Okkens, 2008). During this period, mean circulating FSH concentration, but not LH, is higher than during early- and mid-anoestrus periods (de Gier et al., 2008), suggesting that the rise in circulating FSH is a key event leading to anoestrus termination. Autoradiographic analysis of dog ovarian sections also supports this hypothesis (Saint-Dizier et al., 2008). Specifically, FSH binding sites can be detected exclusively in granulosa cells of preantral and antral follicles whereas LH receptors are only present in >1 mm follicles (Saint-Dizier et al., 2008).

Recently, there have been studies that investigate the roles of kisspeptins in regulating dog reproductive cycle and ovarian function (Albers-Wolthers et al., 2016; Albers-Wolthers et al., 2014; Cielesh, McGrath, Scott, Norman, & Stephen, 2017). Kisspeptins are a family of peptides that bind to G-protein couple receptors that in turn stimulate gonadotropin secretions, primarily through a direct action on GnRH release (Albers-Wolthers et al., 2016; Dungan, Clifton, & Steiner, 2006). Furthermore, studies have shown that kisspeptins regulate ovarian function through their direct actions on the pituitary gland and ovary (Kotani et al., 2001). In the dog, kisspepptin 1 (KISS1) and its receptor (KISS1R) proteins have been observed in the ovary (Cielesh et al., 2017). Specifically, immunolocalization of KISS1 has been observed in the oocytes and granulosa cells of primordial and primary follicles in anoestrous females (Cielesh et al., 2017). Interestingly, this protein is not detected in the ovary of proestrous bitches (Cielesh et al., 2017). Similarly, KISS1R is observed in oocytes and granulosa cells of pre-pubertal and anoestrous females. It also has been shown that adminstration of an exogenous kisspeptin, K-10, increases circulating FSH, LH and estradiol levels in anoestrous females (Albers-Wolthers et al., 2014). Finally, the LH response to K-10 administration is varied among reproductive cycles with anoestrous females being the most sensitive and oestrous individuals being the least responsive to the hormone treatment.

Collectively, it is likely that kisspeptins also play roles in regulating dog reproductive cycle. Yet, future research is needed to fully elucidate the roles of these peptides in regulating dog ovarian function.

**Paracrine and autocrine controls of dog ovarian follicle development**

To date, there is limited information on paracrine and autocrine controls during dog folliculogenesis, particularly those relating to follicle activation and follicle selection. Various growth factors, including insulin-like growth factor (IGF), fibroblast growth factor, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) as well as growth hormone, insulin, activin and anti-Müllerian hormone (AMH) have been shown to be expressed in dog follicles and/or support growth and survival in vitro suggesting their roles in folliculogenesis.

*Insulin-like growth factor system*: The IGF system comprises two ligands (IGF-I and IGF-II) and two receptors (IGF1R and IGF2R) (Silva, Figueiredo, & van den Hurk, 2009; Sirotkin, 2011). There are six IGF binding proteins (IGFBPs) that control the bioavailability of IGF-1 and IGF-II (Silva et al., 2009). IGFBPs control IGFs bioavailability through: (1) acting as transport proteins in plasma, (2) prolonging half-lives of IGFs by regulating metabolic clearance, (3) providing means of tissue and cell type-specific targeting and (4) modulating interactions of the IGFs with their receptors (Silva et al., 2009). In the human, IGF-II is a primary player in controlling reproductive function, whereas IGF-I plays important roles in regulating ovarian folliculogenesis in ruminants and rodents (Silva et al., 2009; Sirotkin, 2011). Both IGF-I and IGF-II stimulate granulosa cell proliferation and steroidogenesis of secondary and antral follicles and increase their sensitivity to FSH stimulation (Silva et al., 2009; Sirotkin, 2011). In the dog, although IGF-I and IGF-II levels in follicular fluid are not linked to follicle size, the concentration of IGF-1 and its binding protein, IGFBP3, are positively correlated with animal's body height that, in turn, is

correlated with the numbers and size of preovulatory follicles (Reynaud, et al., 2010). The level of circulating IGF-I in dogs treated with equine chorionic gonadotropin during late anoestrous is higher during pro-oestrus and oestrous than dioestrus (Stornelli et al., 2016). In preovulatory follicles, immunostaining of IGF-I is stronger in the theca interna than granulosa cells while the expression of IGFIR is more intense in the latter than the former. Collectively, it is likely that IGF-I has a paracrine and autocrine role in dog follicle development. However, the precise roles of this growth factor in dog folliculogenesis remains to be further investigated.

*Fibroblast growth factor family*: Fibroblast growth factor family (FGF) is a group of heparin-binding polypeptides that have been shown to be involved in the regulation of ovarian functions in mammals (Chaves, de Matos, Buratini, & de Figueiredo, 2012). FGFs are potent mitogens involved in cell proliferation, differentiation and migration as well as angiogenesis (Chaves, de Matos, et al., 2012). Within the FGF family, FGF-2 or basic fibroblast growth factor is the most studied, with affinity to all known FGF receptors (Chaves, de Matos, et al., 2012). Studies have shown that FGF-2 supports in vitro activation of primordial follicles enclosed within the ovarian cortex in the goat, but this growth factor exerts no effect in the cow (Chaves, de Matos, et al., 2012). Supplementation of FGF-2 to culture media has also been shown to stimulate growth of isolated preantral follicles during 12-day in vitro culture in the goat (Almeida, Saraiva, Alves Filho, Silva, Goncalves, Brito, Silva, Lima, Cunha, Silva, Figueiredo, 2012). In the dog, FGF-2 is detectable in oocytes and granulosa cells of preantral follicles in all reproductive stages, except during anoestrus (Songsasen et al., 2009). We recently investigated the influence of in vitro survival and growth of dog follicles enclosed within the ovarian cortex. Our preliminary findings showed that FGF-2 did not affect the survival of early stage follicles after in vitro culture for 7

days. However, this growth factor appeared to dose-dependently support the activation of primordial follicles in vitro (N. Songsasen, unpublished data).

Another member of FGF family that has been shown to play roles in ovarian folliculogenesis is FGF-7 (Chaves, de Matos, et al., 2012). Our previous study also has shown that FGF-7 is localised in granulosa cells of dog primary follicles and in both granulosa and theca cells of the secondary follicles in all reproductive stages. Furthermore, FGF-7 is expressed only in the theca layer of antral follicles in pro-oestrous/oestrous females, but is absent in pre-pubertal ovaries, suggesting that this protein may play a role in antral follicle growth in the dog (Songsasen et al., 2009).

*Epidermal growth factor (EGF)*: Epidermal growth factor is a mitogenic polypeptide and member of the EGF-family which includes heparin-binding EGF-like growth factor, transforming growth factor (TGF)-α, amphiregulin, epiregulin, epigen, betacellulin and neuregulins-I-4 (Sirotkin, 2011). Previous studies have shown that EGF regulates cell proliferation and supports in vitro follicle growth in the goat (Celestino et al., 2011) and cow (Wandji, Eppig, & Fortune, 1996), and supports in vitro viability of cat follicles (Fujihara, Comizzoli, Keefer, Wildt, & Songsasen, 2014). In the dog, supplementing culture media with EGF sustains the short-term (3 days) viability of preantral follicle enclosed within the ovarian cortex, but has negligible effect during long-term culture (Thongkittidilok, Wildt, & Songsasen, 2017).

*Vascular endothelial growth factor*: VEGF is a glycoprotein involved in angiogenesis in many tissue and organ systems, including in the ovary (Araujo, Duarte, Bruno, Pinho Lopes, & de Figueiredo, 2013). In the dog, immunolocalization of VEGF has been detected in granulosa and thecal cells of preantral and antral follicles (Abdel-Ghani, Shimizu, & Suzuki, 2014). Furthermore, VEGF dose-dependently affects dog follicle development in vitro. Specifically, a high-dosage (200

ng/ml) of VEGF stimulates the activation of tissue-enclosed primordial follicles after 14 days in vitro culture (Abdel-Ghani et al., 2014). VEGF at 1 ng/ml in the presence of 10 ng/ml EGF protects dog primordial follicles against apoptosis after 7 days in vitro culture; however, this low VEGF dosage has no impact on primordial follicle activation (Thongkittidilok, Wildt & Songsasen, 2017).

*Growth hormone and insulin*: Growth hormone (GH) and insulin are extraovarian factors that have been shown to regulate ovarian folliculogenesis in many mammalian species (van den Hurk & Zhao, 2005). Growth hormone's binding site has been detected in sheep (Eckery, Moeller, Nett, & Sawyer, 1997), cow (Kolle, Sinowatz, Boie, & Lincoln, 1998) and rat ovaries (Zhao, Taverne, van der Weijden, Bevers, & van den Hurk, 2002). mRNA and/or protein expression of insulin receptor have been found in the human (Samoto et al., 1993) and goat follicle (Chaves, Duarte, et al., 2012). Both GH and insulin act synergistically with FSH in promoting ovarian follicle growth and steroidogenesis through increasing sensitivity to gonadotropin stimulation (Chaves, Duarte, et al., 2012). In the dog, addition of growth hormone to culture medium containing FSH sustains survival as well as promotes growth and steroidogenesis of isolated secondary follicles after 18 days in vitro culture (Serafim et al., 2015). Similarly, the presence of insulin, especially at a high concentration (10 μg/ml) in FSH supplemented medium supports in vitro survival and growth of isolated secondary dog follicles after 18 days in vitro (Serafim et al., 2013).

*Activin*: Activins are dimeric polypeptides that belong to the TGF-β superfamily (Bloise et al., 2019) and play key roles in ovarian follicle development. Previous studies have shown that activin A accelerates the progression of mammalian oocytes throughout the meiotic prophase stage (Bloise et al., 2019). In addition to the pituitary cells, activin A and its receptor are expressed in the oocyte and somatic cells of primordial follicles of the mouse, human and baboon (Bloise et al.,

2019). Mouse preantral follicles secrete activin A which, in turn, stimulates granulosa cell proliferation and follicle growth (Bloise et al., 2019). Activin A also enhances aromatase activity and proliferation of granulosa cells, maintains granulosa cell-oocyte communication and promotes in vitro preantral follicle growth in the cow (McLaughlin, Bromfield, Albertini, & Telfer, 2010; Telfer, McLaughlin, Ding, & Joo Thong, 2008). We recently demonstrated that activin A acted synergistically with FSH to support in vitro growth of dog antral follicles during 21-day in vitro culture (Nagashima, Wildt, Travis, & Songsasen, 2019). Furthermore, activin and FSH promotes antral cavity expansion in vitro in early and antral follicles but does not support the transition of the preantral to antral stage (Nagashima et al., 2019). Finally, activin supports in vitro growth and viability of dog oocytes by sustaining bidirectional communication between the gamete and somatic cells during 21-day culture (Nagashima et al., 2019).

*Anti-Müllerian hormone*: AMH is a dimeric glycoprotein in the TGF-β superfamily. AMH is expressed in the granulosa cells of growing follicles (Poole, Ocon-Grove, & Johnson, 2016), and has been shown to inhibit the initiation of primordial follicle activation (Durlinger, Visser, & Themmen, 2002; Josso, 2019) and modulate the stimulating effect of FSH on follicle growth (Durlinger et al., 2002). In the dog, AMH can be weakly detected in the granulosa cells of primordial and primary follicles (Karakas Alkan et al., 2019; Nagashima, Hansen, Songsasen, Travis, & Place, 2016), with the protein expression level increasing significantly in multi-layer secondary follicles (Karakas Alkan et al., 2019; Nagashima et al., 2016). AMH can be detected in the serum of intact, adult female dogs, but is absent in prepubertal and spayed individuals (Place et al., 2011). Evaluation of circulating AMH in dog has demonstrated that there is a two-fold increase in the hormone level 8-9 days before the LH surge (Nagashima et al., 2016). The level of

AMH remains high during oestrous before declining to the baseline level during dioestrous and anoestrous periods (Karakas Alkan et al., 2019; Nagashima et al., 2016).

**Conclusion**:

During the past decades, significant progress has been made to better understand endocrine, paracrine and autocrine controls of ovarian follicle development. Like other mammalian species, dog reproductive cycle is regulated by the hypothalamic-pituitary-gonadal axis with FSH is a key regulator of early follicle development. With the advance in in vitro follicle culture technology, there is increasing evidence that several growth factors and hormone, including IGF system, FGF family, EGF, VEGF, growth hormone, insulin, activin and AMH plays roles in dog folliculogenesis (Fig. 4). Yet, future research is still needed to fully elucidate the mechanisms regulating follicle development in the dog. Such knowledge will be valuable for the ability to effectively control dog reproductive cycle and for developing an in vitro follicle culture system to produce developmentally competent gametes for fertility preservation of genetically valuable research models as well as rare and endangered canids.

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Figure legends:

Figure 1: Diagram illustrates the process of folliculogenesis.

Figure 2: Micrograph of ovarian cortical tissue-enclosed dog (a) primordial and (b) primary follicles, as well as isolated, (c) early secondary, (d) late secondary or preantral and (e) early antral follicles. The photographs were taken by a light microscopy. Bar indicates 10 μm.

Figure 3: Diagram illustartes neuroendocrine controls of ovarian reproductive cycle in the dog.

Figure 4: Diagram illustrates known endocrine, paracrine and autocrine controls of dog ovarian folliculogenesis.