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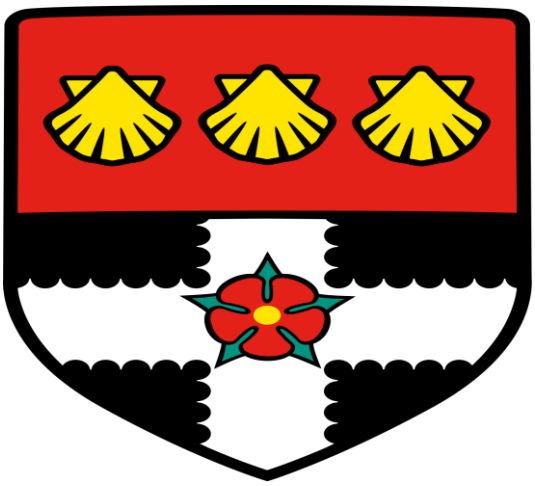

INTRA-OVARIAN FACTORS REGULATING FOLLICULAR DEVELOPMENT, STEROIDOGENESIS AND ANGIOGENESIS

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Dedication

This thesis is dedicated to the memory of my beloved father, Salih Mattar and to the memory of my beloved mother Najat Naitah, both of whom are the reason for what I become today.

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Dareen Salih Mattar

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Finally, I would never have been able to finish without the unceasing encouragement and support from my family, especially my brother Adhum Mattar, my two sisters Noor Mattar and Nahla Mattar, whom always cheered me up and stood by me through the good and bad times. I also place on record, my sense of gratitude to my entire family members and friends who, directly or indirectly, have lent their helping hand and support.

Abstract

Reproduction is an indispensable function that is under the control of a sophisticated network of regulatory signals that originates from and is integrated by the hypothalamic-pituitary-gonadal axis, which is primarily driven by gonadotropin releasing hormone (GnRH) from the hypothalamus. Compelling evidence accumulating over the last few decades has revealed that kisspeptin, a hypothalamic neuropeptide encoded by *KiSS-1* gene, has a key role in promoting the release of GnRH and luteinizing hormone (LH) in various mammalian species. However, the possibility that kisspeptin exerts additional 'peripheral' actions at the level of the gonad has received little attention and no studies have been directed at the bovine ovary. Another neuropeptide, neuromedin B (NMB), belonging to the bombesin-related peptide family, has been shown to have various physiological roles including the regulation of various exocrine and endocrine secretions but its potential action at the gonadal level has not been explored. This thesis reports a series of experiments designed to investigate: (1) whether *KiSS-1* and *NMB* and their cognate receptors are expressed in bovine endocrine tissues; (2) whether kisspeptin and NMB, alone and in combination with their antagonist, can influence the steroidogenesis in cultured ovarian cells; (3) whether expression of *KiSS-1* and *NMB* by cultured ovarian cells is regulated by gonadotropins and other factors; (4) whether NMB, kisspeptin-10, TGF-β-1, BMP-6 and TSP-1 and their respective antagonist modulate capillary network formation in a follicular angiogenesis model; (5) whether TGF-β-1 and BMP-6 (alone and in combination with their antagonists) affect ovarian steroidogenesis *in vitro*; (6) whether expression of steroidogenesis transcripts and other angiogeneic factors by cultured ovarian cells is regulated by TGF-β-1 and BMP-6. The laboratory techniques used to address the above included primary ovarian cell culture systems (bovine ovarian theca and granulosa cells under non-luteinized and luteinized conditions, cell migration and follicular angiogenesis models), steroid immunoassays (androstenedione, oestradiol and progesterone), real-time PCR and immunohistochemistry. The results of RT-PCR confirmed that *KiSS-1* and its receptor (*GPR54*) and *NMB* are expressed in different bovine endocrine tissues including pituitary, adrenal, testis, ovarian corpus luteum, theca cells and granulosa cells. Moreover, changing levels of thecal and granulosal expressions were detected during different stages of follicle development. However,

cell culture experiments offered no evidence to support the hypothesis that kisspeptin and NMB have a direct intra-ovarian role to modulate follicular or luteal steroidogenesis or cell proliferation under basal or gonadotrophin stimulated conditions. Neither did they affect ovarian theca cell migration evaluated using a wound-healing 'scratch' assay. Results from the follicular angiogenesis model indicate that while TGF-β-1 and BMP-6 reduced VEGF/FGF-induced capillary network formation, kisspeptin, NMB and TSP-1 were without effect. In conclusion, the results provided no evidence to support intrafollicular roles of kisspeptin or NMB peptides in modulating steroidogenesis, cell proliferation, cell migration or angiogenesis. However, both TGF-β-1 and BMP-6 were implicated as negative regulators of follicular angiogenesis, a finding that warrants further research, given their inhibitory action on thecal steroidogenesis.

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1. Introduction and Literature Review

1.1. Sexual reproduction

Sexual reproduction of mammalians is defined in biology as a process resulting in the formation of a genetically novel individual (Rastogi, 2007). Reproductive efficiency has a major influence on profitability of dairy farms. Improvement in reproductive performance in dairy cattle encompasses aspects related to resumption of ovarian function, detection of oestrus, and establishment and maintenance of pregnancy (Santos et al., 2009). Reproduction is an indispensable function that is under the control of a sophisticated network of regulatory signals. These regulatory dynamic signals originate from and are integrated by the hypothalamus which is responsible for the secretion of kisspeptin that, in turn, regulates the secretion of gonadotropin releasing hormone GnRH (Pinilla et al., 2012).

1.2. Hypothalamic-pituitary-ovarian (HPO) axis

The oestrous cycle is regulated by a key hormone from the hypothalamus known as GnRH that, in turn, stimulates the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. Progesterone (P4), oestradiol (E2) and inhibins are, in turn, released from the ovaries and prostaglandin F2α (PGF2α) is secreted from the uterus. These hormones function through a system of positive and negative feedback to govern the oestrous cycle. GnRH controls the oestrous cycle via its action on the anterior pituitary to drive the secretion of the gonadotrophs, LH and FSH. The transportation of GnRH from the hypothalamus to the pituitary gland occurs via the hypophyseal portal blood system. GnRH binds to its G-protein coupled receptor on the cell surface of the gonadotroph cells. As a result of this binding, intercellular calcium is released in order to activate intermediaries in the mitogen activated protein kinase (MAPK) signaling pathway which contributes in the release of FSH and LH from storage compartments in the cytoplasm of gonadotroph cells. The storage of FSH is only for short periods, while LH is stored for a longer period through the oestrous cycle (Crowe and Mullen, 2013). During the follicular stage of the oestrous cycle there is a basal level of P4 due to the regression of the CL. The elevated concentrations of E2 is associated with the rapid proliferation of the pre-ovulatory dominant follicle (DF), along with low levels of circulating P4, stimulates a cascade of GnRH leading to an ovulation-inducing LH surges, and allows the display of oestrus behaviour. Ovulation occurs

10-14 hours after oestrus and is followed by the luteal stage of the oestrous cycle. Met-oestrus phase is the beginning of the luteal stage when the CL is formed from the ruptured ovulated follicle and typically lasts for 3-4 days. After ovulation phase, P4 levels is increased due to the formation of the CL in which the GCs and TCs of the ovulated DF lutenize and release P4 in order to establish and maintain pregnancy and/or resume the oestrous cycle. Through the di-oestrous stage, P4 levels remain high and persistent waves of follicle development continue to be started by the secretion of FSH from the anterior pituitary. But these DFs that grow during the luteal stage of the oestrous cycle do not ovulate, due to insufficient LH pulse frequently. During the luteal stage of the oestrous cycle, P4 through its negative feedback action, only permits the production of greater amplitude (but less frequent) LH pulses (one pulse per 3-4 hours) that are insufficient for ovulation of the DF. Lastly, through the pro-oestrous phase, P4 levels plummet when the CL degenerates in response to PGF2α production from the uterus (Figure 1.1) (Forde et al, 2011).

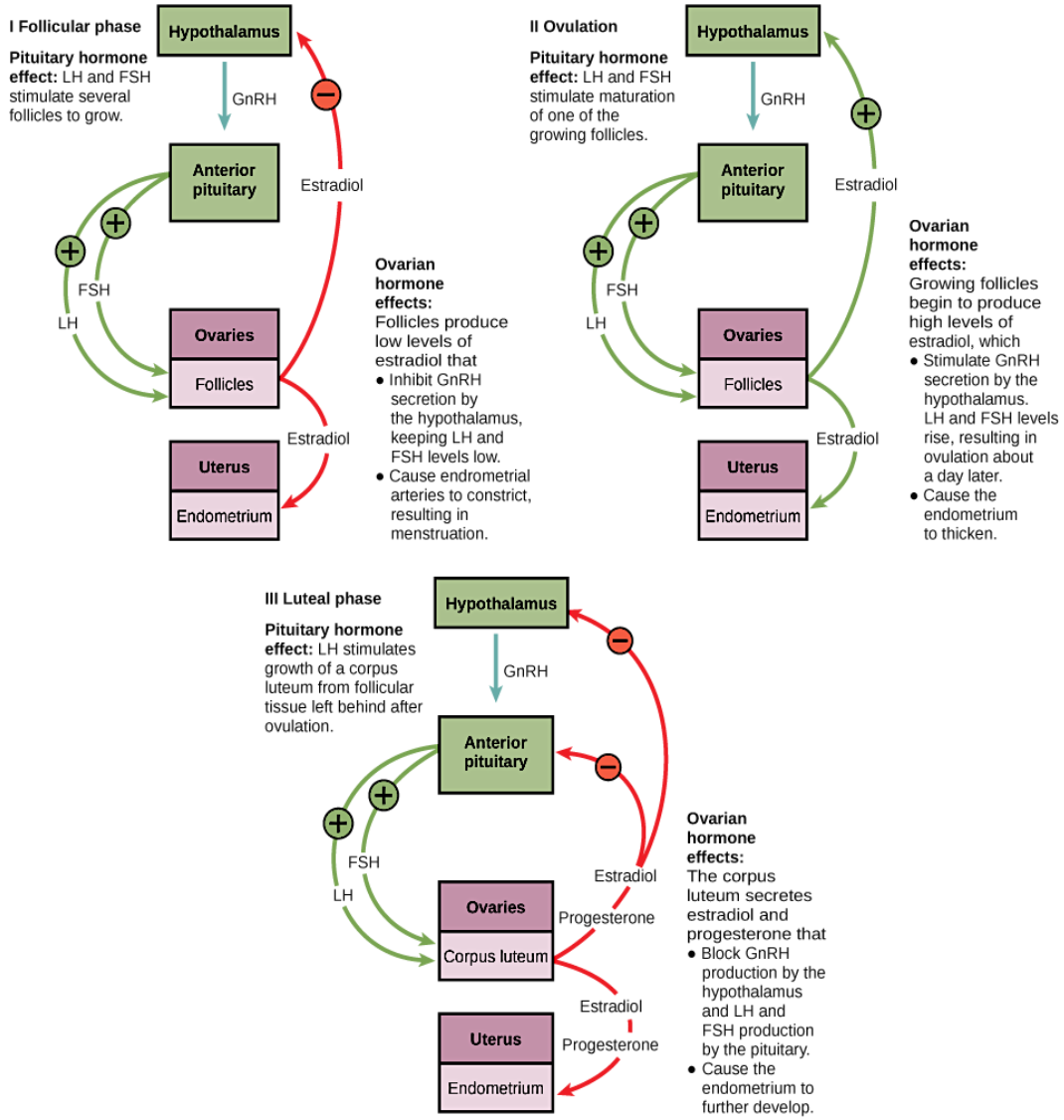


Figure 1.1 Hormonal control of the female reproductive cycle that involves hormones produced by the hypothalamus, pituitary and ovaries. It can be concluded that LH and FSH are secreted from the pituitary while E2 and P4 are secreted from the ovaries. E2 and P4 are secreted from the follicle (and CL) which causes the thickness of the endometrium of the uterus to increase (OpenStax College, 2013).

1.3. Female reproductive organs in cattle

The ovary is the female reproductive organ that functions in the release of unfertilized eggs (oocytes) along with the production of steroid hormones that play a significant role in the reproductive cycle. The genital tract of the female cow exists in the pelvic cavity and consists of vulva, vagina, cervix, uterus, fallopian tubes (oviducts), ovaries and their supporting structures. The ovaries are bean-shaped structures, 1-4 cm long and 1-3 cm in diameter; their size differs according to the stage of the reproductive cycle. They are attached to the uterus by the Fallopian tubes that open anteriorly into the fimbriae, funnel-shaped structures adjacent to, but not directly connected to the ovaries. The fimbriae guide gametes from the ovary into the fallopian tubes (Mukasa-Mugerwa, 1989). The ovary is capsulated within the tunica albugina tissue. It is divided into two zones, the external is known as the cortex and consists of stromal tissue, quiescent primordial follicles and different stages of growing/regressing follicles and corpora lutea. The internal zone is known as the medulla and consists of stromal tissue, blood vessels, nerves and smooth muscle fibres (Figure 1.2). The ovarian follicles mainly produce different steroid hormones such as androgens, oestrogens and progesterone and as well as various growth factors and cytokines including oxytocin, inhibin, activin and insulin-like growth factor (Bloom and Fawcett, 1975).

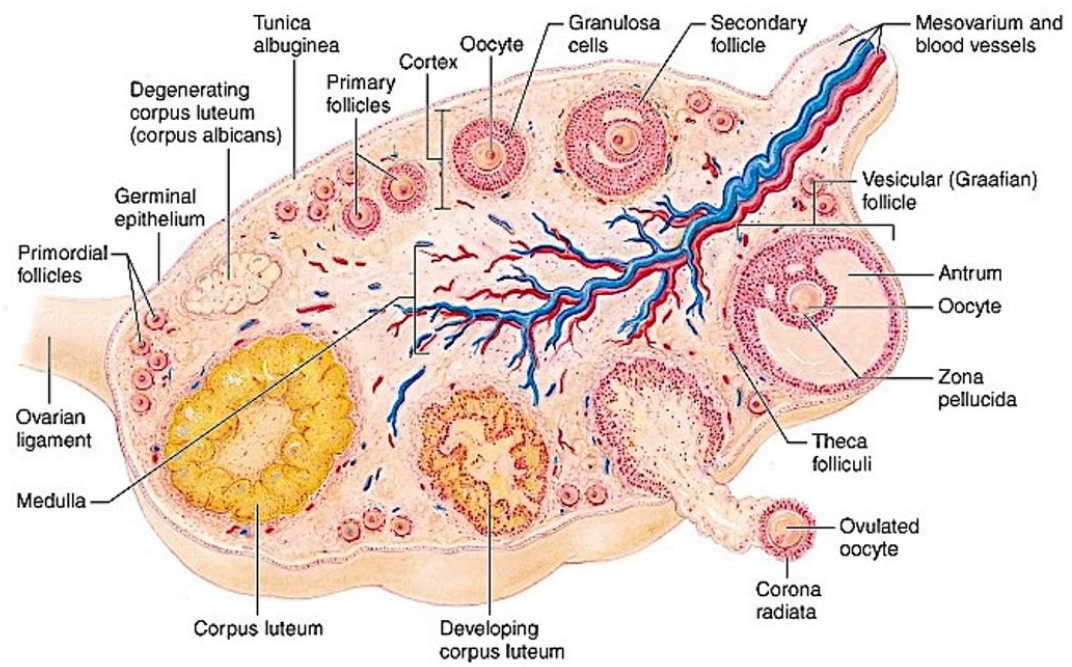


Figure 1.2 Schematic illustration of the morphology and architecture of a mammalian ovary through the reproductive cycle (Copyright 2001 Benjamh Cummings, an imprint of Addison Wesley Longman, Inc).

1.4. Folliculogenesis and the bovine oestrous cycle

1.4.1. Early follicular development

Key structures within the ovary are developing follicle that contain the oocyte and two types of somatic cells, granulosa cells (GC) and theca cells (TC). During the fetal phase, development of oocytes and follicles is started and takes 6-month to be completed. When quiescent primordial follicles activate and become committed to the development pathway, they undergo various sequential phases of development. There are 4 stages of follicle development starting with resting primordial follicles, which consists of an oocyte surrounded by a single layer of flattened pre-GCs. The second stage is the primary follicle, which is referred to as the activated primordial follicle. It has an oocyte, surrounded by a single layer of cuboidal GC. The third stage is the secondary follicle, when GC proliferation continues forming up to 7 layers enclosing the oocyte. At this stage the basement membrane forms around the GC and cells from the outer layer (stroma) condense around this to form TC. This TC becomes vascularized whereas the GC remains avascular. The last stage or antral stage when the secondary follicle enlarges further and a fluid-filled antrum is formed. The continues growth of this follicle is accompanied by further GC and TC

proliferation, increased vascularity of the theca and further growth of the oocyte (Figure 1.3) (Hutt and Albertini, 2007). Eventually, expansion of the antrum leads to a thinning of the follicular wall around the time of ovulation (Knight et al., 2012; Whittier, 1993).

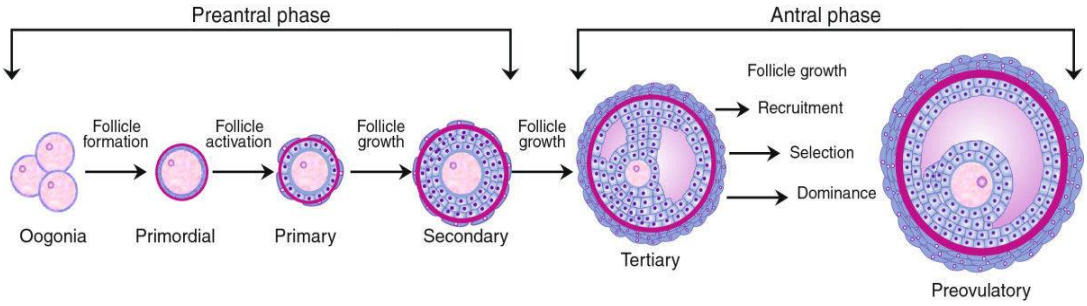


Figure 1.3 A diagram illustrates complete follicular development stages. Preantral phase: characterized by the formation and beginning of growth and activation of primordial follicles and growth of primary and secondary follicles. Antral phase: in which the formation of tertiary follicle (antral-filled follicular fluid cavity). Follicle growth occurs across the stages of recruitment, selection, dominance, and preovulatory phase of follicular waves. Oogonia develop from primordial germ cells and differentiate into oocytes in the ovary. Primordial follicles have a single layer of flattened granulosa cells. Primary follicles develop a single layer of cuboidal granulosa cells. Secondary follicles consist of two or more layers of cuboidal granulosa cells and a small number of theca cells. Oocytes are present in all preantral follicles. Tertiary follicle contains numerous granulosa cell layers, theca cells and primary oocyte and are characterized by the presence of an antral cavity containing follicular fluid. Follicular fluid is a plasma exudate conditioned by secretory products from the granulosa cells and oocyte. The Preovulatory (or Graafian) follicle is the final stage of follicle development; they are larger in size, have additional antral fluid and may have a secondary oocyte (post-LH surge) (Araujo et al., 2014).

1.4.2. Follicle recruitment

The word recruitment has been defined to distinguish two different phases of follicle development. There are two stages of follicle recruitment, ‘initial’ recruitment and ‘cyclic’ recruitment. Initial recruitment is the point at which, the initiation of primordial follicles growth is stimulated by intraovarian and/or other

unknown factors, while, other primordial follicles continue to be quiescent for a long period of time. Initial recruitment is considered as a continuous process that commences after follicle formation in the foetus, long before pubertal onset. Following initial recruitment, the growth of the oocyte becomes a prominent feature of developing follicles; however, oocytes remain arrested in the prophase of meiosis. For primordial follicles that fail to recruit, the default mechanism for them is to remain dormant.

On the other hand, cyclic recruitment is characterized by transient increases of circulatory FSH through each reproductive cycle after pubertal onset that rescue growing antral follicle from atresia. During this stage a limited number of follicles continue to grow and survive with the support of FSH, whereas the default mechanism is to undergo atresia. The growth of oocytes of these follicles has already been largely completed; they are enclosed by a zona pellucida, and are capable of resuming meiosis (Figure 1.4) (table 1.1) (McGee and Hsueh, 2000; Regan et al., 2017).

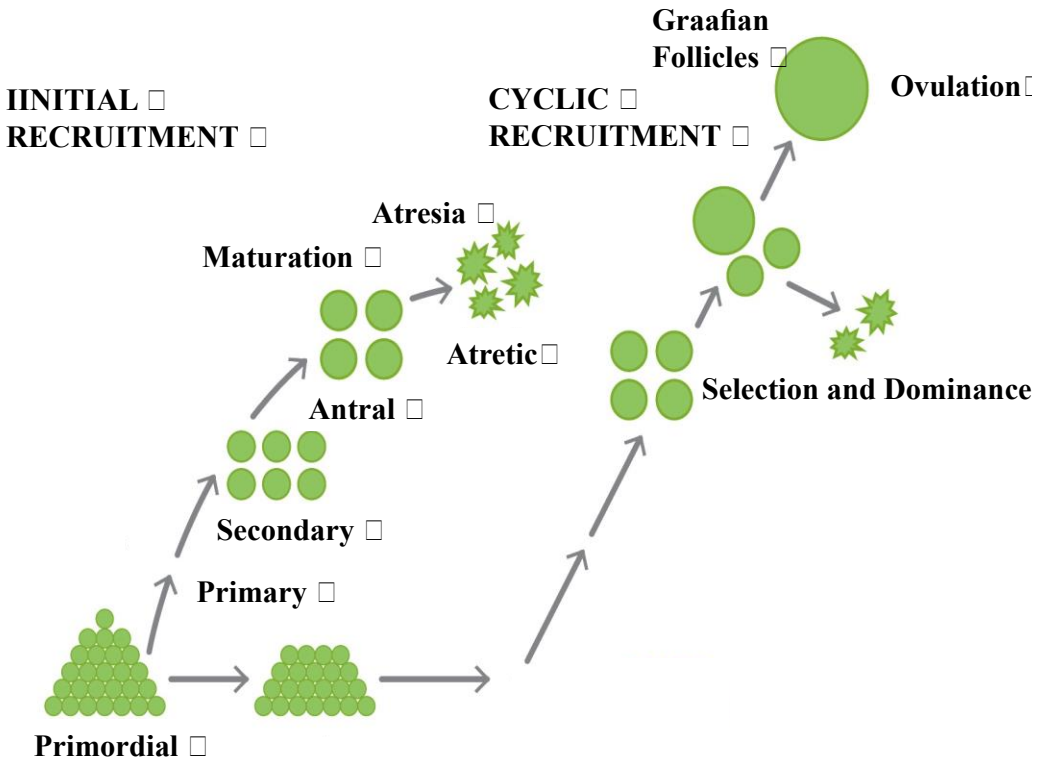


Figure 1.4 A schematic shows follicle initial and cyclic recruitment. A certain number of primordial follicles are endowed in early life, and most of them are maintained in a resting state. Before and throughout reproductive life a number of dormant primordial follicles are activated and initiate growth (Initial recruitment). Activated primordial follicles grow through primary and secondary stages before developing an antral cavity. Most of follicles undergo atresia at the antral stage, however, a few of them are rescued under ideal gonadotropin influence that appears in a cyclic manner after puberty (Cyclic recruitment) to reach the preovulatory phase (modified from McGee and Hsueh, 2000).

Table 1.1 Differences between initial and cyclic recruitment of ovarian follicles (modified from McGee and Hsueh, 2000).

	Initial recruitment (initiation of growth)	Cyclic recruitment (escape from atresia)
Stages	Primordial	Antral follicle
Hormones involved	Not determined	FSH
Default pathway	Remain dormant	Apoptosis
Timing	Continuous throughout life, begins after follicle formation.	Cyclic, starts after puberty onset
Oocyte status	Starting to grow, not capable of undergoing germinal vesicle breakdown	Completed growth, competent to undergo germinal vesicle breakdown

1.4.3. Follicle Selection and dominance

Following initial recruitment into the growth pool, follicular development involves emergence, selection and dominance followed by either atresia or ovulation of the dominant follicle (DF). As is generally known, the synthesis and release of both FSH and LH have a dominant function in ovarian follicle development. As discussed below in more detail (section 1.4.8), cattle typically display 2-3 successive waves of follicle development (i.e. cyclic recruitment) during a single oestrous cycle although only one of these culminates in ovulation. In each wave of follicular development, FSH levels rise as emergence occurs. This becomes closely associated with follicular growth, whereby FSH binds to its receptor FSHR, which is found in the GC by day 3 of follicular wave. As a result, FSH performs its required downstream signaling which affects cellular growth and proliferation. Besides increased FSH levels, the activity of aromatase enzyme in the follicular GC has also risen in order to convert androgen to oestrogen. As the DF is selected from the growing cohort of small antral follicles, its diameter increases faster than other cohort follicles and follicular E2 and inhibin levels are up regulated, identifying this follicle as the healthy dominant follicle of the cohort. When the DF reaches ~9 mm

in diameter, dominance occurs and this leads to the suppression of FSH secretion which limits cohort follicle growth and prevents further follicle wave emergence until the DF undergoes either atresia or ovulates. Basically, the increase in E2 in concert with inhibin plays a key endocrine role which supresses FSH levels from the anterior pituitary through a negative feedback mechanism. The selected DF responds to LH and continues growth in the face of decreasing FSH levels. The switch from FSH to LH dependency is controlled via the acquisition of LH receptors (LHR) on the GC of DF. At different phases of follicular development, LHR is localized to the TC and GC of healthy follicles. As follicle develops, LHR in the theca cells rises and from 8-9mm diameter GC start to express LHR which is required for selection to become the ovulatory DF. It has been confirmed that the increase of LH levels in the circulation during follicle selection, permits the DF to secrete E2 and grow in response with decreasing FSH levels. (Crowe and Mullen, 2013; Singh and Krishna, 2010).

1.4.4. Ovulation and corpus luteum formation

Ovulation is the following stage after the selection of Dominant follicle. During the early luteal stage, LH pulses occur with lesser amplitude and greater frequency between 20 and 30 pulses per 24 hours, while in the mid-luteal phase, the LH pulses are of greater amplitude and lesser frequency from 6 to 8 pulses per 24 hours, this gives insufficient amplitude and frequency for final maturation and further ovulation of the DF. Consequently, the DF produced during the luteal stage of the estrous cycle undergoes atresia, E2 and inhibin secretion declines, and this ends negative feedback block to the hypothalamus-pituitary. Thus, FSH production can rise and a new follicle wave emerges. E2 concentration can be used as a marker to identify the DF, which has greater follicular fluid E2 concentration than other follicles in the cohort. The production of E2 depends on the conversion of TC-derived androgen into estrogen in the neighboring GC in accordance with two cell/two gonadotropin hypothesis referred to earlier. In the TC, the binding between LH and its receptor stimulates the conversion of cholesterol to androgen (androstenedione and testosterone). Then, the produced androgen diffuses into the adjacent granulosa cells where it is converted to estrogen. The role of E2 is not only on follicle development but also it has a positive feedback mechanism action to the hypothalamus-pituitary gland to promote the ovulation-inducing LH surge, and to

induce oestrus behaviour. Thus, the LH pulses achieve sufficient amplitude and frequency to induce final maturation and ovulation of the DF (Crowe and Mullen, 2013; Singh and Krishna, 2010). The ovulatory response to LH involves the resumption of meiosis in the oocyte, the growth of the cumulus, the rupture of the follicle, and the release of a cumulus–oocyte complex (COC) that comprises a fertilizable oocyte. Once the oocyte is released, the remaining follicle cells, the GC and TC in particular, undergo reprogramming of final differentiation to generate the corpus luteum (CL) throughout a mechanism described as luteinization. Thus, the gene expression pathway started by FSH is down regulated and is exchanged for gene pathways that regulate matrix formation and luteinization (Rimon-Dahari et al., 2016).

The CL originates at the site of the ovulated follicle. Luteinizing hormone LH is the main luteotropic hormone, which functions in provoking luteinisation of pre-ovulatory follicle, including GCs and TCs transforming them into granulosa-lutein and theca-lutein cells. The major role of the CL is securing optimum production of progesterone (P4) during the luteal phase of oestrous cycle to support the establishment of pregnancy (Forde et al., 2011). In addition the CL persists and progesterone secretion is sustained throughout gestation if pregnancy is established. CL 'rescue' in cattle is initiated by interferon tau secreted by the trophoblast of the early embryo.

1.4.5. Angiogenesis

Ovarian angiogenesis comprises remodelling of the blood vasculature that takes place concurrently with folliculogenesis at the embryonic stage and remains a highly active process during follicle growth and luteinization. The ovary, like the uterus, has the ability to remodel vascular network through tight regulation within each reproductive cycle. Primordial and primary follicles have no vascular network and depend on the diffusion for oxygen and metabolite transfer. Throughout follicular development stages, growing pre-antral and antral follicles acquire a well-developed vascular network in the internal and external theca layers. The GC layer remains avascular until ovulation (Brown and Russell, 2014). Once ovulation has occurred and CL formation commences, an enormous angiogenic process takes place; breakdown of the basement membrane occurs which leads to the penetration

of newly formed blood vessels into the inner parts of the follicle (Rimon-Dahari et al., 2016).

1.4.6. Atresia

At birth, the number of oocytes in the ovaries far exceeds the number that will actually be ovulated throughout the reproductive lifecycle of the female. As previously mentioned, primordial follicles can be quiescent, committed to growth, ovulatory or atretic. After birth, the number follicle-enclosed oocytes declines gradually and, commencing at puberty, a small proportion of these (<0.1%) will undergo full maturation and ovulation. However, the majority will degenerate in process called atresia (Jablonka-Shar-i. et al., 1994; Greenwald, 1989; Plendl, 2000).

1.4.7. Follicular development and the bovine oestrous cycle

The entire period of ovarian folliculogenesis has been estimated to be 160 days in cattle. This includes the development of activated primordial follicles into tertiary follicles. However, it has been estimated that the time required for follicles to grow from the secondary follicles (pre-antral stage) to a mature ovulatory size is about 42 days. In the bovine, during embryonic development, arrested primordial follicles at meiotic prophase I start to leave the resting pool and enter into the pre-antral growth stage. The wave pattern of antral follicle growth has been observed as early as 2 weeks of age in cattle. The pre-antral phases of follicular development occur independently of gonadotropic hormones support, but follicles become more responsive to GnRH at the early-antral stages. At the age of puberty, the cyclic development of antral follicles takes place as a result of various changes in hypothalamic and pituitary gonadotropin hormones (Baerwald, 2009; Craig et al., 2006).

The oestrous cycle is formally defined as the cyclical pattern of ovarian activity of female animals that ultimately enables mating and consequent establishment of pregnancy. The onset of oestrous cycles occurs at the time of sexual maturity. In cattle, puberty occurs generally at 6-12 months of age and commonly at a body weight of 200-250 kg. The normal duration of a bovine oestrous cycle is approximately 18-24 days. The cycle involves two separate stages: the luteal stage

(14-18) days and the follicular stage (4-6) days. The luteal stage is the phase following ovulation when the CL is formed (known as met-oestrous and di-oestrous), whereas the follicular stage is the phase following the regression of the corpus luteum until ovulation occurs (known as pro-oestrus and oestrus). Through the follicular stage, development and ovulation of the ovulatory follicle takes place, the oocyte is released into the oviduct allowing the potential for fertilization (Forde et al, 2011).

1.4.8. Follicular wave and hormonal profiles during the oestrous cycle in cattle

It is well known that the mammalian ovary is a highly dynamic organ that undergoes different physiological activities including sequential waves of follicular growth and regression, rupture of mature follicles and the ovarian wall during ovulation, repair of the ovulation wound and the formation and subsequent regression of functional corpora lutea (Donadeu et al., 2012). During the bovine oestrous cycle, follicular development occurs in a wave-like fashion with typically 2 or 3 waves through each cycle (Knopf et al., 1989). Some studies reporting >68% of cows with 2 follicular waves pattern whereas others report >70% of cows with 3 follicular waves pattern per oestrous cycle (Ginther et al., 1989 and Jaiswal et al., 2009). The explanation for the prevalence of one wave patthern over the other is not clear, though the wave pattern is slightly repeatable within an animal and 3 wave-patterns are related to a longer oestrous cycle (Jaiswal et al., 2009). In cattle, the first follicular wave is initiated when a group of follicles emerge and grow in response to a transient FSH surge on day 0, the day of ovulation (Adams et al., 1992 and García-Guerra et al., 2017). In two-wave cycles, emergence of the second wave occurs on day 9 or 10, and in three-wave cycles it occurs on day 8 or 9. In three-wave cycles, emergence of the third wave typically occurs on day 15 or 16. Because of raised levels of progesterone during the luteal phase, dominant follicles of successive waves undergo atresia if dominance is achieved prior to luteal regression. At luteolysis progesterone levels fall allowing the dominant follicle to becomes the ovulatory follicle, with ovulation triggered by an LH surge generated by the positive feedback action of oestradiol arising from the dominant follicle. Emergence of the next wave is delayed until the day of ensuing ovulation. In two-wave cycles CL regression occurs on day 16, while in three-wave cycles it usually

occurs around day 19. Thus cows with 2-wave patterns typically have shorter oestrous cycle (19-20 days compared with 22-23 days in 3-wave patterns). Therefore, the so-called 21-day-oestrous cycle of cattle occurs only as an average between two- and three-wave patterns (Figure 1.5) (Adams et al., 2008).

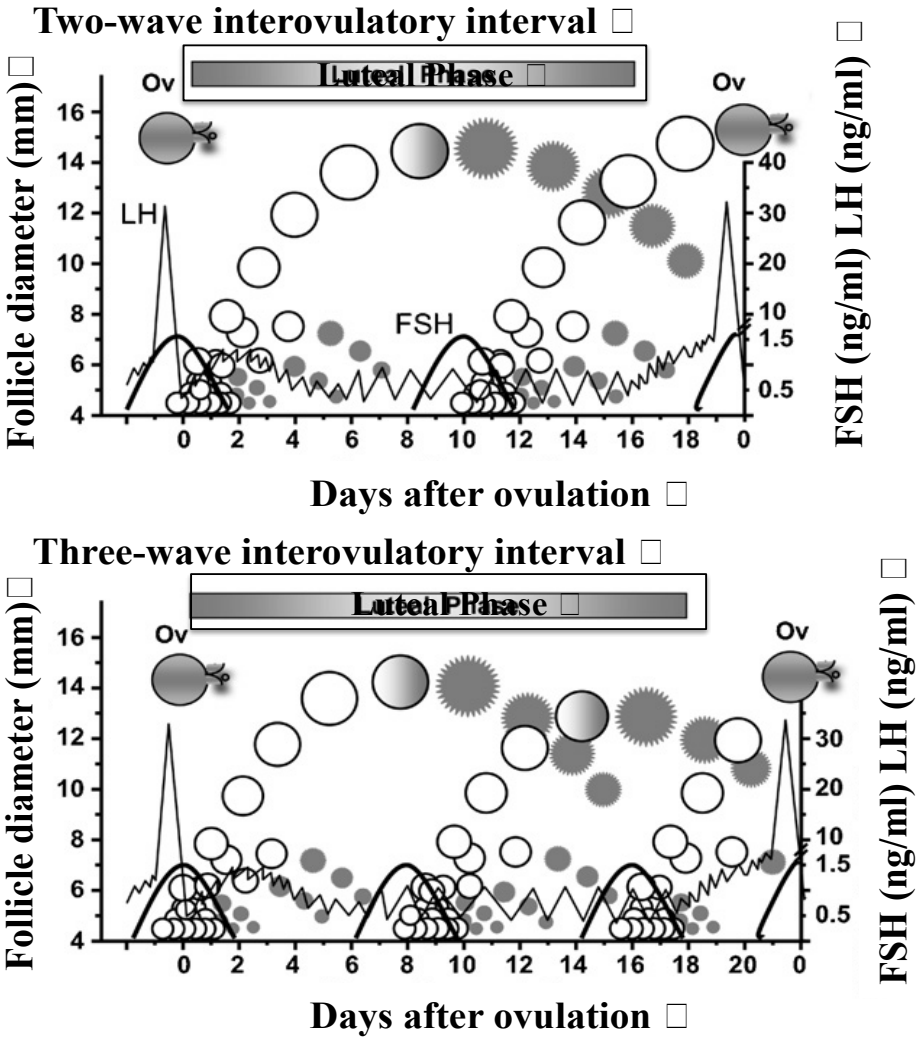


Figure 1.5 Dynamics of ovarian follicular development and gonadotropin secretion during two- and three-wave oestrous cycles in cattle. Dominant and subordinate follicles are shown as open (viable) or shaded (atretic) circles. A surge in circulating FSH levels (thick line) leads emergence of each wave. A surge in circulating LH levels (thin line) leads to ovulation. The LH surge is preceded and succeeded by a period of high-LH pulse frequency as a result of low-circulating progesterone levels (i.e., period of luteolysis and luteogenesis, respectively) (Adams et al., 2008).

1.4.9. Physiological changes associated with the menstrual cycle in human

The menstrual cycle in women is regulated by various endocrine, autocrine and paracrine factors that are responsible for controlling the development of ovarian follicles, ovulation, luteinisation, luteolysis and endometrium remodelling (Golden and Carlson, 2008). The menstrual cycles of women differ in length (26-35 days; average 28 days). During the luteal-follicular transition a rise in FSH level induces continued growth of a cohort of small antral follicle; this is accompanied by increased secretion of inhibin B through the early follicular stage. In the mid-follicular stage (~day 7), the ovulatory DF is selected and as this DF grows, it stimulates the release of E2 and inhibin A which increase in the week prior to ovulation. Evidence suggests that, as in catlle, follicular development occurs in a wave-like pattern with typically 2 or 3 waves through each cycle; two-thirds of women display two follicular waves while one third display three-follicular waves per cycle (Baerwald et al., 2003a and Baerwald et al., 2003b). Women with three-wave pattern show longer cycles, and a later increase in the E2 and LH surge that promote ovulation (Figure 1.6). In response to LH pulses, the CL releases P4, E2 and inhibin A, and reaches its functional peak according to size, secretions, and vascularisation from 6 to 7 days after ovulation. In contrast to cattle, in which uterine pulses of PGF2α initiate luteolysis, cyclic regression of the human CL occurs independently of the uterus. Regression of the CL can be stopped by human chorionic gonadotropin (hCG), the luteotrophic signal from the implanting trophoblast, secreted some 8 days after conception. Cyclic luteal regression is accompanied by as abrupt fall in P4, E2 and inhibin A and menstruation is initiated and executed via uterine prostaglandin E and PGF2α production, vasoconstriction and matrix metalloprotease secretion by leukocytes. With the exception of the luteolytic mechanism, many aspects of ovarian function and hormone changes throughout the human menstrual cycle are similar to the oestrous cycles in cows and mares, justifying research into comparative features of menstrual and oestrous cycles in monovulatory species (Mihm et al., 2011).

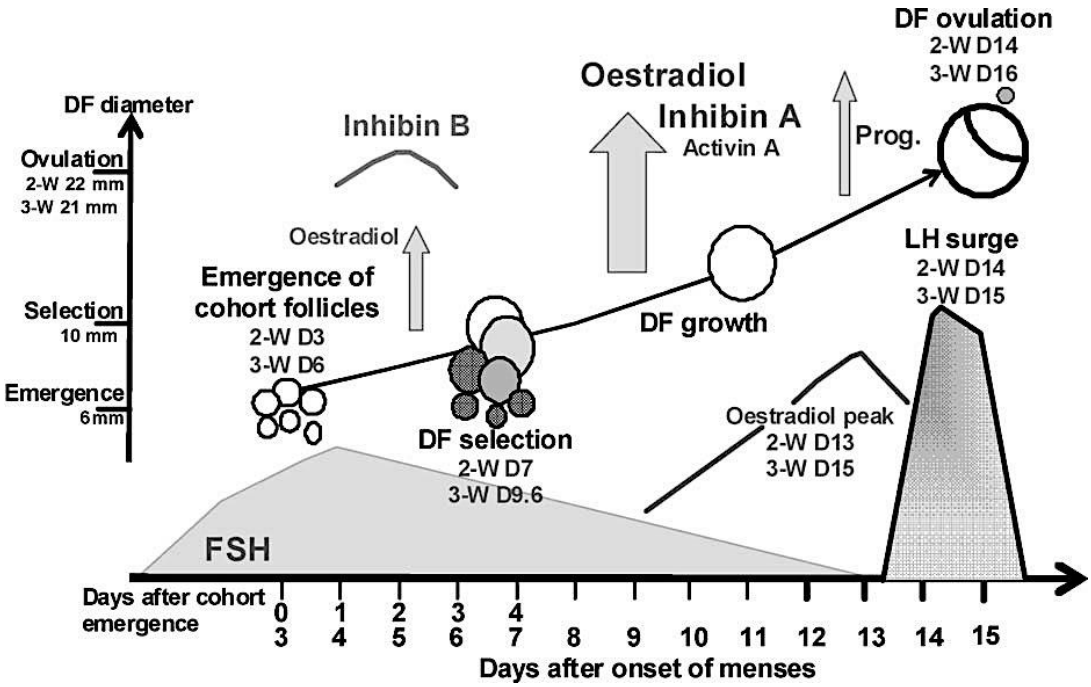


Figure 1.6 A schematic of follicular wave patterns and changes in systemic gonadotrphins, steroids and inhibins through follicular stages of the human menstrual cycle. During the selection of DF, cohort follicles that undergo atresia are shaded. * 2-W (2-wave women), 3-W (3-wave women) and Prog (P4) (Mihm et al., 2011).

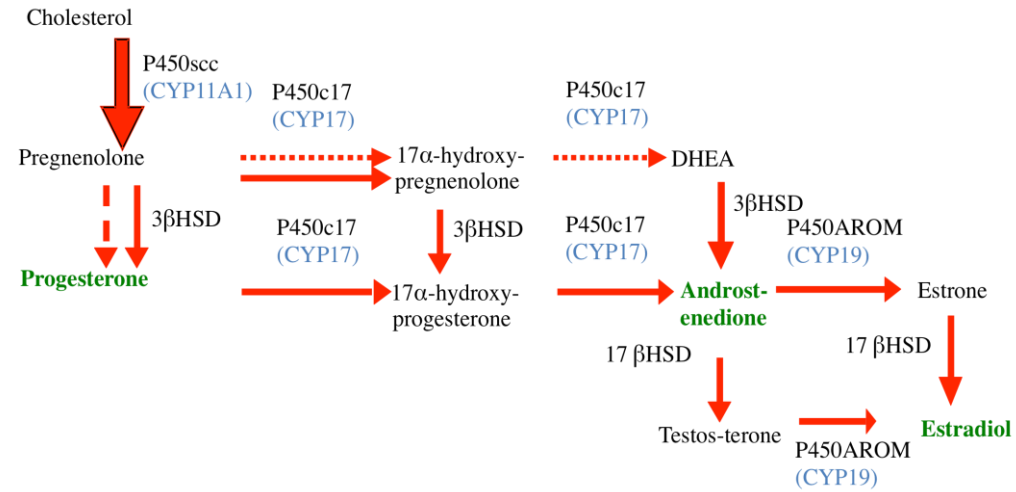
1.5. Ovarian steroidogenesis and its regulation

1.5.1. Steroidogenic pathway in the ovary

Steroidogenic enzymes have a role in the biosynthesis of different steroid hormones that are all derived from cholesterol. They consist of various specific cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenase (HSDs), and steroid reductases. There are several endocrine organs that have the ability to synthesize biologically active steroids such as the ovary, testis and adrenal cortex. The biosynthesis of all steroid hormones initiates from cholesterol which is converted to pregnenolone by CYP11A1 enzyme. This enzyme is bound to the inner membrane of the mitochondria and is expressed in all steroidogenic tissues; however, it is not found in nonsteroidogenic tissues. Then, under the effect of 3β hydroxysteroid dehydrogenase (3β-HSD) enzyme, pregnenolone is converted to progesterone. The enzyme is found in the mitochondria and smooth endoplasmic reticulum, and widely distributed in steroidogenic and non-steroidogenic tissues. Thus,

pregnenolone and progesterone become the precursors for the production of all other steroid hormones (Figure 1.7).

The ovary produces oocytes along with the secretion of steroid hormones for sexual and reproductive function. The basic structure of the developing follicle is an oocyte surrounded by layers of GCs followed by layers of TCs, where steroidogenesis takes place. The theca layer is highly vascularized and TCs secrete P4, as well as A4 and testosterone which act as a precursor for estrogen synthesis in the GC. A4 and testosterone diffuse into the adjacent non-vascularized GC where they are converted into E2 by cytochrome P450 aromatase (CYP19A1) and 17β-HSD enzymes. In the preovulatory follicle, once the follicle reaches the final maturation stage, oestrogen synthesis increased dramatically under the effect of the aromatase enzyme up-regulation, initially by FSH and then by LH. This process is enhanced when the oestrogen up-regulates LH receptors which, as a result, initiate the positive feedback mechanism which generate the preovulatory LH surge, in order to stimulate ovulation. After LH surge, the follicle enters the luteal stage and forms a corpus luteum, which is responsible for the secretion of P4. The subsequent decrease in LH level is associated with a reduced aromatase expression and sharp decline in estrogen production by GC. Correspondingly up-regulation of CYP11A1 and 3β-HSD levels enhances P4 production that then accompanies the stage of follicular rupture and luteinisation (Sanderson, 2006).



(source: Dr AE Michael, St Georges Hospital Medical School; with modifications)

Figure 1.7 The steroid biosynthesis pathway for the conversion of substrate cholesterol to the P4, A4 and E2 in the ovary.

1.5.2. Two-cell two-gonadotropin model

A two-gonadotrophin, two-cell hypothesis has been proposed to explain ovarian steroidogenesis. According to this LH acts directly on TC in order to stimulate biosynthesis of androgen (Androstenedione A4 and testosterone). These molecules diffuse to adjacent GC where the enzyme complex (aromatase) converts them to oestrogens (oestrone, E2), under the influence of FSH (Figure 1.8).

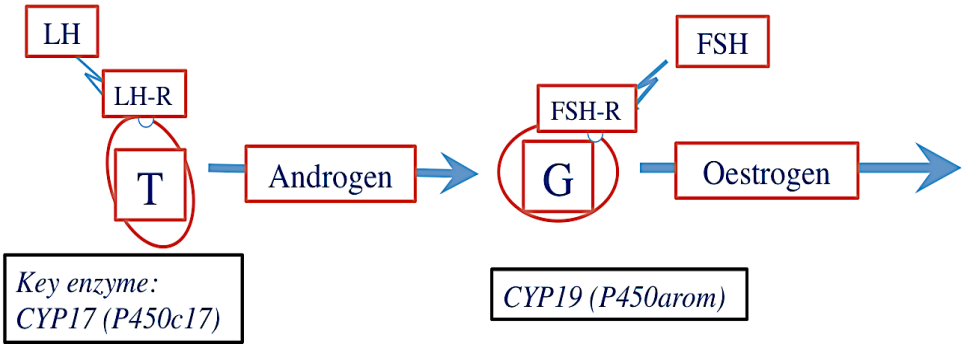


Figure 1.8 Two-cell, two-gonadotrophin model of follicular steroidsynthesis. LH acts on the theca cells to stimulate the synthesis of androgens (A4 and testosterone). Then, these molecules diffuse to the adjacent granulosa cells where the aromatase enzyme converts them to oestrogens (oestrone and E2) under the influence of FSH.

1.6. Intra-ovarian factors regulating follicular function

In addition to steroids there are many other intra-ovarian regulation factors which have either an indirect role via the negative feedback mechanism to the hypothalamus-pituitary glands or direct actions on ovarian cells e.g. through altering the synthesis of E2 by GC, or androgen by TC (Crowe and Mullen, 2013).

1.6.1. TGF-beta super family

Several members of the transforming growth factor-β family (TGF-β) are known to have a key role in follicular and oocyte development (Knight and Glister, 2006). These family members include anti-Mullerian hormone (AMH)/Mullerian inhibiting substance (MIS), activin, follistatin, inhibins, several bone morphogenetic proteins (BMP) and growth differentiation factor (GDF)-9. Several ligands of this family (inhibins, activins) where first discovered in follicular fluid via their effect on the secretion of pituitary FSH (Qiao and Feng, 2010) but they also exert local intra-ovarian actions. Activin acts to up regulate the secretion of E2, while follistatin (activin binding protein) blocks activins positive steroidogenic effects. Inhibins that are secreted by GCs have a function in the suppression of FSH produced in the anterior pituitary that, in turn, regulates the oestrous cycle (Crowe and Mullen, 2013). At a local level inhibin can also stimulate androgen production by TC (knight et al., 2012).

1.6.2. Other growth factors

The epidermal growth factor family (EGF) comprises a range of soluble growth factor molecules that play a significant role in the regulation of cell growth, proliferation and differentiation. In the ovary, EGF is found in the follicular fluid, promoting follicular growth and oocyte meiotic maturation via the binding to its EGF receptor (EGFR) which initiates signaling transduction system (Hsieh et al., 2009). The fibroblast growth factor family (FGFs) are a group of polypeptides that have an important function in development, cell growth, tissue repair and transformation. These factors are expressed in the GCs and TCs of growing follicles, and also have a role in regulating FSH action (Schreiber et al., 2012). The insulin like growth factor family (IGFs) are multifunctional polypeptides with insulin-like action. They consist of two ligands (IGF-I) and (IGF-II), two receptors (IGFR-I) and (IGFR-II), and several binding proteins (IGFBP 1-6) and proteases

inducing pregnancy associated plasma protein-A (PAPP-A) which are implicated in ovarian follicular development (Sudo et al., 2007). Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are major members of the neurotrophin family (NT) which is responsible for the development of central and peripheral nervous systems. These members may also be involved in folliculogenesis and cytoplasmic competence of the oocyte (Linher-Melville and Li, 2013).

1.7. Kisspeptin (KISS-1) and its receptor (GPR54) system

1.7.1. Introduction

A pivotal advance in understanding of the neuronal mechanisms in the hypothalamus regulating GnRH production into the portal vessels, and consequently the final pathway by which the brain controls gonadal function, came with the recognition of the physiological functions of the neural peptide, kisspeptin and its receptor (GPR54) (Ahmed et al., 2009). Kisspeptin, encoded by the *KiSS-1* gene, is a potent endogenous secretagogue of GnRH, and its neuronal system governs both the pulsatile gonadotropin secretion that drives follicular development, spermatogenesis and steroidogenesis, and the preovulatory gonadotropin surge that triggers ovulation in females. The pulsatile mode is controlled by negative feedback mechanism via gonadal steroids, while the surge mode is in response to positive feedback mechanism via estrogen (Ohkura et al., 2009).

1.7.2. Discovery of kisspeptin

The product of the *KiSS-1* gene was originally identified as a tumour metastasis suppressor by a group in Hershy, PA, USA, (Lee et al., 1996). Thereafter, the *KiSS-1* gene and components of this endogenous ligand-G-protein-coupled receptor system were discovered between 1996 and 2001 (Kotani et al., 2001; Ohtaki et al., 2001). In 2003, two independent studies documented the presence of inactive mutations and deletions of the orphan receptor gene called *GPR54* gene in patients suffering from idiopathic hypogonadotropic hypogonadism. These observations from the groups of de Roux and Seminara were the first to highlight the indispensable functions of GPR54 and its ligands in the control of key aspects of reproduction (Pinilla et al., 2012). Such genetic findings in human were substantiated experimentally using mice engineered to lack functional *GPR54*,

which exhibit reduced gonadal size and low levels of gonadotropins and steroids hormones, and failure to undergo puberty (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003; Messager et al., 2005). However, in cattle, the association of *KiSS-1* to the GnRH-gonadotropin response in reproductive physiology remains poorly understood (Ezzat Ahmed et al., 2013; Pinilla et al., 2012). On the other hand, several groups have shown that central or peripheral administration of kisspeptin, stimulates GnRH secretion in mouse (Gottsch et al., 2004), rat (Matsui et al., 2004; Navarro et al., 2004 and 2005) sheep (Messager et al., 2005), monkey (Shahab et al., 2005; Plant et al., 2005) and human (Dhillo et al., 2005) and it seems highly probable that similar mechanisms operate in the bovine.

1.7.3. Amino acid sequence of kisspeptin

The *KiSS-1* gene encodes a precursor peptide that is further processed to generate biologically active forms of kisspeptin of different length (10-13-14-54 amino acids) (Oakley et al., 2009). In human, the kisspeptin precursor includes 145 amino acids, with a putative 19-amino acids signal sequence, two dibasic cleavage sites at amino acids 57 and 67, and one site for terminal cleavage and amidation at amino acids 121-124, which formed the biologically active kisspeptins (Kotani et al., 2001; Muir et al., 2001; Ohtaki tet al., 2001). The proteolysis of prepro-kisspeptin gives kp-54 with a 54-amino acids peptide initially known as metastin. Additionally, the other peptide fragments of the kiss-1 precursor including kp-10, 13 and 14 share the COOH-terminal region of kp-54 (Figure 1.9) (Ohtaki et al., 2001). The COOH-terminal decapeptide of kisspeptin, Kp-10, has been widely used to investigate the physiological actions of kisspeptin-GPR54 signaling. Also, kp-10 is the minimal sequence required for receptor activation in mammals, with higher potency than longer peptides (Pinilla et al., 2012).

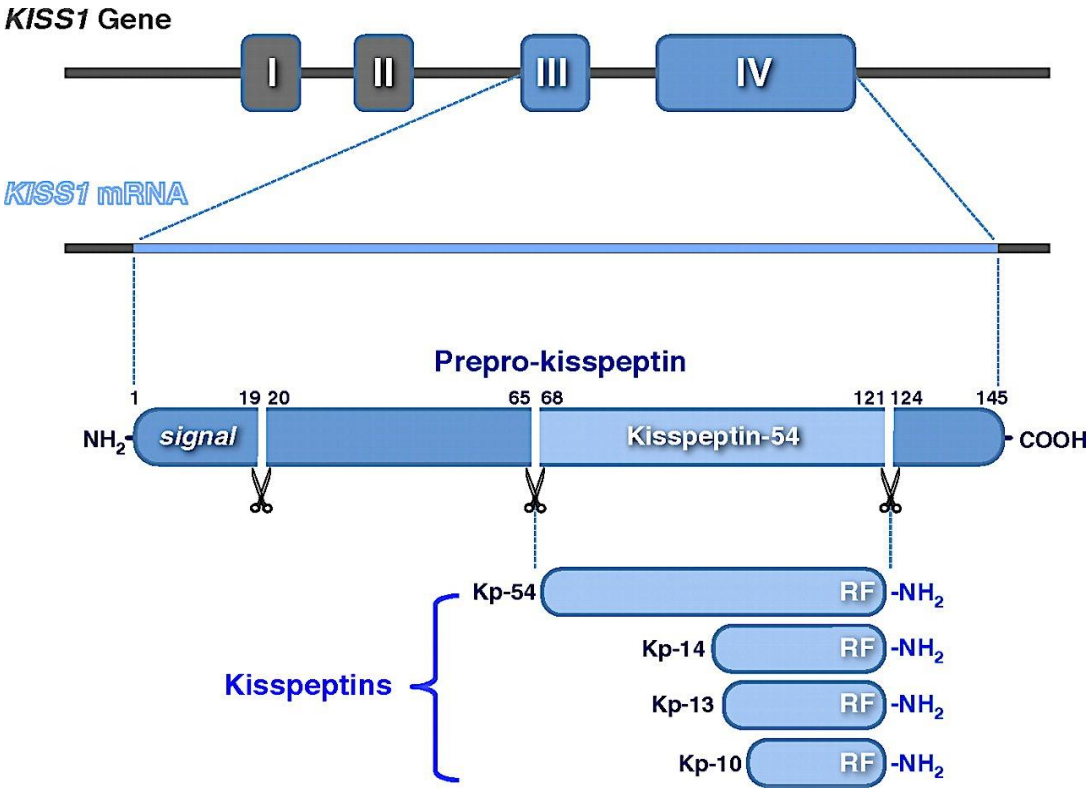


Figure 1.9 Main structural features of *KiSS-1* gene and its products. Several kisspeptins are formed by proteolytic cleavage from prepro-kisspeptin, which is a common precursor, encoded kiss-1 gene. In human, the *KiSS-1* gene is consist of four exon, the first two being non-coding exons (West et al., 1998). Yet, an alternative genomic composition, with three exons, the first one being non-coding, has been also proposed (Luan et al., 2007). The human kisspeptin precursor consists of 145 amino acids, with a 19 amino acids signal peptide and a central region with 54 amino acids. Lower molecular weight forms of kisspeptin including kiss-14, 13, and 10 exist; the latter corresponds to the common COOH-terminal 10-amino acid having the RF-amide motif that is able to completely activate GPR54 (Pinilla et al., 2012).

1.7.4. Biological action of kisspeptin

The effects of centrally or peripherally administrated kisspeptin on GnRH secretion have been reported in various mammals since the initial observation in rodents (Gottsch et al., 2004; Matsui et al., 2004). Kisspeptin consistently promotes the secretion of gonadotropin LH but various studies have reported that it has less effect on FSH secretion (Caraty et al., 2007; Lents et al., 2008; Smith et al., 2009). In reproduction, puberty is considered as a successful event that is associated with the

interaction between the gonadotropic axis and somatotropic axis. It has been postulated that kisspeptin not only has a role in promoting the release of LH but also growth hormone (GH) in prepubertal female cattle. Given its potential role as a metastasis suppressor, the possibility exists that kisspeptin may exert action in other tissues (Ahmed et al., 2009).

1.7.5. Anatomy of kisspeptin neurone distribution

The distribution of KiSS-1 expressing neurons has been examined by two techniques, *in situ* hybridization and/or immunohistochemistry in sheep, goats, pigs and horses (Okamura et al., 2013). The most consistent population of KiSS-1 expressing neurons are found exclusively in two discrete regions within the hypothalamus, rostrally in the preoptic area (POA) and caudally in the arcuate nucleus (ARC) of the forebrain across different mammalian species (Lehman et al., 2010; Smith et al., 2006). Also it was found that *KiSS-1* mRNA is expressed in cells in the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN), the anterodorsal preoptic nucleus (ADP) and the (ARC) (Gottsch et al., 2004 and Smith et al., 2005a,b).

1.7.6. Action of kisspeptin on gonadotropin secretion

KiSS-1 neurons appear to act directly on GnRH neurons to trigger the secretion of GnRH. Areas of kiss-1 neurons including the Arc and AVPV send projections to the medial POA (Canteras et al., 1994; Simonian et al., 1999). As the majority of GnRH neurons express KiSS-1 receptor (GPR54), upon stimulation by kisspeptin, GnRH neurons are stimulated to secrete GnRH (Figure 1.10). The GnRH in turn stimulates the release of gonadotropins LH and FSH from the pituitary. Although the ability of kiss-1, GPR54 and GnRH signaling to simulate the release of LH and FSH under experimental conditions has been shown, it provides little insight into the functional significance of kiss-1 (Smith et al., 2006).

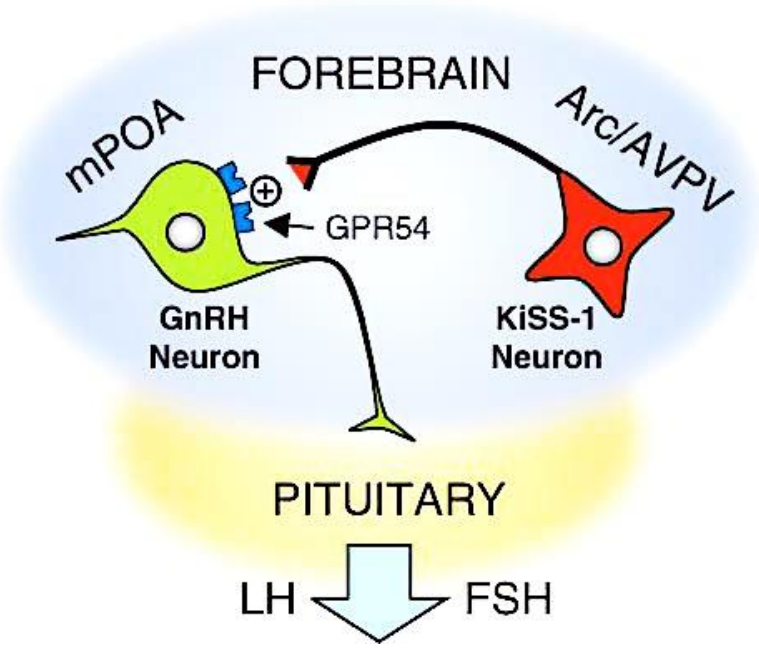


Figure 1.10 Proposed interactions between KiSS-1 secreting neurons and GnRH neurons (Smith et al., 2006).

1.7.7. Other actions of kisspeptin in the control of reproduction

Although the experimental data on kisspeptin-GPR54 signaling points out its primary site of action at the hypothalamus, evidence obtained proposes other regulatory effects of kisspeptin among various mammalian species at other levels of the reproductive system. The possibility of direct actions of kisspeptin on gonads remains barely studied to date and this was identified as one area of investigation to be followed during the current research project utilizing bovine follicle cell culture models. As generally known, the ovary is a complex endocrine organ, which has an essential role in oocyte formation and hormones synthesis. Evidence has been reported for the expression of *KiSS-1* gene and protein in human, rat and monkey ovary (Castellano et al., 2006; Gaytan et al., 2009). Also, the expression of GPR54 at the mRNA and protein level has been reported in some of these previously mentioned species (Pinilla et al., 2012). To our knowledge, detailed expression analysis studies in cattle have not been published and evidence that *KiSS-1* and *GPR54* may be expressed in the adrenal, pituitary, testis and ovary of cow has not been reported.

Regarding the ovary, studies on rats were the first to document *KiSS-1* and *GPR54* expression at different phases of postnatal maturation, through the cycle and in response to hormonal manipulations. Interestingly, studies showing low and stable levels of *GPR54* mRNA in the ovary through the estrus cycle, have also demonstrated an increase in expression of ovarian *KiSS-1* throughout the pubertal transition. In addition, the expression of ovarian kiss-1 peaked at the afternoon of pro-estrus (Castellano et al., 2006). In good agreement, studies on the Siberian hamster documented that kisspeptin immunoreactivity increased through the ovulatory transition including pro-estrus and estrus (Shahed and Young, 2009). It appears that the expression of *KiSS-1* is under the control of pituitary gonadotropins, since protocols of gonadotropin stimulation were able to stimulate *KiSS-1* mRNA levels in immature rat ovary; preventing the preovulatory surge of gonadotropins blocked the rise of ovarian *KiSS-1* expression (Castellano et al., 2006).

In addition, recent analysis of ovulatory dysfunction in a rat model indicated that the inhibition of the synthesis of prostaglandin is associated with a marked suppression of ovarian *KiSS-1* mRNA levels through the ovulatory period. In other word, the expression of *KiSS-1*, which is induced by gonadotropin, was blocked by prevention of prostaglandin synthesis (Gaytan et al., 2009). The above observation suggests a putative role of *KiSS-1* in the regulation of ovulation. The finding of kisspeptin immunoreactivity in different ovarian compartments including theca cells, corpus luteum and the interstitial gland might indicate other ovarian roles of kisspeptin-GPR54 signaling (Castellano et al., 2006 and Gaytan et al., 2009). As mentioned above, this is one of the aspects selected for investigation in the present study.

1.8. Neuromedin B (NMB) and its receptor (NMBR)

In relation to a potential role of NMB in ovarian function, a theca cell microarray study carried out in this laboratory (Glister et al., 2013), indicated that *NMB* is expressed in these cells and was amongst the most highly down-regulated transcripts in bovine TCs in response to BMP-6 treatment (~10-fold suppression). This observation drew our attention to NMB as another potential intrafollicular regulatory factor and prompted experiments on NMB reported in this thesis.

1.8.1. Discovery

In 1970, Erspamer et al. discovered bombesin and the related peptide ranatensin in frog skin. The first mammalian bombesin-like peptide was purified from porcine non-antral gastric and intestinal tissue and known as gastrin-releasing peptide for its effective gastrin releasing activity. Gastrin- releasing peptide which consist of 27-amino acid peptide and bombesin share the same seven C-terminal amino acid sequence. Neuromedin B (NMB), a highly conserved member of a family of bombesin-related decapeptide in mammals, was originally isolated from porcine spinal cord and is involved in the neural communication system. Since the C-terminal seven amino acid sequence is identical, this peptide is believed to be the mammalian homologue of ranatensin (Zhao et al., 2012). Human cDNA encoding *NMB* was firstly isolated by Krane et al in 1988 using screening of human hypothalamic libraries that lead to identification of its molecular structure. Consequently, the *NMB* gene was purified from rats, and a 117-amino acid prepro-*NMB* was revealed through nucleotide sequence analysis. It has been reported that the Neuromedin (NMBR) is part of the G protein-coupled receptor (GPCR) family which, when activated by NMB, plays several physiological functions. NMBR amino acid sequence is well conserved among different species (Ma et al., 2016).

1.8.2. Tissue-specific expression of *NMN* and *NMBR*

Since its discovery, *NMB* has been shown using RT-PCR and *in situ* hybridization to be widely expressed in central nervous system (CNS) and in peripheral organs. In humans, *NMB* is largely expressed in the hypothalamus, stomach and colon and to a smaller degree in the cerebellum, pancreas and adrenal glands, in adipose tissues and in the urinary tract. In rats, *NMB* mRNA has been highly identified in the dentate gyrus, olfactory bulb, dorsal root ganglion and brain stem. In peripheral

tissues and organs, *NMB* has been identified in the esophagus, stomach, intestines, uterus, urinary bladder, lungs, gall bladder, adipose tissues, gastrointestinal tissues, pancreas and pituitary (Ma et al., 2016; Kameda et al., 2014).

Expression of *NMBR* mRNA has been found in human, rats, mouse and monkeys. Highly expression levels of *NMBR* mRNA are reported in the CNS and in various peripheral tissues. In the CNS, the *NMBR* is extensively expressed in several brain regions, including the caudate nucleus, amygdala, thalamus, hippocampus, brain stem, hypothalamus, spinal cord and olfactory region in rat and mouse. In peripheral tissues, broad distribution of the *NMBR* has been reported in the testis, urogenital smooth muscles, gastrointestinal system, esophagus and adipose tissues. Additionally, NMB receptors have been reported on several types of tumors, such as CNS tumors, small cell and nonsmall cell lung cancers, carcinoids (intestinal, thymic, and bronchial), human pancreatic cancer cell lines and ovarian epithelial cancers (Ma et al., 2016).

As previously mentioned, molecular structure of NMB was initially discovered in human by Krane et al. (1988) and its human cDNA isolated by screening human hypothalamic libraries. *NMB* is encoded in a prepro-NMB, a 76-amino acid precursor which has a 24-amino acid signal peptide, a NMB-32 and a 17-amino acid carboxyl-terminal extension peptide. The carboxyl terminal of NMB-32 coding region is flanked by glycine a-amidation donor and a dibasic (Lys-Lys) cleavage recognition site which doubtless divides mature NMB-32 from its carboxyl-terminal extension peptide through proteolytic processing. Two *NMB* transcripts of 750-850 bases were revealed by northern blot analysis in human brain and gastrointestinal tissues with great expression levels in hypothalamus, stomach, colon and low levels in cerebellum, pancreas and adrenals. Hybridization analysis of human genomic DNA with NMB probe is reliable with a single human NMB-encoding gene. Analysis of human-mouse somatic cell hybrids specified the localization of this gene on chromosome 15, q11-qter region (Ohki-Hamazaki, 2000).

1.8.3. Biological action of Neuromedin B

The binding between NMB and its receptor is involved in several has important physiological regulations such as smooth muscle contraction, glucose metabolism and pancreatic endocrine or exocrine function. Also, NMB activates the

proliferation of carcinoma cells in prostate cancer, nonsmall-cell lung cancer, and colon cancer, as well as the differentiation of rat osteoblasts. In the pituitary gland, the expression and function of *NMB* have been studied, particularly in thyrotrophs (Kameda et al., 2014).

NMB/NMBR also a role in various behaviors such as fear and anxiety, stress, itching and scratching behavior, feeding, and thermoregulation. It also appears to have regulatory roles in reproduction, blood pressure, blood glucose, energy balance and cell growth (Ma et al., 2016).

1.8.4. Ligand binding

It has been proven in some studies that NMBR rapidly internalize and cleave bound agonist. At 22℃, NMB and its receptor showed maximal binding after 5 and 15 minutes, and in cells expressing native or transfected receptors more than 70% of the binding between the NMB and its receptor was internalized by 6o minutes (Benya et al., 1992). Continuous exposure to agonist resulted in receptor suppression due to NMBR internalization and consequence elimination of agonist caused receptor recycling from an intracellular site to cell surface which is not rely on protein synthesis (Ohki-Hamazaki, 2000).

1.8.5. Signaling transduction

Upon binding of NMB to its receptor, several intracellular signaling mechanisms are activated. Phospholipase C is activated and lead to the increase of cellular inositol phosphate and cytosolic Ca^{2+} levels in different NMBR-expressing cells such as rat C6 glioma cells, small cell lung cancer cell lines, NMBR transfected broblasts and cultured brain microvascular endothelial cells (Kroog et al., 1995; Wang et al., 1992; Benya et al., 1992 and 1994; Moody et al., 1995; Vigne et al., 1995). Cellular responses are mediated via heterotrimeric G-protein comprised monomeric $G\alpha_q$ and dimeric Gβγ linked to NMBR (Shapira et al., 1994; Jian et al., 1999). NMB/NMBR binding catalyses the exchange of GDP bound to the $G\alpha$ subunit for GTB. After detachment from Gβγ subunits, functional GTP bound $G\alpha$ subunit then stimulates βisoform of phospholipase C which catalyses the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2) in the cell membrane. Subsequent products, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) mediate

signaling pathways as second messengers. Then, the binding between IP3 and the receptors in the endoplasmic reticulum (ER) activates the release of Ca^{2+} from intracellular stores resulting in the increase of cytosol Ca^{2+}. Another molecule of second messenger, DAG, stimulates PKC. PKC signaling cascades probably control arachidonic acid secretion from NMB stimulation, and this leads to increase intracellular cAMP (Moody et al., 1995; Rozengurt, 1998). NMB also triggers tyrosine phosphorylation of multiple proteins involving $p125^{FAK}$ and paxillin, one of the main adhesion substances (Lach et al., 1995). Additionally, $p74^{raf\text{-}1}$ and $p42^{mapk}$ stimulation can appear in a PKC-independent manner, indicating that several signaling mechanisms may be activated following NMBR stimulation (Ohki-Hamazaki, 2000; Charlesworth and Rozengurt, 1997).

2. Aims of Study

1. Investigate whether *KiSS-1* and *NMB* and their cognate receptors are expressed in bovine endocrine tissues with particular focus on the ovary.

2. Explore whether kisspeptin and NMB, alone and in combination with their antagonists, can influence the steroidogenic pathway in cultured ovarian cells *in vitro*.

3. Investigate whether expression of *KiSS-1*, kisspeptin receptor and *NMB* by cultured ovarian cells is regulated by gonadotropins and other factors.

4. Examine the effect of kisspeptin-10 on bovine ovarian cortical stromal cell migration using an *in vitro* wound healing 'scratch' assay.

5. Investigate whether NMB, kisspeptin-10, TGF-β-1, BMP-6 and TSP-1 and their respective antagonist modulate capillary network formation in a follicular angiogenesis model *in vitro*.

6. Investigate whether TGF-β-1 and BMP-6 (alone and in combination with their antagonists) affect ovarian steroidogenesis in the above-mentioned follicular angiogenesis model.

7. Explore whether expression of steroidogenic transcripts and other angiogeneic factors by cultured ovarian cells is regulated by TGF-β-1 and BMP-6.

3. Materials and Methods

3.1. Bovine ovarian cell culture

3.1.1. Materials

Unless otherwise state, all materials were purchased from Sigma UK Ltd. (Poole, Dorest, UK), Life Technologies Ltd. (Paisley, UK), Fisher Scientific Ltd. (Loughborough, Leicestershire, UK), TOCRIS bioscience by R&D systems (Abingdon, UK) and Lonza (Wokingham.UK).

3.1.2. Bovine ovary collection

In all experiments, bovine ovaries at random stages of the oestrous cycle in cattle were collected from a local abattoir in Guildford, Surrey and delivered back to the laboratory within 50 minutes in medium-199 (M-199) containing antibiotic/antimycotic solution 1% v/v (Sigma, A9909). These ovaries came from animals of unknown breed, age, health and reproductive cycle background.

3.1.3. Media preparations

3.1.3.1. Culture media

3.1.3.1.1. McCoy's

Culture media were freshly prepared on the day of ovary collection under aseptic conditions in a laminar flow hood. This consisted of McCoy's 5A (500ml) modified medium (Sigma, M-8403), supplemented with antibiotic/antimycotic solution 1% v/v (Sigma, A9909), TSS (apo-transferrin) 5µg/ml (Sigma, T-2036) and sodium selenite 5ng/ml (Sigma, S-9133), Insulin 10ng/ml (bovine pancreas, Sigma, I-1882), HEPES 20mM (Sigma, H-4034) and bovine serum albumin (BSA) 0.1% (Sigma, A-9418). Cultured medium used for granulosa cells was also supplemented with 10^{-7}M androstenedione. For cells cultured under serum-supplemented conditions, 2% fetal calf serum (FCS) was included in addition to the other supplemented mentioned above.

3.1.3.1.2. Endothelial cell culture medium

Culture media were freshly prepared on the day of ovary collection under aseptic conditions in a laminar flow hood. This consisted of EBM-2 endothelial cell basal medium (500ml; Larnzo, CC-4176), supplemented with undefined concentration of Hydrocortisone (Lonza, CC-4112A), R3- insulin like growth factor-1 (Lonza, CC-

4115A), Ascorbic acid (Lonza CC-4116A), Human epidermal growth factor (hEGF) (Lonza CC-4317A), Antibiotics (GA-1000) (Lonza CC-4381) and Heparin (CC-4396A. In-house supplements including Apo-transferrin (TSS) 5µg/ml (Sigma, T-2036) and sodium selenite 5ng/ml (Sigma, S-9133), Insulin 10ng/ml (bovine pancreas, Sigma, I-1882) and BSA 0.1% (Sigma, A-9418) were also added. BSA was supplemented to the media after day 1 until the end of the culture period. Cells were cultured under serum-supplemented conditions (2% FCS) in addition to the other supplemented mentioned above for day 1 only.

3.1.4. Ovary processing and follicle selection

From the time of collection from a local abattoir the bovine ovaries were immersed in sterile supplemented M-199. On arrival in the laboratory, ovaries with a healthy appearance were washed in 70% ethanol for approximately 30 seconds then the excess tissues were trimmed off and the ovaries were maintained in fresh supplemented M-199. Medium sized follicles with a morphologically normal healthy appearance were dissected out aseptically in a laminar flow cabinet and transferred into petri-dish containing 5ml Dulbecco's phosphate buffer saline (DPBS). Follicle diameter was measured using a ruler and follicles 4-8 mm in diameter were selected for further processing to recover GC and TC for cell culture.

3.1.4.1. Granulosa cell preparation (GC)

Follicular fluid, cumulus-oocyte complex and loose GCs were aspirated using a 19-gauge needle attached to a 5ml syringe. Follicles were hemisected in a petri-dish contains DPBS and then cut in half to scrap the inner surface (i.e. GCs) using a sterile inoculating loop. After that, the cell suspension was transferred into a centrifuge tube, filled up with DPBS, centrifuged at 1500rpm for 10 minutes; then the supernatant was discarded and the pellet ready for osmotic shock treatment to lyse any red blood cells present (see section 3.1.5. below). Scraped follicles 'shells' were retained in a tube containing supplemented M-199 ready for theca cell preparation.

3.1.4.2. Theca cell preparation (TC)

Hemisected follicle 'shells' that had been gently scraped to remove GCs were put into a 50ml centrifuge tube containing supplemented M-199. They were shaken

vigorously for approximately 20 seconds to remove any remaining GCs and then the 'shells' were allowed to settle down. The supernatant was removed and replaced with fresh supplemented M-199 and the previous step was repeated twice. At this time, follicles were placed in a petri-dish containing supplemented M-199 using watchmaker's forceps. The theca internal layer was peeled away from stromal tissue under a dissecting microscope (Wild M8, Leica) with a uniform light. The collected TC layers were incubated in 10ml cultured medium containing 1% (v/v) trypsin inhibitor and (10mg/ml stock) and 10% (v/v) collagenase (10mg/ml stock) in a shaking water bath at 37 ℃ for 45 minutes. The dispersed tissue was triturated after 30 minutes with a sterile Pasteur pipette for 5 minutes and returned back into water bath. After the trituration step, any large debris was allowed to settle down and the TC supernatant was placed into a new centrifuge tube which was centrifuged at 1500rpm (400 g) for 10 minutes. The supernatant was removed and the TC pellet retained for osmotic shock treatment.

3.1.4.3. Stromal cell preparation (SC)

Bovine stromal tissue slices (~0.5mm) were dissected from the ovarian cortex up to about 1mm depth; all visible follicles and CL were avoided. Slices were collected in a sterile petri dish and cut by sterile blades in to small pieces (0.5mm). They were collected in 18 ml of medium-199 in a universal tube supplemented with 1% (v/v) trypsin inhibitor (10mg/ml stock), 10% (v/v) collagenase (10mg/ml stock) and 2% (v/v) DNase (2.5mg/ml) in a shaking water bath at 37℃ for 1 hour. Note, trypsin inhibitor was added to the collagenase dissociation medium to eliminate any contaminating trypsin present in the collagenase preparation. SCs were triturated after 1 hour with a sterile Pasteur pipette for 5 minutes and returned back to the shaking water bath for 30 minutes. Cells were triturated again for 5 minutes and then any large debris was allowed to settle down and SC supernatant was placed into a new 50ml centrifuge tube. Then, the tube was centrifuged at 1500rpm for 10 minutes. The supernatant was removed and the SC pellet retained for osmotic shock treatment.

3.1.5. Osmotic shock and cell seeding procedure

The cell pellets obtained from the above procedures were resuspended in 5ml PBS. Any contaminating red blood cells were removed by adding 10ml double distilled

water for 10 seconds followed immediately by addition of 10ml of 2x PBS to restore isotonicity. The cells were centrifuged at 1500rpm for 10 minutes and then the supernatant was removed. Each cell pellet was diluted in pre-equilibrated culture medium for a cell counting step using the trypan blue dye exclusion method.

3.1.6. Trypan blue dye exclusion method

This method was used to determine cell viability on the principle that only dead (non-viable) cells take up the dye while live (viable) cells remain clear. 50µl of cell suspension was diluted 1:10 using 250µl of trypan blue solution 0.4% (w/v) and 200µl of culture medium. The solution was mixed thoroughly and incubated at room temperature for 3 minutes. Cells number was then counted under a microscope using a haemocytometer.

3.1.7. Cell plating and culture

Cells were seeded and cultured into either 96-well or 24-well tissue culture plates (Nunclon, Life Technologies Ltd, Paisley, UK). Plates were incubated at 38.5℃ with saturating humidity in 5% CO_2 in air. Based on cell counting by trypan blue dye exclusion method, the serum free cell suspension was seeded either at a density of 500,000 viable cells/250µl (96-well plates), 363,000 viable cells/1ml (24-well plates), or 100,000 cells/1ml (24-well plates). For serum supplemented cell culture (2%), cell suspension was seeded at a density of 100,000 viable cells/250µl (96-well plates). However, SC were seeded into (12-well plates) at a density of 100,000 viable cells/1ml with (10% FCS). Note, culturing GC and TC using defined serum-free preserves a non-luteinized phenotype reflected by LH-induced A4 secretion by TC and FSH-induced E2 secretion by GC. Culturing GC and TC under serum-supplemented conditions promote spontaneous luteinisation, as indicated by reduced A4/E2 secretion and greatly increased secretion of P4 (Glister et al., 2001, Glister et al., 2005 and Kayani et al., 2009).

3.1.8. Media change during the experiments

3.1.8.1. McCoy's

After an initial 48h incubation period, culture medium was removed and replaced with fresh medium with/without treatments in this manner every 48 hours for 7 days of culture. Disturbance of cells was kept to minimum by removing only 70% of the

total volume in each well. Plates to which the spent medium had been transferred were then sealed with Parafilm and stored at -20℃ until used for hormonal assay.

3.1.8.2. Endothelial cell medium

After an initial ~6 hours incubation period, culture medium was removed and replaced with serum free fresh medium supplemented with BSA and with/without treatments every 24 hours for 7 days of culture. During media replacement, cells were washed (x1) with (DPBS). Plates to which the spent medium had been transferred were sealed with Parafilm to be stored at -20℃ until used for hormonal assay. Plates with adherent cell monolayers were either fixed for immunostaining or lysed for RNA extraction (see below).

3.1.9. Neutral red assay

This technique was performed for the estimation of viable cell number at the end of culture using the vital dye neutral red (3-Amino-7-dimethyl-amino-2-methylphenazine hydrochloride) described by Campbell, Scaramuzzi and Webb (1996). Viable cells readily take up the dye, thus the amount of dye consequently released upon cell lysis is directly proportional to the number of viable cells. Briefly, after 7 days of culture, 175µl of conditioned media were removed and kept in 96-well plates at 20℃. The culture medium was replaced with (200µl/well) of pre-warmed culture medium containing 50µg/ml neutral red dye. The plates were incubated at 38.5℃ for 3 hours. After this incubation period, the medium was discarded and replaced with 4% paraformaldehyde solution for 3 minutes. Paraformaldehyde was discarded and 200µl of lysis buffer containing 1% (v/v), glacial acetic acid and 50% ethanol in water was added to each well. The cells were lysed and released the neutral red. Due to the loosely clumped nature of the cultured cells, the plates were centrifuged briefly at each stage where the reagent was discarded from the cells, to minimize any cell loss and counting error. The plates were incubated overnight at 4℃ and then the absorbance was determined at 540nm (with a 600nm reference filter) using a microwell plate reader. Absorbance units were converted into cell number using a calibration curve generated by incubating known number of viable cells with neutral red solution (performed by Dr Clair Glister in this laboratory). The cell number was calculated using the following formula derived from this calibration experiment:

Cell number = (-20150+720800)*(absorbance-0.05)

3.1.10. ApoTox-Glo TM Triplex Assay

The ApoTox-Glo™ Triplex Assay combines three Promega assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. Reagent preparation, storage and use were done in accordance with the manufacturer's instructions. A black 96-well plate was used containing <20,000 cells with a final volume of 100µl/well. A 20µl of viability/cytotoxicity reagent contained both glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) substrate and bis-alanylalanyl-phenylalanyl-rhodamine 110 (bis-AFF-R110) substrate was added to each well, and briefly mixed by orbital shaking (300-500rpm) for 30 seconds. The plate was incubated for 2 hours at 37℃ and fluorescence measured at the following two wavelength sets; $400_{ex}/505_{em}$ (viability) and $485_{ex}/520_{em}$ (cytotoxicity). After that, a 100µl of Caspase-Glo®3/7 reagent was added to all wells and briefly mixed by orbital shaking (300-500rpm) for 30 seconds. The plate was incubated for 30 minutes at room temperature and luminescence was measured.

3.2. Peptides

3.2.1. Kisspeptin-10

The kisspeptin-10 (rat), endogenous ligand for the rodent kiss-1 receptor was purchased from Tocris Bioscience. Kisspeptin-10 corresponds to the C-terminal region of the kiss-1 peptide, kiss-1 (-112-121) and its sequences is shown in table 3.1. Note that rodent and bovine sequences are identical.

3.2.2. Kisspeptin 234

The Kiss-1/GPR54 antagonist kisspeptin 234 was purchased from Tocris Bioscience. It inhibits kisspeptin-10 stimulation of inositol phosphate (IP) and release of GnRH; its peptides sequence is listed in table 3.1.

3.2.3. Neuromedin B

Neuromedin B (porcine), is a mammalian bombesin-like peptide and endogenous ligand for the neuromedin B receptor; it was first isolated from pig spinal cord and has a role in regulating endocrine and exocrine secretion, smooth muscle contraction, blood pressure, feeding, and cell growth. Neuromedin B was purchased

from Tocris Bioscience. This peptide sequences is identical to the corresponding bovine peptide and listed in table 3.1.

3.2.4. BIM 23042

BIM 23042, a selective neuromedin B receptor (NMB-R_1 and BB_1) antagonist, was purchased from Tocris Bioscience and its peptide sequence is listed in table 3.1.

3.2.5. TGF-β-1

TGF-β-1 is a multifunctional protein that controls proliferation, differentiation and other functions in many cell types. TGF-β1-1 is involved in hematopoiesis and endothelial differentiation. Recombinant Human TGF-β1-1 Protein was purchased from R&D systems (ProDots formulation).

3.2.6. SB 431542

This is a potent and selective inhibitor of the transforming growth factor-β (TGF-β) type 1 receptor activin receptor- like kinase ALK5 and its relatives ALK4 and ALK7. It supresses TGF-β-induced proliferation of human osteosarcoma cells. It stimulates proliferation, differentiation and sheet formation of ESC-derived endothelial cells. Also it inhibits TGF-β-induced EMT, migration, invasion and VEGF secretion in several human cancer cell lines. SB 431542 was purchased from Tocris Biosciences.

3.2.7. BMP-6

Bone Morphogenetic Protein 6 (BMP-6), also known as Vgr-1, is a member of the BMP subfamily of TGF-beta superfamily proteins. BMPs are involved in a wide range of processes including embryogenesis, tissue morphogenesis, cell differentiation and migration, and tumorigenesis. Recombinant human BMP-6 was purchased from R&D systems.

3.2.8. K02288

K02288, a potent and selective inhibitor of type I bone morphogenic protein (BMP) receptors, was purchased from Tocris Biosciences.

3.2.9. FGF

Fibroblast growth factor is a member of the FGF family that involved angiogenesis, wound healing, embryonic development and various endocrine signaling pathways. Bovine Recombinant FGF was purchased from R and D systems.

3.2.10. VEGF

Vascular endothelial growth factor also known as vascular permeability factor (VPF), is a potent mediator of both angiogenesis and vasculogenesis. Bovine Recombinant VEFG was purchased from R and D systems.

3.2.11. Thrombospondin-1

TSP-1 regulates a wide range of cellular functions including their interactions with other cells and with the extracellular matrix (ECM). Recombinant human Thrombospondin-1 was purchased from R and D systems.

Table 3. 1 Peptide sequences.

Product name	**Peptide sequence**
Kisspeptin-10 rat	Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr-NH_2
Kisspeptin 234	Ac-D-Ala-Asn-Trp-Asn-Gly-Phe-Gly-D-Trp-Arg-Phe-NH_2
Neuromedin B porcine	Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2
BIM 23042	D-Nal-Cys-Tyr-D-Trp-Lys-Val-Cys-Nal-NH_2

3.3. Steroid hormone assays

3.3.1. Competitive enzyme-linked immunosorbant assay (ELISA)

The steroid enzyme-linked immunosorbant assay (ELISA) methods used for steroid measurements throughout this project were developed ‘in-house’ in Professor Knight’s laboratory. The principle is based on competition between variable amount of unlabelled steroid (antigen) in sample and fixed amount of labelled steroid enzyme-conjugated hormone (antigen) which is the detection reagent. The test sample and a fixed amount of enzyme-conjugated antigen were added into the antibody-coated well of microtiter plate. Antigen and enzyme-conjugated antigen

compete to bind to bind to the limited amount of antibody in the well surface. The more antigen in the sample the more it will compete with enzyme-conjugated antigen and less enzyme-conjugated antigen will bind to the plate, resulting in a lower color signal begin generated. A quantitative hormone measurement can be made from a standard curve of known dilution of the desired hormone.

3.3.1.1. Androstenedione (A4) ELISA

3.3.1.1.1. Assay buffers

3.3.1.1.1.1. Plate coating buffer

The plate coating solution buffer made up of 0.17mM sodium acetate in dH_2O.

3.3.1.1.1.2. Assay diluent buffer

Gelatin phosphate buffer with proclin (GPBP) was used as assay diluent buffer. The assay diluents were made up of 10% of 10X PBS (v/v), 90% of distilled water and 0.1% gelatine (w/v); gelatine was heated and dissolved in 200ml distilled water with constant stirring on the hotplate stirrer. Then Proclin 2000 was added at 0.05% (v/v) as a preservative and the diluent was stored at 4℃.

3.3.1.1.1.3. 10X azide-free PBS stock (PH 7.2)

10X azide-free PBS solution was used as a stock for the preparation of washing buffer assay and assay diluent. This buffer contains 80g of NaCl, 2g of KCl, 16.9g of $Na_2HPO_42H_2O$ and 2.4g of KH_2PO_4, which were dissolved in 1 litre of distilled water. The buffer was stored at room temperature for several months.

3.3.1.1.1.4. Wash buffer (0.1% Tween)

Wash buffer (PBS+0.1% tween) consisted of 250ml of 10X PBS stock, 2250ml distilled water and 2.5ml of tween. This buffer was stored in 4℃ for up to 2 weeks.

3.3.1.1.1.5. Substrate buffer (0.05M) citrate-phosphate buffer (PH 5.0)

This buffer comprised 2.58g citric acid (anhydrous), 3.62g disodium hydrogen orthophosphate (anhydrous) dissolved in 500ml of distilled water and store at 4℃ for up to 1 month.

3.3.1.1.2. Preparation of antibody-coated microtitre plates and enzyme-labelled antigen

Gelatine phosphate buffer (GPB) was used as the assay buffer for diluting samples and standards. The standards were prepared in the range 1.5-10,000pg/ml as 3 fold serial dilutions and stored at 4℃ while the samples were diluted to appropriate concentration in GPB before running the assay. The goat anti-androstenedione serum (IR637; gift from the late Prof GS Pope, National Institute for Research in Dairying, Shinfield, Berkshire, UK) was diluted 1:10,000 in 0.17mM sodium acetate buffer. 96 well microtiter plates (Nunc Maxisorb, Life Technologies) were coated with 100µl/well of A4 antibody and incubated at room temperature for 24 hours in a moist sealed box. Before the assay, the antibody was aspirated, plates washed (x3) with wash buffer and any free sites on the plates were blocked with 250µl/well GPB. Then, plates were kept in a moist, sealed box and stored at 4℃ until next day.

3.3.1.1.3. Assay protocol

At this stage, plates were washed (x3) with wash buffer on an automatic plate washer (Wellwash 4MK 2, Denly) and banged dry on paper towel before use. The assay was run with duplicate wells for standards, samples and QC samples which were added before and after samples for checking the intra assay accuracy. 100µl and 50µl of GPB were added to wells as non-specific binding (NSB) and maximum binding (B_{max}) respectively. 50µl of standards and diluted samples were added to wells. 50µl of Androstenedione-horseradish peroxidase conjugate (CAL Bioreagents, Los Angeles, CA, USA) was diluted 1:10,000 in GPB buffer and applied to each well except NSB and mixed thoroughly. The plates were incubated at room temperature in a moist box for 4 hours. After 4 hours incubation, the plates were washed with wash buffer and 200µl of freshly prepared o-phenylenediamine (OPD) horseradish peroxidase substrate was added to each well and the plates were incubated at room temperature in a dark box to allow the color to develop depending on the reaction for 2 hours. The absorbance was read at 450nm (600nm reference filter) on a microplate reader (Emax, Molecular Device). A standard curve was constructed using SoftMax Pro V5 program. A typical standard curve is shown in (Figure 3.1).

3.3.1.1.4. Sensitivity and reproducibility

The minimum detection level of the assay was approximately 10pg/ml and inter- and intra- assay CVs were <15%.

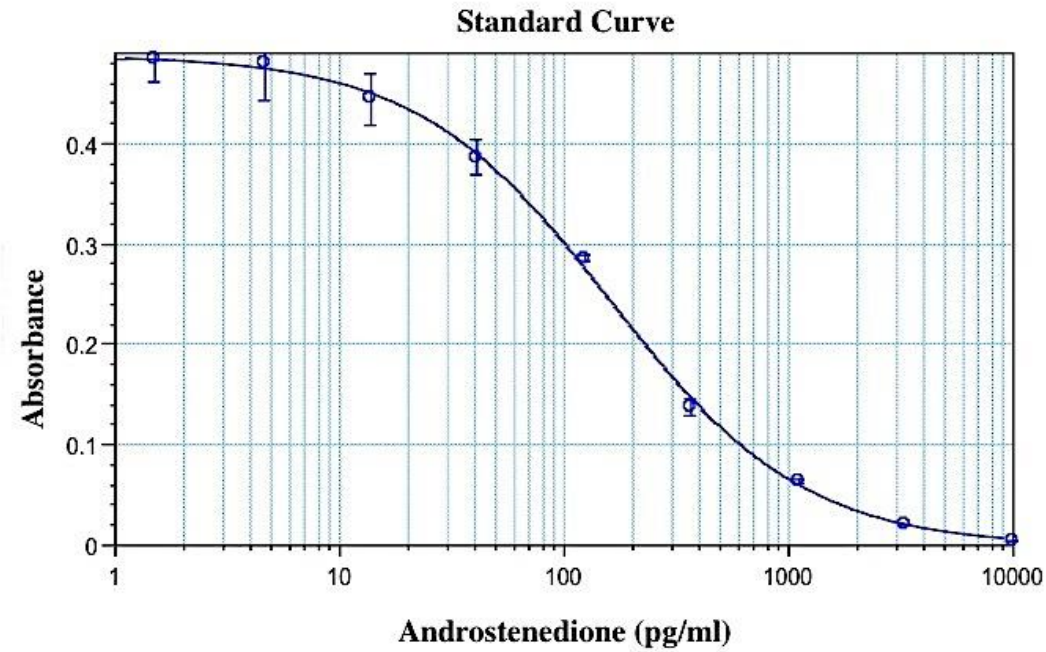


Figure 3. 1 Typical standard curve obtained in androstenedione ELISA.

3.3.1.2. Oestradiol (E2) ELISA

3.3.1.2.1. Assay buffers

3.3.1.2.1.1. Plate coating buffer

Carbonate buffer was used as plate coating buffer. This buffer contains 1.55g of Na_2CO and 2.965g of $NaHCO_3$, dissolved in 1 litre of distilled water and was stored at 4℃.

The rest of the buffers are the same as those used in Androstenedione assay.

3.3.1.2.2. Preparation of antibody-coated microtitre plates and enzyme-labelled antigen

Gelatine phosphate buffer (GPB) was used as the assay buffer for diluting samples and standards. The standards were prepared in the range 1.5-10000pg/ml as three fold serial dilutions and stored at 4℃ while the samples were diluted to appropriate concentration in GPB before running the assay. The goat anti-estradiol serum (510/6; gift from the late Prof GS Pope, National Institute for Research in Dairying, Shinfield, Berkshire, UK) was diluted 1:10,000 in 0.05M carbonate buffer (pH 9.6). 96 well microtiter plates (Nunc Maxisorp, Life Technologies) were coated with 100µl/well of E2 antibody and incubated at room temperature for 24 hours in a moist sealed box. After incubation period, the antibody was aspirated, plates washed with wash buffer and any free sites on the plates were immediately blocked with 250µl/well GPB. Then, plates were kept in a moist, sealed box and stored at 4℃ until next day.

3.3.1.2.3. Assay protocol

At this stage, plates were washed (x3) with wash buffer on an automatic plate washer (Wellwash 4MK 2, Denly) and banged dry on paper towel before use. The assay was run with duplicate wells for standards, samples and QC samples which were added before and after samples for checking the intra assay accuracy. 100µl and 50µl of GPB were added to wells as non-specific binding (NSB) and maximum binding (B_{max}) respectively. 50µl of standards and diluted samples were added to wells. 50µl of Oestradiol-horseradish peroxidase conjugate (Fitzgerald Industries, MA, USA) was diluted 1:2,000 in GPB buffer and applied to each well except NSB well and mixed thoroughly. The plates were incubated at room temperature in a moist box for 4 hours. After 4 hours incubation, the plates were washed and 200µl of freshly prepared o-phenylenediamine (OPD) horseradish peroxidase substrate was added to each well and the plates were incubated at room temperature in a dark box to allow the color to develop depending on the reaction for 2 hours. The absorbance was read at 450nm (600nm reference filter) on a microplate reader (Emax, Molecular Device). A standard curve was constructed using SoftMax Pro V5 program. A typical standard curve is shown in (Figure 3.2).

3.3.1.2.4. Sensitivity and reproducibility

The minimum detection level of the assay was approximately 10pg/ml and inter- and intra- assay CVs were <15%.

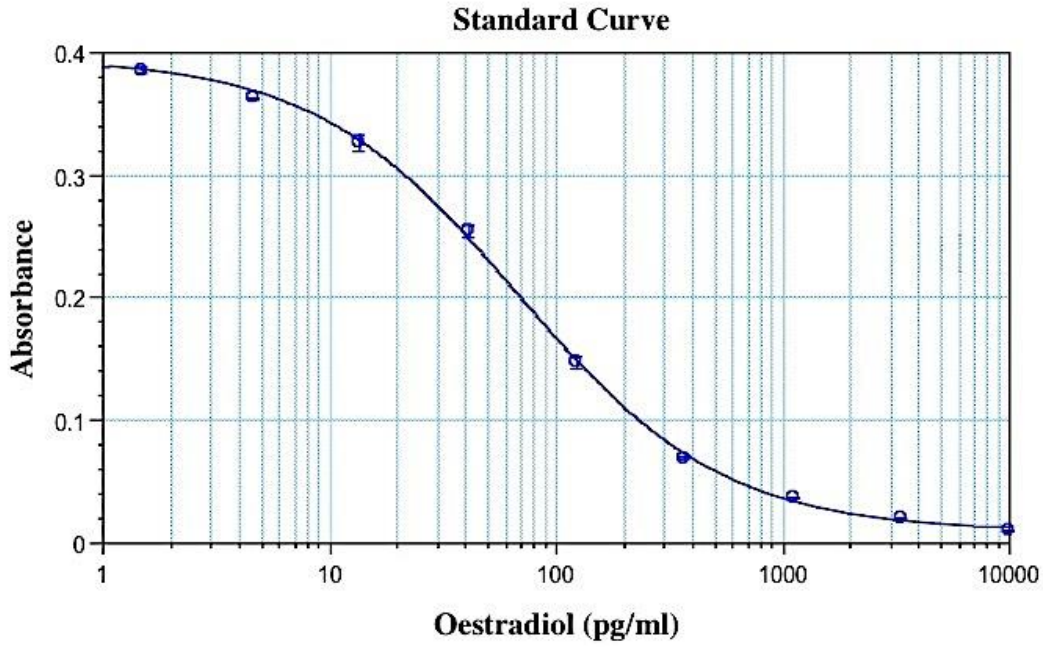


Figure 3. 2 Typical standard curve obtained in oestradiol ELISA.

3.3.1.3. Progesterone (P4) ELISA

3.3.1.3.1. Assay buffers

3.3.1.3.1.1. Plate coating buffer

The same assay buffer was used as in the Androstenedione assay.

3.3.1.3.1.2. Assay diluent buffer (PH 7.0)

Gelatine phosphate buffer (GPB) was used as the assay buffer. This buffer contains 0.1% (w/v) gelatin, 0.05M NaH_2PO_4, 0.05M Na_2HPO_4, 0.15M NaCl and 0.1% (w/v) NaN_3. The pH was adjusted to 7.0 and the buffer was stored at 4℃.

3.3.1.3.1.3. Wash buffer (PH 7.5)

The wash buffer used was 0.05M Tris HCL containing 0.15M NaCl, 0.1% NaN_3 and 0.05% (v/v) Tween 20. The pH was adjusted to 7.5 and the buffer was stored at room temperature.

3.3.1.3.2. Preparation of antibody-coated microtitre plates and enzyme-labelled antigen

Gelatine phosphate buffer (GPB) was used as the assay buffer for diluting samples and standards. The standards were prepared in the range 0.008-50ng/ml as 3-fold serial dilutions and stored at 4℃ while the samples were diluted to appropriate concentration in GPB. The goat anti-progesterone serum (711/12; gift from the late Prof GS Pope, National Institute for Research in Dairying, Shinfield, Berkshire, UK) was raised against progesterone 11-∝-succinyl-BSA and diluted 1:4,000 in 17mM sodium acetate buffer. A 96 well microtiter plates (Nunc Maxisorp, Life Technologies) were coated with 100µl/well of P4 antibody and incubated at room temperature for 24 hours in a moist sealed box. After incubation period, the antibody was aspirated, plates were washed with wash buffer containing azide and any free sites on plates were blocked immediately with 250µl/well GPB with azide. Then, plates were kept in a moist sealed box and stored at 4℃ until used.

3.3.1.3.3. Assay protocol

At this stage, plates were washed (x3) with wash buffer containing azide on an automatic plate washer (Wellwash 4MK, Denly) and banged dry on paper towel before use. The assay was run with duplicate wells for standards, samples and QC samples which were added before and after samples for checking the intra assay accuracy. 100µl and 50µl of GPB were added to wells as non-specific binding (NSB) and maximum binding (B_{max}) respectively. 50µl of standards and diluted samples were added to wells. 50µl of progesterone alkaline phosphate conjugate which was diluted 1:2000 in GTB buffer and applied to each well except NSB well and mixed thoroughly. Progesterone in standards and samples was competing with progesterone-alkaline phosphate conjugate for binding to the “solid phase” antibody. The plates were incubated at room temperature in a moist box for overnight. After overnight incubation, the plates were washed and 200µl of freshly

prepared p-nitrophenylphosphate (pNPP) substrate solution (Sigma) was added to each well and the plates were incubated at room temperature in a dark box to allow the colour to develop depending on the reaction for ~2 hours. The absorbance was read at 450nm (600nm reference filter) on a microplate reader (Emax, Molecular Device). A standard curve was constructed using SoftMax Pro V5 program. A typical standard curve is shown in (Figure 3.3).

3.3.1.3.4. Sensitivity and Reproducibility

The minimum detection level of the assay was approximately 20pg/ml and inter- and intra- assay CVs were <15%.

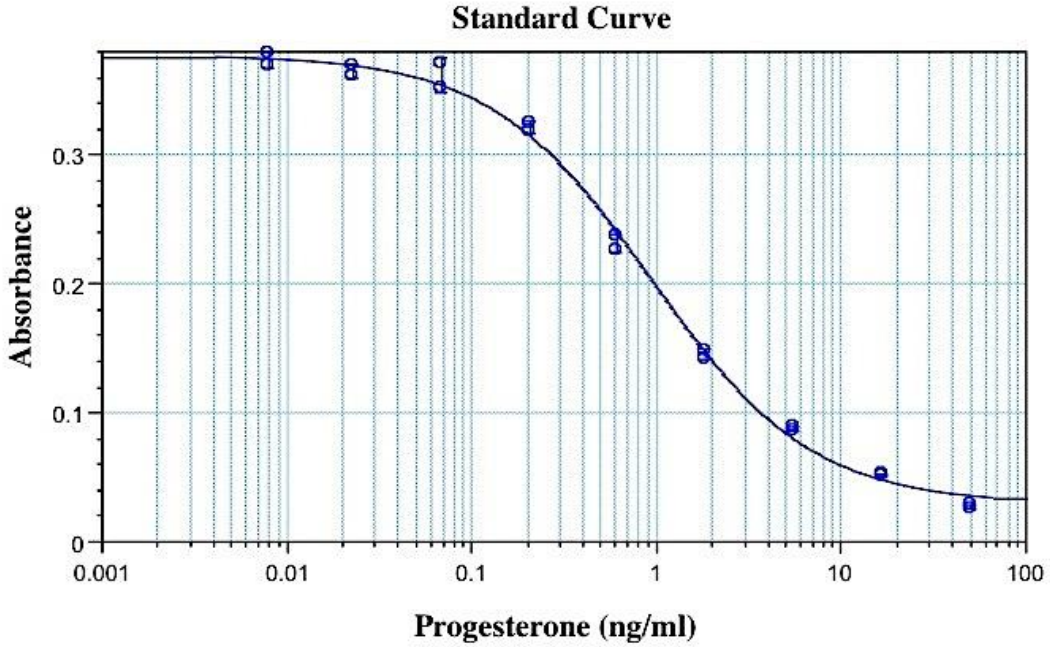


Figure 3. 3 Typical standard curve obtained in progesterone ELISA.

3.4. Gene expression analysis

3.4.1. Sample preparation

3.4.1.1. Total RNA extraction from bovine endocrine tissues

Bovine adrenal, pituitary, testis and corpus luteum were processed for RNA isolation using TRI-reagent procedure according to the manufacturer's instructions. Tissue samples were homogenized in 500µl of TRI reagent and allowed to stand at room temperature for 5-10 minutes. Samples were centrifuged at 12,000g, 4℃ for

10 minutes. The aqueous supernatant was removed to a clean tube and samples were preceded to RNA extraction procedure. Then 50µl of bromo-chloropropane was added to each homogenate, caped and inverted repeatedly to mix for 15 seconds. The mixtures were allowed to stand for 15 minutes at room temperature following by centrifugation at 12,000g, 4℃ for 15 minutes. After centrifugation, 3 separate phases were formed; the colorless upper aqueous phase which contained RNA, a white cloudy interphase which contained the DNA and a red organic phase which contained protein. The upper colourless aqueous phase was removed into new DNase-RNase-free microcentrifuge tube. RNA was precipitated by adding 250µl of isopropanol, mixed and stood at room temperature for 15 minutes then centrifuged to form pellet at 12,000g, 4℃ for 25 minutes. After that, the supernatant was discarded and 500µl of 75% ethanol was added to wash RNA pellet then the tube was centrifuged at 12,000g, 4℃ for 15 minutes. The supernatant was removed and the pellet was re-suspended in 50µl RNase-free water.

Removal of genomic DNA-DNase treatment

The extracted RNA samples were treated with RNase-free DNase kit (RQI; Promega, UK LtD) to get rid of any contaminating genomic DNA that may have been presented and cause false positive in PCRs. Briefly, 5µl of DNase reaction buffer and 2.5µl of DNase enzyme were added into each sample then mixed and incubated at 37℃ for 15 minutes. The TRI method was repeated in section 3.4.1.1. and the treated RNA was re-suspended in 50µl RNase-free water. The samples were kept in -80℃ freezer for subsequent cDNA synthesis.

3.4.1.2. Total RNA extraction from cultured cells

TC culture cells were prepared as previously described in Materials and method section 3.1.4.2. The prepared cell suspensions were seeded into 24-well tissue culture plates at a density of 100,000/ml. Plates were incubated at 38℃ with saturating humidity in 5% CO_2 in air. At the end of culture, media were collected for hormonal assay. Cells were lysed and RNA extraction was performed by using RNeasy Mini Kit (Qiagen, UK) and treated using the RNase free DNase set (Qiagen, UK) to eliminate any potential genomic DNA contamination. The kit was

used according to the manufacturer's instruction. The samples were stored in -80℃ for subsequent cDNA synthesis.

3.4.2. Quantification and purity assessment of RNA

The RNA quality and quantity were determined by spectrophotometer (NanoDrop2000, ThermoScientific, UK) at 260/280. 1µl was added to the machine to check the RNA in each sample. Nucleic acid absorbs light in the UV range with the optimum wavelength (λ_{max}) at 260nm. However, λ_{max} is dependent on the state of nucleotides. λ_{max} absorbance values are highest for free nucleotides, lower for single-stranded DNA (ss-DNA) or RNA, lowest for double-stranded DNA (ds-DNA). 1 absorbance unit at A260 from 1mm pathlength is equivalent to 40µg/ml. Since contaminating protein that remains from nucleic acid extraction absorbs the light at 280nm, samples purity were calculated by ratio of absorbance at 260 and 280nm (A_{260}/A_{280}). Pure RNA sample gives an A_{260}/A_{280} approximately 2. A ratio of 1.8 to 2.0 indicated good quality RNA.

3.4.3. RNA integrity analysis using agarose gel electrophoresis

The most common technique used to analyse total RNA integrity is to run an aliquot of samples on agarose gel stained with ethidium bromide (EtBr). Ethidium bromide enables the visualization of fragments by the interaction between the nucleic acids and the fluorescent molecule under the ultra violet (UV) light. Agarose gel electrophoresis separates RNA and DNA fragments according to size and by intensity of EtBr staining indicates the amount of nucleic acid present. 1% (w/v) agarose was mixed with 1x of Tris Acetate (TAE) buffer and heated in microwave until the agarose powder was dissolved. The mixture was allowed to cool to approximately 50℃ and 0.001% EtBr was added and gently mixed. Then, the mixture was poured into gel tray in gel tank (Bio-Rad) with an appropriate comb and allowed to set for approximately 30 minutes. After solidifying, the gel was placed in a submarine gel tank (Bio-Rad Laboratories Ltd., Hemel Hempsted, Hertfordshire, UK) filled up with TAE buffer. Samples and a 100bp DNA ladder were mixed with 20% (V/V) Blue/Orange 6X loading dye (Promega, Madison, WI, USA) in a volume ratio of 5:1 (sample: dye) and loaded to each well. The gel electrophoresis principle is based on the fact that when an electric field is applied across the gel, negatively charged DNA or RNA migrates toward cathode electrode.

The rate of migration depends on size and shape (smaller fragments migrate faster than bigger fragments). Gel was run at 50V until separation of bands was achieved (30-45 minutes). Lastly, the gel was visualized under a UV transilluminator-imager, U:Genius 3 (Syngene, Cambridge, UK) using built-in software U;Genius V3.0.7.0. Intact, non-degraded RNA appears as sharp, clear bands of 28S and 18S ribosomal RNA in a 2:1 ratio.

3.4.4. cDNA synthesis

Complementary DNA (cDNA) was synthesized from total RNA template (1µg per reaction) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cheshire, UK) according to the manufacturer's instruction. The reaction plates were placed in an Eppendorf's Mastercycler Gradient thromocycler cDNA for 10 minutes at 25℃, 120 minutes at 37℃, 5 minutes at 85℃ and then on hold at 4℃.

3.4.5. Quantitative RT-PCR

3.4.5.1. Primer validation

Primers were designed using their accession number for each gene through online design tool (http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?ORGANISM=9913&INPUT_SEQUENCE=AC_000164.1&LINK_LOC=nuccore). They were specifically checked against all know bovine (Bos Taurus) transcripts as part of the design procedure. All primers were designed to anneal to the target sequence and amplify regions within the coding sequence of key genes of interest as well as for the housekeeping gene, β-actin (ACTB) which was used for normalization of gene expression analysis by real-time PCR.

Prior to using in the experiments, the primers for the gene of interest were verified and selected according to their melting curve, PCR efficiency >85% that assessed by the slope of the cDNA template dilution plot and the presence of single amplicon product of the predicted size which was indicated by agarose gel electrophoresis (100µM stock in 1x TE buffer). The primers were prepared according to the manufacturer's instruction. The mixture of forward and reverse primers was diluted in Tris-EDTA (TE) buffer to a concentration of 2.5µM. The cDNA samples were

diluted 1:10, 1:50, 1:250, 1:1250 and 1:6250 in TE buffer. The list of primers sequences and accession numbers used for the experiments is show in Table 2.

3.4.5.2. Amplification of target sequences

The quantitative RT-PCR was performed by applying 5µl cDNA templates (typical dilution 1:40), 1µl each of forward and reverse primers and 7µl QuantiTect SYBER Green QPCR 2X 'hot start' Master Mix (Qiagen). Samples were processed on an AB StepOne Plus real-time PCR machine (Applied Biosystem, UK) using the following protocol: 1 cycle only at 95℃ for 15 minutes, then 40 cycles of 95℃ for 15 seconds and 60℃ for 1 minute. A melt curve was included at the end of each run. The method that was used to semi-quantitatively compare the difference between the abundance of each mRNA transcript was $\Delta\Delta C_t$ method using β-actin as the housekeeping control. C_t values for each transcript in a given sample were first normalized to β-actin C_t value (which was uniform across all experimental group: ANOVA $P>0.1$). For tissue samples, ΔC_t values for each transcript in a given sample were normalised to the average ΔC_t value for that transcript in all tissue samples. For cell culture experiments, ΔC_t values for each treatment were normalised to the ΔC_t value of the respective vehicle-treated control group. Finally, $\Delta\Delta C_t$ values were converted to fold difference for graphical presentation using the formula $2^{(-\Delta\Delta^{Ct})}$.

Table 3. 2 List of primers used for Quantitative RT-PCR

Target	Accession number	Forward primer 5`-3`	Reverse primer 5`-3`	Amplicon size (bP)
***KiSS-1* v1**	AB466319.1	TCAGGACACAGCCAAGGCAAGG	TGAAGGCGGTGGCACAAAGG	108
***KiSS-1* v2**	AB466319.1	AAGGCAAGGGCACTTCCAAGACC	TTTCCAGTGTCTCCCTGAAGGCG	110
***KiSS-1R* v1**	XM_003582417.2	TTCGTCATCTGCCGCCACAAGC	TGCACATGAAGTCGCCCAGAACC	154
***KiSS-1R* v2**	XM_003582417.2	TGTTGCTCGGGTGAACAGTGG	AGCCACTGCGCGTTTATACCCC	112
ACTB	NM_173979.3	ATCACCATCGGCAATGAGCGGTTC	CGGATGTCGACGTCACACTTCATGA	128
NMB	NM_001075270.2	ATGGGCAAGAAGAGCCTGGAGC	AGCTTGCTTTTGCAGGAGGACCC	126
NMBR	NM_001205710.1	AAAGGGATTTCCTACCCGCCCC	TGATGTTGCCCAGCAAGCCC	111
BEX2	NM_001077087.1	ACGGTCACCCTCTTGCTTCTTGG	GCAACACTTCGACTCAGACCTGC	116
STAR	NM_174189	TTTTTTCCTGGGTCCTGACAGCGTC	ACAACCTGATCCTTGGGTTCTGCACC	103

HSD3B1	NM_174343.2	GCCACCTAGTGACTCTTTCCAACAGCG	TGGTTTTCTGCTTGGCTTCCTCCC	111
CYP11A1	NM_176644	CAGTGTCCCTCTGCTCAACGTCC	TTATTGAAAATTGTGTCCCATGCGG	103
LHR	NM_176644	CAGTGTCCCTCTGCTCAACGTCC	TTATTGAAAATTGTGTCCCATGCGG	103
VEGF	NM_174304	GACAAAGGCACAGACGTTGTGGTCA	TGATCTGCAAGACGAGACTGGCATG	301
FGF	NM_174305.1	CGCCACTGAGTTGATTTTTGCTGAGA	TAAGGCTTTGCGCATGACCAGGTC	301
INHA	NM_174381.1	ATTGCCTCAGTCGATGCCCAGACC	AAAAAGCCAGCCGCGCTGC	92
NR5A1	M31836.1	CAAGAAAATCCCTGTGGGCCTTGC	TTAACTCAAGCTGCCTCGCCTTGC	124
TGFB1	M13440.1	CCAAGCGGCTGTACTGCAAGAACG	TGATGTGTGGGTCGCTCTTCTCGC	96
TGFB2	NM_174094.3	GAGCCCGAGGACCAAGATGTCTCC	CCTCAGCCTCTCCAGCATCTGGC	91
TGFB3	S45997.1	CGGGCTACCACTACGGGCTGC	CGGGCTACCACTACGGGCTGC	125
INSL3	XM_592497.2	TGGCTGACCCGCAGAGAGGAAATAGA	CCGGAACTGAACCCGTTAATGTCCAC	107

3.5. Wound Healing "Scratch" assay to evaluate cell migration

The *in vitro* scratch assay is a simple method used to investigate the effect of various treatments on cell migration. This assay was carried out to test the effect of (kisspeptin-10) on ovarian SC migration. The technique is based on the observation that, upon creation of a new artificial gap, a so called "scratch", on a confluent monolayer, the cells on the edge of the newly created gap will move toward the opening to close the "scratch" until new cell-cell contacts are established. Comparison of images captured at the beginning and at intervals during cell migration to close the scratch, allows one to determine the degree of cell migration. It is important to select a time point that does not result in either complete closure or zero closure. An 18 h time point was chosen for evaluating the extent of SC migration. This was based on previous experiments in the laboratory conducted by Dr Moafaq Samir and was judged to be appropriate as the extent of scratch closure in control SC was around 60-70 %.

3.6. Immunohistochemistry

Bovine ovaries were dissected into segments and fixed in formalin for 48 hours, before being dehydrated through an alcohol series, embedded in wax and sectioned (5µm) onto Superfrost charged slides (VWR, Lutterworth, UK). Sections were dewaxed and rehydrated prior to boiling in citrate buffer (10mM citric acid, pH6.0), blocking of endogenous peroxidase (3% H_20_2 in methanol) and blocking of nonspecific binding with 20% normal goat serum (NGS, Vector Laboratories Ltd, Peterborough, UK). After this, sections were incubated in rabbit polyclonal antibody against NMBR (1:100; ab188807, Abcam, USA) diluted in 2% NGS. The primary antibodies were incubated overnight at 4°C and then detected using biotinylated goat anti-rabbit diluted 1:250 in 2% NGS and Vector Elite ABC reagents (Vector), prepared as per manufacturer's instructions. Visualization of bound antibodies was determined using 3,3'–diaminobenzidine tetrahydrochloride (DAB; Vector), prior to slides being counterstained with haematoxylin, dehydrated through an alcohol series and mounted with coverslips using DPX mounting medium. Sections were imaged using a Zeiss Axioscop 2 microscope and AxioCam digital camera under a 20x objective lens.

3.7. Follicular angiogenesis cell culture system

A novel *in vitro angiogenesis* system, which utilizes primary cells derived from the theca interna, was developed based on the method described by Robinson et al (2008) for bovine early CL tissue. In this system, tubule-like structures are produced and after 7 days in culture, a network of endothelial cells has developed, which resembles a capillary bed.

3.7.1. Coating of coverslips

Sterile coverslips (circular, 19mm diameter x 0.25mm thick) obtained from (Thermo Scientific, Rochester, NY) were transferred to wells of a 24-well plate (Nunclon, Life Technologies Ltd, Paisley, UK). 1ml of Attachment Factor 1X was added to each well and incubated at 38.5℃ with saturating humidity in 5% CO_2 in air until used.

3.7.2. Isolation of theca layers

The preparation of bovine TC was described in section 3.1.4.2.

3.7.3. Preparation of cells for immunostaining studies

3.7.3.1. Fixation of cells

At the end of culture, media were either discarded or kept for hormonal assay. Cells were fixed immediately and permeabilized in acetone:methanol (1:1) at 4℃ for 5 minutes then washed with 1X PBS (3 x 5 minutes).

3.7.3.2. Blocking

Endogenous peroxidase blocking prevented unrelated peroxidases from being visualized. An endogenous peroxidase block 3% (v/v) hydrogen peroxide in methanol was applied for 10 minutes at room temperature. Plates were washed in 1X PBS buffer (3 x 5 minutes). Followed by serum blocking with 20% (v/v) normal goat serum for 30 minutes at room temperature, to prevent any binding to non-target sites.

3.7.3.3. Von Willebrand Factor (vWF) antibody stage

Polyclonal rabbit anti-human vWF antibody (Dako, High Wycombe, UK) was used at 5µg/ml diluted in 2% (v/v) normal goat serum in 1X PBS. A 200µl of the antibody solution was applied to each well and then incubated in a humidifier box for overnight at 4℃.

3.7.3.4. Secondary antibody stage

On the second day, plates were washed in 1X PBS (3 x 5 minutes). The primary antibodies were detected using Vector ABC Elite method as follows: biotinylated secondary goat ant-rabbit antibody was diluted 1:250 with 2% (v/v) normal goat serum in 1X PBS and incubated for 30 minutes at room temperature. Plates were then washed in 1X PBS (3 x 5 minutes). The avidin-biotin complex was then prepared according to manufacturer's instructions and applied to each well. After that, plates were incubated for 30 minutes at room temperature followed by further washes in 1X PBS (3 x 5 minutes).

3.7.3.5. Antigen detection stage

Visualisation of bound antibodies was determined using 3,3'-diaminobenzidine tetrahydrochloride (DAB). The DAB solution was prepared according to the manufacturer's instructions and incubated for 2 minutes, after which the reaction was stopped by washing the wells in distilled water.

3.7.3.6. Counterstaining and mounting stage

Plates were counterstained with haematoxylin for 20 seconds, washed in tap water and then washed once in water before being dehydrated through a series of alcohols (70% ethanol (v/v) 1 x 5 minutes), (90% ethanol (v/v) 1 x 5 minutes and 100% ethanol (v/v) 2 x 5 minutes. Coverslips were placed in histoclear for (2 x 20 seconds), removed (with cells attached) from the 24-well plates and then mounted on slides using DPX mountain medium. Images of all sections were visualised under 5x objective lens and then captured using an inverted microscope (Zeiss A1 Inverted Epifluorescent Microscope) fitted with a camera (Nikon NIS Elements).

3.7.3.7. Image analysis of von Willebrand factor immunostaining

A quantification method was developed, based on a protocol previously used to quantify area of vWF staining in a luteal endothelial cell culture (Robinson et al., 2008). All image analysis was performed using ImageJ 2.0.0. The areas of brown staining (vWF) were highlighted and only areas stained positively for vWF with endothelial cell cluster were recorded. This was repeated for a total of 25 fields of view across the whole coverslip. Two coverslips were examined for each treatment and from this the mean area average of vWF staining was recorded.

4. Does kisspeptin exert a local modulatory effect on ovarian steroidogenesis?

4.1. Introduction

Antral follicle growth in the ovaries of cows is regulated by the action of gonadotropins. The pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus into the hypophyseal portal circulations promotes tonic gonadotropin secretion from the pituitary, leading to follicular development and steroidogenesis in the ovaries (Naniwa et al., 2013). The ovarian steroids including oestrogen and progesterone control the central female reproductive axis through feedback actions. A positive feedback mechanism action on the hypothalamus involves the secretion of oestrogen in the late follicular phase that is necessary for the release of the GnRH-mediated preovulatory luteinizing hormone (LH) surge. In contrast, oestrogen and progesterone exerts negative feedback actions on the release of GnRH during the rest of reproductive cycle (Gal et al., 2016). In the last decade, kisspeptins, the product of the *KiSS-1* gene that act via the surface G-protein-coupled receptor-54 (GPR-54), has attracted attention as having a pivotal neuroendocrine role in the regulation of GnRH/LH release and hence ovulation, in many mammalian species including rodents, ruminants and primates. Kisspeptin was originally discovered as a metastasis suppressor and shown to prevent tumor spread (Mead et al., 2007). It has been also suggested that kisspeptin controls trophoblast invasion (Bilban et al., 2004). Additional actions of kisspeptin at other levels of the hypothalamic-pituitary-gonadal axis, in particular the ovaries, have been suggested but remain under-investigated (Merhi et al., 2016, Gaytan et al., 2014, Naniwa et al., 2013; Gaytan et al., 2009). Moreover, the expression of *KiSS-1* and *GPR54* genes has been reported in the ovary of some species. Kisspeptin and GPR54 immunoreactivity was detected in ovarian tissues and their gene expression levels evidently fluctuate in a cyclic-dependant manner under the control of pituitary LH. Taken together, these observations suggested a potential role of kisspeptins in the local control of ovarian function. However, the physiological relevance of an ovarian kiss-1/GPR54 system remains under-explored to date (Gaytan et al., 2009). The aims of the study reported in this chapter were firstly to investigate whether *KiSS-1* and its receptor (*GPR54*) are expressed in the bovine ovary and other endocrine tissues. Secondly, to examine whether kisspeptin can influence the steroidogenic pathway in cultured ovarian cells. Thirdly, to investigate the effect of a kisspeptin

antagonist (alone and in combination with kisspeptin) on ovarian steroidogenesis *in vitro*. Fourthly, to investigate whether expression of *KiSS-1* and its receptor by cultured ovarian cells is regulated by gonadotropins and other factors that were previously shown to modulate follicle steroidogenesis including BMPs (Glister et al., 2003 and 2005) and TNFα (Glister et al., 2014 and Samir et al., 2017). The laboratory techniques that have been used to address the above include a primary ovarian cell culture system (bovine ovarian theca and granulosa cells under non-luteinized and luteinized conditions), steroid immunoassay (androstenedione, oestradiol and progesterone) and real-time qPCR.

4.2. Materials and methods

4.2.1. Cell culture

Randomly cycling bovine ovaries were collected from a local abattoir then granulosa, theca and stromal cells were isolated and cultured according to section 3.1.4.

All treatments were applied as stated below. The spent culture medium was collected and retained for hormonal assay and replaced with fresh medium with the appropriate treatments every 48 hours. Viable cell number at the end of the culture was determined by neutral red assay (see section 3.1.9).

4.2.2. Preparation and administration of treatments

Kisspeptin-10 and kisspeptin 234 (antagonist) were dissolved in water and 20% (w/v) acetonitrile / water respectively to give a stock concentration of 10^{-3}M. Then stocks were diluted in sterile medium to give desired concentrations of the working solutions. A set of 5 dose-levels was prepared and a 25 µl applied to each well to give a final concentration of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}M respectively.

LH and FSH were prepared from frozen stock solutions of 100µg/ml that were pre-aliquoted into cryovials and stored under liquid nitrogen. Then LH and FSH stock solutions were diluted in complete medium supplemented with 0.3% (w/v) of BSA to give final stocks concentration of 500ng/ml.

FSK was prepared from a stock solution of 10mM in DMSO and diluted in complete medium supplemented with 0.3% (w/v) of BSA to give desired concentrations. Prior to conducting the experiments optimal concentrations of LH, FSH and FSK were established in pilot studies testing effects of 8 serial concentrations of LH and FSH on A4 and E2 secretion by non-luteinized cells. Also 3 does-levels of FSK were selected as optimal concentrations to give a maximum stimulation of A4, E2 and P4 secretions respectively. The initial stocks solution were diluted in sterile medium and filtered with 0.2µm membrane filter. A 25µl of each treatment were applied to wells to give final concentrations of LH, FSH and FSK (100pg/ml, 0,33ng/ml and 10µM) respectively.

4.2.3. Gene expression analysis

4.2.3.1. Sample preparation, total mRNA extraction, purification, cDNA synthesis, and RT-PCR

Samples were collected and processed according to section 3.4.1. Total RNA extracts were quantified as in section 3.4.2 and tested for integrity as in section 3.4.3. First-strand cDNA was synthesized as described in section 3.4.4 following by RT-qPCR analysis as described in section 3.4.4 using specific primer pairs as shown in table 3.2.

4.2.3.2. The expression of *KiSS-1* and *GPR54* mRNA in different bovine endocrine and ovarian tissues

Bovine adrenal, pituitary, testis and ovarian GC, TC and CL tissues were processed for RNA isolation using Tri-reagent procedure and cDNA synthesis using the AB high capacity cDNA synthesis kit according to the manufacture instructions as described in section 3.4.1.1 and 3.4.4. The cDNA samples from GC, TC and CL at different stages of follicle and luteal development were kindly provided by my laboratory colleague Dr Warakorn Cheewasopit and Dr Moafaq Samir. Then, cDNA samples were used for RT-qPCR for the detection of the expression of kiss-1 and its receptor using their designed primers and β-actin for normalization of gene expression as described in section 3.4.5 The set of primers used to detect the desired genes as well as housekeeping gene are listed in table 3.2. Melt curve analysis and agarose gel electrophoresis were used to verify that each selected primer pair gave a single amplicon of the predicted size and Tm. After that, cDNA samples were diluted 1:10 and 5µl of these diluted cDNA samples were used for qPCR. Volume of 2µl of forward and reverse primers, and 7µl of QuantiTect SYBR Green 2X "hot start" Master Mix (Qiagen) were added. Samples were run for 40 cycles on an AB StepOne plus real-time PCR instrument (Applied Biosystems). The method that was used to compare the difference between each mRNA transcript was ΔΔCt method using β-actin as the house keeping control. Resultant ΔC_t values for individual replicates within each tissue group were then normalised to the average ΔC_t value of these different tissues to give $\Delta\Delta C_t$ values. Finally, $\Delta\Delta C_t$ values were converted to fold difference for graphical presentation using the formula $2^{(-\Delta\Delta^{Ct})}$.

4.2.3.3. The expression of *KiSS-1* in ovarian cells treated with LH, FSH, FSK and other factors

The cDNA samples from cultured ovarian cells analysed for *KiSS-1* expression were kindly provided by my laboratory colleagues Dr Moafaq Samir and Dr Claire Glister. Cells which had been treated with LH, FSH, TNFα, BMPs and FSK for 7 days were used for RNA extraction procedure using Qiagen RNeasy mini-column kits followed by cDNA synthesis using the AB high capacity cDNA synthesis kit; qPCR was used for the detection of the expression of kiss-1 using as described in sections 3.4.1.2, 3.4.4 and 3.4.5.

4.2.3.4. The expression of *KiSS-1* and *GPR54* in stromal cells (SC) treated with kisspepin-10

Cortical stromal cells which had been treated with kisspeptin-10 for 18 hours in the wound healing assay as described in 3.1.4.3 and 3.5 were lysed and used for RNA extraction procedure using Qiagen RNeasy mini-column kit followed by cDNA synthesis using the AB high capacity cDNA synthesis kit, and RT-qPCR for the detection of the expression of *KiSS-1* and its *GPR54* using their designed primers and β-actin for normalization of gene expression as described in sections 3.4.1.2, 3.4.4 and 3.4.5. The wound healing assay data was analysed by one-way analysis of variance (ANOVA) using the statistical software StatView.

4.2.4. Hormone immunoassays

A4, E2 and P4 concentrations in retained spent media were determined by ELISA as described in section 3.3.

4.2.4.1. The effect of kisspeptin-10, kisspeptin antagonist, LH and FSK on TCs (A4 and P4 secretion)

Bovine ovarian TCs were cultured in 96 well plates as described in section 3.1.4.2. After 48 hours incubation period, 175µl of cultured medium were removed and replaced with control and treatment (containing medium), and then plates were placed in the incubator. Media were applied every 48 hours for 6 days. At two time points (96h, 144h) medium were collected for hormonal assay.

Depending on the type of cells whether they are non-luteinized or luteinized with 2% serum, 25µl of LH and FSK were added to wells to give final concentration of 100pg/ml and 10µM/ml respectively. 25µl of kisspeptin-10 and its antagonist were applied to wells to give final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M. There were two experimental designs carried out; (a) the presence/absence of LH, kisspeptin-10 and kisspeptin antagonist and (b) the presence/absence of LH or FSK and kisspeptin-10 along with expected optimum dose of kisspeptin-10 (10^{-7}M) in each well. Conditioned media were assessed for A4 and P4 by ELISA assay. The A4 and P4 production data were analysed by two-way analysis of variance (ANOVA).

4.2.4.2. The effect of kisspeptin-10, kisspeptin antagonist, FSH and FSK on GCs (E2 and P4 secretion)

Bovine ovarian GCs were cultured in 96 well plates as described in section 3.1.4.1. After 48 hours incubation period, 175µl of cultured medium were removed and replaced with control and treatment (containing medium), and then plates were placed in the incubator. Media were applied every 48 hours for 6 days. At two time points (96h, 144h) medium were collected for hormonal assay.

Depending on the type of cells whether they are non-luteinized or luteinized with 2% serum, 25µl of FSH and FSK were added to wells to give final concentration of 0.33ng/ml and 10µM/ml respectively. Kisspeptin-10 and its antagonist were prepared as mentioned above to give final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M. There were two experimental designs carried out; (a) the presence/absence of LH, kisspeptin-10 and kisspeptin antagonist and (b) the presence/absence of LH or FSK and kisspeptin-10 along with expected optimum dose of kisspeptin-10 (10^{-7}M) in each well. Conditioned media were assessed for E2 and P4 by ELISA assay. The E2 and P4 production data were analysed by two-way analysis of variance (ANOVA).

4.2.4.3. The effect of kisspeptin-10 on migration of bovine ovarian cortical stromal cells (SCs) assessed using a wound healing assay

Bovine ovarian cortical SCs were cultured in 24 well plates with 10% serum until confluence as described in section 3.1.4.3 950µl of medium was discarded from each well and cells were treated with new medium supplemented with mytomycin C (5µg/ml, inhibitor of cell division) at the same day of making wound scratches as following: (a) control cells (vehicle treated). (b) Cells treated with 50µl of kisspeptin-10 (Sigma) diluted to achieve 10^{-8}M, 10^{-7}M and 10^{-6}M as final concentrations. Using a 200µl pipette tip, the straight wound scratch was made, keeping the pipette tip at an angle of around 30 degrees to keep the scratch width limited. Then, using the inverted microscope (Zeiss A1 Inverted Epifluorescent Microscope) fitted with a camera (Nikon NIS Elements), images were taken for both wound edges of each well using a 10x objective lens. After 18 hours of incubation, pictures of the wound were taken again.

4.3. Statistical analysis

The effects of the various treatments on hormone secretion and gene expression were evaluated by two-way analysis of variance (2-way ANOVA). Individual pairwise comparisons within different treatments range were subsequently made by Fisher's PLSD. In order to reduce heterogeneity of variance, some dates were log-transformed prior to statistical analysis. Unless otherwise stated, results are presented mean ±SEM of ≥ 3 independent batches of cultured cells.

4.4. Results

4.4.1. The expression of *KiSS-1* and *GPR54* in different bovine endocrine tissues

The expression of *KiSS-1* in different bovine endocrine tissues including pituitary, testis, theca cells and granulosa cells and corpus luteum varied significantly (P<0.0001) with adrenal gland having the lowest level (figure 4.1a). Also the expression of *GPR54* varied significantly (P<0.0001), being highest in pituitary and lowest in testis (figure 4.1b).

Follicular expression of *KiSS-1* showed a highly significant effect by follicle category, with maximum expression in the small size class analyzed (Figure 4.2a). Likewise, the expression of *GPR54* was also significantly affected by follicle size class (Figure 4.2b). In addition, the expression of *KiSS-1* and its receptor was significantly different between TC and GC.

As shown in figure 4.3a and 4.3b, the expression of *KiSS-1* and *GPR54* in CL tissue tended to be greater in mid-luteal stage compare with growing or regression stages. However, the difference is not significant.

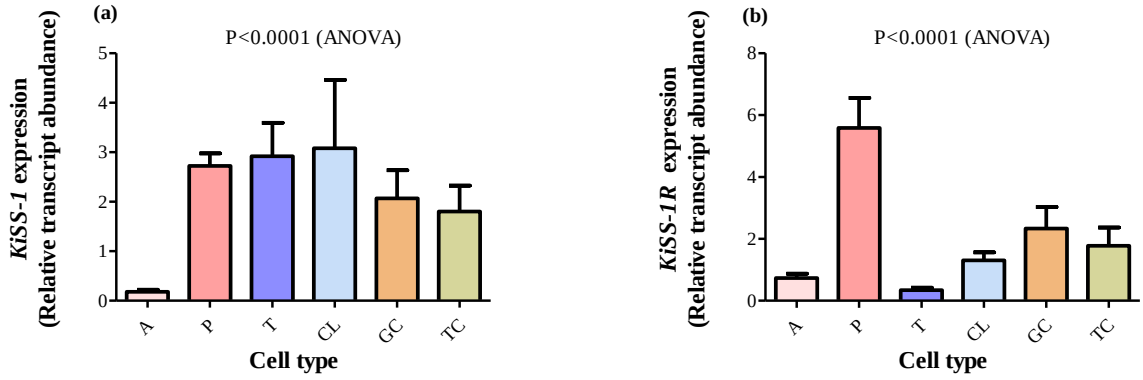


Figure 4. 1 Comparison of the relative abundance of mRNA transcripts for (a) *KiSS-1* and (b) its receptor in different bovine endocrine tissues including adrenal gland (A, n=6), corpus luteum (CL, n=13), granulosa cell (GC, n=38), pituitary gland (P, n=6), testis (T, n=6) and theca cell (TC, n=43). Values are means ±SEM and one-way ANOVA results are shown.

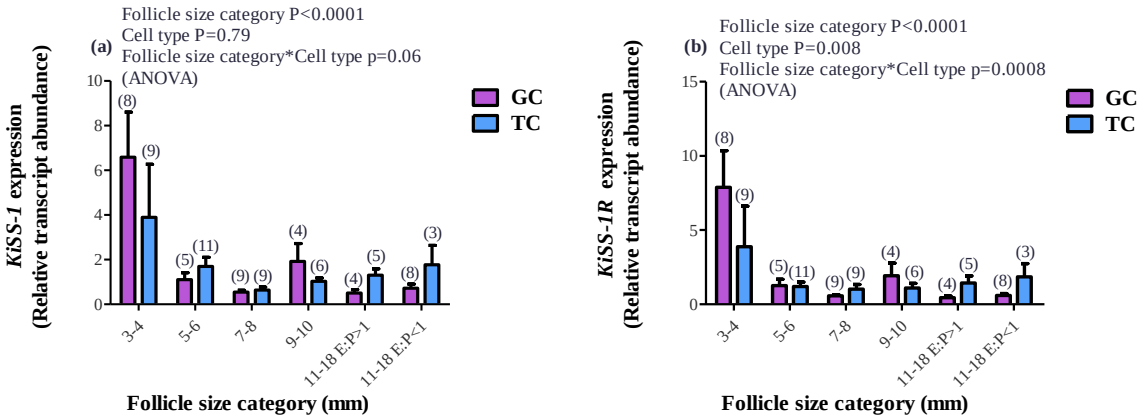


Figure 4. 2 A comparison of the relative abundance of mRNA transcript for (a) *KiSS-1* and (b) *GPR54* in GC and TC from ovarian follicles. Values are means ±SEM and summarized two-way ANOVA results are shown. *Numbers in parenthesis above bars are n-values.

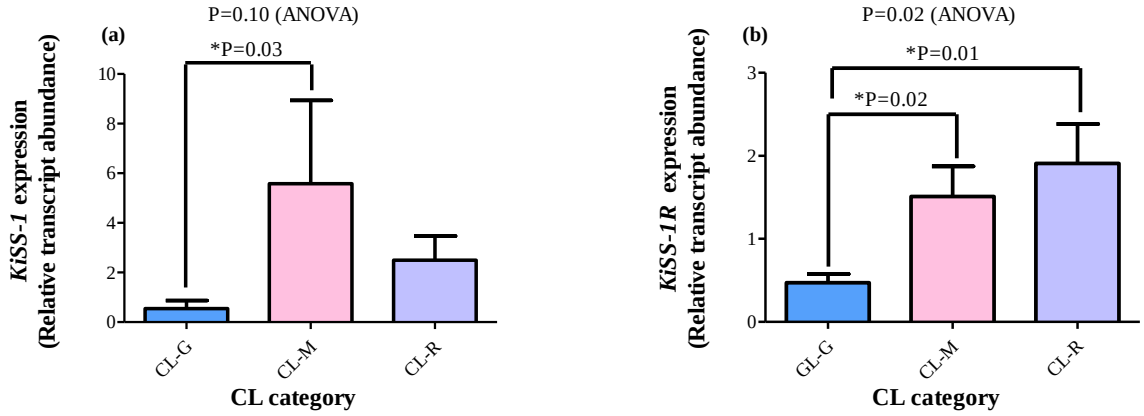


Figure 4. 3 The expression of (a) *KiSS-1* and (b) its receptor in CL tissue at growing (G, n=4), mid-luteal (M, n=5) and regressing (R, n=4) stages. Values are means ±SEM and results of one-way ANOVA and pairwise comparisons are shown.

4.4.2. The expression of *KiSS-1* mRNA in cells treated with LH, FSH, FSK and other factors

The expression of *KiSS-1* in cultured TC treated with/without LH and different BMPs (2ng/ml) showed a varied effect of BMPs ($p<0.0003$). LH tended to increase *KiSS-1* expression but the effect was not significant (Figure 4.4a). Likewise, expression of *KiSS-1* in LTC treated with FSK was not affected (Figure 4.4c).

The expression of *KiSS-1* in cultured GC treated with TNFα (10ng/ml) was not affected under both basal and FSH-induced conditions (Figure 4.4b). Likewise expression of *KiSS-1* by LGC was not affected by FSK (Figure 4.4d).

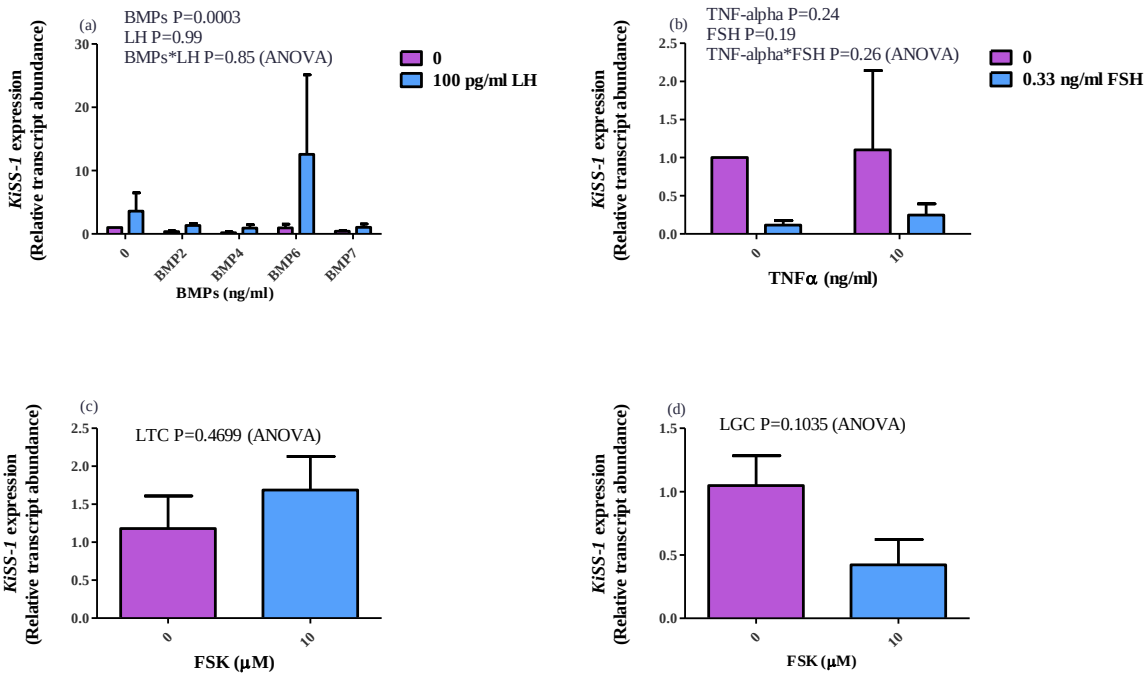


Figure 4. 4 Comparison of the relative abundance of mRNA for *KiSS-1* in (a); non-luteinized TC cultured in the presence/ absence of LH and different BMPs ligand at concentrations of 100pg/ml and 2ng/ml respectively, (b); non-luteinized GC cultured in the presence/absence of FSH and TNFα at concentrations of 0.33ng/ml and 10ng/ml respectively, (c); luteinized TC and (d) luteinized GC cultured in the presence/absence of 10μM of FSK. Values are means ±SEM (n=3-7 in dependant batches of cell) and two-way ANOVA results are shown.

4.4.3. The effect of kisspeptin-10 on SC migration assessed by wound healing assay

As shown in Figure 4.5, there was no significant effect of kisspeptin-10 on the percentage of wound closure by cultured SCs.

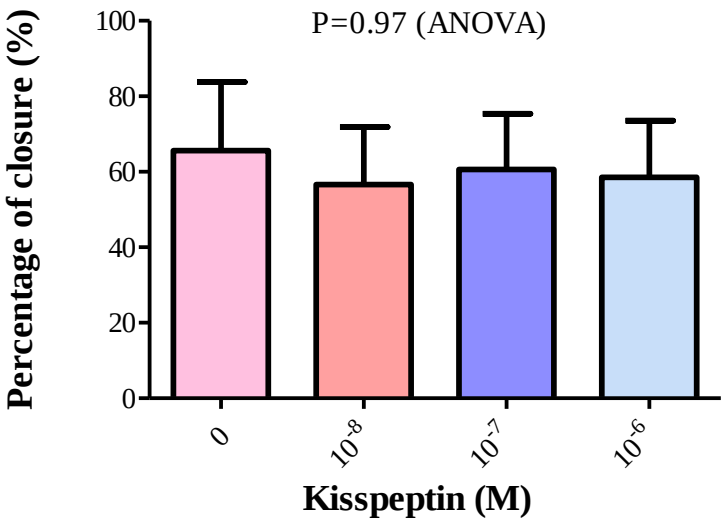


Figure 4. 5 Lack of effect of kisspeptin-10 on the percentage of wound closure by cultured SCs. Values are means ±SEM (n=3 independent batches of cells).

4.4.4. The expression of *KiSS-1* and *GPR54* in SC used for the wound healing assay

The statistical analysis showed no significant difference in the expression of *KiSS-1* and its receptor in cultured SCs exposed to kisspeptin-10 (Figure 4.6).

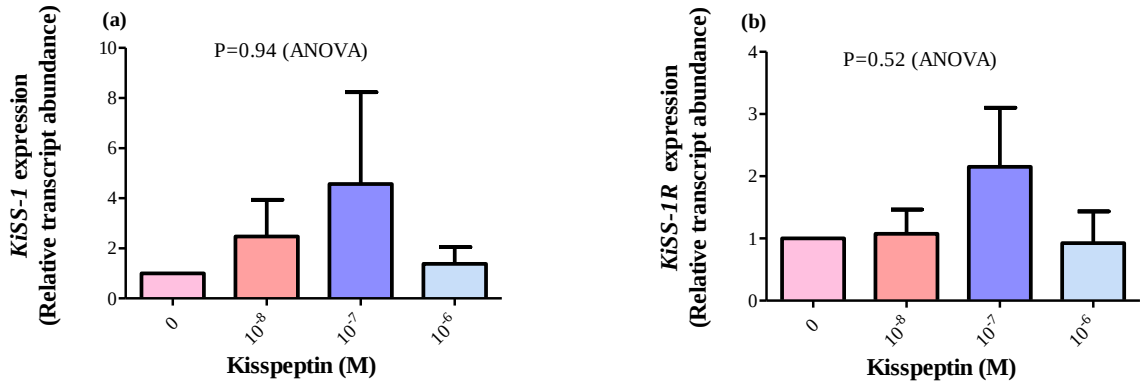


Figure 4. 6 Relative abundance of mRNA transcript for (a) *KiSS-1* and (b) *GPR54* in bovine SCs treated with kisspeptin-10. Values are means ±SEM (n=4 independent batches of cells).

4.4.5. The effect of kisspeptin-10 and kisspeptin antagonist on basal and LH-induced A4 and P4 secretion by non-luteinized bovine TC

As shown in Figures 4.7, 4.8 and 4.9 LH promoted a significant increase in secretion of both A4 and P4 and a small though significant decrease in viable cell number at the end of culture. However basal and LH-stimulated production of A4 and P4 by non-luteinized TC was not affected by kisspeptin-10 or kisspeptin antagonist. Likewise, there was no effect on viable cell number at the end of the culture period.

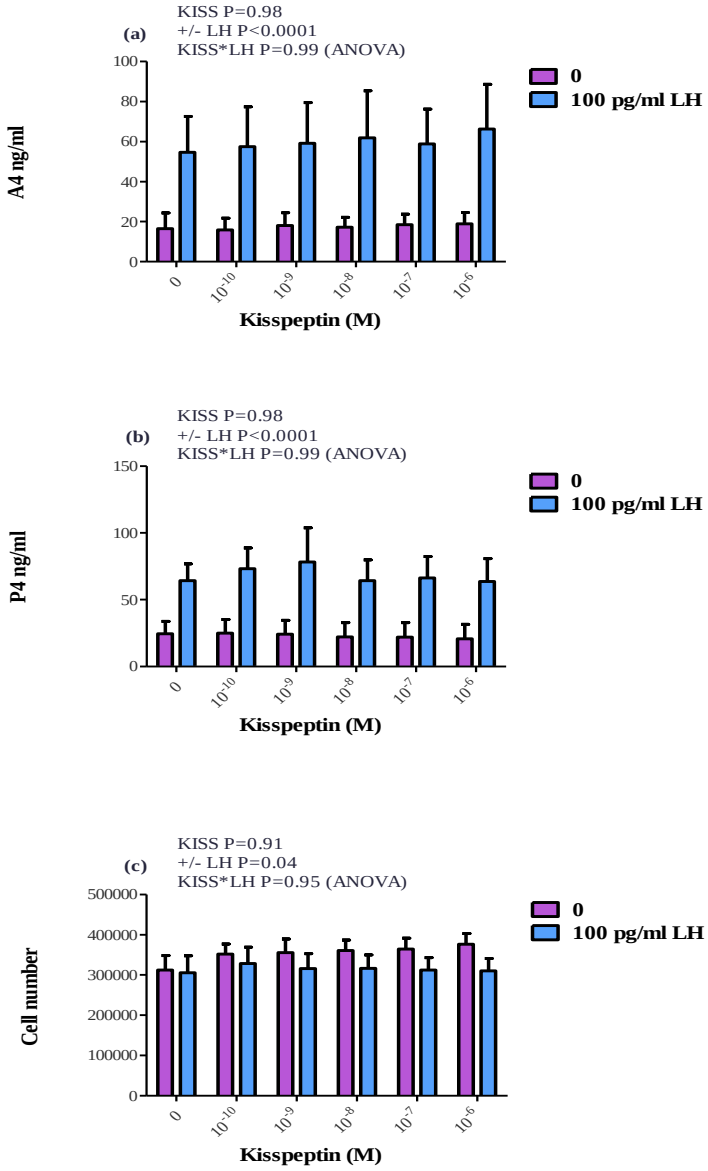


Figure 4. 7 The effects of LH and kisspeptin-10, alone and in combination, on the production of (a) A4 and (b) P4 by non-luteinized bovine TC; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=6 independent batches of cells) and two-way ANOVA results are shown.

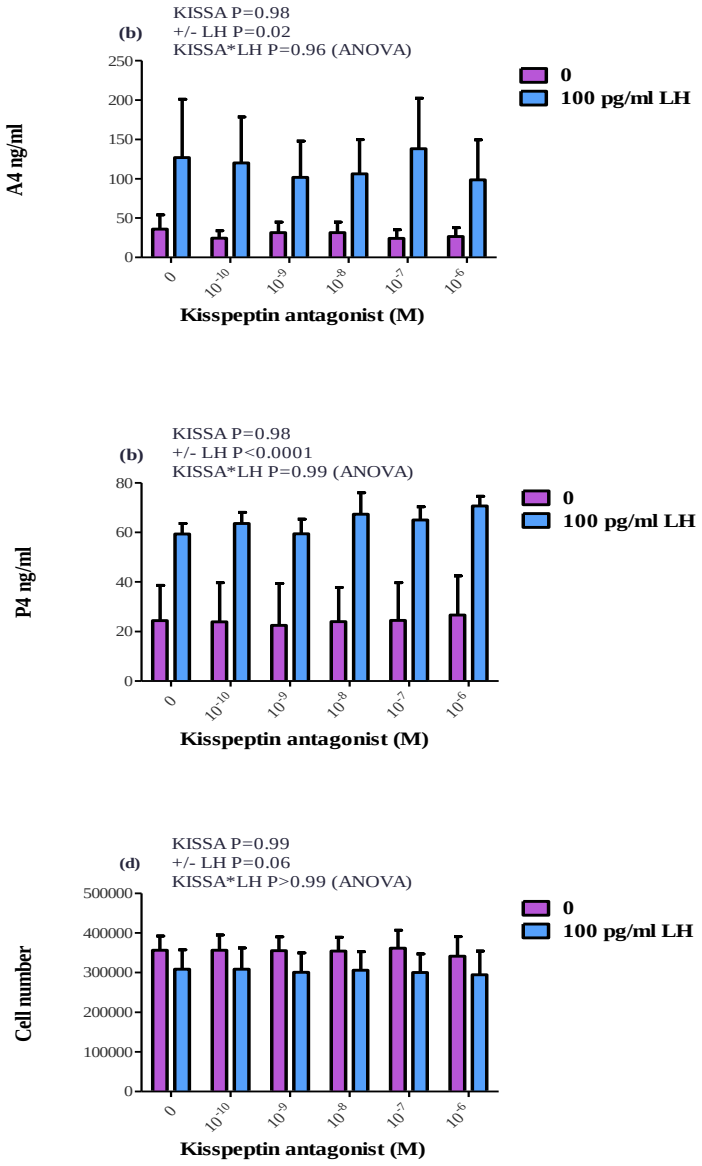


Figure 4. 8 The effects of LH and kisspeptin antagonist, alone and in combination, on the production of (a) A4 and (b) P4 by bovine non-luteinized TC; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=4 independent batches of cells) and two-way ANOVA results are shown.

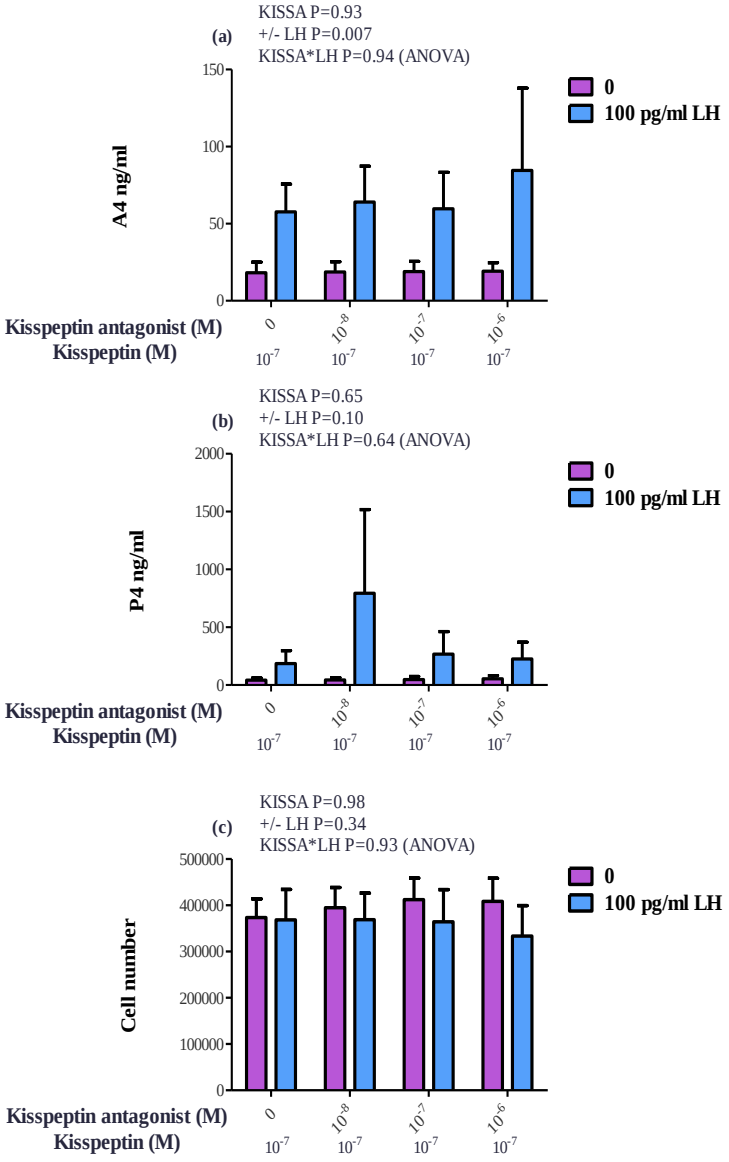


Figure 4. 9 The effects of LH and kisspeptin antagonist in combination with a fixed concentration of kisspeptin-10 (10^{-7}M), on the production of (a) A4 and (b) P4 by bovine non-luteinized TC under basal and LH-stimulated conditions; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown.

4.4.6. The effect of kisspeptin-10 and kisspeptin antagonist on basal and FSK-induced secretion of P4 by luteinized TC

As shown in Figure 4.10 kisspeptin-10 and kisspeptin antagonist did not modify basal or FSK-induced secretion of P4 by luteinized TC, or change viable cell number. Beside, Figure 4.10 demonstrates the release of (e) P4, and (f) cell number at the end of the culture period.

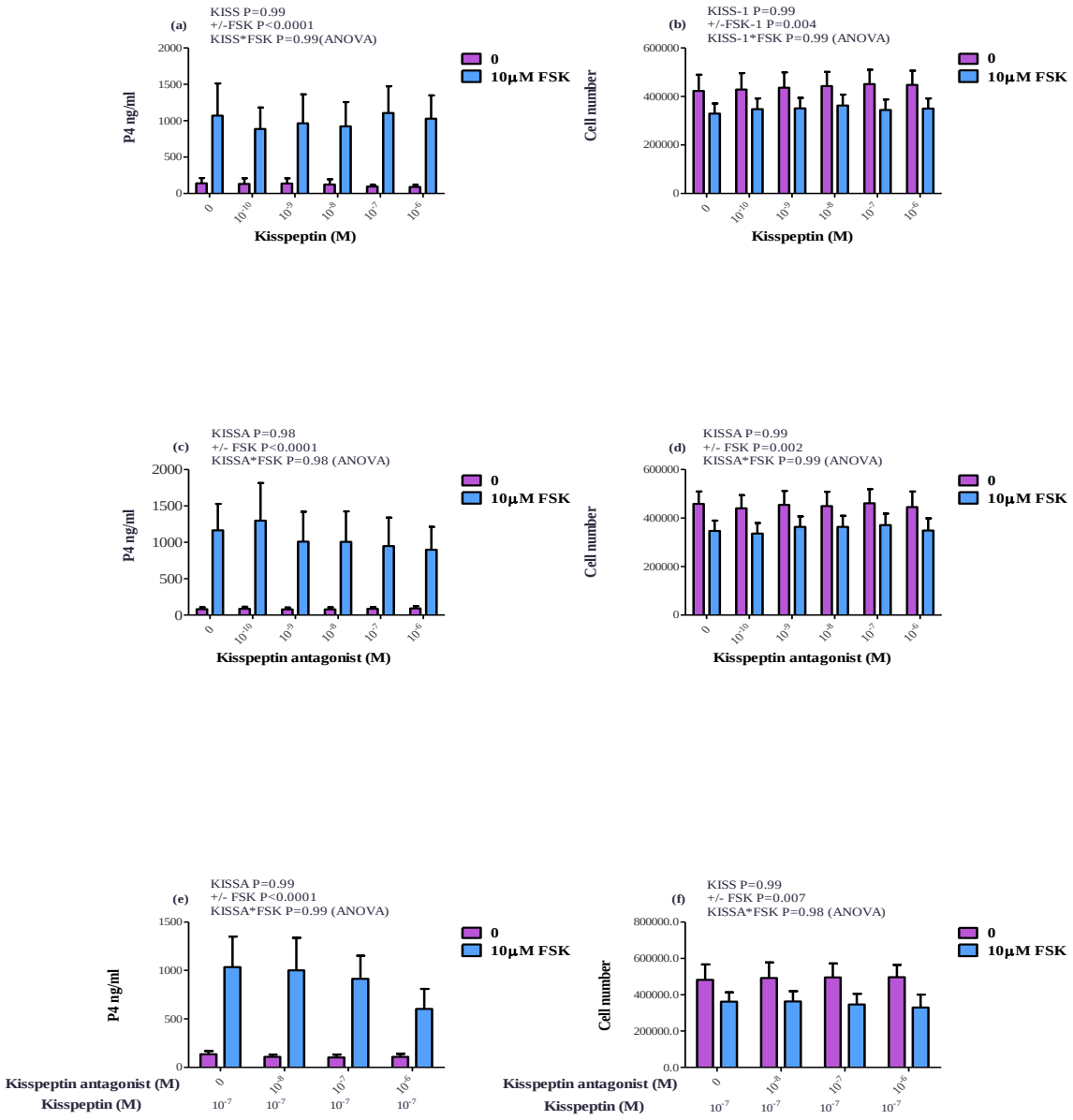


Figure 4. 10 Lack of effect of kisspeptin-10 and kisspeptin antagonist, alone and in combination, on basal and FSK-stimulated production of P4 by luteinized bovine TC (a, c and e) and on viable cell number at the end of the culture (b, d and f). In (e) and (f) all cells were treated with a fixed concentration of kisspeptin-10 (10^{-7}M). Values are means ±SEM (n=5-6 independent batches of cells) and two-way ANOVA results are shown.

4.4.7. The effect of kisspeptin-10 and kisspeptin antagonist on basal and FSH-induced E2 and P4 secretion by non-luteinized bovine GC

As shown in Figure 4.11 basal and FSH-stimulated production of E2 and P4 by non-luteinized GC was not affected by kissspeptin-10 or kisspeptin antagonist. Likewise, there was no effect on viable cell number at the end of the culture period. Treatment of GC with a kisspeptin antagonist also has no effect on steroid secretion or cell number (Figure 4.12 and 4.13).

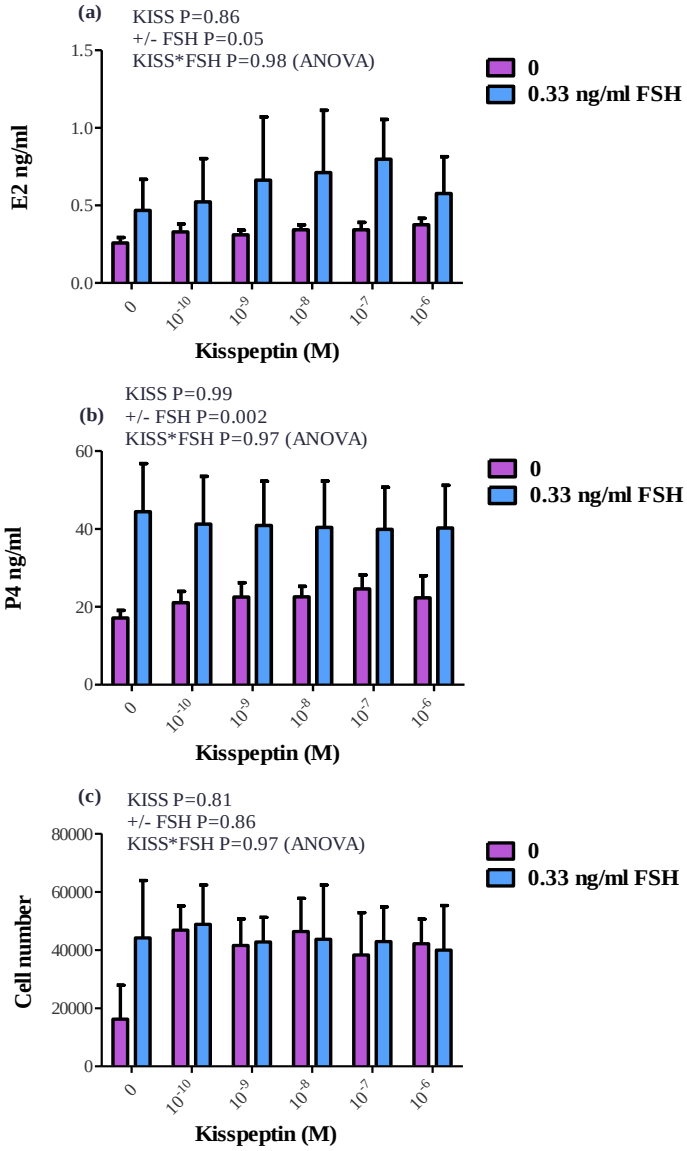


Figure 4. 11 The effects of FSH and kisspeptin-10, alone and in combination, on the production of (a) E2 and (b) P4 by non-luteinized bovine GC; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

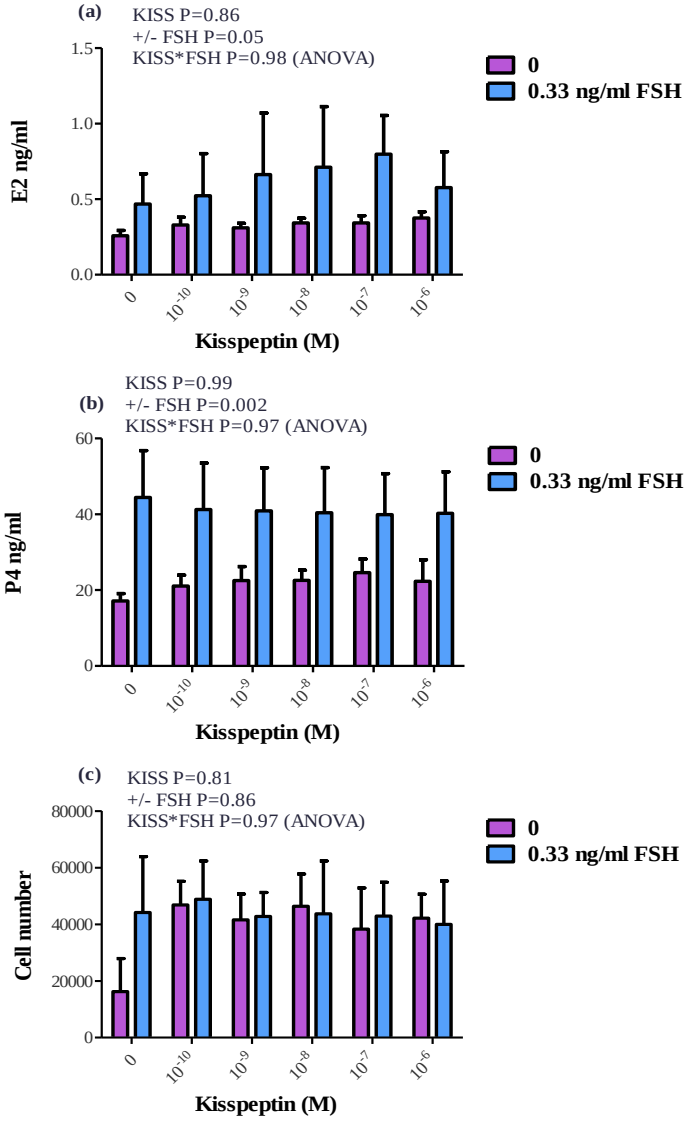


Figure 4. 12 The effects of FSH and kisspeptin antagonist, alone and in combination, on the production of (a) E2 and (b) P4 by non-luteinized bovine GC; panel (c) the viable cell number at the end of the culture. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

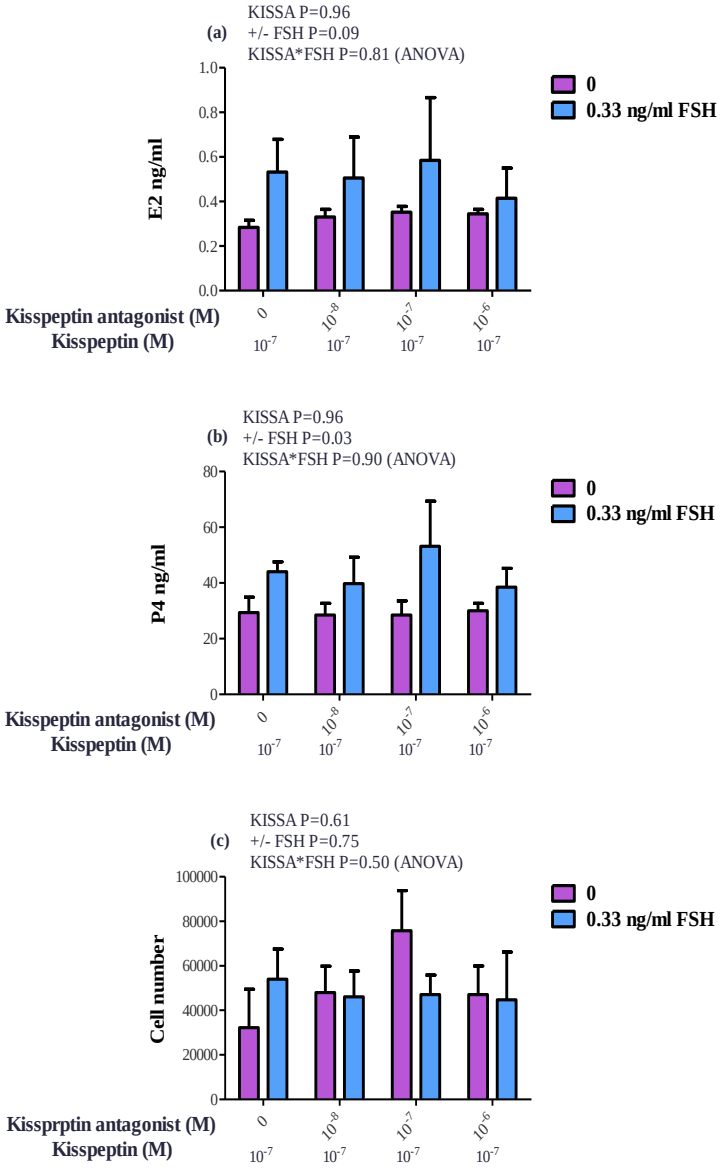


Figure 4. 13 The effects of kisspeptin antagonist in combination with a fixed concentration of kisspeptin-10 (10^{-7}M), on the production of (a) E2 and (b) P4 by bovine non-luteinized GC under basal and FSH-stimulated conditions; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

4.4.8. The effect of kisspeptin-10 and kisspeptin antagonist, on basal and FSK-stimulated P4 secretion by luteinized bovine GC

As shown in Figure 4.14 kisspeptin-10 and kisspeptin antagonist did not modify basal or FSK-induced secretion of P4 by luteinized GC or change viable cell number at the end of the culture period.

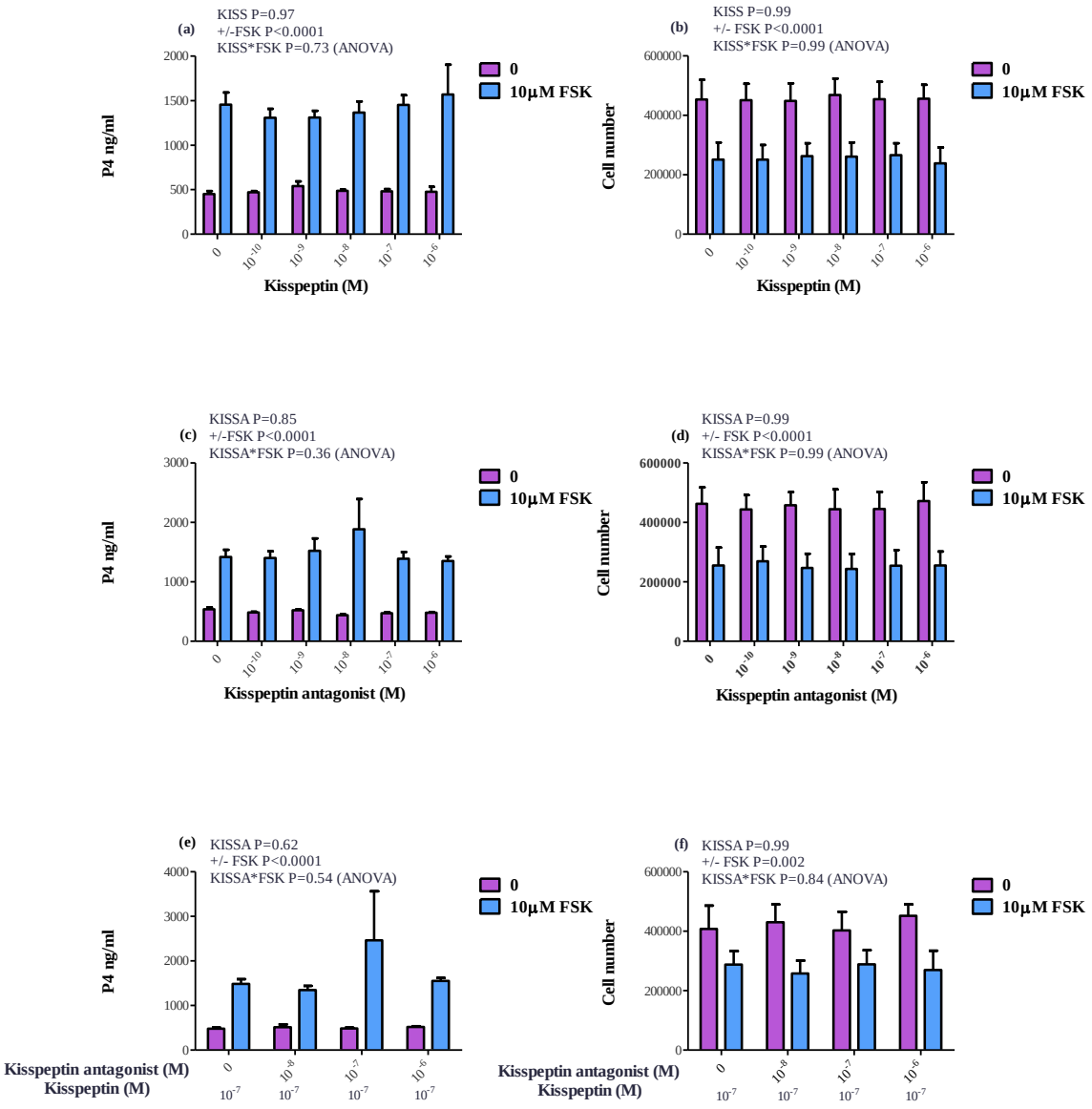


Figure 4. 14 Lack of effect of kisspeptin-10 and kisspeptin antagonist, alone and in combination, on basal and FSK-stimulated production of P4 by luteinized bovine GC (a, c and e) and on viable cell number at the end of the culture (b, d and f). In (e) and (f) all cells were treated with a fixed concentration of kisspeptin-10 (10^{-7}M). Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

4.5. Discussion

Compelling evidence has now established the vital role of the kiss-1/GPR54 system in the hypothalamus, which is integrally involved in the regulation of development and function of the male and female reproductive axis. On the basis of genetic, molecular and pharmacological methods, this indispensable role was expected to be primarily or entirely conducted at hypothalamic levels, in which *KiSS-1* neurons have been suggested as gatekeepers of the GnRH system (Dungan et al., 2006). Although many studies now support this contention, evidence has also been reported indicating expression of *KiSS-1* and *GPR54* genes in different peripheral tissues, including placenta and other reproductive organ, for instance the gonads. However, the physiological functions of kisspeptin in peripheral tissues remain uncertain (Ohtaki et al., 2001; Terao et al., 2004). Based on preliminary evidence showing expression of *KiSS-1* gene and *GPR54* in various bovine endocrine tissues including the ovary, the present study aimed to determine (i) whether *KiSS-1* and *GPR54* gene expression is sensitive to changes in ovarian physiology (i.e. follicle development stage, CL stages) (ii) whether kisspeptin-10 and a kisspeptin antagonist can modulate steroid production by cultured bovine theca and granulosa cells and (iii) whether gonadotropins and other regulatory molecules can influence expression of *KiSS-1* and *GPR54* in cultured ovarian cells.

Our RT-qPCR results conclusively showed that the genes encoding *KiSS-1* and its receptor *GPR54* are indeed expressed in different bovine tissues including pituitary, adrenal, testis, corpus luteum, theca cell and granulosa cell. Thus, the adrenal gland seems to show the lowest level of the expression of *KiSS-1* gene. While the expression of *KiSS-1* receptor varied significantly, being by far the highest in pituitary and lowest in testis. More interestingly, our current data demonstrated that the profiles of ovarian *KiSS-1* gene and *GPR54* expression in theca and granulosa cells from follicles at different stages of development are clearly distinct. Therefore, a series of experiments have been conducted using the agonist and antagonist (kisspeptin-10 and kisspeptin 234) to determine the potential function of kisspeptin signaling in ovarian follicles.

As previously mentioned, *KiSS-1* was originally identified as a metastasis suppressor in melanoma and referred to as ‘metastin’. A subtractive hybridization study involving human melanoma cell lines that differed in their metastatic capacity revealed that tumor cells with low invasiveness selectively overexpressed *KiSS-1*

gene (Lee et al., 1996). Furthermore, the direct administration of the kiss-1 peptide inhibited pulmonary metastasis of melanoma cells in mice (Ohtaki et al., 2001). In the present studies, we have used kisspeptin-10 (metastin-45-54), a short 10 amino acid peptide of the carboxy-terminal region that is proteolytically cleaved from metastin. Kisspeptin-10 is 10 times as active as metastin and considered as a candidate for clinical use (Tomita et al., 2007). Migration of cow SCs was not affected by kisspeptin-10 in the *in vitro* wound-healing 'scratch' assay in which cell proliferation was inhibited using mitomycin C. Inclusion of mytomycin C in the medium precluded an assessment of whether kisspeptin-10 affected cell proliferation. Furthermore, to evaluate if metastin treatment alters gene expression pattern, we conducted real-time PCR analysis of SCs that were treated with or without kisspeptin-10. There was no significant difference in the expression of kiss-1 and GPR54 in SCs treated with and without kisspeptin-10. However, Kang et al (2011) used human endometrial cancer cell line to examine if the mestastin-GPR54 axis influences the migration and invasion of the cells in vitro. They showed that the migration of these cell line was significantly inhibited by kisspeptin-10 in the wound-healing assay, whereas proliferation was not affected. In addition, a recent study has reported that kisspeptin-10 inhibited *in vivo* and *in vitro* breast cancer and human umbilical vein endothelial cell (HUVEC) growth (Song and Zhao, 2015). This suggests that primary cultures of bovine ovarian SC cells do not respond to kisspeptin in the same manner as several cancer cell lines used as models for cell migration/metastasis. It should be noted that the amino acid sequence of bovine and human kisspeptin-10 are identical and so species differences in biopotency could not explain this lack of effect on bovine cells.

Our RT-qPCR analysis demonstrated that *KiSS-1* and *GPR-54* expression in the cow follicle was follicle size dependent and different between TC and GC. In the corpus luteum, the expression was also shown to vary in a stage- dependent manner. In the rat ovary, the expression of *KiSS-1* and its receptor at the protein level were also reported to be stage dependent, with immunostaining detected in the theca layer of growing and pre-ovulatory follicles from oestrus to early pro-oestrus, which then moved to the granulosa cell layer of preovulatory follicles in late pro-oestrus. This pattern different from that observed in the cow ovary in the present study. After ovulation in the rat, expression of *KiSS-1* and *GPR54* was found in the theca-lutein cells of the corpus luteum and expression decreased as the corpus luteum regressed

(Castellano et al., 2006 and Roseweir and Millar, 2008). Some of these findings was in agreement with the marked fall in *KiSS-1* expression observed in regressing bovine CL in the present study.

A physiological action of kiss-1/receptor in the ovary can be suggested by the presence of receptor and ligand. As previously mentioned the expression of *KiSS-1* and its receptor has been reported in several tissues including adipose tissue, pancreas, liver, small intestine, peripheral blood lymphocytes, testis, lymph nodes, aorta, coronary artery, and umbilical vein, female tract, with highest expression in placenta and the central nervous system (Terao et al., 2004; Roman et al., 2012; Hussain et al., 2015). The mRNA *KiSS-1* expression in the ovary was firstly reported by Terao et al (2004) in a rat study, suggesting a local role of kisspetin in reproductive tissues (Terao et al., 2004). The expression of kisspeptin/receptor was found in rat theca cells, corpora lutea and interstitial tissues (Castellano et al., 2006). There are some inconsistent studies even with the same species regarding cellular expression of kiss-1/receptor (Shahed and Young, 2009; Laoharatchatathanin et al., 2015; García-Ortega et al., 2016; Mondal et al., 2016). For instance, the absence of kiss-1/receptor has been demonstrated in GC of rat ovary (Castellano et al., 2006; Zhou et al., 2014). whereas, highly expression of kiss-1/receptor was found in GC of rat ovary (Peng et al., 2013; Laoharatchatathanin et al., 2015; Ricu et al., 2012). According to Ricu et al (2012), the expression of *KiSS-1* mRNA was strongly expressed in rat GC compared with TC and other ovarian cells (Ricu et al., 2012). Whereas, other findings showed that the expression of *KiSS-1* receptor was found in GC and other cells of the ovary. The apparent inconsistencies of the expression of kiss-1/receptor in the ovary may relate to the variety of methods used to assess their existence. Other elements can considerably affect the patterns of expression of the kiss-1/receptor system include age and ovarian tissues and cells being obtained from different oestrous/menstrual cycle (Castellano et al., 2006; Shahed and Young, 2009; Gaytan et al., 2009; Ricu et al., 2012; Mondal et al, 2015; Merhi et al., 2016). It has been confirmed in humans that the expression of kiss-1/receptor gradually increased as follicles grow, with a peak level at the preovulatory stage (Shahed and Young, 2009; Mondal et al., 2015; Mondal et al., 2016). According to the previously mentioned observations, the increased expression was due to the stimulatory effect of gradually increased gonadotropins (Castellano et al., 2006).

For many years, it has been known that the steroid hormone feedback from the gonads has a major controlling influence on the HPG axis. Several studies have documented the stimulatory action of kisspeptin-10 administration on GnRH, LH and FSH secretion (George et al., 2011). As far as we know, no study had examined the direct effect of kisspeptin-10 in ovarian tissues. Therefore, the current study appears to be the first to examine *in vitro* whether kisspeptin-10 and a kisspeptin antagonist modulate basal and LH-induced secretion of A4 and P4 by TC, basal and FSH-induced secretion of E2 and P4 by GC and basal and FSK- induced secretion of P4 by luteinized TC and GC. Despite an initial indication that basal A4 secretion was increased by kisspeptin treatment, this effect was not significant when results from six replicate experiments using independent batches of cells were combined. Overall, the results from this series of experiments were negative; they showed that, in response to kiss-1 and its antagonist, there was no significant change in the production of A4, P4 and E2 by the cells in the presence/ absence of LH (TC), FSH (GC) and FSK (luteinized TC/GC). Furthermore, there was no effect of kisspeptin-10 and its antagonist on viable cell number at the end of culture. Thus, although kisspeptin-10 did not alter the secretion of sex steroid hormones or cell number, it may possibly have other intra-ovarian roles. However, exogenously administered kisspeptin-10 exerts a profound stimulatory effect on pituitary gonadotropin secretion in several species and this, in turn, would lead to stimulation of gonadal function *in vivo* (Thompson et al., 2004; Dhillo et al., 2005; Dungan et al., 2006). In summary, the results show that kiss-1 and its receptor are expressed in different bovine endocrine tissues including pituitary, adrenal, testis, ovarian corpus luteum, theca cells and granulosa cells. Moreover, changing levels of expression were detected during different stages of follicle development. However, the cell culture experiments offered no evidence to support the hypothesis that kisspeptin has a direct intra-ovarian role to modulate follicular or luteal steroidogenesis.

5. Does neuromedin B exert a local modulatory effect on ovarian steroidogenesis?

5.1. Introduction

Although the involvement of GnRH in the reproductive axis is fully understood, the neuronal system operating upstream of GnRH neurons to regulate the hypothalamic-pituitary-gonadal axis via GnRH are still uncertain. The stimulation of GnRH release has been shown to be modulated by numerous peptides in *in vivo* and *in vitro* experimental models; however, their precise physiological roles and relative importance is difficult to establish (Boughton et al., 2013). One of these neuropeptides is neuromedin B (NMB), a highly conserved decapeptide isolated from porcine spinal cord in 1983 by Minamino et al (1983), which is a member of the bombesin-related peptide family in mammals, and shown to have various physiological effects, both in the central nervous system (CNS) and periphery, including the regulation of exocrine and endocrine secretions (Ohki-Hamazaki, 2000). With regard to a potential role of NMB in ovarian function, a theca cell microarray study carried out in this laboratory (Glister et al., 2013), showed that NMB is expressed in these cells and was amongst the most highly down-regulated transcripts in bovine TCs in response to BMP6 treatment (~10-fold suppression). This unexpected observation prompted the current study reported in this chapter.

NMB peptide consists of His-Phe-Met residues at its C-terminal and is categorized as a candidate of the ranatensin family (Minamino et al., 1983). A number of molecular studies have succeeded in defining a high affinity receptor for NMB. The NMB receptor (NMBR) is a member of a G-protein coupled receptor with seven membrane-spanning regions. The NMBR has a well-conserved amino acid sequence within various species (Ohki-Hamazaki et al., 2005). The binding between NMB and its cell surface receptor (NMBR) leads to activate several intracellular signaling pathways including phospholipase activation, calcium mobilization and protein kinase C (PKC) activation; these are responsible for altering the expression of multiple genes, DNA synthesis or cellular effects such as secretion. The distribution of NMB as well as its receptor is overlapping in various brain areas and digestive tissues (Ohki-Hamazaki, 2000). Genes encoding *NMB* and *NMBR* and their roles especially in reproduction are currently unclear (Ma et al., 2016). In humans, the expression of *NMB* is particularly high in the hypothalamus, stomach and colon with low expression

levels reported in cerebellum, pancreas and the adrenal glands (Krane et al., 1988). CNS expression of *NMB* mRNA is particularly high in the medial preoptic area and the arcuate nucleus of the hypothalamus. Amongst peripheral tissues testis and the gastrointestinal smooth muscle cells (Ohki-Hamazaki et al., 1997; Boughton et al., 2013) also shown high expression levels.

The effect of NMB reported in peripheral tissues and organs includes an involvement in regulation of smooth muscle contraction (Von Schrenck et al., 1989; Jensen et al., 2008). Reported roles of NMB in the CNS include modulation of satiety (Ladenheim et al., 1994), reproduction (Boughton et al., 2013) and thermoregulation (Ohki-Hamazaki et al., 1999) along with stress, fear and other behavioral responses (Merali et al., 2006; Bédard et al., 2007; Jensen et al., 2008; Guo et al., 2015). Additionally, NMB appears to have a significant role in immune cells including lymphocytes and leukocytes (Ruff et al., 1985; Narayan et al., 1990) as well as promoting the growth and proliferation of different types of tumour cell including colon cancer (Narayan et al., 1990), lung carcinoma (Viallet and Minna, 1989) and prostate cancer (Bologna et al., 1989; Gajjar and Patel, 2017).

In this chapter, the aims of the study were firstly to investigate whether *NMB* and its receptor are expressed in a range of bovine endocrine tissues including different ovarian compartments. Secondly, to examine whether NMB can influence the steroidogenic pathway in cultured ovarian cells. Thirdly, to investigate the effect of an NMB antagonist (alone and in combination with NMB) on ovarian steroidogenesis *in vitro*. Fourthly, to investigate whether expression of *NMB* by cultured ovarian cells is regulated by gonadotropins and other factors. The laboratory techniques that have been used to address the above include a primary ovarian cell culture system (bovine ovarian theca and granulosa cells under non-luteinized and luteinized conditions), steroid immunoassay (androstenedione, oestradiol and progesterone), immunohistochemistry and real-time PCR.

5.2. Materials and methods

5.2.1. Cell culture

Ovaries from randomly cycling cattle were collected from a local abattoir then granulosa and theca cells were isolated and cultured according to section 3.1.4. All treatments were applied as stated in next section 5.2.2. The spent culture medium was collected and retained for hormonal assay and replaced with fresh medium with the appropriate treatments every 48 hours. Viable cell number at the end of the culture was determined by neutral red assay in section 3.1.9.

5.2.2. Preparation and administration of treatments

NMB and NMB (antagonist) were dissolved in water and 0.1% (w/v) acetic acid respectively to give a stock concentration of 10^{-3}M. Then stocks were diluted in sterile medium to give desired concentrations of the working solutions. A set of 5 doses was prepared and a 25 µl applied to each well to give a final concentration of 10^{-6}, 10^{-7},10^{-8}, 10^{-9} and 10^{-10}M respectively.

LH and FSH were prepared from frozen stock solutions (100µg/ml) that was pre-aliquoted into cryovials and stored under liquid nitrogen. Then LH and FSH stock solutions were diluted in complete medium supplemented with 0.3% (w/v) of BSA to give a 'top'concentration of 500ng/ml.

FSK and DMSO were prepared from stocks of 10mM and dissolved in complete medium supplemented with 0.3% (w/v) of BSA to give desired concentrations. Prior to the experiments optimum concentration was obtained by test 8 serial concentrations of LH and FSH on A4 and E2 secretions. Also 3 concentrations of FSK and DMSO were tested to obtain the optimum concentration. 100pg/ml of LH, 0.33 ng/ml of FSH and 10µM of FSK and DMSO were considered as an optimum concentrations to give a maximum stimulation of A4 (TC), E2 (GC) and P4 (LTC, LGC) secretions by the respective cell-types cultured. The initial stock solution were diluted in sterile medium and filtered with a 0.2µm membrane filter. A 25µl of each treatment were applied to wells to give final concentrations of LH, FSH and FSK of 100pg/ml, 0.33ng/ml and 10µM respectively.

5.2.3. Gene expression analysis

5.2.3.1. Sample preparation, total mRNA extraction, purification, cDNA synthesis and RT-PCR

Samples were collected and processed for according to section 3.4.1. Total RNA extracts were quantified in section 3.4.2 and tested for integrity in section 3.4.3. First-strand cDNA was synthesized in section 3.4.4 following by RT-PCR analysis as described in section 3.4.5 using specific primer pairs as show in table 3.2.

5.2.3.2. The expression of NMB gene and NMBR in different bovine endocrine and ovarian tissues

Bovine adrenal, pituitary, testis and ovarian GC, TC and CL tissues were processed for RNA isolation using Tri-reagent and cDNA synthesis using the AB high capacity cDNA synthesis kit as described in section 3.4.1.1, 3.4.1.2 and 3.4.4. The cDNA samples from GC, TC and CL at different stages of follicle and luteal development were kindly provided by my laboratory colleague Dr Warakorn Cheewasopit and Dr Moafaq Samir. cDNA samples were used for Quantitative RT-PCR for the detection of the expression of *NMB* gene and *NMBR* using their designed primers; β-actin was used for normalization of gene expression as described in section 3.4.5. Melt curve analysis and agarose gel electrophoresis were used to verify that each selected primer pair gave a single amplicon of the predicted size and Tm. However, it was not possible to generate satisfactory template dilution curves for the NMBR primers as the C_t values obtained for pooled cDNA sample were >30; this indicated either that *NMBR* expression levels are very low, or that neither of the designed primer sets were adequate. cDNA samples were diluted either 1:10 or 1:50 and 5µl of these diluted cDNA samples were used for qPCR. Volume of 2µl of forward and reverse primers, and 7µl of QuantiTect SYBR Green 2X "hot start" Master Mix (Qiagen) were added. Samples were run for 40 cycles on an AB StepOne plus real-time PCR instrument (Applied Biosystems). The method that was used to compare the difference between each mRNA transcript was the ΔC_t method using β-actin as the house keeping control. Resultant ΔC_t values for individual replicates within each tissue group were then normalised to the average ΔC_t value of these different

tissues to give $\Delta\Delta C_t$ values. Finally, $\Delta\Delta C_t$ values were converted to fold difference for graphical presentation using the formula $2^{(-\Delta\Delta^{Ct})}$.

5.2.3.3. The expression of *NMB* gene in ovarian cells treated with LH, FSH, FSK and other factors

The cDNA samples from cultured ovarian cells analysed for *NMB* expression were kindly provided by my laboratory colleagues Dr Moafaq Samir and Dr Claire Glister. Cells which had been treated with LH, FSH and FSK for 7 days were used for RNA extraction procedure using Qiagen RNeasy mini-column kits followed by cDNA synthesis using the AB high capacity cDNA synthesis kit; qPCR was used for the detection of the expression of *NMB* using as described in section 3.4.1.2, 3.4.4 and 3.4.5.

5.2.4. Hormone immunoassays

A4, E2 and P4 concentrations in retained spent media were determined by ELISA as described in section 3.3.

5.2.4.1. The effect of NMB, NMB antagonist, LH and FSK on TCs (A4 and P4 secretion)

Bovine ovarian TCs were cultured in 96 well plates as described in section 3.1.4.2. After 48 hours incubation period, 175µl of cultured medium were removed and replaced with control and treatment (containing medium), and then plates were placed in the incubator. Media were applied every 48 hours for 6 days. At two time points (96h, 144h) medium were collected for hormonal assay by ELISA.

Depending on the type of cells (non-luteinized, TC or serum-luteinized, LTC), 25µl of LH and FSK were added to wells to give final concentration of 100pg/ml (TC) and 10µM/ml (LTC) respectively. 25µl of NMB and its antagonist were applied to wells to give final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M. The experimental design evaluated the effect of the presence/absence of LH, NMB and NMB antagonist. The A4 and P4 secretion data and viable cell number data were analysed by two-way analysis of variance (ANOVA) using the statistical program StatView v.5.0.1.

5.2.4.2. The effect NMB, NMB antagonist, FSH and FSK on GCs (E2 and P4 secretion)

Bovine ovarian GCs were cultured in 96 well plates as described in section 3.1.4.1. After 48 hours incubation period, 175µl of cultured medium were removed and replaced with control and treatment (containing medium), and then plates were placed in the incubator. Media were applied every 48 hours for 6 days. At two time points (96h, 144h) medium were collected for hormonal assay by ELISA.

Depending on the type of cells (non-luteinized, GC or serum-luteinized, LGC), 25µl of FSH and FSK were added to wells to give final concentration of 0.33 ng/ml (GC) and 10µM/ml (LGC) respectively. 25µl of NMB and its antagonist were applied to wells to give final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M. The experimental design evaluated the effect of the presence/absence of FSH, NMB and NMB antagonist. The E2 and P4 secretion data and viable cell number data were analysed by two-way analysis of variance (ANOVA) using the statistical program StatView v.5.0.1.

5.2.5. ApoTox-GloTM Triple assay

Bovine ovarian nonluteinized GCs were cultured in 96 well plates as described in section 3.1.4.1. Cells were treated with/without FSH (0.33ng/ml) and NMB ($10^{-10}$and 10^{-9}M). At the end of the culture period 150µl of the culture media was removed to leave a final volume of 100µl/well. The Promega ApoTox-Glo assay was then applied to the cells described in section 3.1.10. The data were analysed by two-way analysis of variance (ANOVA) using the statistical program StatView v.5.0.1.

5.2.6. Immunohistochemistry

The experiment was carried out as described in section 3.6. Formalin-fixed, wax-embedded bovine ovary sections were kindly prepared and provided by Dr Mhairi Laird in this laboratory.

5.3. Statistical analysis

The effects of the various treatments on hormone secretion and gene expression were evaluated by two-way analysis of variance (2-way ANOVA). Individual pairwise comparisons within different treatments range were subsequently made by Fisher's PLSD. In order to reduce heterogeneity of variance, some dates were log-transformed prior to statistical analysis. Unless otherwise stated, results are presented mean ±SEM of ≥ 3 independent batches of cultured cells.

5.4. Results

5.4.1. The expression of *NMB* and it receptor in different bovine endocrine tissues

The relative expression of *NMB* mRNA in different bovine endocrine tissues including pituitary, testis, TC, GC and CL varied significantly ($P<0.0001$), being highest in testis and lowest in pituitary (Figure 5.1). NMBR expression was only examined in TC and GC as described below.

Analysis of follicular GC and TC layers from different size follicles revealed that expression of *NMB* showed significant variation by both follicle category and cell type. *NMB* expression in TC tended to increase with follicles size while the opposite trend was observed with GC (Figure 5.2a). Expression of *NMBR* was also significantly affected by follicle size class, being highest in both TC and GC of the smallest follicle class and lowest in large regressing follicles with E;P ratio <1 (Figure 5.2b). In addition, the expression of *NMB* was significantly different between TC and GC while expression of *NMBR* was not.

As shown in Figure 5.3, the expression of *NMB* in CL tissue was significantly higher in the mid-luteal stage compared with regression stage. However, the difference between early (growing) and mid-luteal stages was not significant.

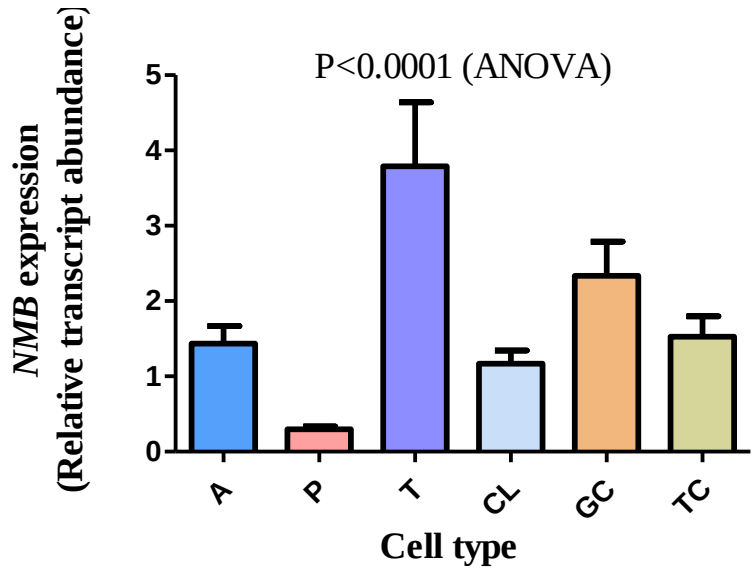


Figure 5. 1 The relative abundance of mRNA transcripts *NMB* in different bovine endocrine tissues including adrenal gland (A, n=6), corpus luteum (CL, n=17), granulosa cell (GC, n=39), pituitary gland (P, n=6), testis (T, n=6) and theca cell (TC, n=44). Values are means ±SEM and one-way ANOVA results are shown.

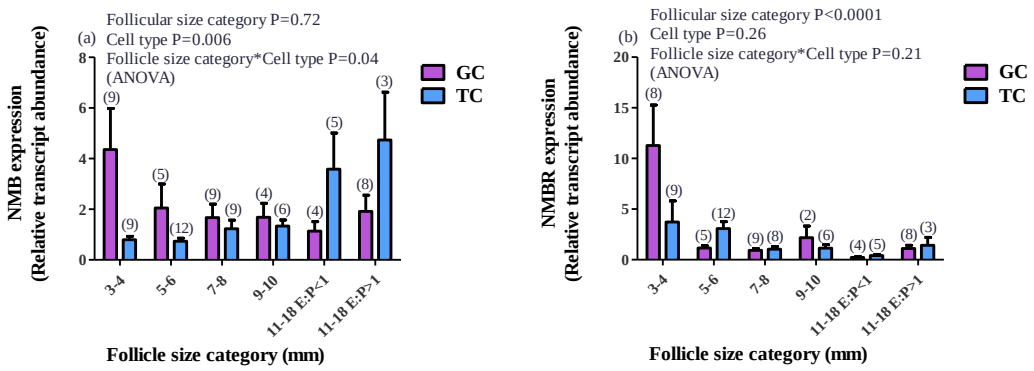


Figure 5. 2 A comparison of the relative abundance of mRNA transcript for (a) *NMB* and (b) *NMBR* in GC and TC from ovarian follicles. Values are means ±SEM and summarized two-way ANOVA results are shown. *Numbers in parenthesis above bars are n-values.

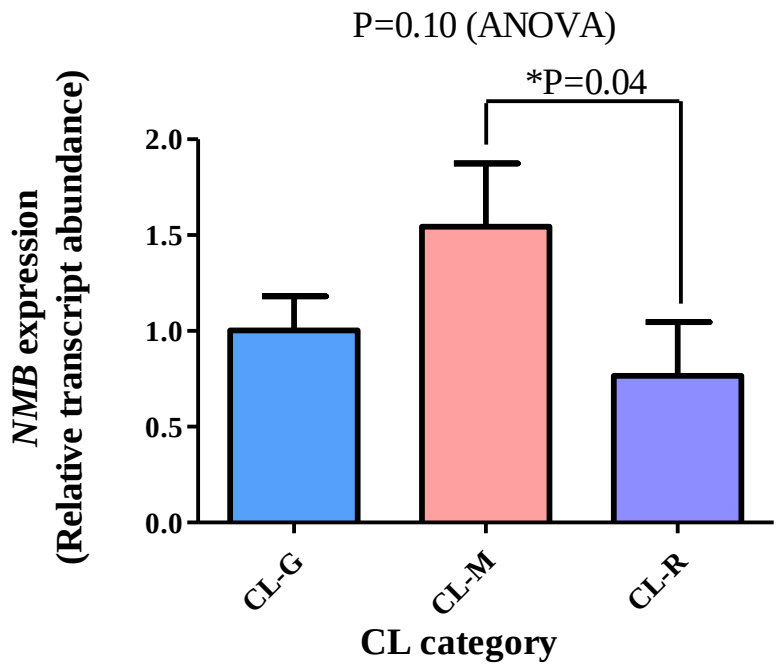


Figure 5. 3 The expression of NMB in CL tissue at growing (G, n=6), mid-luteal (M, n=7) and regressing (R, n=4) stages. Values are means ±SEM and results of one-way ANOVA and pairwise comparisons are shown.

5.4.2. The expression of *NMB* mRNA in cells treated with LH, FSH, FSK and other factors

The expression of *NMB* in cultured cells treated with/without LH and different BMPs (2ng/ml) showed a marked suppressive effect of BMPs ($p<0.001$) with BMP6 evidently the most potent. LH tended to increase *NMB* expression but the effect was not significant (Figure 5.4a). However, expression of *NMB* in LTC treated with FSK was ~3-fold higher compared to control ($P<0.0001$) (Figure 5.4c).

On the other hand, the expression of *NMB* in cultured GC treated with TNFα (10ng/ml) was significantly inhibited under both basal and FSH-induced conditions; FSH tended to increase *NMB* expression but the effect was not significant (Figure 5.4b). Likewise expression of *NMB* by LGC was not affected by FSK (Figure 5.4d).

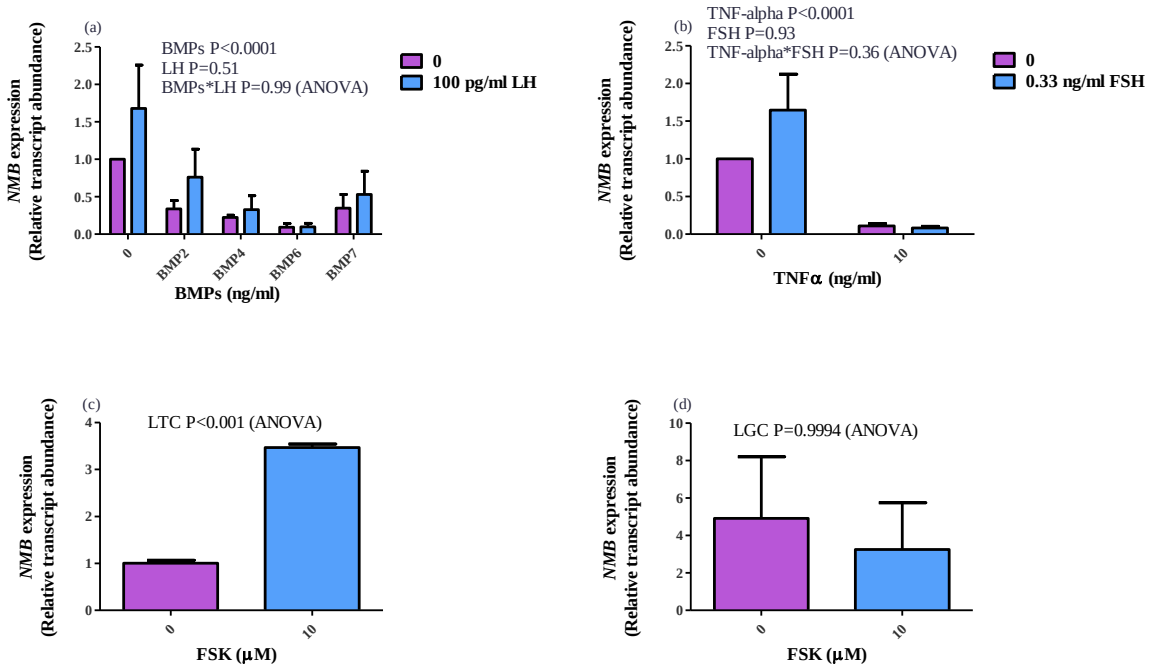


Figure 5. 4 Comparison of the relative abundance of mRNA for *NMB* in (a); non-luteinized TC cultured in the presence/ absence of LH and different BMPs ligand at concentrations of 100pg/ml and 2ng/ml respectively, (b); non-luteinized GC cultured in the presence/absence of FSH and TNF α at concentrations of 0.33ng/ml and 10ng/ml respectively, (c); luteinized TC and (d) luteinized GC cultured in the presence/absence of 10μM of FSK. Values are means ±SEM (n=3-7 in dependant batches of cell) and two-way ANOVA results are shown.

5.4.3. The effect of NMB and its antagonist on basal and LH-induced A4 and P4 secretion by non-luteinized bovine TC

As shown in Figures 5.4 and 5.6 basal and LH-stimulated productions of A4 and P4 by non-luteinized TC were not affected by NMB or its antagonist. Likewise, there was no effect on viable cell number at the end of the culture period. As expected, TC responded to LH with increased steroid secretion (P<0.0001).

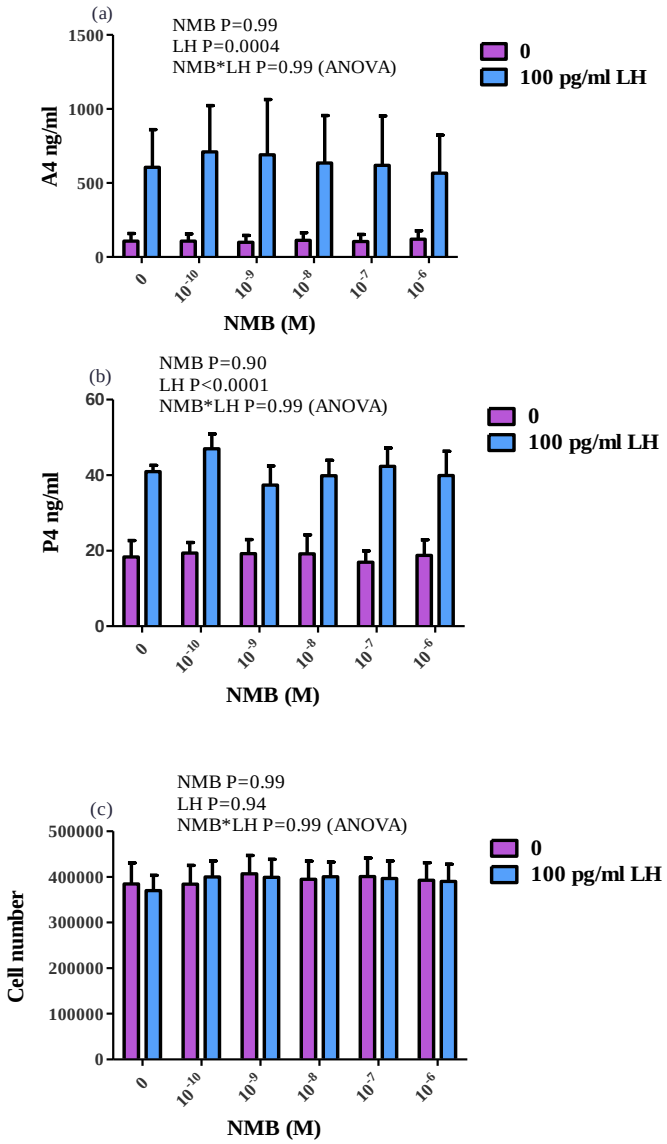


Figure 5. 5 The effects of LH and NMB, alone and in combination, on the production of (a) A4 and (b) P4 by non-luteinized bovine TC; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

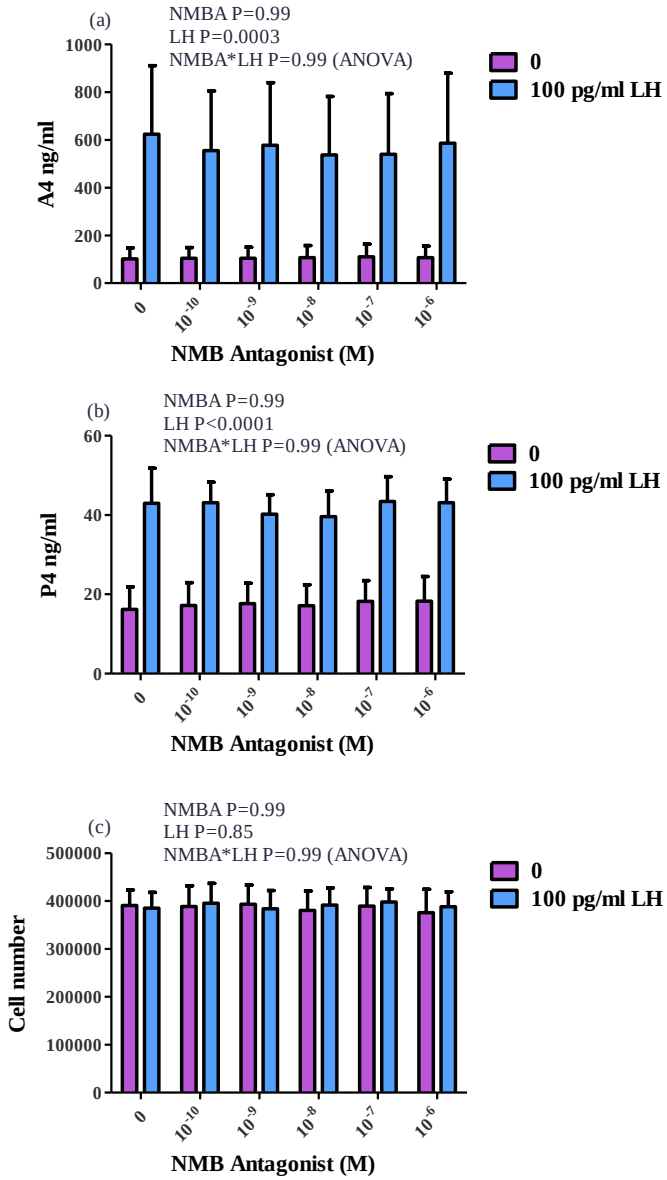


Figure 5. 6 The effects of LH and NMB antagonist, alone and in combination, on the production of (a) A4 and (b) P4 by non-luteinized bovine TC; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown. *NMBA referred to NMB antagonist.

5.4.4. The effect of NMB and its antagonist on basal and FSK-induced secretion of P4 by luteinized TC

As shown in Figure 5.7 NMB and its antagonist did not modify basal or FSK-induced secretion of P4 by luteinized TC, or change viable cell number. However, FSK greatly increased P4 secretion and also reduced cell number (P<0.0001).

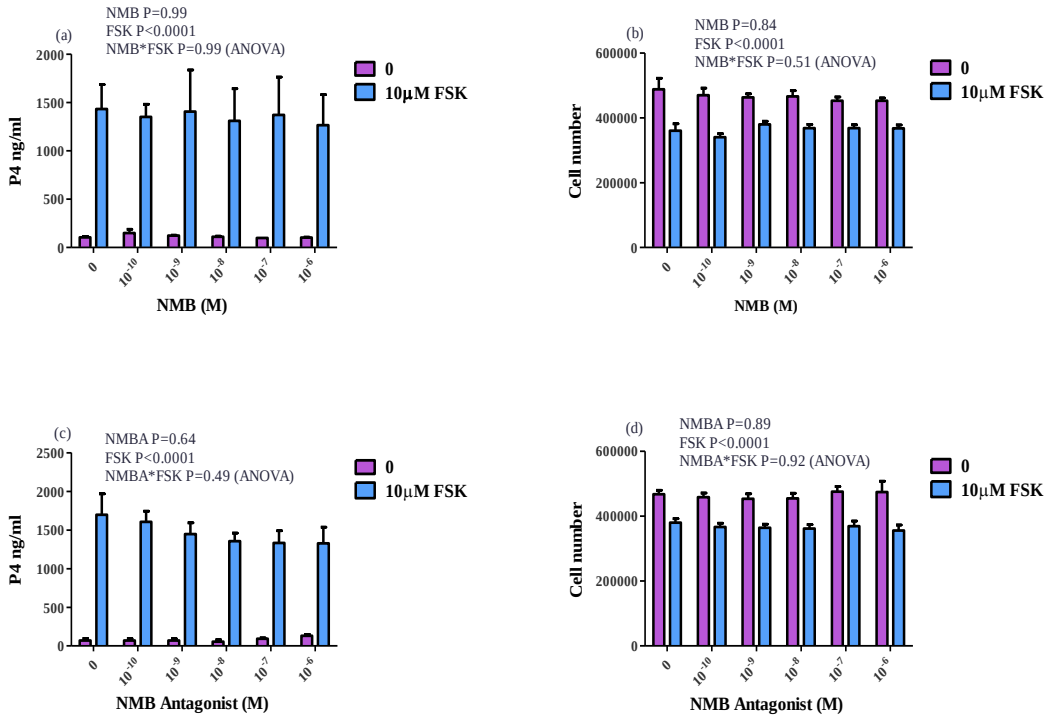


Figure 5. 7 The effects of NMB and its antagonist, alone and in combination, on basal and FSK-stimulated production of P4 by luteinized bovine TC (a and c) and on viable cell number at the end of the culture (b and d). Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

5.4.5. The effect of NMB and its antagonist on basal and FSH-induced E2 and P4 secretion by non-luteinized bovine GC

As shown in Figure 5.8 and 5.9 basal and FSH-stimulated productions of E2 and P4 by non-luteinized GC was not affected by NMB or its antagonist. NMB tended to increase viable cell number at the end of the culture period but the effect was not significant ($p=0.06$). As expected, FSH greatly increased E2 secretion (>20-fold increase) with little effect on P4 secretion.

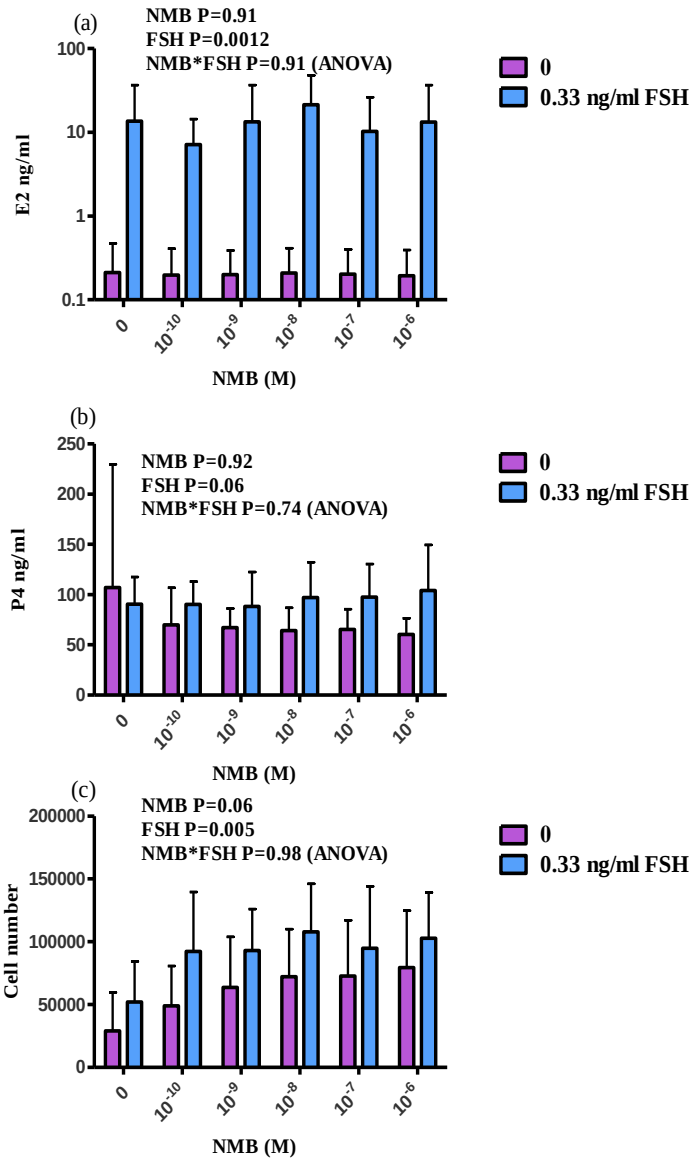


Figure 5. 8 The effects of FSH and NMB, alone and in combination, on the production of (a) E2 and (b) P4 by non-luteinized bovine GC; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown.

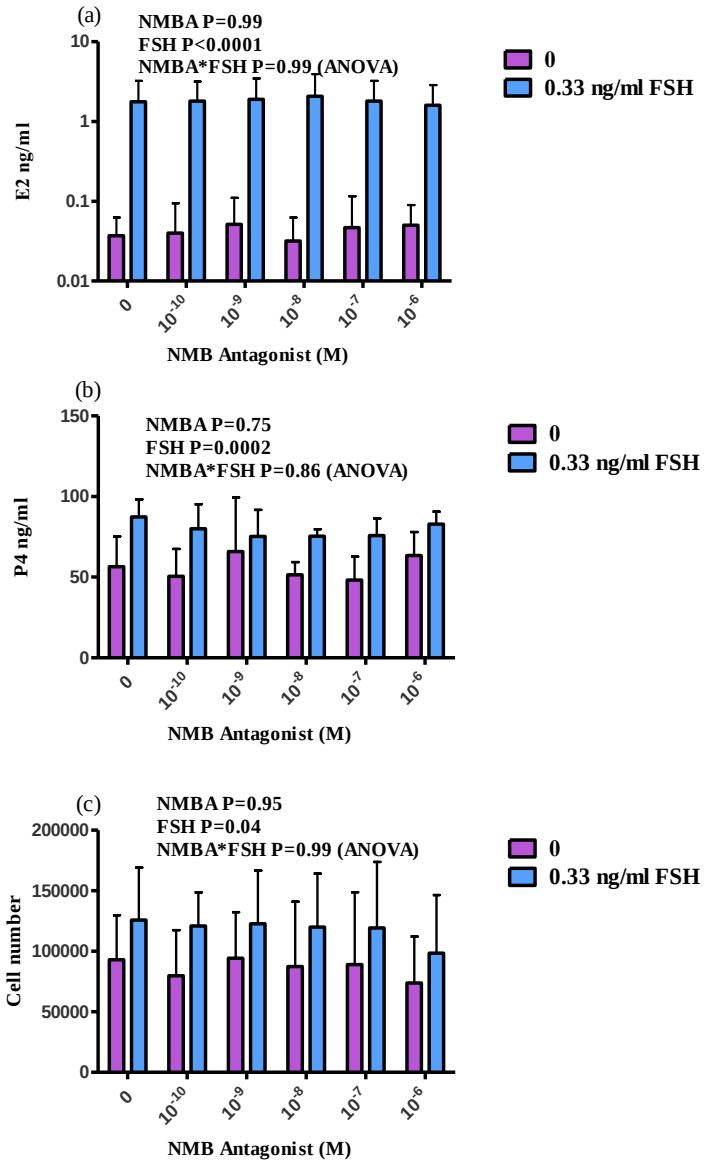


Figure 5. 9 The effects of FSH and NMB antagonist, alone and in combination, on the production of (a) E2 and (b) P4 by non-luteinized bovine GC; panel (c) shows the viable cell number at the end of the culture. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

5.4.6. The effect of NMB and its antagonist, on basal and FSK-stimulated P4 secretion by luteinized bovine GC

As shown in Figure 5.10 NMB and its antagonist did not modify basal or FSK-induced secretion of P4 by luteinized GC or change viable cell number at the end of the culture period. However, FSK significantly increased P4 secretion while reducing viable cell number.

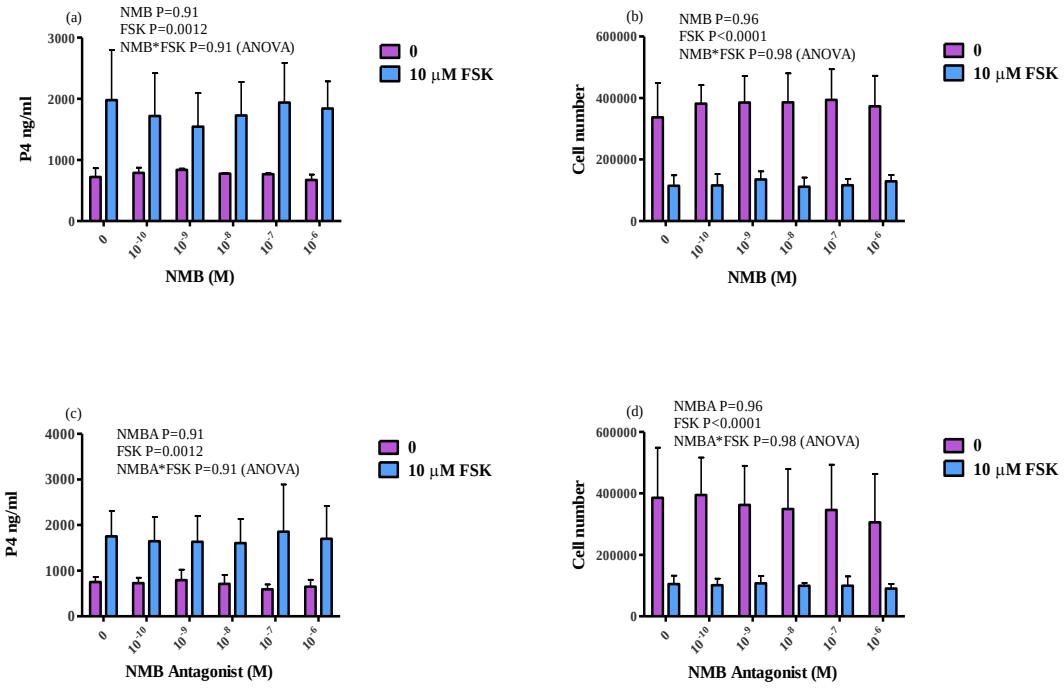


Figure 5. 10 The effects of NMB and its antagonist, alone and in combination, on basal and FSK-stimulated production of P4 by luteinized bovine GC (a and c) and on viable cell number at the end of the culture (b and d). Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

5.4.7. The effect of NMB and FSH on cell viability, cytotoxicity and apoptosis by non-luteinized GC

As shown in Figure 5.11, there were no significant effects of either NMB or FSH on GC viability, cytotoxicity and apoptosis as indicated by the Promega ApoTox Glo assay.

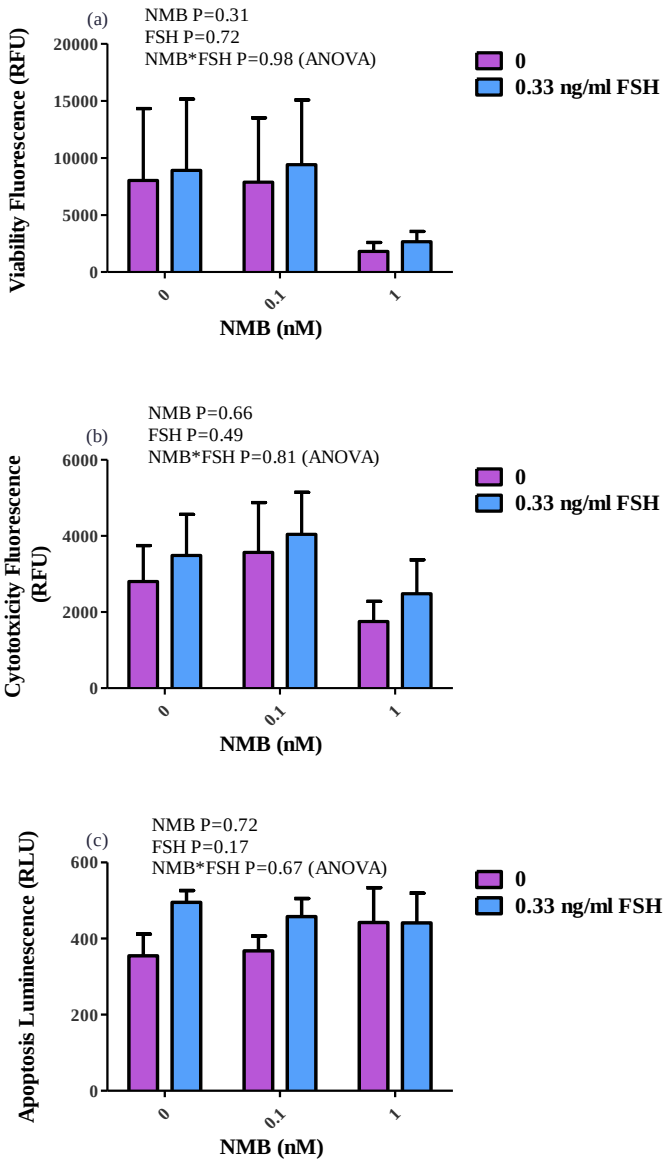


Figure 5. 11 The effect of NMB and FSH on cell (a) viability, (b) cytotoxicity and (c) apoptosis by non-luteinized GC. Values are means ±SEM (n=5-6 independent batches of cells) and two-way ANOVA results are shown.

5.4.8. The presence of NMBR in bovine ovary sections

As shown in Figure 5.12, the expression of NMBR was found in different follicle stages including primordial, primary, secondary and antral. Also, NMBR was found in both GC and TC at all stages of examined follicles, however, the expression appeared to be higher in granulosa than theca layer.

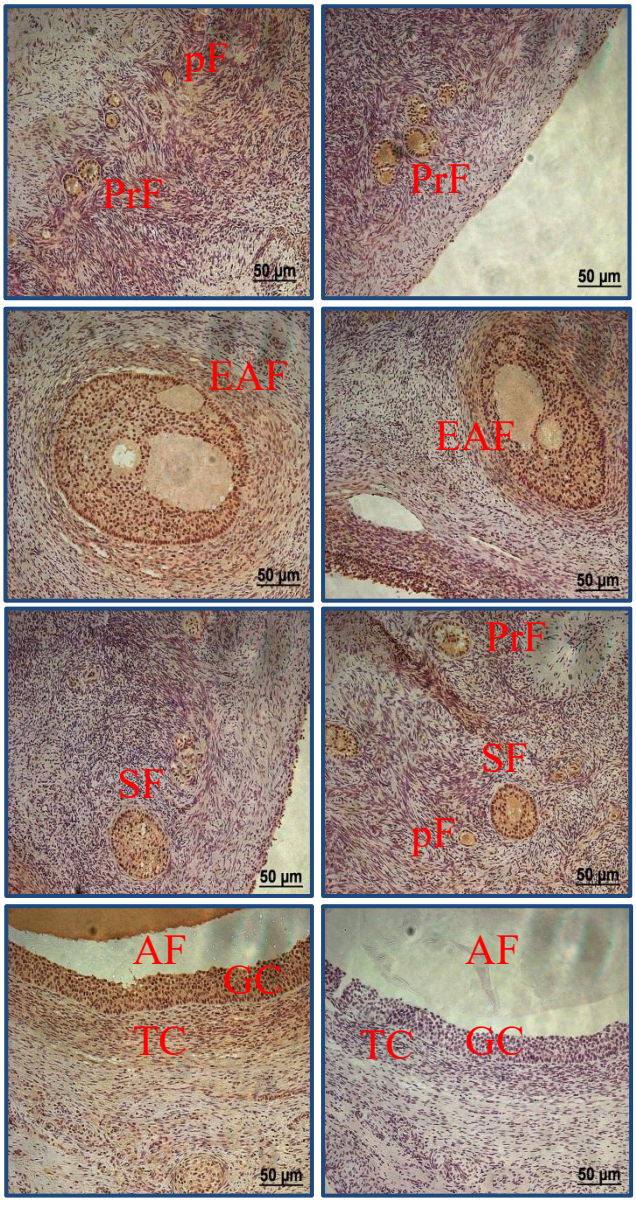


Figure 5. 12 Immunohistochemical staining of bovine ovary sections (20x objective lens) showing NMBR immunereactivity (brown) in primordial (pF), primary (PrF), secondary (SF), early antral (EAF), antral follicles (AF) and in thecal (TC) and (GC). No staining was observed in control section treated with non-immune rabbit IgG normal (bottom right).

5.5. Discussion

Neuromedin peptides are categorized into 4 classes including (1) Bombesin family that involving Neuromedin B and Neuromedin C, (2), Kanassin family that involving Neuromedin K and Neuromedin L, (3) Neurotensin family that involving Neuromedin N and (4) Neuromedin U and Neuromedin S. These various neuromedins have several physiological functions including constrictive roles on smooth muscles, control of blood pressure, pain sensations, hunger/satiety, bone metastasis and release and regulation of hormones. The identification of various physiological functions for neuromedins suggests that pharmalogical agonists and antagonists may have potential for development as novel therapeutic treatments for various conditions (Gajjar and Patel, 2017). The NMB ligand/receptor system is implicated in controlling several physiological activities in both human and animals (Ma et al., 2016). Yet, information on the anatomical distributions and physiological roles of NMB/NMBR system is still limited (Boughton et al 2013; Ma et al., 2016) and to my knowledge there are no reports regarding its potential regulatory role in the bovine ovary.

The experiments reported in this chapter were prompted by the findings of a global microarray study carried out in this laboratory showing that *NMB* expression by cultured bovine TC was markedly reduced by BMP-6 treatment (Glister et al 2013). Here, we first studied the expression levels of *NMB* and *NMBR* mRNA among different bovine endocrine tissues with particular emphasis on the ovary and its principle cellular compartments (GC, TC, and CL). Expression of mRNA for *NMB* and its receptor were detected by RT-qPCR in all endocrine tissues examined and found to vary between different tissues. In the ovary, *NMB* and *NMBR* expression was found to vary in a cell-type and follicle stage-dependent manner supporting the possibility of a functional involvement in aspects of follicle function such as cell proliferation/survival and steroidogenesis. This study also confirmed and extended the findings from the above-mentioned TC microarray study (Glister et al., 2013) with mRNA expression of both *NMB* and *NMBR* identified in GC and CL. As mentioned in section 5.2.3.2. no satisfactory template dilution curves for the NMBR primers were generated using pooled bovine ovarian cDNA. The ideal positive control for evaluating the efficiency and specificity of the NMBR primers would be bovine brain tissue.

However, it was not possible to obtain bovine brain tissue from the slaughterhouse because of the UK Specified Bovine Offals Order (1995) that was introduced in response to the Bovine Spongiform Encephalopathy outbreak in the late 1980s. This bans the collection and supply of bovine brain and spinal chord tissue. Although two different sets of NMBR primers were designed and tested with limited success, it is possible that additional primer sets may have been more effective. Theoretically, an increased amount of cDNA could have been included in each qPCR reaction. In practice, however, the limited amount of cDNA available for these analyses excluded this option. Despite these doubts about the quality of the NMBR primers, and the failure to detect NMBR expression in the bovine endocrine tissue sample set, qPCR assays did yield low but measurable detection levels in the GC and TC samples from developing follicles. To provide more evidence that NMBR is indeed expressed in bovine ovary, an NMBR antibody was sourced and used for immunohistochemical staining of bovine ovary sections. Positive NMBR immunistaining was evident, supporting the tentative mRNA expression data for follicular GC and TC samples analysed by qPCR.

These initial results led us to carry out further investigations on the potential multifunctional role of the NMB/NMBR system in the ovary. These studies utilised several in vitro culture models in which TC and GC harvested from fresh bovine ovaries are maintained under defined serum-free conditions to preserve a non-luteinized phenotype (Glister et al., 2001; Glister et al., 2004). In addition, the same cell-types were cultured under serum-supplemented conditions to promote spontaneous luteinisation (Kayani et al., 2009) characterised by massively increased P4 output and diminished E2 and A4 output by GC and TC respectively. Disappointedly, neither NMB nor its antagonist modulated follicular or luteal steroidogenesis or cell proliferation/survival in the series of experiments that used four different bovine primary cell culture models (non-luteinized and serum-luteinized GC and TC). To my knowledge, there are no comparable studies in other species exploring direct intra-ovarian role(s) of the NMB/NMBR system to modulate follicular or luteal steroidogenesis. However, it is clear from the study of Boughton et al (2013) that NMB has a neuroendocrine role to control hypothalamic GnRH secretion Nevertheless, the present findings lead us to reject

the hypothesis that NMB and its antagonist modulate follicular or luteal steroidogenesis or cell proliferation/survival in vitro.

In the reproductive axis of female pigs, the expression pattern of *NMB*/*NMBR* mRNA in the hypothalamus and pituitary was measured across the oestrous cycle (Ma et al., 2016). Regarding the hypothalamus, the expression patterns of *NMB*/*NMBR* mRNA were high during pro-oestrus; however, the expression levels were not significantly different compared to estrus. Result of study in rats suggested a role of NMB to regulate the release of GnRH via regulating the secretion of NMBR (Boughton et al., 2013). NMB was found to stimulate GnRH release from hypothalamic explants and from hypothalamic GT1-7 cells. In regards to the pituitary, a low level of *NMB* mRNA expression was found during estrus, whereas, a high level of *NMBR* mRNA expression was also detected during the same stage. Thus, the suggested effect of NMB on the rat H-P-G axis may be to stimulate the GnRH secretion rather than exert a direct effect on the pituitary to modulate gonadotrophin secretion (Boughton et al., 2013). According to Ma et al., (2016) expression of *NMB* in porcine ovary peaked during pro-oestrus, whereas peak *NMBR* expression occurred during oestrus. This led to the suggestion that NMB may regulate ovaries through binding to its own receptor. Thus, the previously mentioned data provides some albeit incomplete, evidence suggesting roles for NMB in the regulation of the reproductive axis. Additional experimental work is needed to further elucidate the potential physiological functions performed by the NMB/NMBR system in gonadal regulation.

Experiments involving an NMBR antagonist revealed an inhibitory effect on cell proliferation in different type of cells (Moody et al, 2000; Moody et al., 2010). Our results suggested that NMB may up regulate the cell proliferation and/survival of non-luteinized GC although the effect was not significant ($p=0.06$). To follow up this tentative finding, a more detailed evaluation of the effect of NMB treatment on GC proliferation, viability and apoptosis was undertaken using the Promega ApoTox Glo assay kit. However, no significant effect of NMB on any of these parameters was identified and so the above tentative finding remains unsubstantiated.

In conclusion, we found *NMB* mRNA expression in the bovine adrenal gland,

pituitary gland, testis and ovarian GC, TC and CL. *NMBR* expression was tentatively identified in GC and TC although it was not possible to confirm the efficiency of the primers used. Despite this, immunohistochemistry supported the presence of NMBR protein expression in these ovarian tissues. The presence of *NMB*/*NMBR* expression in ovarian cell-types suggests that NMB may directly influence ovarian follicle function. However, an extensive series of experiments on non-luteinized and serum-luteinized GC and TC in primary culture found no evidence that NMB or its antagonist modulate follicular or luteal steroidogenesis or cell proliferation/survival *in vitro*. Therefore, further work is needed to examine whether NMB can directly affect ovarian function in cattle. High expression levels of *NMB* mRNA were also detected in the testis which may imply that NMB functions through autocrine and paracrine in the male gonad. Collectively, the results presented here are inconclusive but provide a basis for future studies on the functions of NMB in gonadal regulation.

6. Role of kisspeptin, neuromedin B and other peptides in regulating follicular angiogenesis

6.1. Introduction

Formation of the circulatory system (vascular system) involves two distinct mechanisms known as vasculogenesis and angiogenesis (Robinson, 2013). The ovary is a very dynamic organ with considerable tissue turnover and remodelling. A coordinated interaction of various autocrine, paracrine and endocrine regulators is required during ovarian follicular development, ovulation and CL formation to control physiologic process such as angiogenesis that underpin tissue turnover and remodelling (Osz et al., 2014). The formation of new blood vessels (angiogenesis) is a process involving the migration and proliferation of endothelial cells from pre-existing ones; this involves a complex series of cellular processes and molecular changes. In adults, angiogenesis is largely limited to pathological conditions such as tumour growth and wound healing. However, in the reproductive tract and especially the ovary it is well established that continual angiogenesis is of great importance for ovarian development and function (Robinson et al., 2009). The characteristics of ruminants ovarian cycles include the recurrent patterns of specific cellular proliferation, differentiation and transformation that leads to folliculogenesis, ovulation and formation and function of the CL (Berisha et al., 2016). An established vasculature consists of an inner lining of endothelial cells linked to mural cells such as pericytes and vascular smooth muscle cells, which are ideally located to take an active part in the angiogenic process. These vessels continue to be inactive until an angiogenic stimulus occurs, involving local upregulation of proangiogenic factors including vascular endothelial growth factor A (VEGF-A) and fibroblast growth factor 2 (FGF-2). In response to the stimulus, the existing vessels turn to dislocate throughout the endothelial and mural cellular contacts. During this mechanism, several proteases and cytokines are stimulated and the extracellular matrix is disrupted (Plendl, 2000). At this point, the endothelial cells migrate and directionally in response to the angiogenic stimuli, and proliferate under the effect of proangiogenic influences. Once connected and aligned, the endothelial cells form a tube with a lumen and the newly formed vessel is then stabilised by the recruitment of pericytes. Consequently, angiogenesis is a highly regulated mechanism including a balance between a plethora of pro- and anti-angiogenic factors (Robinson et al., 2009; Gerhardt and Betsholtz, 2003). Studies in different

mammalian species suggests that, during the bovine ovarian cycle, many vascular changes in the follicle facilitate the development of the follicle's delivery of the nutrient and hormonal transportation to maintain oocyte development process (Fraser and Lunn, 2000; Ferrara et al., 2003). Coordinated expression of both extraovarian factors and intrafollicular regulatory factors produced by theca and granulosa cells in an autocrine and paracrine manner are involved in ovarian angiogenesis (Greenaway et al., 2005).

As mentioned previously, VEGF is considered to be a potent regulator for the proliferation and migration of vascular endothelial cell (Ferrara, 2004). VEGF is found in the bovine CL at high levels and it has a stimulatory role throughout the process of luteal angiogenesis *in vitro* (Robinson et al., 2007; Robinson et al., 2008). According to several studies, the inhibition of VEGF during the pre-ovulatory stage leads to suppression of *in vivo* luteal function and vascularity in different species including the cow (Fraser et al., 2000; Wulff et al., 2001; Yamashita et al., 2008). The other potent regulator is FGF2, which has a series of pro-angiogenic activities (Presta et al., 2005). FGF has a role in the simulation of endothelial cells proliferation in bovine corpora lutea and its expression is particularly high through the bovine follicular-luteal transition (Gospodarowicz et al., 1986; Robinson et al., 2007). Furthermore, the local neutralisation of FGF action by direct injection of FGF-2 antibody leads to an alteration of luteal growth and function of the developing bovine CL, as observed by the reductions of luteal volume and steroidogenesis (Yamashita et al., 2008; Woad et al., 2012).

Another important regulator of angiogenesis is transforming growth factor-β-1 (TGF-β), which is involves in several biological mechanisms such as embryonic development, cell proliferation and migration, extracellular matrix production and differentiation of a numerous cell types (Peshavariya et al., 2014). Bone morphogenetic proteins (BMPs) are also implicated in the regulation of angiogenesis. BMPs and TGF-β belong to a large family of structurally and multifunctional proteins termed the TGF-β superfamily (Massague, 2000). BMPs have been found to be expressed by endothelial cells and vascular smooth muscle cells (Schluesener and Meyermann, 1995; Glienke at al., 2000). It has been reported that BMPs can inhibit the proliferation of vascular smooth muscle cells

and increase the expression of some markers, including those for smooth muscle differentiation (Nakaoka et al., 1997; Willette et al., 1999; Valdimarsdottir et al., 2002). Furthermore, thrombospondin-1 (TSP-1), an extracellular matrix glycoprotein that plays a role in cellular phenotype and the structure of the extracellular matrix, is also implicated in tissue remodeling that is linked to angiogenesis and neoplasia (Lawler, 2002).

In this chapter, the aims of the study were firstly to investigate the potential role of NMB, kisspeptin-10, TGF-β-1, BMP6 and TSP-1 in modulating capillary network formation in follicular angiogenesis. Secondly, to examine the effect of antagonists of the previously mentioned peptides on follicular angiogenesis. These were tested alone and in combination with agonist. The rationale of testing the effect of antagonist alone was to unmask the effect of endogenous ligand secreted by the cultured cells. Thirdly, to investigate the effect of TGF-β-1 and BMP6 (alone and in combination with their antagonists) on ovarian steroidogenesis in *vitro*. Fourthly, to investigate whether expression of steroidogenic transcripts and other angiogenic factors by cultured ovarian cells is regulated by TGF-β-1 and BMP6. The laboratory techniques that have been used to address the above include a primary ovarian angiogenic cell culture system (bovine ovarian theca internal layer), steroid immunoassay (androstenedione and progesterone), immune-staining and real-time PCR.

6.2. Material and methods

6.2.1. Cell culture

Randomly cycling bovine ovaries were collected from a local abattoir and thecal interna layers were recovered and cells isolated and cultured according to section 3.1.4.2.

All treatments were applied as stated in next section 6.2.2. The spent culture medium was collected and retained for hormone assay and replaced with fresh medium with the appropriate treatments every 24 hours.

6.2.2. Preparation and administration of treatments

FGF and VEGF were dissolved in sterile PBS containing at least 0.1% bovine serum albumin and 4 mM HCl. Two concentrations of the angiogenic factors were prepared to achieve final concentrations of 1 or 10ng/ml respectively as used previously in a bovine luteal cell angiogenesis model (Robinson et al., 2008).

Kisspeptin-10 and kisspeptin 234 (antagonist) were dissolved in water and 20% (w/v) acetonitrile / water respectively to give a stock concentration of 10^{-3}M. Then stocks were diluted in sterile medium to give desired concentrations of the working solutions. A set of 5 doses was prepared and a 25 µl applied to each well to give a final concentration of 10^{-8}, 10^{-7} and 10^{-6}M respectively.

Neuromedin B was dissolved in water while BIM 23042 (NMB antagonist) was dissolved in 0.1% acetic acid to give a stock concentration of 10^{-3}M for both of them. Then stocks were diluted in sterile medium to give desired concentrations of the working solutions. A set of 3 doses of NMB was prepared and a 15µl applied to each well to give a final concentration of 10, 100 and 1000ng/ml respectively. Another concentration of NMB was prepared (being an optimal dose from preliminary results) and 50 µl applied to each well to give a final concentration of 10nM. Two doses of the antagonist were prepared and 50µl applied to each well to give a final concentration of 10^{-8} and 10^{-7}M respectively.

TGF-β-1 was dissolved in 4mM HCL to give a stock concentration of 10000ng/ml whereas SB 431542 (TGF-β antagonist) was dissolved in ethanol to give a stock concentration of 10mM. Then stocks were diluted in sterile medium

to give desired concentrations of the working solutions. A set of 3 doses of TGF-β was prepared and a 50µl applied to each well to give a final concentration of 0.1, 1 and 10ng/ml respectively. According to the dose response curve a 5ng/ml of TGF-β was considered as being the optimal effective dose. However, two doses of the antagonist were prepared and 50µl applied to each well to give a final concentration of 2µM and 10µM respectively.

BMP-6 was dissolved in sterile 4 mM HCl containing at least 0.1% human or bovine serum albumin to give a stock concentration of 20000ng/ml while K02288 (BMP-6 antagonist) was dissolved in ethanol to give a stock concentration of 10mM. Then stocks were diluted in sterile medium to give desired concentrations of the working solutions. A set of two concentrations of BMP-6 were prepared and 50µl applied to each well to give a final concentration of 1 and 5ng/ml respectively. According to the dose respond curve a 5ng/ml of BMP6 was considered as being the optimal effective dose. However, two doses of the antagonist were prepared and 50 µl applied to each well to give a final concentration of 2µM and 10µM respectively.

Thrompospondin-1 was dissolved in sterile PBS to give a final concentration of 100 µ g/ml. Then stocks were diluted in sterile medium to give desired concentrations of the working solutions. A set of three concentrations of thrompospondin-1 were prepared and 50µl applied to each well to give a final concentration of 1, 10 and 100ng/ml respectively.

6.2.3. Follicular angiogenesis cell culture system

Theca interna cells were seeded at a density of 1×10^5/ml and maintained for 7 days. Medium was changed and treatment was applied on day 1, 3 and 5. On day 7 media either discarded or collected for hormonal assay and the endothelial cells were stained for vWF and the degree of network formation determined as described in section 3.7.3. Bovine theca layer were cultured in 24 well plates with 2% of fetal calf serum for the first day of the culture and then grown in serum free media until the end of the culture period. Media were removed and cells were washed with 1ml of PBS. Then, cells were treated with new medium supplemented with desired treatments as described below. At the end of culture,

media were either discarded or kept for hormonal assay as described in section 3.3. Cells were fixed immediately and stained for vWF as described in section 3.7.3.

6.2.3.1. Effect of NMB and its antagonist on endothelial cells network formation

Bovine theca cells were treated with NMB and its antagonist along with VEGF and FGF (Figure 6.1 and 6.2). A mixture of angiogenic factors (1 ng/ml VEGF/FGF and 10 ng/ml VEGF/FGF) was tested and prepared as described in section 6.2.2. NMB ligand was added at either 0, 10, 100, 1000ng/ml or 10nM as described in section 6.2.2 according to the designed experiment. Then, NMB antagonist was prepared as described in section 6.2.2 and after that cells were treated in the presence/absence of NMB ligand, NMB antagonist and VEGF/FGF as mention in section 6.2.3.

VF / NMB	0 ↓	0 ↓	1ng/ml ↓	1ng/ml ↓	10ng/ml ↓	10ng/ml ↓
0 →	Control 1500µl M	Control 1500µl M	1µl V/F + 1499µl M	1µl V/F + 1499µl M	15µl V/F + 1485µl M	15µl V/F + 1485µl M
10ng/ml →	15µl NMB + 1485µl M	15µl NMB + 1485µl M	1µl V/F + 15µl NMB + 1484µl M	1µl V/F + 15µl NMB + 1484µl M	15µl V/F + 15µl NMB + 1470µl M	15µl V/F + 15µl NMB + 1470µl M
100ng/ml →	15µl NMB + 1485µl M	15µl NMB + 1485µl M	1µl V/F + 15µl NMB + 1484µl M	1µl V/F + 15µl NMB + 1484µl M	15µl V/F + 15µl NMB + 1470µl M	15µl V/F + 15µl NMB + 1470µl M
1000ng/ml →	15µl NMB + 1485µl M	15µl NMB + 1485µl M	1µl V/F + 15µl NMB + 1484µl M	1µl V/F + 15µl NMB + 1484µl M	15Vµl /F + 15µl NMB + 1470µl M	15µl V/F + 15µl NMB + 1470µl M

Figure 6. 1 Diagram demonstrates experiment set up in 24-well plate for NMB effect on endothelial cells network formation. * NMB (Neuromedin B ligand),V/F (VEGF/FGF) and M (media).

V/F + A / NMB	0 + 0	0 + 10^{-8}M	0 + 10^{-7}M	1ng/ml + 0	1ng/ml + 10^{-8} M	1ng/ml + 10^{-7}M
0	Control 1000µl M	50µl A + 950µl M	50µl A + 950µl M	1µl V/F + 999µl M	1µl V/F + 50µl A + 949µl M	1µl V/F + 50µl A + 949µl M
0	Control 1000µl M	50µl A + 950µl M	50µl A + 950µl M	1µl V/F + 999µl M	1µl V/F + 50µl A + 949µl M	1µl V/F + 50µl A + 949µl M
10 nM/ml	50µl NMB + 950µl M	50µl NMB + 50µl A + 900µl M	50µl NMB + 50µl A + 900µl M	1µl V/F + 50µl NMB + 949µl M	1µl V/F + 50µl A + 50µl NMB + 899µl M	1µl V/F + 50µl A + 50µl NMB + 899µl M
10 nM/ml	50µl NMB + 950µl M	50µl NMB + 50µl A + 900µl M	50µl NMB + 50µl A + 900µl M	1µl V/F + 50µl NMB + 949µl M	1µl V/F + 50µl A + 50µl NMB + 899µl M	1µl V/F + 50µl A + 50µl NMB + 899µl M

Figure 6. 2 Diagram demonstrates experiment set up in 24-well plate for NMB antagonist effect on endothelial cells network formation. *NMB (neuromedin B ligand), V/F (VEGF/FGF), A (neuromedin antagonist) and M (media). Note, only images generated for treatments shown in the first two rows were statistically evaluated and presented in results. Treatments indicated in the two bottom rows were included in the experiments performed but image analysis was not undertaken due to lack of time.

6.2.3.2. Effect of kisspeptin-10 and its antagonist on endothelial cells network formation

Theca interna cells were treated with Kisspeptin-10 and its antagonist along with VEGF and FGF (Figure 6.3). A 1ng/ml of the angiogenic factors VEGF and FGF was tested and prepared as described in section 6.2.2. Both Kisspeptin-10 ligand and it antagonist were tested at different concentrations including 0, 10^{-8}, 10^{-7} and 10^{-6}M as described in section 6.2.2. After that cells were treated with VEGF and FGF and in the presence/absence of kisspeptin-10 and its antagonist as mentioned in section 6.2.3.

V/F Kiss-10 Kiss A	1ng/ml V/F + Kiss-10 ↓	1ng/ml V/F + Kiss-10 ↓	1ng/ml V/F + Kiss A ↓	1ng/ml V/F + Kiss A ↓		
0 →	1µl V/F + 999µl M	1µl V/F + 999µl M	1µl V/F + 999µl M	1µl V/F + 999µl M		
10^{-8}M →	1µl V/F + 50µl Kiss-10 + 949µl M	1µl V/F + 50µl Kiss-10 + 949µl M	1µl V/F + 50µl Kiss A + 949µl M	1µl V/F + 50µl Kiss A + 949µl M		
10^{-7}M →	1µl V/F + 50µl Kiss-10 + 949µl M	1µl V/F + 50µl Kiss-10 + 949µl M	1µl V/F + 50µl Kiss A + 949µl M	1µl V/F + 50µl Kiss A + 949µl M		
10^{-6}M →	1µl V/F + 50µl Kiss-10 + 949µl M	1µl V/F + 50µl Kiss-10 + 949µl M	1µl V/F + 50µl Kiss A + 949µl M	1µl V/F + 50µl Kiss A + 949µl M		

Figure 6. 3 Diagram demonstrates experiment set up in 24-well plate for kiss-10 and its antagonist effect on endothelial cells network formation. *Kiss-10 (kisspeptin-10 ligand), kiss A (kisspeptin antagonist), V/F (VEGF/FGF) and M (media).

6.2.3.3. Effect of thrombospondin-1 on endothelial cells network formation

Theca interna cells were treated with thrmbospondin-1 along with VEGF and FGF (Figure 6.4). A 1ng/ml of the angiogenic factors VEGF and FGF was tested and prepared as described in section 6.2.2. Thrombospondin-1 was added at a range of concentrations (0, 1, 10 and 100ng/ml) as described in section 6.2.2. After that cells were treated in the presence/absence thrombosponsin-1 and VEGF and FGF as mentioned in section 6.2.3.

V/F / TSP-1	0 ↓	0 ↓	0 ↓	1ng/ml ↓	1ng/ml ↓	1ng/ml ↓
0 →	Control 1000µl M	Control 1000µl M	Control 1000µl M	1µl V/F + 999µl M	1µl V/F + 999µl M	1µl V/F + 999µl M
1ng/ml →	50µl TSP-1 + 950µl M	50µl TSP-1 + 950µl M	50µl TSP-1 + 950µl M	1µl V/F + 50µl TSP-1 + 949µl M	1µl V/F + 50µl TSP-1 + 949µl M	1µl V/F + 50µl TSP-1 + 949µl M
10ng/ml →	50µl TSP-1 + 950µl M	50µl TSP-1 + 950µl M	50µl TSP-1 + 950µl M	1µl V/F + 50µl TSP-1 + 949µl M	1µl V/F + 50µl TSP-1 + 949µl M	1µl V/F + 50µl TSP-1 + 949µl M
100ng/ml →	50µl TSP-1 + 950µl M	50µl TSP-1 + 950µl M	50µl TSP-1 + 950µl M	1µl V/F + 50µl TSP-1 + 949µl M	1µl V/F + 50µl TSP-1 + 949µl M	1µl V/F + 50µl TSP-1 + 949µl M

Figure 6. 4 Diagram demonstrates experiment set up in 24-well plate for thrombospondin-1 effect on endothelial cells network formation. *TSP-1 (thrombospondin-1 ligand), V/F (VEGF/FGF) and M (media).

6.2.3.4. Effect of TGF-β-1 and its antagonist on endothelial cells network formation

Theca interna cells were treated with TGF-β-1 and its antagonist along with VEGF and FGF (Figures 6.5 and 6.6). A 1ng/ml of the angiogenic factors VEGF and FGF was tested and prepared as described in section 6.2.2. TGF-β-1 was tested at 0, 0.1, 1 and 10ng/ml while two different concentrations (2 and 10µM) of its antagonist were tested as described in section 6.2.2. After that cells were treated without/with TGF-β-1, its antagonist, VEGF and FGF and in combination with ligand optimal doses of ligand and antagonist as mentioned in section 6.2.3.

V/F / TGF-β	0 ↓	0 ↓	0 ↓	1ng/ml ↓	1ng/ml ↓	1ng/ml ↓
0 →	Control 1000µl M	Control 1000µl M	Control 1000µl M	1µl V/F + 999µl M	1µl V/F + 999µl M	1µl V/F + 999µl M
0.1ng/ml →	50µl TGF-β + 950µl M	50µl TGF-β + 950µl M	50µl TGF-β + 950µl M	1µl V/F + 50µl TGF-β + 949µl M	1µl V/F + 50µl TGF-β + 949µl M	1µl V/F + 50µl TGF-β + 949µl M
1ng/ml →	50µl TGF-β + 950µl M	50µl TGF-β + 950µl M	50µl TGF-β + 950µl M	1µl V/F + 50µl TGF-β + 949µl M	1µl V/F + 50µl TGF-β + 949µl M	1µl V/F + 50µl TGF-β + 949µl M
10ng/ml →	50µl TGF-β + 950µl M	50µl TGF-β + 950µl M	50µl TGF-β + 950µl M	1µl V/F + 50µl TGF-β + 949µl M	1µl V/F + 50µl TGF-β + 949µl M	1µl V/F + 50µl TGF-β + 949µl M

Figure 6. 5 Diagram demonstrates experiment set up in 24-well plate for TGF-β-1 effect on endothelial cells network formation. * TGF-β (transforming growth factor- β-1), V/F (VEGF/FGF) and M (media).

VF	0 ↓	0 ↓	0 ↓	1ng/ml ↓	1ng/ml ↓	1ng/ml ↓
	Control 1000µl M	Control 1000µl M	50µl TGF-β (5ng/ml)+ TGF-β A (2µM) + 950µl M	1µl V/F + 999µl M	1µl V/F + 999µl M	1µl V/F + 50µl TGF-β (5ng/ml) + 50µl TGF-β A (2µM) + 949µl M
	50µl TGF-β (5ng/ml)+ 950µl M	50µl TGF-β (5ng/ml)+ 950µl M	50µl TGF-β (5ng/ml)+ TGF-β A (2µM) + 950µl M	1µl V/F + 50µl TGF-β (5ng/ml) + 949µl M	1µl V/F + 50µl TGF-β (5ng/ml) + 949µl M	1µl V/F + 50µl TGF-β (5ng/ml) + 50µl TGF-β A (2µM) + 949µl M M
	50µl TGF-β A (2µM) + 950µl M	50µl TGF-β A (2µM) + 950µl M	50µl TGF-β (5ng/ml)+ TGF-β A (10µM) + 950µl M	1µl V/F + 50µl TGF-β A (2µM) + 949µl M	1µl V/F + 50µl TGF-β A (2µM) + 949µl M	1µl V/F + 50µl TGF-β (5ng/ml) + 50µl TGF-β A (10µM) + 949µl M
	50µl TGF-β A (10µM) + 950µl M	50µl TGF-β A (10µM) + 950µl M	50µl TGF-β (5ng/ml)+ TGF-β A (10µM) + 950µl M	1µl V/F + 50µl TGF-β A (10µM) + 949µl M	1µl V/F + 50µl TGF-β A (10µM) + 949µl M	1µl V/F + 50µl TGF-β (5ng/ml) + 50µl TGF-β A (10µM) + 949µl M

Figure 6. 6 Diagram demonstrates experiment set up in 24-well plate for TGF-β-1 and its antagonist effect on endothelial cells network formation. * TGF-β (transforming growth factor- β-1), and TGF-β A (TGF-β antagonist), V/F (VEGF/FGF) and M (media). Note, wells in each row were treated differently to one another as indicated in the corresponding cell; hence the empty cells in the left hand column.

6.2.3.5. Effect of BMP-6 and its antagonist on endothelial cells network formation

Theca interna cells were treated with BMP-6 and its antagonist along with VEGF and FGF (Figures 6.7 and 6.8). A 1ng/ml of the angiogenic factors VEGF and FGF was tested and prepared as described in section 6.2.2. BMP-6 was tested at 0, 1, 5ng/ml while two different concentrations (2 and 10µM) of its antagonist were tested as described in section 6.2.2. After that cells were treated without/with BMP-6, its antagonist, VEGF and FGF and in combination with optimal doses of ligand and antagonist as mentioned in section 6.2.3.

V/F / BMP-6	0 ↓	0 ↓	1ng/ml ↓	1ng/ml ↓		
0 →	Control 1000µl M	Control 1000µl M	1µl V/F + 999µl M	1µl V/F + 999µl M		
1ng/ml →	50µl BMP-6 + 950µl M	50µl BMP-6 + 950µl M	1µl V/F + 50µl BMP-6 + 949µl M	1µl V/F + 50µl BMP-6 + 949µl M		
5ng/ml →	50µl BMP-6 + 950µl M	50µl BMP-6 + 950µl M	1µl V/F + 50µl BMP-6 + 949µl M	1µl V/F + 50µl BMP-6 + 949µl M		

Figure 6. 7 Diagram demonstrates experiment set up in 24-well plate for BMP-6 effect on endothelial cells network formation. * BMP-6 (bone morphogenetic protein-6), V/F (VEGF/FGF) and M (media).

VF	0 ↓	0 ↓	0 ↓	1ng/ml ↓	1ng/ml ↓	1ng/ml ↓
	Control 1000µl M	Control 1000µl M	50µl BMP-6 (5ng/ml)+ BMP-6 A (2µM) + 950µl M	1µl V/F + 999µl M	1µl V/F + 999µl M	1µl V/F + 50µl BMP-6 (5ng/ml) + 50µl BMP-6 (2µM) + 949µl M
	50µl BMP-6 (5ng/ml)+ 950µl M	50µl BMP-6 (5ng/ml)+ 950µl M	50µl BMP-6 (5ng/ml)+ BMP-6 A (2µM) + 950µl M	1µl V/F + 50µl BMP-6 (5ng/ml) + 949µl M	1µl V/F + 50µl BMP-6 (5ng/ml) + 949µl M	1µl V/F + 50µl BMP-6 (5ng/ml) + 50µl BMP-6 (2µM) + 949µl M
	50µl BMP-6 A (2µM) + 950µl M	50µl BMP-6 A (2µM) + 950µl M	50µl BMP-6 (5ng/ml)+ BMP-6 A (10µM) + 950µl M	1µl V/F + 50µl BMP-6 A (2µM) + 949µl M	1µl V/F + 50µl BMP-6 A (2µM) + 949µl M	1µl V/F + 50µl BMP-6 (5ng/ml) + 50µl BMP-6 (10µM) + 949µl M
	50µl BMP-6 A (10µM) + 950µl M	50µl BMP-6 A (10µM) + 950µl M	50µl BMP-6 (5ng/ml)+ BMP-6 A (10µM) + 950µl M	1µl V/F + 50µl BMP-6 A (10µM) + 949µl M	1µl V/F + 50µl BMP-6 A (10µM) + 949µl M	1µl V/F + 50µl BMP-6 (5ng/ml) + 50µl BMP-6 (10µM) + 949µl M

Figure 6. 8 Diagram demonstrates experiment set up in 24-well plate for BMP-6 and its antagonist effect on endothelial cells network formation. * BMP-6 (bone morphogenetic protein-6), BMP-6 A (BMP6 antagonist), V/F (VEGF/FGF) and M (media). Note, well in each row were treated differently to one another as indicated in the corresponding cell; hence the empty cells in the left hand column.

6.2.4. Hormone immunoassays

6.2.4.1. The effect of TGF-β-1, BMP6 and their antagonist on TCs (A4 and P4 secretion)

A4 and P4 concentrations in retained spent media were determined by ELISA as described in section 3.3.

6.2.5. Gene expression analysis

6.2.5.1. Sample preparation, total mRNA extraction, purification, cDNA synthesis and RT-PCR

Samples were collected and processed according to section 3.4.1. Total RNA extracts were quantified in section 3.4.2 and tested for integrity in section 3.4.3. First-strand cDNA was synthesized in section 3.4.4 following by RT-PCR analysis as described in section 3.4.5 using specific primer pairs as show in table 3.2.

6.2.5.2. The expression of steroidogenic and other transcripts in theca interna angiogenic culture system

Cultured cells were processed for RNA isolation using Qiagen RNeasy mini kits and cDNA was synthesized using the AB high capacity cDNA synthesis kit according to the manufacture instructions as described in section 3.4.1.2 and 3.4.4. Then, cDNA samples were used for Quantitative RT-PCR for the detection of the expression of *StAR*, *CYP11A1*, *CYP17*, *HSD3B1*, *LHR*, *INSL3*, *INHA*, *NR5A1*, *TGF-β-1*, *TGF-β-2*, *TGF-β-3*, *VEGFR* and *FGFR* using their designed primers and β-actin (ACTB) for normalization of gene expression as described in section 3.4.5. The set of primers used to detect the desired genes as well as housekeeping gene are listed in table 3.2. Melt curve analysis and agarose gel electrophoresis were used to verify that each selected primer pair gave a single amplicon of the predicted size and Tm. After that, samples were diluted into 1:10 and 5µl of these diluted cDNA samples were used for qPCR. Assays were carried out in a 142µl volume comprising 2µl of forward and reverse primers, 5µl cDNA and 7µl of QuantiTect SYBR Green 2X “hot start” Master Mix (Qiagen). Samples were run for 40 cycles on an AB StepOne plus real-time PCR instrument (Applied

Biosystems). The method that was used to compare the difference between each mRNA transcript was the ΔΔCt method using β-actin as the house keeping control. Finally, ΔΔCt values were converted to fold difference for graphical presentation using the formula $2^{(-\Delta\Delta^{Ct})}$.

6.3. Statistical analysis

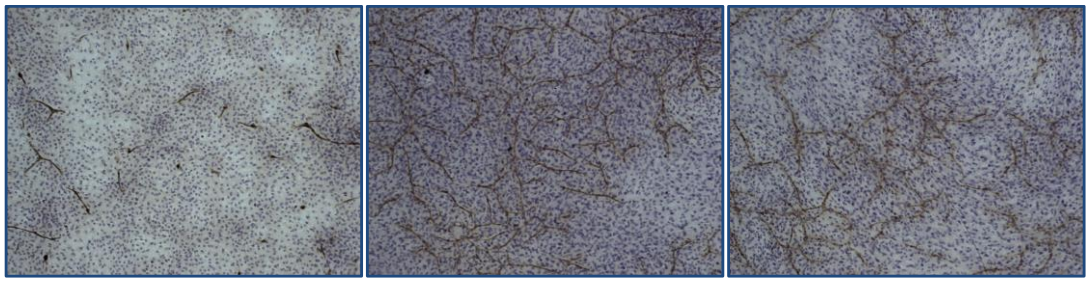
The effects of the various treatments on endothelial network formation, hormone secretion and gene expression were evaluated by two-way analysis of variance (2-way ANOVA). Individual pairwise comparisons within different treatments range were subsequently made by Fisher's PLSD. In order to reduce heterogeneity of variance, some data were log-transformed prior to statistical analysis. Unless otherwise stated, results are presented mean ±SEM of ≥ 3 independent batches of cultured cells.

6.4. Results

6.4.1. The effect of NMB and its antagonist on endothelial network formation

Staining for endothelial cells counter in brown using vWF as a marker, revealed that a number of networks had formed in each culture (Figure 6.9). Each network had a central body of endothelial cells from which a number of branches had developed. These networks appeared to be at different stages of development, with varying size and degree of branching. Also, the growth of other cell types was observed, the nuclei of which could be seen when cells were counter in blue.

Statistical analysis showed that there was extensive formation of endothelial networks with 1 and 10ng/ml of VEGF and FGF (hereafter referred to as V/F) as indicated by the % in area of vWF stating when comparing to basal level ($P<0.0001$) (Figures 6.10 and 6.11). However, the effect of NMB and its antagonist on basal and V/F induced network formation was not statistically significant as shown in (Figures 6.10 and 6.11).



(a) (b) (c)

Figure 6. 9 Development of endothelial cell network in theca interna system in response to two concentrations (1 and 10 ng/ml) of V/F. Endothelial cells were stained brown with vWF antibody. (a) Representative control cells; (b) representative cells treated with 1ng/ml V/F; (c) representative cells treated with 10ng/ml V/F.

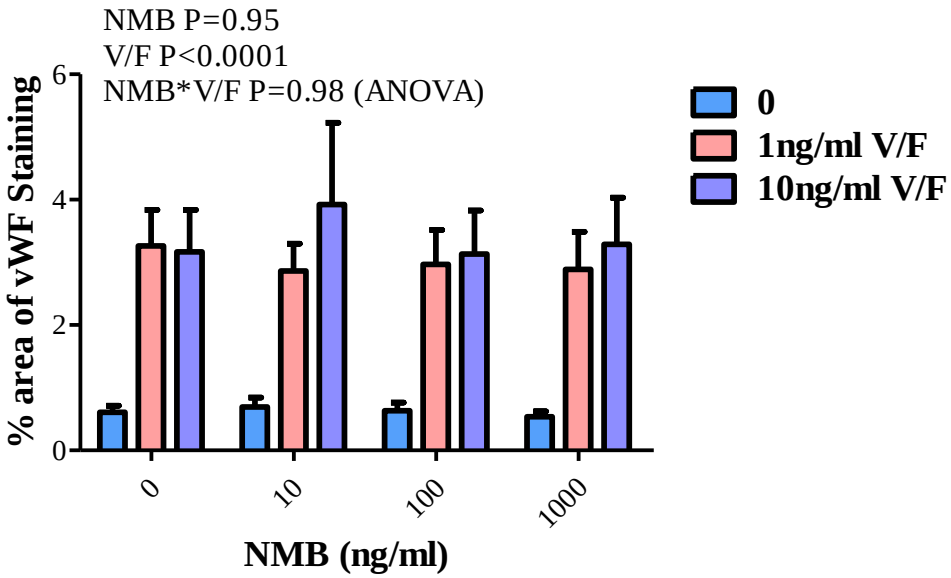


Figure 6. 10 The effect of NMB alone and in combination with VEGF and FGF, on network formation by cultured theca interna cells. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown.

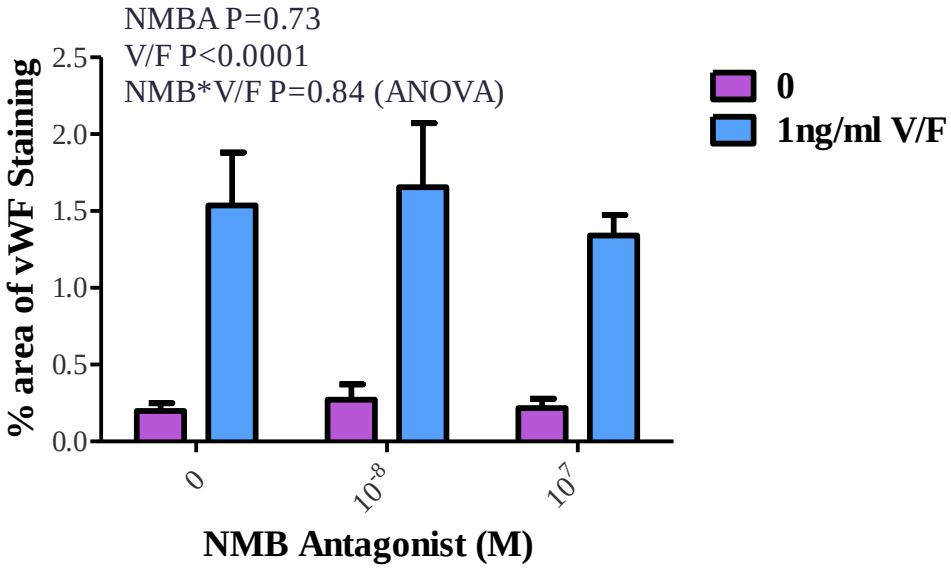


Figure 6. 11 The effect of NMB antagonist alone and in combination with VEGF and FGF, on network formation by cultured theca interna cells. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

6.4.2. The effect of kiss-10 and its antagonist on endothelial network formation

As shown in Figures 6.12 and 6.13, there was no significant effect of kisspeptin-10 or its antagonist on endothelial network formation.

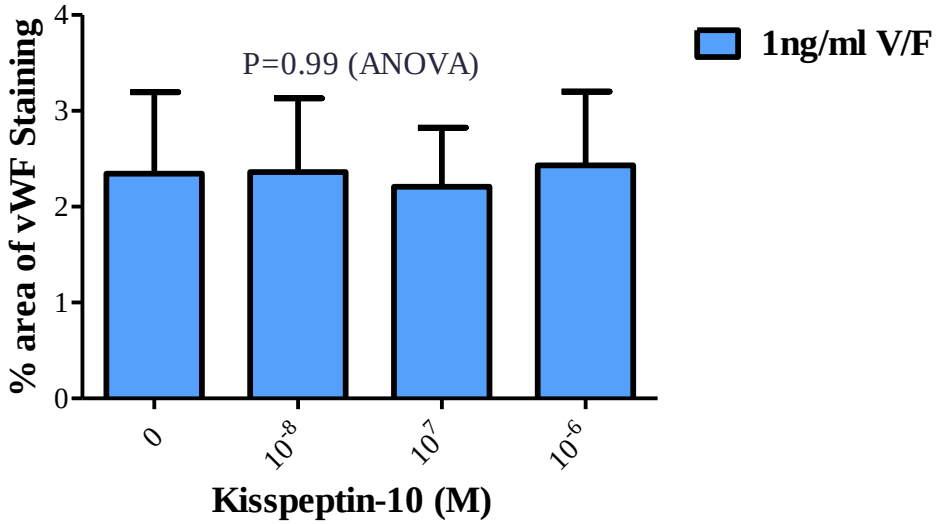


Figure 6. 12 The effect of kisspeptin-10 in the presence of VEGF and FGF, on network formation by cultured theca interna cells. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

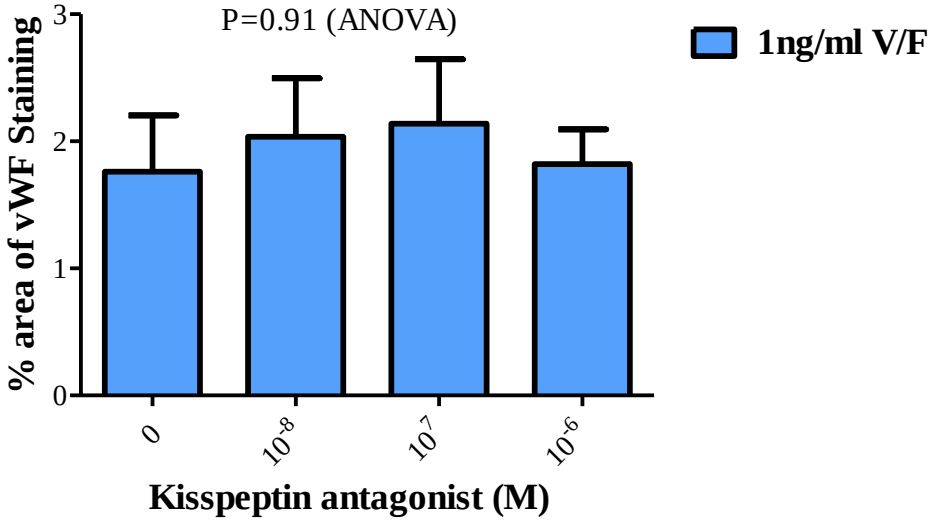


Figure 6. 13 The effect of kisspeptin-10 antagonist in the presence of VEGF and FGF, on network formation cultured theca interna cells. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

6.4.3. The effect of thrombospondin-1 on endothelial network formation

Statistical analysis showed that there was extensive formation of endothelial network with 1ng/ml doses of V/F in area of vWF staining when compared to basal level (P<0.0001). However, the effect of thrombospondin-1 on basal and V/F induced network formation was not statistically significant as shown in (Figure 6.14).

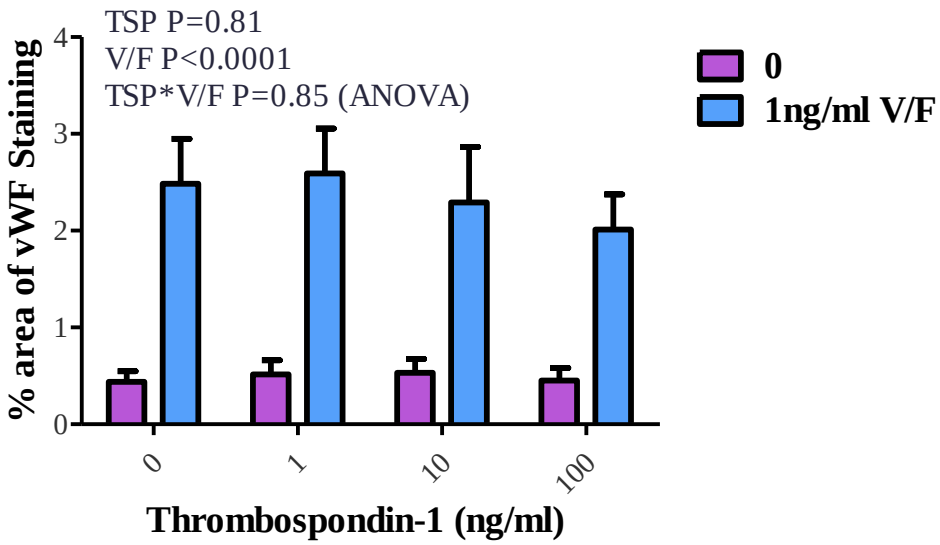


Figure 6. 14 The effect of thrombospondin-1 alone and in the presence/absence of VEGF and FGF, on network formation by cultured theca interna cells. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

6.4.4. The effect of TGF-β-1 and its antagonist on endothelial network formation

As previously mentioned, staining for endothelial cells using vWF as a marker, showed that a number of networks had formed in each culture. However, TGF-β-1 significantly reduced endothelial cell network formation under both basal and V/F induced conditions (P=0.0049) (Figure 6.15). Also the statistical analysis showed that there was significantly extensive formation of endothelial network with 1ng/ml dose of V/F in area of vWF staining when compared to basal level (P<0.0267) in (Figure 6.15) and (P<0.0001) in (Figure 6.16).

As shown in figure 6.15, the effect of different treatments on endothelial cell network formation was significant (P=0.0002). Furthermore, TGF-β-1 inhibitor at 2 and 10μM significantly reversed the inhibitory effect of TGF-β-1 ligand on network formation on basal and V/F induced endothelial network formation (P<0.0001) and (P=0.0007) respectively. Moreover, the combination between the ligand and inhibitors doses showed a significant increase in network formation in comparison to 5ng/ml dose of TGF-β-1 ligand alone under basal and V/F induced conditions (P=0.0033) and (P=0.0002) respectively (Figure 6.16).

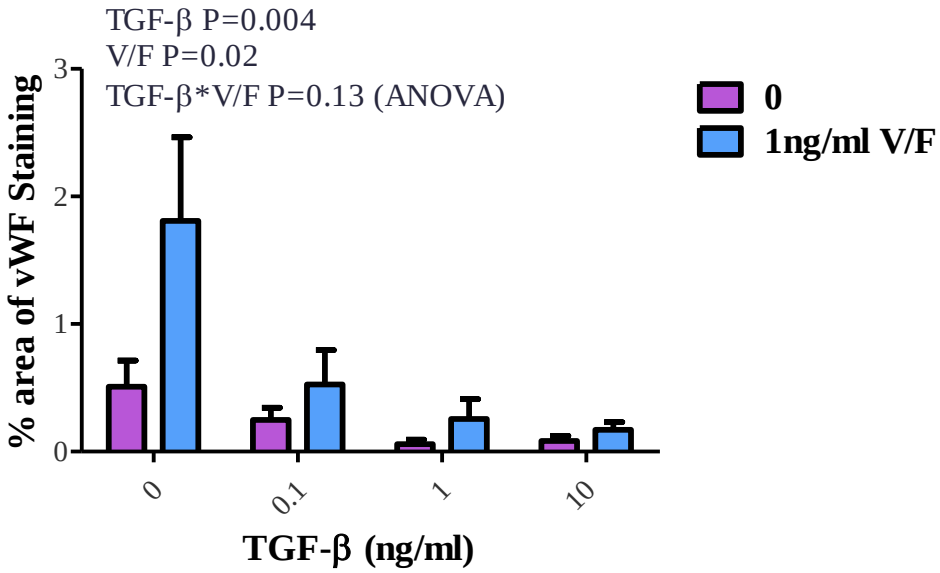


Figure 6. 15 The effect of TGF-β-1 alone and in combination in the presence/absence of VEGF and FGF, on network formation by cultured theca interna cells. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

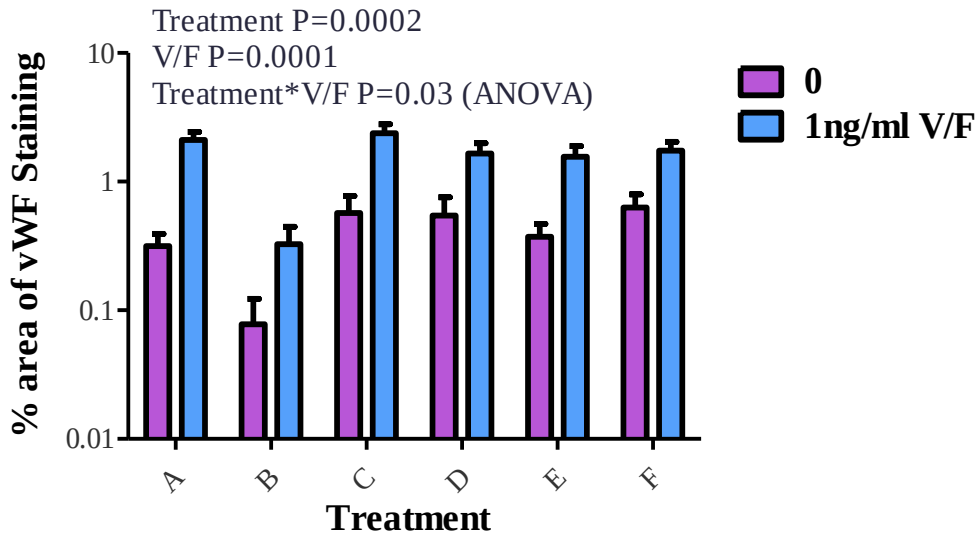


Figure 6. 16 The effect of TGF-β-1 and its antagonist alone or in combination on basal and VEGF/FGF-induced network formation cultured theca interna cells. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only), D (10μM of TGF-β-1 antagonist only), E (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist) and F (5ng/ml of TGF-β-1 + 10μM of TGF-β-1 antagonist).

6.4.5. The effect of BMP-6 and its antagonist on endothelial network formation

Figure 6.17 shows that BMP-6 dose dependently decreased V/F induced endothelial cell network formation (P=0.0117). Also the statistical analysis showed that there was significantly increased formation of endothelial network with 1ng/ml dose of V/F in comparison to basal level (P<0.0001).

According to Figure 6.18, the effect of different BMP-6 and BMP-6 antagonist treatments on endothelial cell network formation was not significant overall. However, V/F had a highly significant stimulatory effect as seen previously.

Additional pairwise comparisons indicated that co-treatment with BMP-6 (5ng/ml) and 10μM induced a significant increased in network formation in comparison to 5ng/ml dose of BMP-6 alone, under both basal and V/F conditions (B versus E; P=0.016) (Figure 6.18).

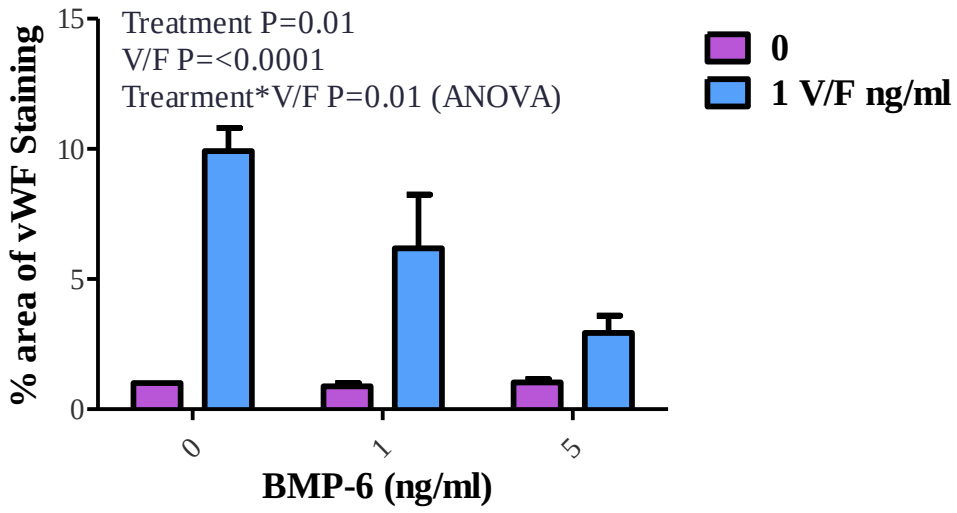


Figure 6. 17 The effect of BMP-6 in presence/absence of VEGF and FGF, on network formation by cultured theca interna cells. Values are means ±SEM (n=3 independent batches of cells) and two-way ANOVA results are shown.

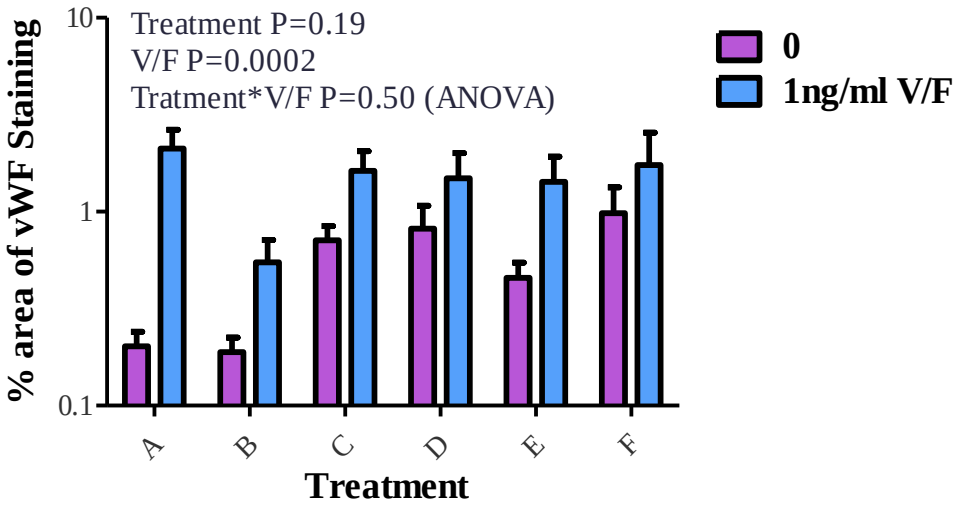


Figure 6. 18 The effect of BMP-6 and its antagonist alone or in combination with VEGF and FGF, on network formation cultured theca interna cells. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP6 only), C (2μM of BMP6 antagonist only), D (10μM of BMP6 antagonist only), E (5ng/ml of BMP6 + 2μM of BMP6 antagonist) and F (5ng/ml of BMP6 + 10μM of BMP6 antagonist).

6.4.6. The effect of TGF-β-1 and its antagonist on A4 and P4 secretion by TCs

As shown in Figure 6.19, the production of both (a) A4 and (b) P4 was significantly affected in cultured cells treated with different treatments combinations (P=0.0009) and (P<0.001) respectively. However, there was no overall difference between basal and V/F stimulated cells.

In Figure 6.19 (a), there was an increase in A4 production under both basal and V/F induced conditions between controls and cells treated with 2µM of TGF-β-1 inhibitor only (P=0.0002)**.** Additionally, there was a decreased in A4 production in cells treated with 10µM TGF-β-1 inhibitor, 5ng/ml TGF-β-1 with 2 and 10µM of the inhibitor in comparison with cells treated with 2µM of TGF-β-1 inhibitor (P=0.006), (P=0.0002) and (P=0.0003) respectively.

Figure 6.19 (b) showed a significant decrease in P4 production between control cells and cells treated with 5ng/ml TGF-β-1 only (P<0.0001). Whereas, there was a significant increase in P4 production in cells treated with two doses of the inhibitor alone and in combination with 5ng/ml TGF-β-1 according to basal and V/F induced tube formation compared to cells treated with a 5ng/ml of TGF-β-1 (P=0.01), (P=0.01), (P<0.001) and (P<0.0001) respectively.

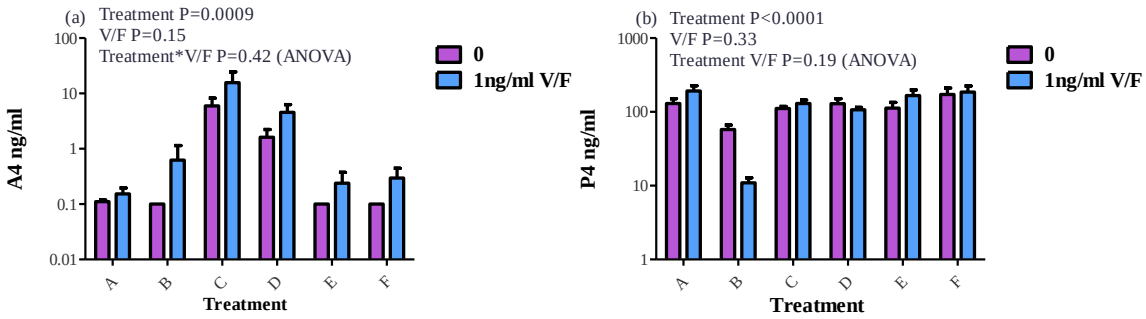


Figure 6. 19 The effect of TGF-β-1 and its antagonist alone or in combination on basal and VEGF/FGF-induced production of (a) A4 and (b) P4 cultured bovine theca interna cells. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only), D (10μM of TGF-β-1 antagonist only), E (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist) and F (5ng/ml of

TGF-β-1 + 10μM of TGF-β-1 antagonist). Note, error bars that are not visible are smaller than the width of the line drawn.

6.4.7. The effect of BMP-6 and its antagonist on A4 and P4 secretion by TCs

As demonstrated in Figure 6.20 the production of (a) A4 was not significantly affected in cells treated with different treatments while production of (b) P4 was affected (P<0.0003). Neither, V/F alone or in interaction with various treatments showed a significant effect on either A4 or P4 production.

Figure 6.20 (a) there was an increase in A4 production between control cells and cells treated with 10µM of BMP-6 inhibitor only (P=0.0246)**.** 10µM of BMP6 inhibitor promoted a significant increase in A4 production (in basal and V/F induced conditions) compare to 5ng/ml of BMP-6 treatment (P=0.02). Additionally, there was a significant decrease in A4 production in cells treated with the combination of the ligand and the inhibitor compared to 10µM BMP-6 inhibitor alone (P<0.025).

On the other hand, Figure 6.20 (b) showed a significant increase in P4 production between control cells and cells treated with 10µM BMP-6 inhibitor only (P=0.0001). Similarly, there was a significant increase in P4 production in cells treated with 10µM BMP-6 inhibitor alone compared to cells treated with 5ng/ml BMP-6 (P<0.0001). Likewise, 10µM BMP-6 inhibitor promoted a significant increase in P4 production compared to 2µM inhibitor (P=0.0003) Moreover, 10µM BMP-6 inhibitor alone increased P4 production in comparison to cells treated with the combination of ligand and inhibitor, under both basal and V/F induced network conditions (P<0.0001) and (P=0.0004) respectively.

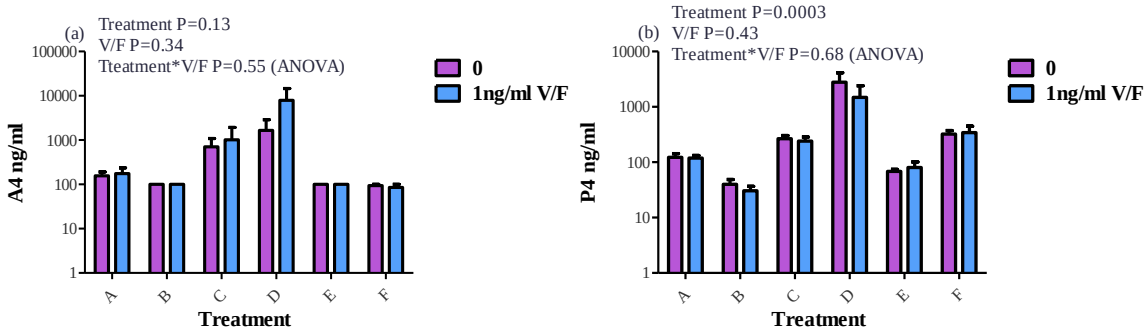


Figure 6. 20 The effect of BMP-6 and its antagonist alone or in combination on the presence/absence of VEGF and FGF, on the production of (a) A4 and (b) P4 by bovine theca layer cultured cells. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP6 only), C (2μM of BMP6 antagonist only), D (10μM of BMP6 antagonist only), E (5ng/ml of BMP6 + 2μM of BMP6 antagonist) and F (5ng/ml of BMP6 + 10μM of BMP6 antagonist). Note, error bars that are not visible are smaller than the width of the line drawn.

6.4.8. The expression of steroidogenic and other transcripts in cultured theca interna cells treated with TGFβ-1 and its inhibitor

6.4.8.1. StAR

The expression of *StAR* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P=0.0004) among different treatments under basal and V/F induced conditions (Figure 6.21). However, there was no significant effect of V/F treatment on *StAR* expression; nor was there a statistical interaction between treatment and V/F. TGF-β-1 inhibitor alone significantly increased *StAR* expression compared to control under both basal and V/F induced conditions (P=0.0012). TGF-β-1 reversed the increase in *StAR* expression induced by 2μM of TGF-β-1 inhibitor (P=0.0150).

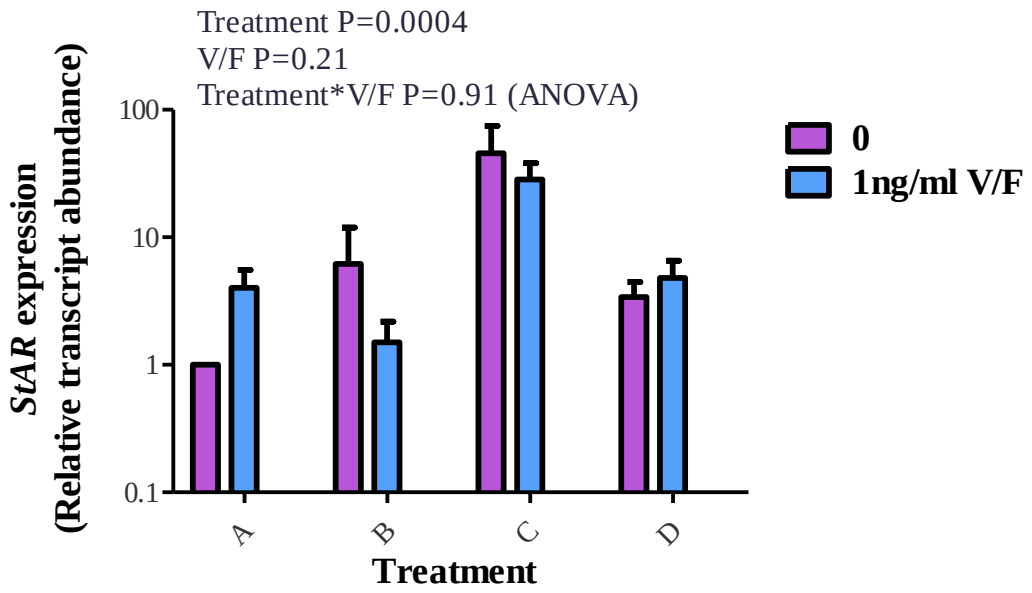


Figure 6. 21 The expression of *STAR* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.2. CYP11A1

The expression of *CYP11A1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P<0.0001) among different treatments but there was no significant effect of V/F (Figure 6.22). However, the interaction between treatment and V/F effect was significant (P=0.0345). TGF-β-1 induced significantly the decrease compared to control (P<0.0001). TGF-β-1 inhibitor alone increased significantly the expression of *CYP11A1* compared to control (P<0.0001). TGF-β-1 in combination with TGF-β-1 inhibitor decreased significantly the expression of *CYP11A1* compared to TGF-β-1 treatment alone (P<0.0001).

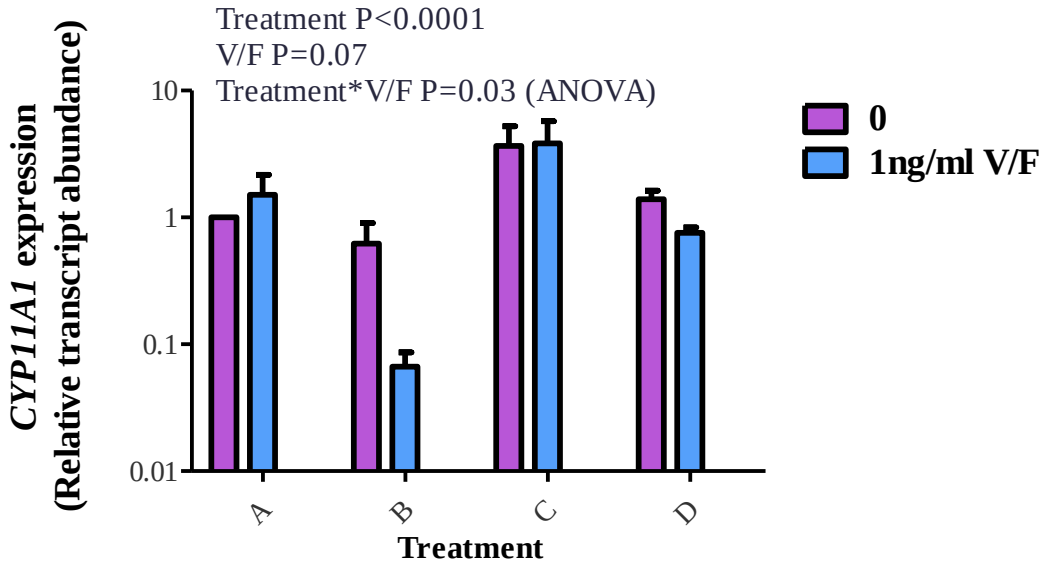


Figure 6. 22 The expression of *CYP11A1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.3. CYP17A1

The expression of *CYP17A1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P<0.0001). However, neither V/F alone nor the interaction between the V/F and treatments showed a significant effect (Figure 6.23). There was a significant decrease in response to TGF-β-1 in V/F induced endothelial cells (P=0.0276). In contrast, TGF-β-1 inhibitor increased significantly the expression of CYP17A1 (P=0.0005). Also, TGF-β-1 reversed the stimulatory effect of TGF-β-1 inhibitor on *CYP17A1* expression under both basal and V/F-induced endothelial (P=0.0006).

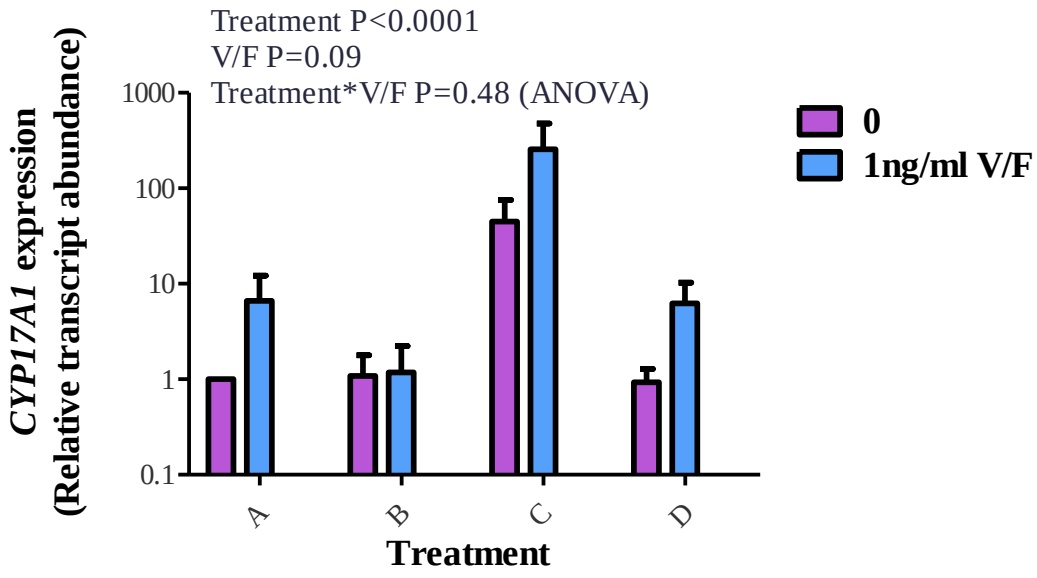


Figure 6. 23 The expression of *CYP17A1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.4. HSD3B1

The expression of *HSD3B1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P<0.0001) in both basal and V/F-treated cells (Figure 6.24). However, there was no significant effect of V/F or interaction between treatments and V/F. There was a significant TGF-β-1 induced decrease under both basal and V/F induced conditions (P=0.0018). TGF-β-1 inhibitor alone significantly expression of *HSD3B1* in both basal and V/F-treated cells (P=0.0240). Also, TGF-β-1 inhibitor alone increased expression of *HSD3B1* compared to TGF-β-1 alone under basal and V/F induced conditions (P<0.0001). Likewise, TGF-β-1 in combination with TGF-β-1 inhibitor increased expression of *HSD3B1* compared to TGF-β-1 alone, but only V/F treated cells (P=0.0003).

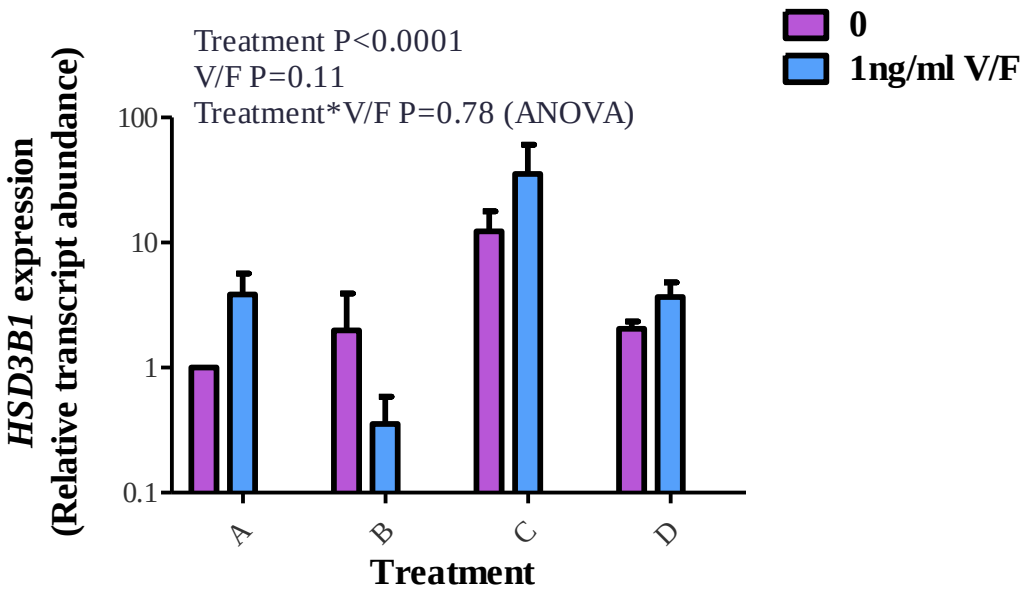


Figure 6. 24 The expression of *HSD3B*1 in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.5. LHR

The expression of *LHR* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P<0.0001) among different treatments but there was no significant effect of V/F treatments, or interaction between treatments and V/F (Figure 6.25). TGF- β -1 inhibitor increased significantly the expression of *LHR* and this effect was reversed by TGF-β-1 co-treatment (P<0.0001).

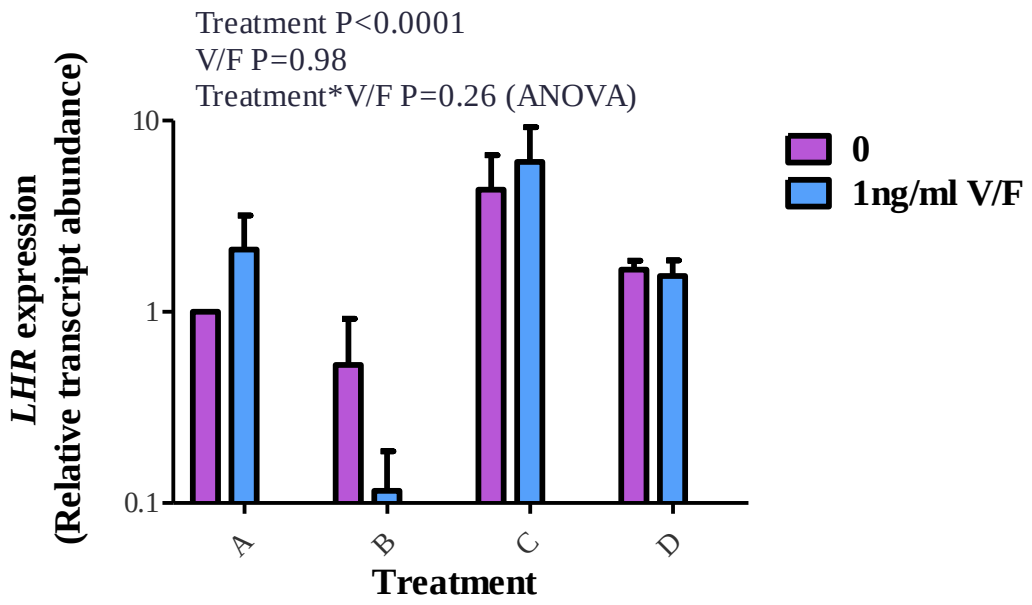


Figure 6. 25 The expression of *LHR* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.6. INSL3

The expression of *INSL3* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P<0.0001) among different treatments under basal and V/F-induced conditions (Figure 6.26). There was a significant effect of V/F treatment on *INSL3* expression (P=0.0419) while, the interaction between treatments and V/F was not significant. TGF-β-1 alone increased expression of *INSL3* compared to controls under basal conditions whereas it decreased expression in V/F- treated cells (P=0.0148). Also, there was a significant increased TGF-β-1 inhibitor under both basal and V/F induced conditions (P=0.0029). TGF-β-1 inhibitor also reversed the suppressive effect of TGF-β-1 under basal and V/F induced conditions (P=0.0033).

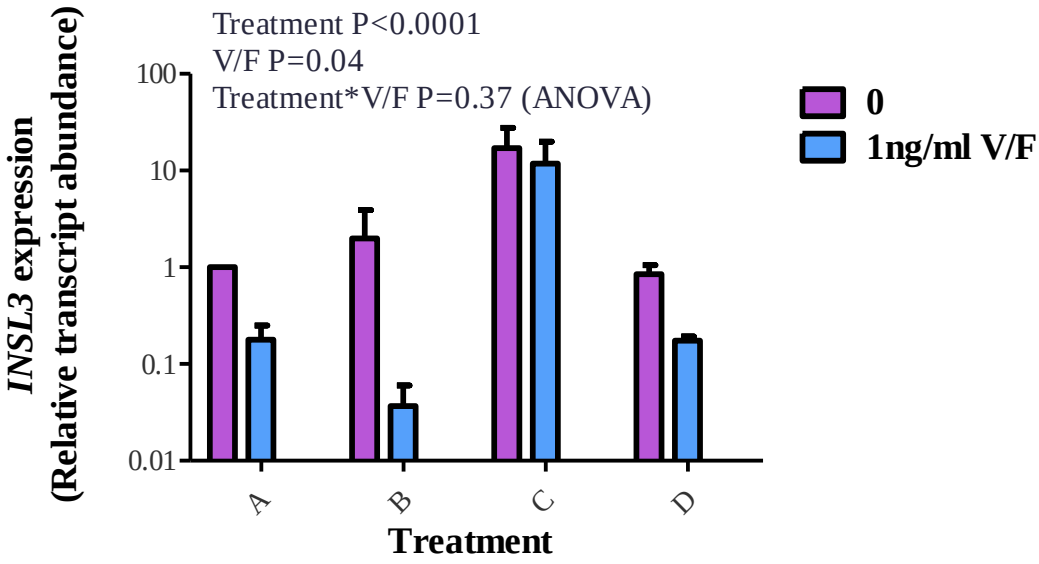


Figure 6. 26 The expression of *INSL3* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.7. INHA

The expression of *INHA* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P=0.0062) among different treatments under both basal and V/F induced conditions (Figure 6.27). Likewise, there was a significant effect of V/F treatment on *INHA* expression although no interaction between treatment effect and V/F was observed. TGF-β-1 alone decreased expression of *INHA* compared to control under basal conditions (P=0.0053). In contrast TGF-β-1 inhibitor increased expression of *INHA* under basal conditions (P=0.0011) and reversed the suppressive effects of TGF-β-1 (P=0.0294).

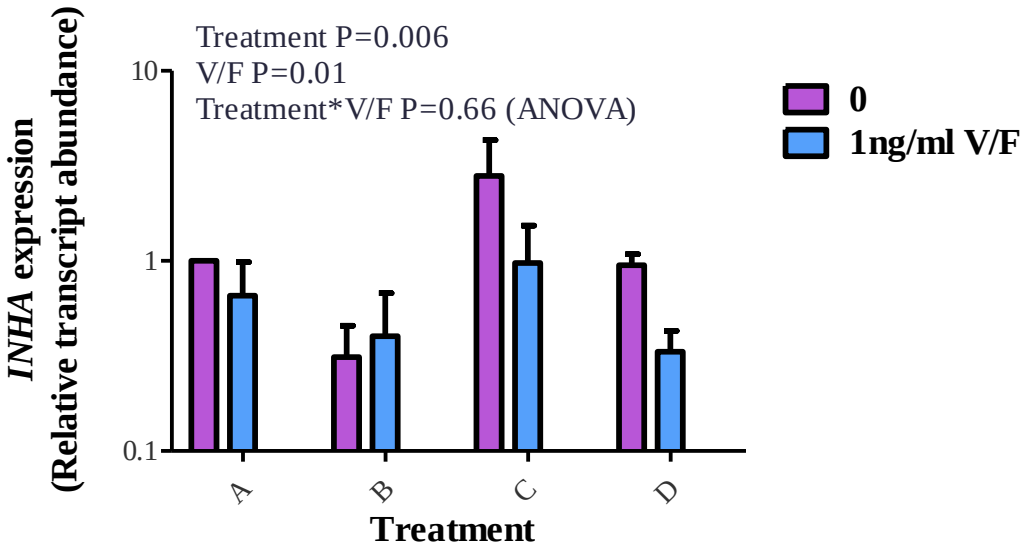


Figure 6. 27 The expression of *INHA* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.8. NR5A1

The expression of *NR5A1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P=0.0035) among different treatments under basal and V/F induced conditions (Figured 6.28). *NR5A1* expression was also affected by V/F treatment (P=0.0277), but the interaction between treatments and V/F was not significant. A small though significant decrease in expression of *NR5A1* was induced by TGF-β-1 under basal conditions (P=0.0331) whereas TGF-β-1 inhibitor alone increased expression of *NR5A1* (P=0.0003).

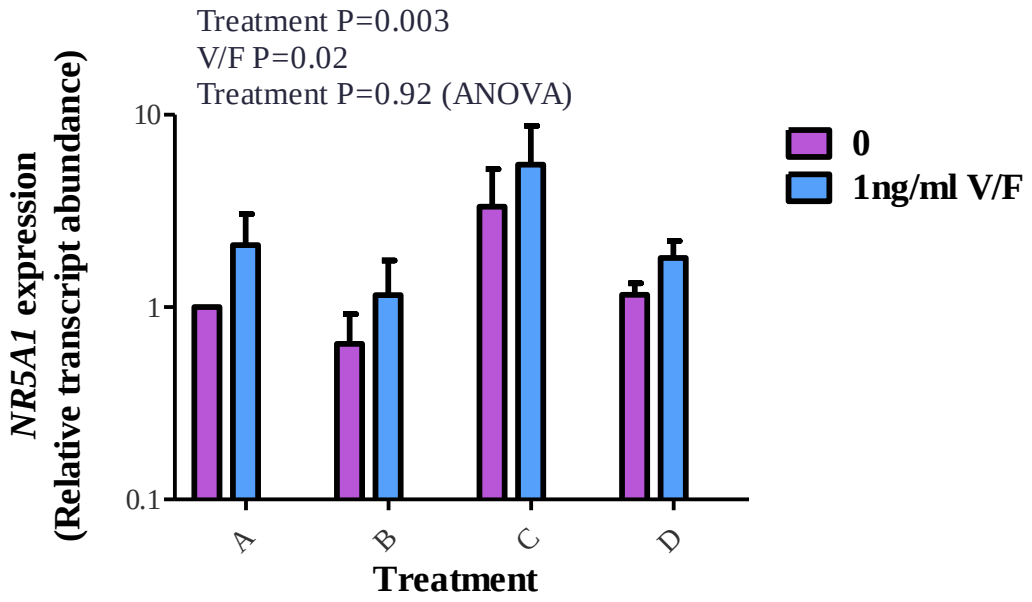


Figure 6. 28 The expression of *NR5A1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.9. TGFβ1

The expression of *TGFβ1* was not affected by TGF-β-1 and its inhibitor alone and in combination, nor by V/F treatment (Figure 6.29).

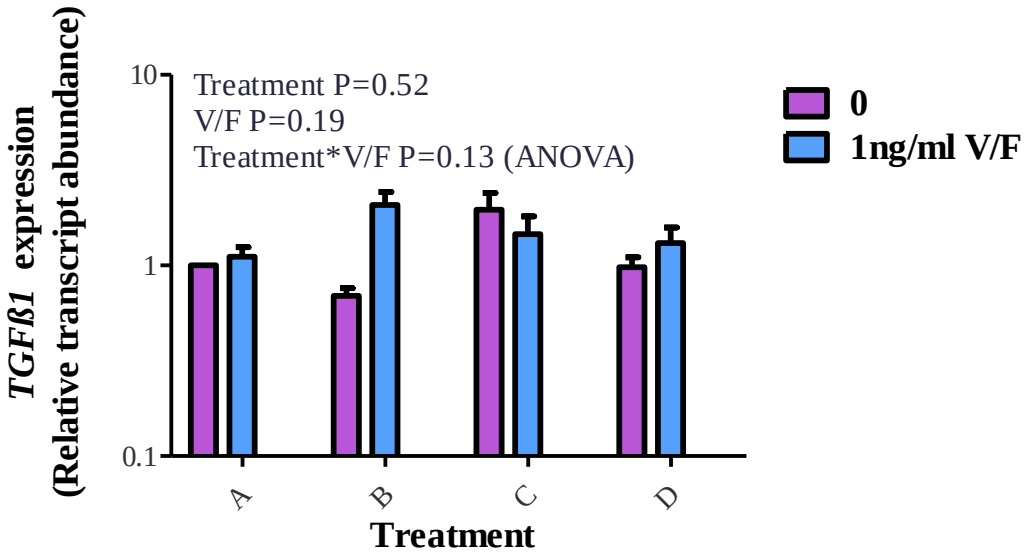


Figure 6. 29 The expression of *TGFβ1* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.10. TGFβ2

The expression of *TGFβ2* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P=0.0153) among different treatments (Figure 6.30). However, there was no significant effect of V/F, or interaction between treatments and V/F. TGF-β-1 inhibitor alone decreased expression of *TGFβ2* under both basal and V/F induced conditions (P=0.0397), and under basal conditions, this effect was reversed by TGF-β-1 (P=0.0020).

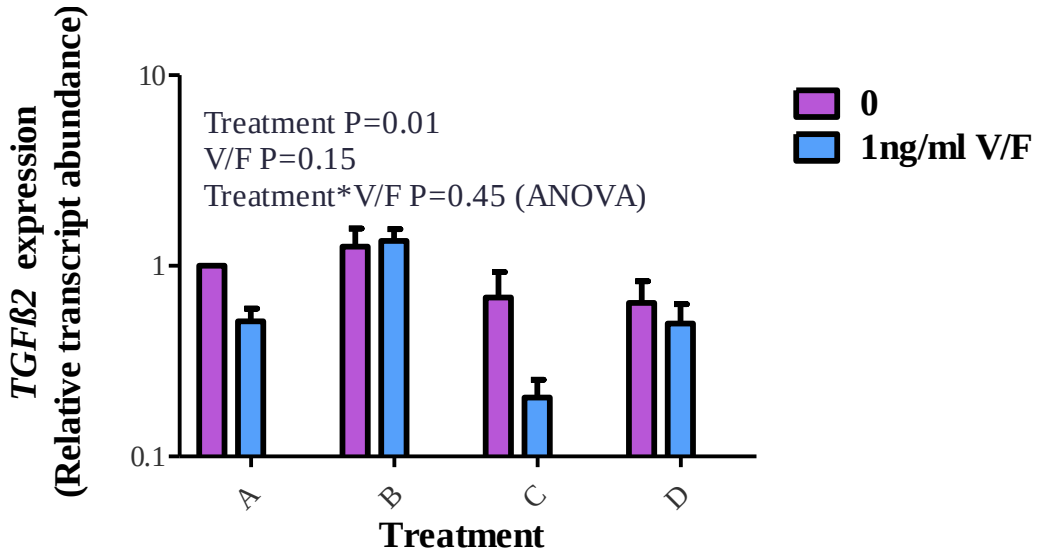


Figure 6. 30 The expression of *TGFβ2* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.11. TGFβ3

The expression of *TGFβ3* was not affected by TGF-β-1 and its inhibitor alone and in combination, nor by V/F treatment (Figure 6.31).

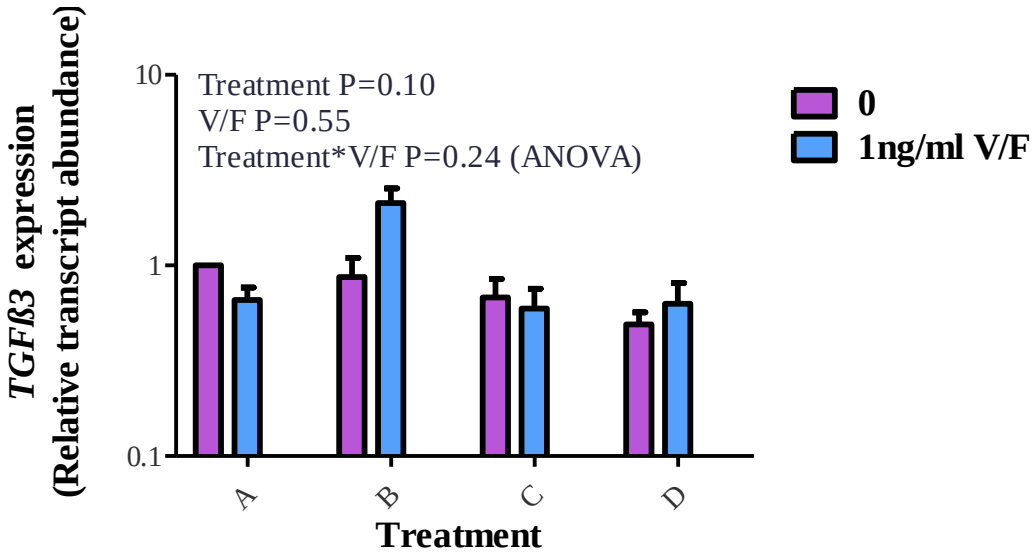


Figure 6. 31 The expression of *TGFβ3* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.12. FGFR

The expression of *FGFR* was not affected by TGF-β-1 and its inhibitor alone and in combination, nor by V/F treatment (Figure 6.32).

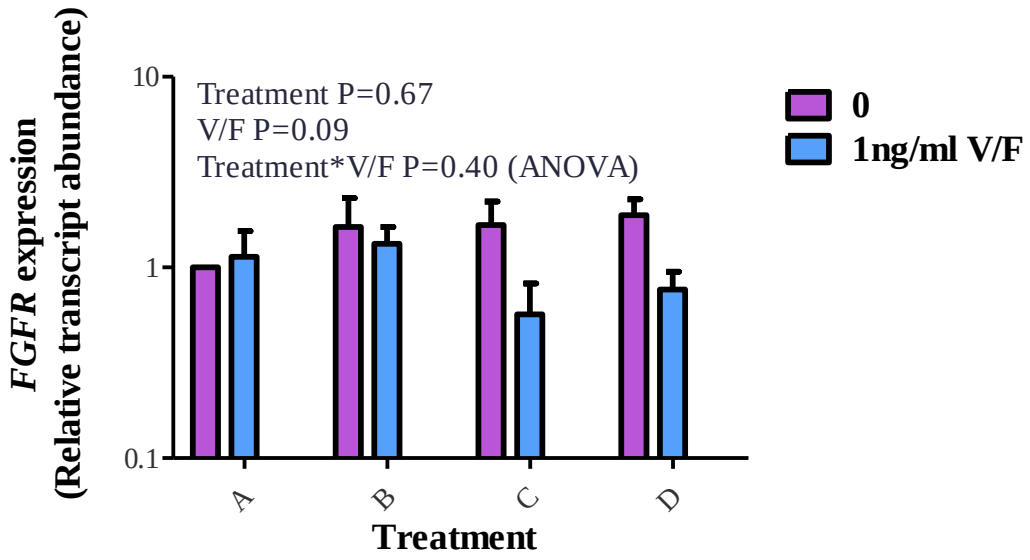


Figure 6. 32 The expression of *FGFR* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2μM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2μM of TGF-β-1 antagonist).

6.4.8.13. VEGFR

The expression of *VEGFR* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination varied significantly (P=0.0138) among different treatments under basal and V/F induced conditions (Figure 6.33). Although, there was a significant effect of V/F on *VEGFR* expression (P=0.0432), the interaction between treatments and V/F was not significant. It can be seen that TGF-β-1 alone increased significantly the expression of *VEGFR* compared to controls under both basal and V/F induced conditions (P=0.0033). However, expression of *VEGFR* was also increased by TGF-TGF-β-1 inhibitor alone and in combination with TGF-β-1 under both basal and V/F induced conditions (P=0.0078).

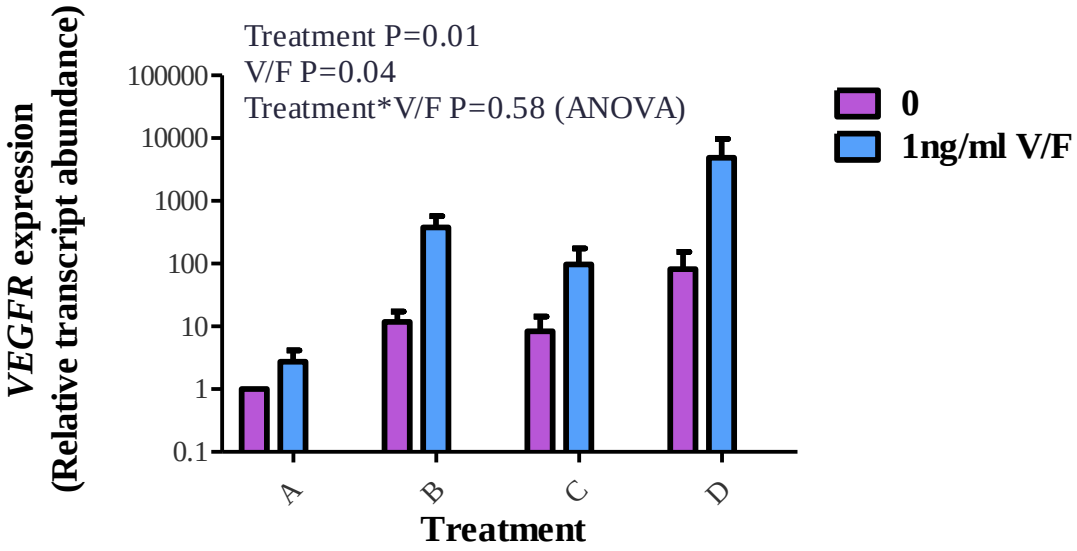


Figure 6. 33 The expression of *VEGFR* in cultured cells treated with TGF-β-1 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of TGF-β-1 only), C (2µM of TGF-β-1 antagonist only) and D (5ng/ml of TGF-β-1 + 2µM of TGF-β-1 antagonist).

6.4.9. The expression of steroidogenic and other transcripts in cultured theca interna cells treated with BMP-6 and its inhibitor

6.4.9.1. StAR

The expression of *StAR* in cultured cells treated with BMP-6 and its inhibitor alone and in combination varied significantly (P=0.0019) among different treatments under basal and V/F-induced conditions (Figure 6.34). However, there was no significant effect of V/F treatment on *StAR* expression; nor was there a statistical interaction between treatment and V/F. BMP-6 inhibitor alone significantly increased *StAR* expression compared to controls under both basal and V/F-induced conditions (P=0.0074). BMP-6 reversed the increase in *StAR* expression induced 10µM of BMP-6 inhibitor (P=0.0218).

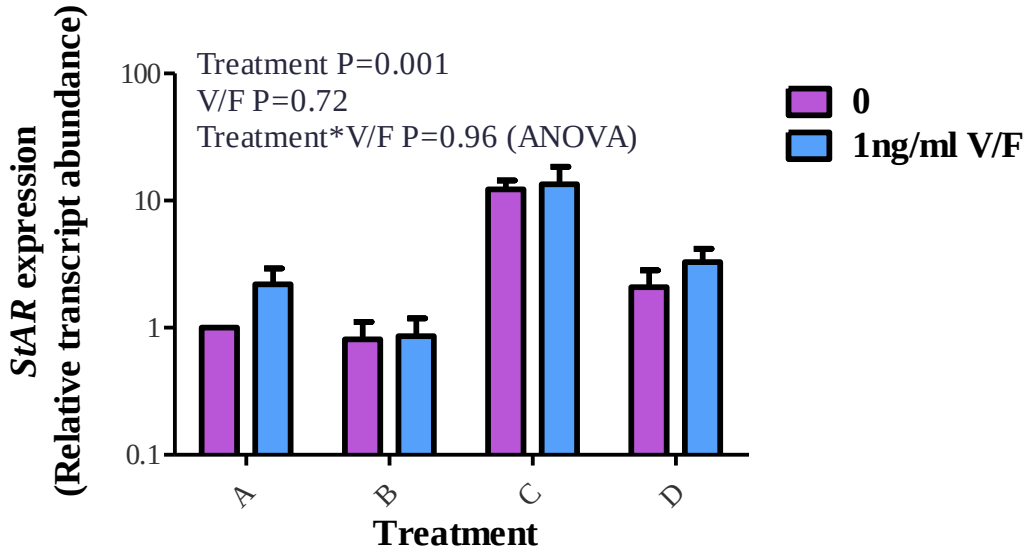


Figure 6. 34 The expression of *STAR* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.2. CYP11A1

The expression of *CYP11A1* in cultured cells treated with BMP-6 and its inhibitor alone and in combination varied significantly (P=0.0013) among different treatments under basal and V/F-induced conditions (Figure 6.35). However, there was no significant effect of V/F treatment on *CYP11A1* expression; nor was there as statistical interaction between treatment and V/F. There was a significant BMP-6- induced decrease compared to control (P=0.0089). BMP-6 inhibitor alone increased significantly the expression of *CYP11A1* compared to BMP-6 only (P=0.0001). BMP-6 in combination with BMP-6 inhibitor decreased significantly the expression of *CYP11A1* compared to BMP-6 inhibitor treatment only (P=0.0342).

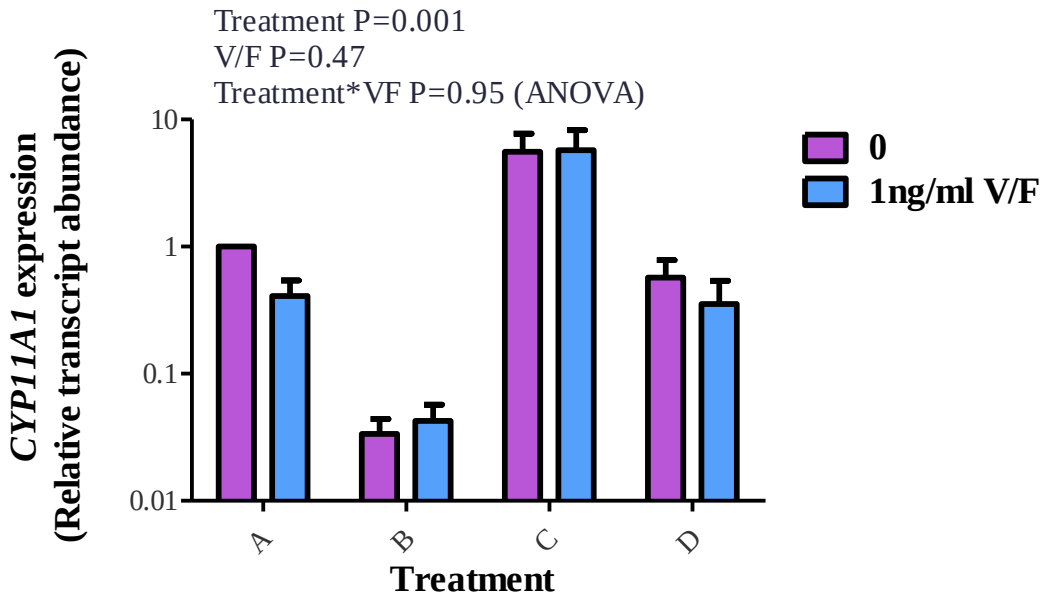


Figure 6. 35 The expression of *CYP11A1* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.3. CYP17A1

The expression of *CYP17A1* in cultured cells treated with BMP-6 and its inhibitor alone and in combination varied significantly (P=0.0349). However, neither V/F alone nor the interaction between the V/F and treatments showed a significant effect (Figure 6.36). There was a significant increased in response to BMP-6 inhibitor in both basal and V/F-induced conditions (P=0.0486). Also, BMP-6 reversed the stimulatory effect of BMP-6 inhibitor on *CYP17A1* expression under both basal and V/F-induced conditions (P=0.0056).

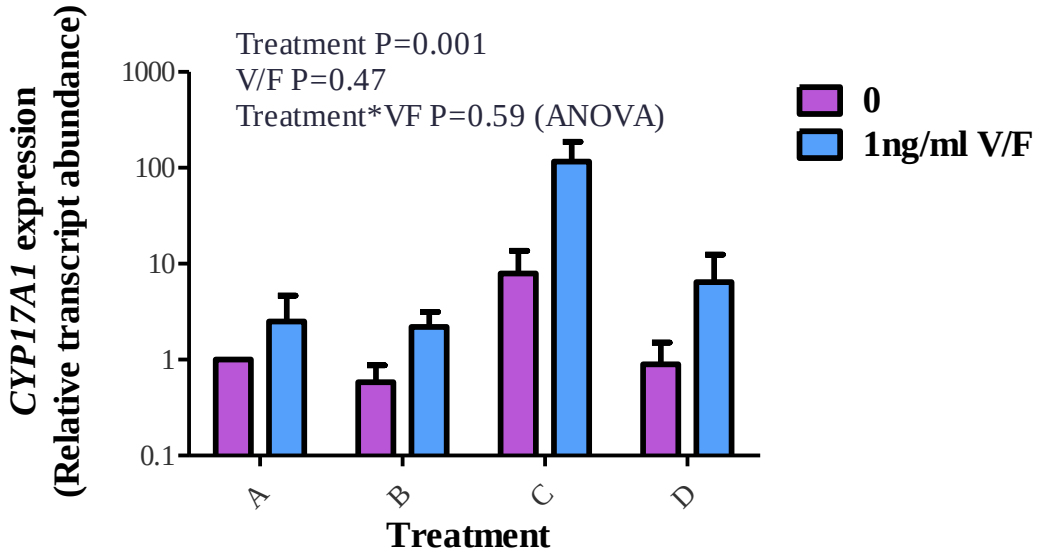


Figure 6. 36 The expression of *CYP17A1* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.4. HSD3B1

The expression of *HSD3B1* in cultured cells treated with BMP-6 and its inhibitor alone and in combination varied significantly (P=0.0016) in both basal and V/F-treated cells (Figure 6.37). However, there was no significant effect of V/F or the interaction between treatments and V/F. BMP-6 inhibitor alone compared to control increased significantly the expression of *HSD3B1* in both basal and V/F-treated cells (P=0.0080). Also, BMP-6 inhibitor alone increased expression of *HSD3B1* compared to BMP-6 alone under basal and V/F-induced conditions (P=0.0010).

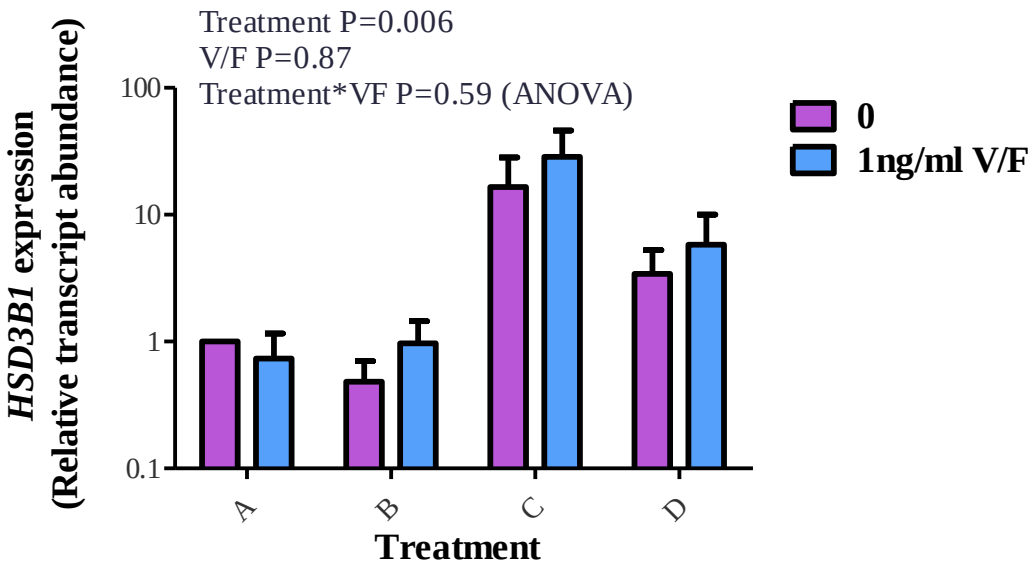


Figure 6. 37 The expression of *HS*D3*B*1 in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.5. LHR

The expression of *LHR* in cultured cells treated with BMP-6 and its inhibitor alone and in combination varied significantly (P<0.0001) among different treatments but there was no significant effect of V/F treatments, or the interaction between treatments and V/F (Figure 6.38). BMP-6 significantly decreased LHR expression under V/F-stimulated conditions (P=0.0029). BMP-6 inhibitor increased significantly the expression of LHR and this effect was reversed by BMP-6 co-treatment (P=0.0270).

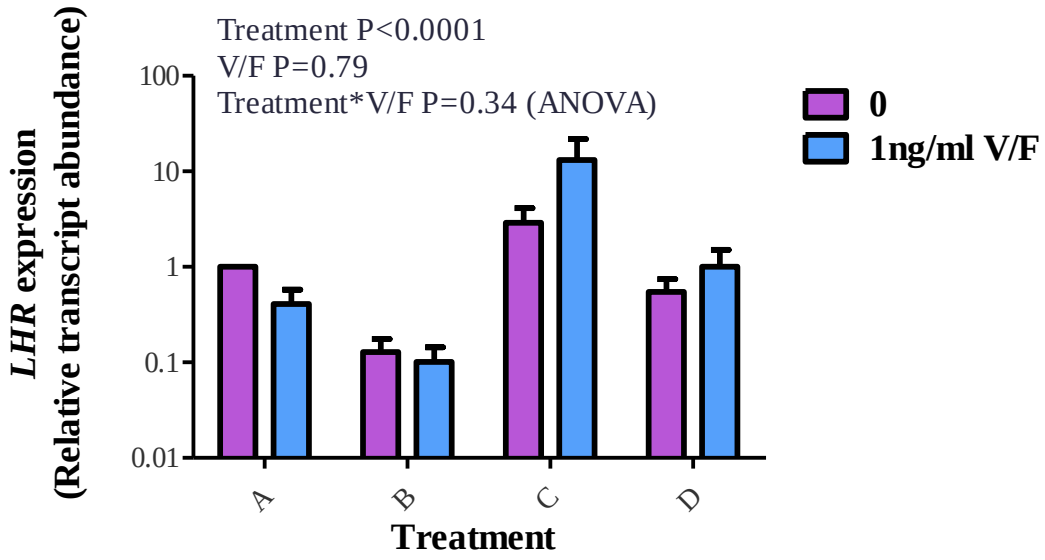


Figure 6. 38 The expression of *LHR* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.6. INSL3

The expression of *INSL3* in cultured cells treated with BMP-6 and its inhibitor alone and in combination varied significantly (P<0.0001) among different treatments but there was no significant effect of V/F treatment, or interaction between treatments and V/F (Figure 6.39). BMP-6 inhibitor alone increased expressions of *INSL3* compared to controls under basal and V/F-induced conditions (P=0.0019). Also, there was a significant increased with BMP-6 inhibitor under both basal and V/F-induced conditions (P<0.0001). BMP-6 inhibitor also reversed the suppressive effect of BMP-6 under basal and V/F-induced conditions (P=0.0002).

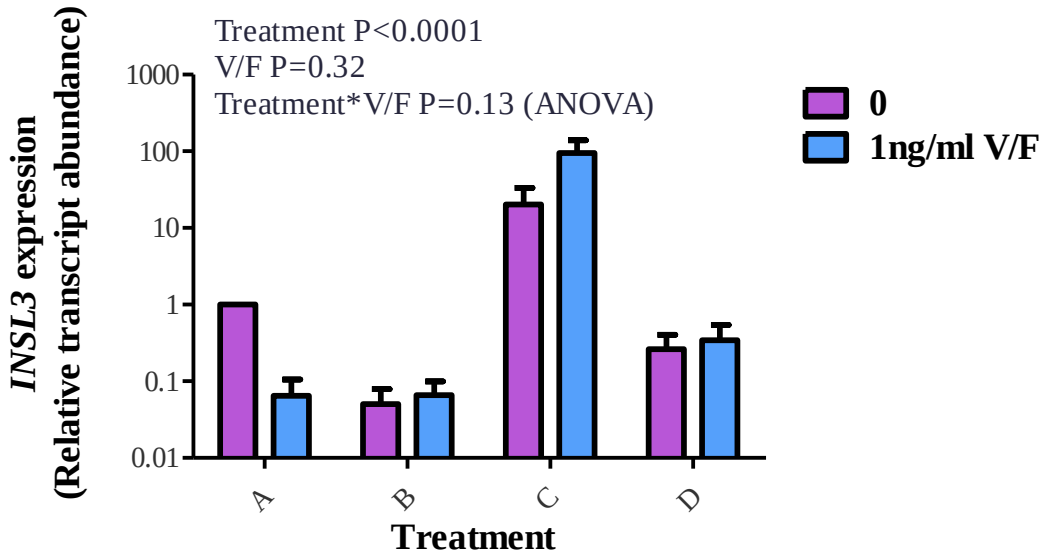


Figure 6. 39 The expression of *INSL3* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.7. INHA

The expression of *INHA* was not effected by BMP-6 and its inhibitor alone and in combination, nor by V/F treatment (Figure 6.40).

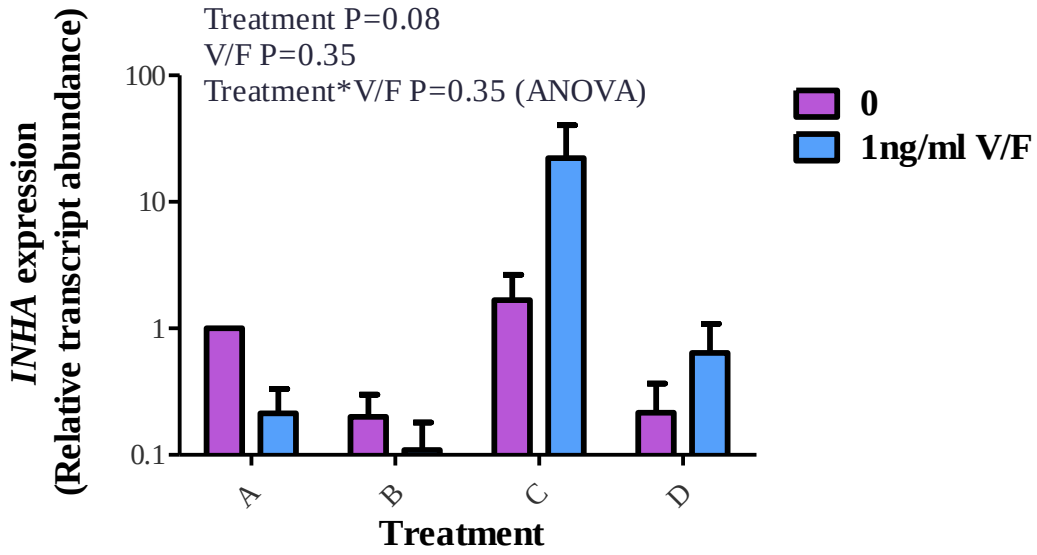


Figure 6. 40 The expression of *INHA* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.8. NR5A1

The expression of *NR5A1* was not effected by BMP-6 and its inhibitor alone and in combination, nor by V/F treatment (Figure 6.41).

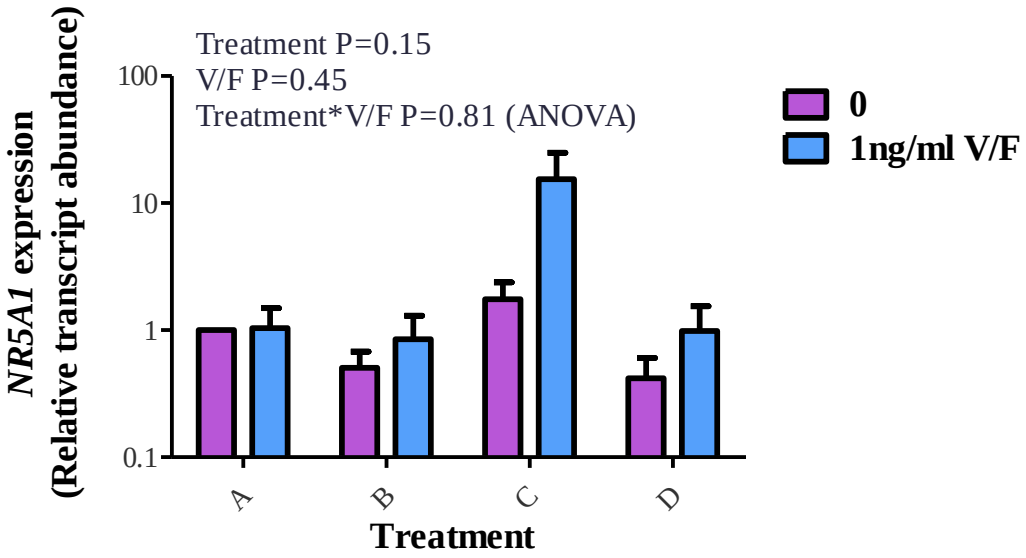


Figure 6. 41 The expression of *NR5A1* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.9. FGFR

The expression of *FGFR* was not effected by BMP-6 and its inhibitor alone and in combination, nor by V/F treatment (Figure 6.42).

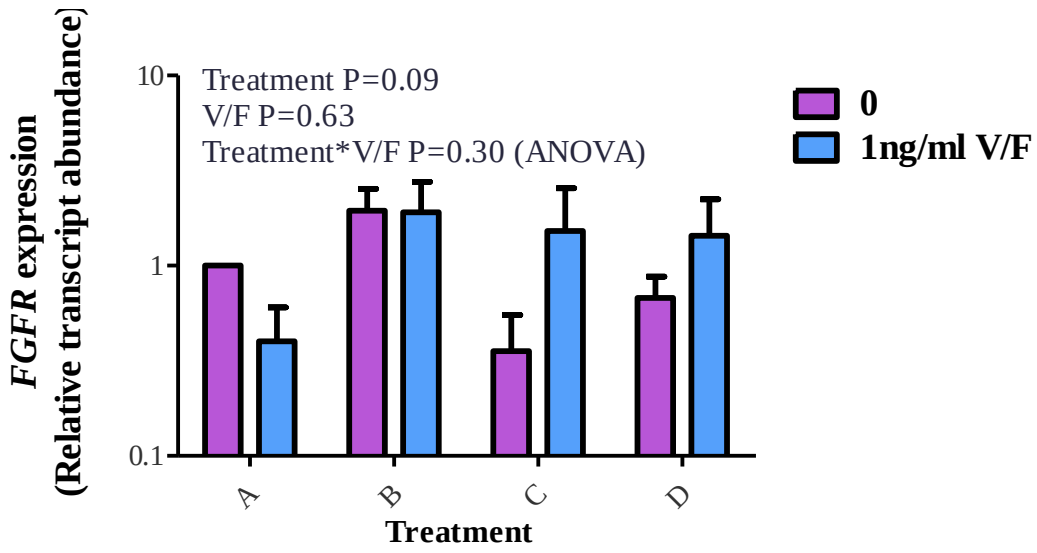


Figure 6. 42 The expression of *FGFR* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.4.9.10. VEGFR

The expression of *VEGFR* in cultured cells treated with BMP-6 and its inhibitor alone and in combination varied significantly (P=0.017) among different treatments under basal and V/F-induced conditions (Figure 6.43). Although, there was a significant effect of V/F on *VEGFR* expression (P=0.0180), the interaction between treatments and V/F was not significant. It can be seen that BMP-6 inhibitor alone increased significantly the expression of *VEGFR* compared to controls under both basal and V/F-induced conditions (P=0.0448). However, expression of *VEGFR* was also increased by BMP-6 inhibitor alone and in combination with BMP-6 under both basal and V/F-induced conditions (P=0.0006).

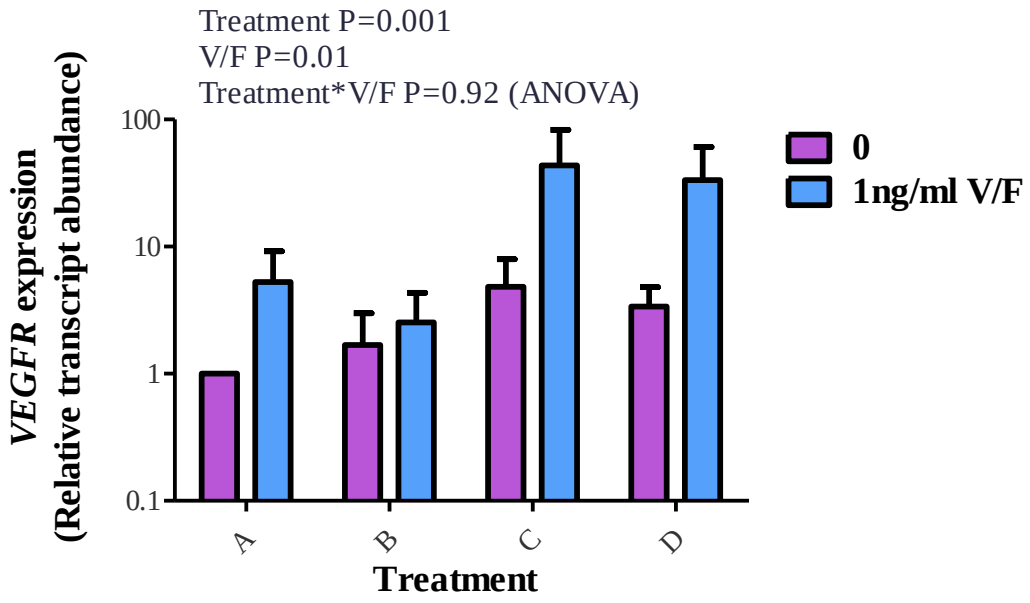


Figure 6. 43 The expression of *VEGFR* in cultured cells treated with BMP-6 and its inhibitor alone and in combination. Values are means ±SEM (n=5 independent batches of cells) and two-way ANOVA results are shown. *A (control), B (5ng/ml of BMP-6 only), C (10μM of BMP-6 antagonist only) and D (5ng/ml of BMP-6 + 10μM of BMP-6 antagonist).

6.5. Discussion

This chapter documents findings on the role of various peptides including NMB, kisspeptin-10, TGF-β-1, BMP6 and TSP-1 in modulating basal and FGF/VEGF-induced ovarian angiogenesis. Although some of the peptides were not of bovine source/origin, they either shared identical amino acid sequences or were close orthologs of the same biologically active peptides in cattle.

The most exciting finding from this chapter was that the ability of endothelial cells from the theca interna layer of follicles to re-assemble and form capillary-like networks *in vitro*. The experimental culture system involved seeding collagenase-digested theca interna layer, containing steroidogenic cells as well as endothelial cells, in monolayer culture. In order for the endothelial cells network to form, a commercial endothelial cell growth supplemented medium with various proprietary factors was utilized.

In vivo, ovarian angiogenesis takes place concurrently with folliculogenesis and continues through follicle growth and luteinization (Brown and Russell, 2013). It is well established that during development of the bovine CL, both angiogenesis and the synthesis of progesterone are enhanced, whereas during subsequent CL regression degeneration of the vascular bed is accompanied by a decrease of progesterone secretion initiated by the luteolytic action of prostaglandin F2α (PGF2α). In CL, the tissue growth rate and angiogenesis rate are comparable to those observed in rapidly growing tumors. VEGF and bFGF are considered to be major angiogenic factors amongst several factors produced by bovine CL (Shirasuna et al., 2009). Research exploring the regulatory roles of different factors in the control of ovarian angiogenesis has increased significantly in the past decade. While both VEGF and FGF undoubtedly have important roles, there are likely many other factors that play integral roles in vascular remodeling involved in follicle growth and CL formation/regression.

6.5.1. The role of neuromedin B in modulating basal and FGF/VEGF-induced ovarian angiogenesis

As it is previously mentioned, the neuropeptides of the bombesin family involves amphibian bombesin and two mammalian bombesin-like peptides, GRP and NMB (Erspamer, 1988; Minamino et al., 1983). The action of theses peptides is achieved by the specific binding to their respective membrane-bound receptors in an autocrine, paracrine or endocrine context. Boughton et al (2013) showed that NMB stimulates the reproductive axis through the IVC injection that significantly increased plasma LH level in male rats. Also NMB increased GnRH release from hypothalamic explants and GT1-7 cells *in vitro*. It has been reported that most of bombesin family members bind in an autocrine manner as a growth factors to stimulate directly the proliferation of various human cancer cells. Also bombesin and GRP have a role in the regulation of tumor growth through paracrine stimulation of angiogenesis. However, the potential stimulatory effect of NMB on endothelial angiogenesis has not been fully characterized (Park et al., 2009). Park et al (2009) demonstrated that NMB has a novel role as being a pro-angiogenic factor. However, potential physiological functions of NMB and NMBR in the bovine ovary remain unknown. It has been proposed that the binding between NMB and it receptor activates multiple intracellular pathways in various cell lines (Ohki-Hamazaki et al., 2003). Some studies have shown that NMB and NMBR activate MEK/ERK 1/2 PI3K/Akt pathways, which are related to cell proliferation, survival, angiogenesis and tumorigenesis. Also, it has been observed that NMB enhances the capillary-like network formation of HUVECs and sprouting from aortic rings; whereas a potent selective NMB receptor antagonist completely blocked this action (Charlesworth and Rozengurt, 1997; Park et al., 2011; Park et al., 2009). In the current study, the expression levels of *NMB* and *NMBR* mRNA were detected in different bovine endocrine tissues including ovarian bovine tissues. In the light of these findings, we hypothesized an intrafollicular role(s) of NMB in modulating cell proliferation and angiogenesis *in vitro*. Despite the evidence that NMB is a pro-angiogenic factor, no effect of either NMB agonist or antagonist on tube formation was observed when results from six replicate experiments using independent batches of cells were combined. However, VEGF/FGF mixture had a marked stimulatory effect, being capable of

promoting capillary-like tubular structures in the cultured theca internal model. This confirms previous findings based on a bovine CL angiogenesis culture model (Woad et al., 2012).

6.5.2. The role of kisspeptin in modulating basal and FGF/VEGF-induced ovarian angiogenesis

Another neuropeptide implicated in the regulation of reproduction is kisspeptin, which has a central role in the regulation of GnRH and gonadotrophin secretion. It is well known that kisspeptin has a potent inhibitory role in tumour metastasis and placentation, which both involve intense angiogenesis (Martino et al., 2015). It has been published that kisspeptin-10 significantly inhibited angiogenesis in placental vasculature and tube-like structure formation of HUVEC (Ramaesh et al., 2010). According to this study, kisspeptin receptor was found in in the placental vascular wall and in endothelial cells of the umbilical cord of human. Ex vivo techniques were used as a model for angiogenesis and demonstrated that kisspeptin-induced new vessels sprouting from the placental artery. Thus, a physiological role of kisspeptin in placental angiogenesis was suggested. Multiple processes are involved in angiogenesis including endothelial cell proliferation, migration and tube formation. This has been investigated using *in vitro* model to define the role of kisspeptin-10 in endothelial cells angiogenesis. According to Ramaesh at al (2010), results showed that the morphogenetic differentiation of HUVEC leading to tube formation was inhibited by kisspeptin-10 in a concentration dependent manner. It also inhibited their in vitro proliferation and migration without affecting their viability or apoptosis (Ramaesh et al., 2010). Another study showed that, the expression level of a proangiogenic VEGF-A was down-regulated by kisspeptin in human trophoblast cells. This suggested that kisspeptin-induced VEGF-A inhibition could adversely affect angiogenesis (Francis et al., 2014). We, therefore, hypothesised that kisspeptin-10 plays a role in regulating ovarian follicular angiogenesis and aimed to examine its effects *in vitro* on key angiogenic steps. However, the combined results of three replicate experiments using independent batches of cells led us to reject the hypothesis as neither kisspeptin-10 nor its antagonist had any modulatory effect on tube

formation despite our finding (chapter 3) that kiss-1 and its receptor are indeed expressed in bovine endocrine tissues including the ovary.

6.5.3. The role of TGF-β in modulating basal and FGF/VEGF-induced ovarian angiogenesis

6.5.3.1. TGF-β family

A growing body of evidence suggests that numerous growth factor peptides have wide-ranging roles on different tissue and organ systems including the ovary. Among these peptides are member of the transforming growth factor-β (TGF-β) superfamily. Various TGF-β superfamily member are expressed in ovarian tissues including granulosa and theca cells and have their roles as intraovarian regulatory molecules in follicle recruitment, proliferation/atresia of granulosa and theca cells, steroidogenesis, oocyte maturation, ovulation and luteinisation (Knight and Glister, 2003; Knight and Glister, 2006). The actions of TGF-β superfamily ligands are accomplished via the binding to specific transmembrane kinase receptors type I and type II Ser/Thr. The differentiation between the two receptors is that type I receptors activate downstream of type II receptors and control the signaling specificity in the receptor complex. The binding between ligand/type II receptor lead to the formation of a heteromeric complex, that transphosphorylates and activates the type I receptor, which consequently direct the signal via phosphorylating specific receptor-regulated (R-) SMAD transcription factors at the two C-terminal Ser residues (Figure 6.44). Upon the activation, heteromeric complexes formed via R-SMADs bind to release partner molecules, the Co-SMAD (SMAD4 in mammals), and accumulate in the nucleus where they contribute in the transcriptional control of target genes. Although, there are over 30 TGF-β family members with diverse roles in mammals, there is a massive convergence in signaling to only five type II receptors, seven type I receptors (also defined as activin-receptor-like kinases; ALKs) and two main SMAD intra cellular pathways (Ten Dijke and Arthur, 2007; Massague and Gomis, 2006; Feng et al., 2005).

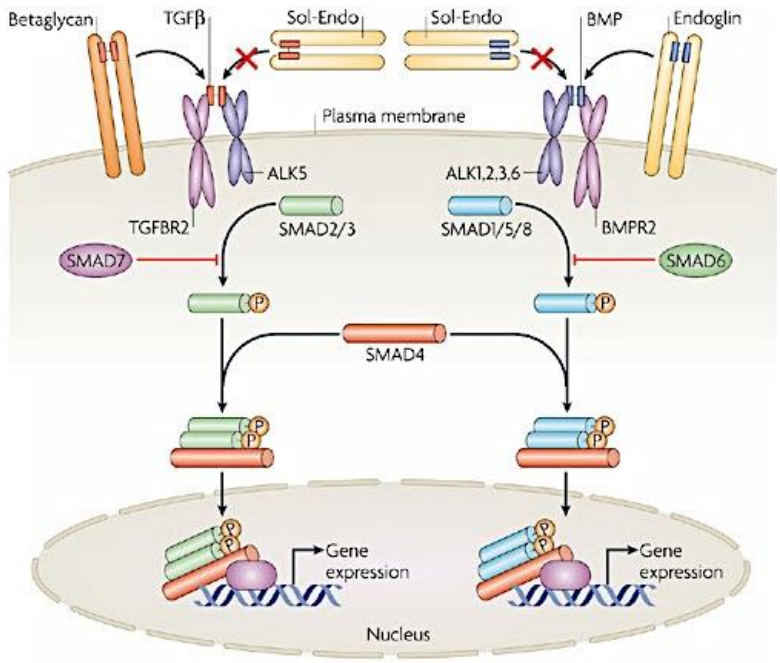


Figure 6. 44 Signal transduction by TGF superfamily members. The signaling pathways of transforming growth factor (TGF) super family divided into two main pathways according to the SMAD mediators: either SMAD2/3 or SMAD1/5/8. The TGF family members bind to specific Ser/Thr kinase type II and I receptors; in most cells, TGF signals via TGF-βR2 and ALK5 (also identified as TGF receptor-1; TGF-βR1), and bone morphogenetic proteins (BMPs) signal via the BMP type II receptor (BMPR2) and ALK1, -2, -3 and -6. The other receptors betaglycan and endoglin can regulate signaling through the type II and type I receptors. Betaglycan stimulates the binding of TGF2 to TGF receptors, while endoglin may act to achieve a similar role for particular TGF family members and their receptors. Soluble endoglin (Sol-Endo) is supposed to sequester ligand and thus inactivates receptor binding; whereas, the exact pathway where this occurs is controversial as endoglin associates with TGF-βR2 for TGF binding (Venkatesha et al., 2006; Barbara et al., 1999). The stimulated type I receptors lead to the phosphorylation of specific receptor regulated (R-) SMADs, which are the intracellular regulators of TGF members. Activated type I receptors induce the phosphorylation of specific receptor regulated (R-) SMADs, which are the intracellular effectors of TGF family members. Among most of cell types, TGF stimulates SMAD2/3 phosphorylation and BMPs stimulate SMAD1/5/8 phosphorylation. Activated R-SMADs form heteromeric complexes with SMAD4 that accumulate in the nucleus, where they control the expression of target genes

such as SERPINE1 (plasminogen activator inhibitor) and ID1 (inhibitor of DNA binding-1) in cooperation with transcription factors, co-activators and co-repressors1, 2 (Feng et al., 2005; Massague and Gomis, 2006). Inhibitory SMADs, including SMAD6 and SMAD7, can antagonize TGF signaling by inhibiting the activation of R-SMADs (Feng et al., 2005).

6.5.3.2. TGF-β-1

This chapter will also discuss findings relating to the potential intraovarian roles of TGF-β superfamily members (TGF-β-1 and BMP6) in bovine ovarian angiogenesis. In angiogenesis, the role of TGF-β ligands raises the question of which ligand isoforms bind to TGF-β receptors to regulate new vessel formation. TGF-β-1 is one of the three mammalian isoforms; it is localized to endothelial cells during embryogenesis, suggesting it is the most likely of the three isoforms to be involved in angiogenesis (Akhurst et al., 1990). TGF-β-1 was therefore studied as being a potent stimulus for the proliferation and differentiation of endothelial cells, formation of vascular bed and maintaining the integrity of vessel walls. In the vascular biology field, the recognition and identification of mutations of TGF-β receptor genes has revealed links with hereditary vascular pathologies (Ten Dijke and Arthur, 2007). It is evident that dysregulation of TGF-β-1 signaling mechanisms contributes to the pathology of fibrosis, neointima formation and cancer progression and metastasis (Peshavariya et al., 2014).

Our data confirmed that TGF-β-1 induced a dose dependant inhibition of basal and VEGF/bFGF-induced endothelial network formation, an action that was reversed by a TGF-β-1 inhibitor. Moreover, since treatment with the TGF-β-1 inhibitor alone increased endothelial cell network formation, this suggests neutralisation of an endogenous ligand, perhaps TGF-β-1 itself that was shown to be expressed by the cultured cells. However, expression of other ligands including TGF-β-2 and TGF-β-3 was also detected so it is possible that multiple endogenous TGF-β ligands contribute to the suppression of endothelial cell network formation. Further work to explore this possibility could use an RNA interference approach to selectively knockdown individual ligands and determine how this affects endothelial network formation in this *in vitro* model. Despite the

above finding, some *in vitro* studies demonstrated that low extracellular TGF-β-1 levels evoke the proliferation and migration of cells that promote proliferation of new vessels in angiogenesis (Lebrin et al., 2004). On the other hand, high concentrations of extracellular TGF-β-1 have been associated with cytostasis and synthesis of extra cellular matrix proteins that regulated mature or differentiating vessels. It is also known that TGF-β family members can function in a paracrine manner by activating the production of pro-angiogenic cytokines, including (VEGF), TGF-α and monocyte chemo-attractant protein-1 (MCP1) (Vinals and Pouyssegur, 2001; Deckers et al., 2002; Ma et al., 2007). Additionally, TGF-β family members may regulate the function of other factors such as switching the VEGF pro-survival function into an apoptotic factor for endothelial cells (Ferrari et al., 2006; Ten Dijke and Arthur, 2007).

It is acknowledged that TGF-β, through its surface receptor signaling, can exert a dual role in either enhancing pro-angiogenic activity or inhibiting vascular angiogenesis mechanisms (Pepper et al., 1993; Mustafa et al., 2012). Interestingly, TGF-β-1 along with VEGF acts to modulate apoptosis mechanisms preventing excessive vascular sprouts and can even be responsible for initial developed sprouting from an existing vascular bed (Ferrari et al., 2006; Ferrari et al., 2012). The classic angiogenic response pathway to TGF-β relies on the balance between ALK1 and ALK5 signaling input. The nature function of ALK1 is predominantly to stimulate sprouting while ALk5 favors the resolution/stabilization stage of angiogenesis (Holderfield and Hughes, 2008). Consequently, TGF-β can either be pro- or anti-angiogenic according to which TGF-RII/Smad mechanism is involved (Kumar et al., 2014). It has been reported that TGF-β-1 (5ng/ml) has an inhibitory effect in bovine endothelial cord formation via modulating endothelial angiogenic receptor expression in an ALK5 dependant fashion *in vitro*. Thus, this inhibitory effect occurred via significant upregulation of the TGF-β accessory receptor endoglin, and SMAD2 phosphorylation without altering Smad1/5 activation pathway. Our result is in agreement with this study (Jarad et al., 2017) and shows that TGF-β-1 dose dependently induced the inhibition of endothelial cells network formation under both basal conditions and in the presence of the known pro-angiogenic factors VEGF and bFGF. It has been suggested that the down regulation of cell surface

VEGFR2 and concomitant upregulation of secreted VEGFR2 levels in endothelial cell-conditioned medium can be a direct response to ALK5-mediated TGF-β signaling (Jarad et al., 2017).

6.5.4. The effect of TGF-β-1 on the expression of steroidogenic transcripts and productions of steroids

Steroid hormone biosynthesis occurs mainly in the adrenal cortex, testis, ovary and placenta (Bhangoo et al., 2006). As is well known, the first stage of the synthesis of steroids is the conversion of cholesterol to pregnenolone via cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme that is encoded by the *CYP11A1* gene. Then, 3β-hydroxy steroid dehydrogenase/Δ^5-Δ^4 isomerases 3β -HSD which is encoded by *HSD3B* genes metabolized pregnenalone to progesterone. The rate-limiting of steroidgenesis is the transportation of cholesterol from the outside of the mitochondrial membrane to the inside where P450scc resides. This stage is regulated by the steroidogenic acute regulatory protein (StAR) which is encoded by the *STARD1* gene (Clark et al., 1994; Stocco and Clark, 1996). The steroid synthesis stimuli provoke rapidly the expression of StAR which consequently catalyzes intermembrane delivery of cholesterol to P450scc and thereby the steroidogenesis pathway is initiated (LaVoie and King, 2009).

There is growing evidence that locally secreted growth factors alone or in combination with gonadotropins function to regulate the production of steroids in theca cells (Ruutiainen and Adashi, 1993; Young and McNeilly, 2012; Knight and Glister, 2014). TGF-β superfamily proteins particularly TGF-β-1 and BMPs appear to modulate theca cells steroid production (Sawetawan et al., 1996). Ovarian TGF-β-1 expression has been reported in many different species including human, rodents, sheep, pigs and cows; it is mainly produced by theca cells (Juengel et al., 2004). It has been reported that TGF-β-1 dose dependently inhibits the production of androstenedione and increases progesterone production in human ovarian theca like tumor cells via the downregulation of 17-α-hydroxylase (CYP17A1) expression (Carr et al., 1996). Previously, TGF-β-1 was proven to downregulate steroidgenic acute regulatory protein (StAR) expression in a human adrenocortical carcinoma cell line (Brand et al., 1998). The effect of

TGF-β-1 on StAR is significant as StAR is a regulatory protein engaged in the transportation of cholesterol from the external to the internal membrane of the mitochondria in the adrenal and gonads where it is converted to pregnenolone (Sugawara et al., 1995; Stocco et al., 1996). The conversion of cholesterol to pregnenolone is a rate limiting stage in steroidogenesis as StAR expression is critical in this stage. Thus, the regulation of StAR protein expression mechanism is of primary importance. The coloclization of TGF-β-1 and StAR in theca cells indicates that TGF-β could regulate the expression of StAR.

In the present study, we also investigated the effect of TGF-β-1 on the production of steroids and expression of steroidogenesis-related genes in our bovine theca interna angiogenesis model. Our observation appeared to conflict with the previously mentioned studies as TGF-β-1 along with the angiogenic factors decreased significantly the production of progesterone but did not affect androstenedione. This suppression of progesterone secretion was reversed by a pharmacological inhibitor of TGF-β-1 action. Moreover, treatment with the inhibitor alone increased significantly both androstenedione and progesterone production, implying a tonic suppressive action of endogenous TGF-β ligands on steroidogenesis. This was supported by the finding that expression of several key steroidogenesis-related transcripts, including *StAR*, *CYP11A1*, *HSD3B1* and *CYP17A1* was increased in response to the pharmacological inhibitor alone. The effect of TGF-β-1 on steroidogenic gene expression was less clear and appeared to vary according to whether the cells were co-treated with the angiogenic (V/F). In the presence of V/F, TGF-β-1 treatment reduced *CYP11A1*, *HSD3B1* and *LHR* expression about 10-fold and this matched the approximate 10-fold reduction in progesterone secretion under the same conditions. These effects were all reversed by the pharmacological inhibitor, offering further evidence that the responses to TGF-β-1 were specific. In the absence of V/F, only marginal changes in progesterone secretion and steroidogenic gene expression were observed in response to TGF-β-1. This suggests an interaction between TGF-β, VEGF and/or bFGF signalling pathways involved in the regulation of endothelial network formation. A preliminary attempt to investigate this was made by examining whether TGF-β-1 and the pharmacological inhibitor affected expression of

bFGFR or VEGFR. While there was no effect on bFGFR expression, TGF-β-1 elicited a marked (>10-fold) increase in VEGFR expression suggesting a potential upregulation of VEGF signalling. However, this effect was not reversed by the pharmacological inhibitor which actually increased VEGFR expression further. Further experiments, beyond the scope of the present study, are needed to unravel these potentially complex interactions.

6.5.5. The role of BMP in modulating basal and FGF/VEGF-induced ovarian angiogenesis

6.5.5.1. BMPs

Other important member of the TGF-β superfamily include the BMPs, which play an essential role in the stimulation of osteoblast differentiation, leading to the differentiation of progenitor cells into chondrocytes and osteoblasts, endochondral ossification and bone formation (Liao et al., 2018). BMPs play numerous functions including the regulation of growth, differentiation and apoptosis of multiple cell types. It has been reported that various BMPs are associated with ovarian follicular development as being autocrine/paracrine regulators of cell proliferation and steroidogenesis (Elvin et al., 1999; Shimasaki et al., 1999; Knight and Glister, 2006). Similarly to other TGF-β superfamily members, BMPs signal by binding to two types of receptors on the cell surface namely serine/threonine kinase receptor (type-I and type-II) and forming heteromeric complexes with the common partner Smad-4 (Massague and Chen, 2000; Miyazono et al., 2000; Miyazawa et al., 2002). A type-I receptor is transphosphorylated via the type-II recptor, which consequently stimulates transcripitional regulators termed Smads, which regulate gene expression through transducing the signal to the nucleus. The activation of BMPs can be achieved through the binding to one of three type-II receptors including (BMPRII, activin receptor ActRIIA or ActRIIB) and one of three type-I receptors (BMPRIA, BMPRIB or ActRIA). Note that these BMP type I receptors are also referred to as activin receptor-like kinase (ALK) 3, ALK 6, and ALK2, respectively. The specificity of the signal transduction in terms of which Smad pathway is triggered is mainly depended on the type-I receptor engaged (Macias-Silva et al., 1998; Ebisawa et al., 1999; Valdimarsdottir et al., 2002). Smad-1, -5 and -8 pathways

are activated by BMPs while Smad-2 and -3 pathways are activated via activin and TGF-β (Miyazono et al., 2000; Miyazawa et al., 2002).

6.5.5.2. BMP-6

Previous *in vivo* and *in vitro* studies in mice and human reported that BMP receptor/Smad activation stimulated endothelial cells migration and tube formation. Consequently, a role for BMPs in regulating the organization and differentiation of the newly formed capillary network from endothelial cells was indicated. It has been suggested that the activation of endothelial cells through BMP/Smad was critically reliant on BMP/Smad-stimulated up-regulation of the target gene Id1 that is involved in angiogenesis. This finding suggested that BMPs are potent proangiogenic factors. Also, they reported that BMP-6 induced phosphorylation of Smad-1, Smad-5, and/or Smad8 in endothelial cells. However, this mechanism was distinct from that of TGF- β , which stimulated phosphorylation of Smad-2 and Smad-5 through ALK5 and ALK1 respectively, and of activin which stimulated only the phosphorylation of Smad2 that was possibly regulated through ALK4. The kinetics of TGF-β – stimulated versus BMP-stimulated Smad-5 phosphorylation is different; TGF- β -stimulated phosphorylation of Smad-5 is transient, while BMP-6-stimulated phosphorylation of Smad-5 is very stable. It is known that BMP-6 and other related family members are expressed by vascular system cells including endothelial cells and smooth muscle cells that suggested their stimulatory effect on the endothelium in an autocrine or paracrine manner (Valdimarsdottir et al., 2002). Thus, BMP6 was suggested to stimulate migration and tube formation of bovine aortic endothelial cells (Valdimarsdottir et al., 2002). In addition, BMP-6 induced the proliferation and migration of mouse embryonic endothelial cells, as well as network formation and microvessel development in aortic rings (Ren et al., 2007; David et al., 2009).

Here, we studied the function of BMP-6 in cultured bovine theca interna cells and examined the effect of BMP-6 on endothelial cells tube formation behaviour. Interestingly, our observation was not in agreement with the above mentioned reports since BMP6 dose-dependently inhibited endothelial cells network formation in VEGF/bFGF treated cells. The mechanism by which BMP-6 modulates tube formation is unknown. However, it is possible that BMP-6 affects

transcriptional factors activity that leads to changes in gene expression of angiogenesis-related proteins.

The existence of an intra-ovarian BMP system was first documented in 1999; however, the majority of findings have focussed on granulosa cells which were considered a key potential target for BMP activities in the ovary (Shimasaki et al., 1999). A study conducted by Glister et al (2005), was the first to examine the roles of BMPs on basal and LH-induced steroidogenesis using primary cultures system of bovine theca interna cells. Their findings indicated that several BMP ligands including BMP-6 are potent regulators of basal and LH-induced androgen production and cell proliferation/ survival, thus demonstrating intra-follicular regulatory actions of BMPs on theca cells. It has been confirmed that ovarian follicles expressed several BMPs including BMP-6 in a cell dependant fashion and in particular bovine theca cells were shown to express different BMP receptors including BMPRIB, BMPRII, ActRIIA and ActRIIB. Besides, BMP-6 is expressed by granulosa cells and oocyte, which supports its potential role as an intra-follicular paracrine regulator of theca cell action (Glister et al., 2004). While Glister et al (2005) found that BMP-6 dose-dependently inhibited basal and LH-induced A4 production, it moderately increased basal P4 production. As previously mentioned, the binding between BMPs ligand and type I and type II receptors on the cell surface activates an intracellular signaling mechanism involving Smad-1, Smad-5 and/or Smad-8. However, the activation of Smad-2/-3 mechanism resulted from the activation of activin signaling (Miyazono et al., 2000; Miyazawa et al., 2002). Consistent with this, Glister et al (2005) found that the exposure of bovine theca cells to BMPs including BMP-6 promoted the accumulation of phosphorylated (p)Smad-1 (not pSmad-2) in the nucleus, whereas, exposure to activin-A led to nuclear accumulation of pSmad-2 (not pSmad-1). However, it was not known which combination of type I or II receptors were recruited by BMPs or activin in order to achieve Smad activation and the steroidogenic response Glister et al., 2005). Nonetheless, at least two type I receptors including (BMPRIB and ActRIA) and three type II receptors including (BMPRII, ActRIIA and ActRIIB) are expressed by bovine theca cells and potentially mediate the actions of BMPs (Glister et al., 2004). Interestingly, Glister et al (2005) also noted that the accumulation of pSmad-2 was significantly

lower in BMP-treated cells than in control cells. This raises the possibility that exogenous BMP may compete for 'common' receptor binding with an endogenous ligand (perhaps activin or TGF-β) leading to a reduced activin/ TGF-β signaling through the Smad-2/Smad3 mechanism. We also measured the steroid hormones (A4 and P4) in cultured theca cells treated in the presence and absence of BMP-6 and its inhibitor. However, our findings were not in agreement with the above-mentioned studies since neither A4 nor P4 production were significantly affected by BMP-6 in this angiogenesis culture system. A4 production was slightly suppressed (non significant) and this is in slight agreement with Glister et al (2005). It appears that theca interna cells cultured in the present angiogenesis culture model (using proprietary supplemented media) behave differently from cells cultured in the defined serum-free culture conditions used by Glister et al (2005). This is likely due to the proprietary supplements added by the supplier, the identity of which is not known to us. Interestingly, although exogenous BMP-6 did not significantly affect A4 or P4 production in the present culture system, blocking endogenous ligand using a BMP inhibitor significantly increased production of both A4 and P4 under both basal and VEGF/bFGF-stimulated conditions. This implies that endogenous BMP(s) do indeed exert a suppressive action on thecal steroidogenesis in our angiogenesis culture model. This fits with previous observation that bovine TC express various BMPs including BMP-6 (Glister et al., 2010). An inhibitory action of BMP-6 on basal and forskolin-induced progesterone secretion has also been reported by Kayani et al (2009) who cultured bovine theca cells in serum-supplemented culture conditions.

6.5.6. The effect of BMP6 on the expression of steroidogenic transcripts and productions of steroids

Glister et al (2005) also conducted a semiquantitative RT-PCR experiment for mRNA expression levels to examine the mechanism through which several BMPs including BMP-6 inhibited A4 and raised P4 production through key regulatory proteins and enzymes involved in the steroidogenesis pathway such as *StAR*, *CYP11A1*, *HSD3B1*, *CYP17A1* and *LHR*. BMPs including BMP-6 promoted a down-regulation in the expression of several transcripts especially *CYP17A1*, which is considered as being a key target for BMP action. Due to the suppression

of *CYP17A1* expression by BMP-6, the conversion of C21 steroids to C19 would be blocked. Expression of *StAR*, *CYP11A1* and *HSD3B1* were also inhibited but to a lesser extent in comparison to *CYP17A1*. Therefore, activation of the BMP signaling pathway appears likely to affect theca steroidogenesis at various stages (Glister et al., 2005). It has been shown that BMP-6 inhibited the expression of *StAR* by cultured rat granulosa cells (Otsuka et al., 2001). The finding in which the basal level of P4 secretion was increased in response to cell treated with BMP, suggested that, under culture cells environments, these other potential inhibitory actions were not rate limiting with respect to thecal C21 steroids synthesis, at least in the absence of LH stimulation. Hence, the raised production of P4 in response to BMP likely resulted from the reduction rate of the conversion to androgen rather than compensating for any reduction in the synthesis rate of P4 that resulted from the reduction of *StAR* expression. Also, the steady-state level of *StAR* mRNA expression does not reflect the functionally active StAR protein which was found to be controlled at the post-translational level (Arakane et al., 1997; Clark et al., 2001).

In the current angiogenesis culture model, we used real-time PCR to analyse expression of steroidogenic and other transcripts in theca cells treated with BMP-6 and its inhibitor. Our findings were in agreement with the previously mentioned findings for *CYP11A1* and *LHR* mRNA expression level, which were both reduced by BMP-6 treatment. The reduced expression of *CYP11A1* would fit with observed reduction in P4 secretion. The other transcripts examined, including *CYP17A1* showed little change in response to BMP6 and the differences were not statistically significant, perhaps reflecting the lack of effect on A4 secretion we observed. However, blocking of endogenous ligand using a BMP inhibitor promoted significant increases in mRNA expression for all steroidogenesis-related transcripts except *CYP11A1*, and this is consistent with the finding of increased P4 and A4 secretion by the cells treated with BMP inhibitor alone. As such, these observation reinforce the view that endogenous BMPs expressed by the cultured cells exert a suppressive action both angiogenesis and steroidogenesis.

6.5.7. The role of TSP-1 in modulating basal and FGF/VEGF-induced ovarian angiogenesis

The physiological angiogenesis mechanisms operating in the ovary likely involve a complex cross-talk between pro and anti-angiogenic factors. Several naturally-occurring angiogenesis inhibitors have been identified in mammalian tissues and these are thought to control normal vascular quiescence (Hanahan and Folkman, 1996). One of these regulatory factors is the thrombospondin (TSP) family that includes TSP-1 (Thomas et al., 2007). TSP-1 is a large multimodular glycoprotein (450 kDa) encoded by the *THSB1* gene that was originally known as a key factor of platelet α-granules (Lawler, 2000 and 2002). TSP-1 has been found to be an important matricellular protein involved in different processes including cell signaling, wound healing, cell adhesion and angiogenesis (Adams and Lawler, 2004 and 2011). The most notable biological function of TSP-1 is the suppression of angiogenesis mechanisms in animal models (Silverstein, 2002; Osz et al., 2014). The biological effects of TSPs are mediated through the binding to the cell surface receptors including CD36 and integrin-associated protein known as CD47 (Gao et al., 1996; Carron et al., 2000; Thomas et al., 2007). In fact, a novel pattern of expression of TSP family members has been discovered in the bovine ovary through follicle growth stages (Greenaway et al., 2005). The expression level of TSP was lower in medium and large bovine follicles than in small follicles associated with an increase in the expression level of VEGF. TSP protein is present in follicular fluid and granulosa cells lining the follicular antrum express TSP protein and mRNA. Some TSP-immunopositive theca cells were also noted in developing follicles, however, the involvement of these cells in the accumulation of TSP in follicular fluid remain unclear (Greenaway et al., 2005). This observation was in agreement with reports for other species including human and porcine ovarian tissues (Barboni et al., 2000; Kamat et al., 1995). According to different studies, VEGF is involved in many biological functions within the ovary. VEGF is a stimulator of angiogenesis mechanism in follicles and corpora lutea among some mammals (Ferrara et al., 1998; Shimizu et al., 2002). It has also been shown that VEGF increases vascular permeability and due to the alteration of the vascular permeability within ovarian follicles, the expression of VEGF increased in response to exogenous gonadotropins *in vivo* (Gomez et al., 2003;

Wang et al., 2002). Increased expression of VEGF which increases the vascular permeability appears to contribute to normal healthy follicles development in cows and other species (Petrik et al., 2002; Ferrara et al., 1998; Shimizu et al., 2002; Greenaway et al., 2005). Given the above, we decided to examine the effects of TSP-1 on basal and VEGF/bFGF-induced angiogenesis in our culture model. However, combined results of three replicates experiments using independent batches of cells produced no evidence to support a role for TSP-1 on endothelial tube formation, despite the fact that its expression was confirmed in various bovine endocrine tissues including the ovary.

6.6. Conclusions

1. VEGF/FGF mixture had a marked stimulatory effect, being capable of promoting capillary-like tubular structures in the cultured theca internal model.

2. Despite previous evidence that NMB is a pro-angiogenic factor, no effect of either NMB agonist or antagonist on tube formation was observed when results from six replicate experiments using independent batches of cells were combined.

3. The combined results of three replicate experiments using independent batches of cells led us to reject the hypothesis that kisspeptin influences thecal angiogenesis; neither kisspeptin-10 nor its antagonist had any modulatory effect on tube formation despite our finding (chapter 4) that *KiSS-1* and its receptor are indeed expressed in bovine endocrine tissue including the ovary.

4. Our data confirmed that TGF-β-1 induced a dose dependant inhibition of basal and VEGF/bFGF-induced endothelial network formation, an action that was reversed by a TGF-β-1 inhibitor. The stimulatory effect of treatment with the inhibitor alone suggests neutralization of an endogenous TGF-β ligand that exerts an inhibitory influence on endothelial network formation.

5. We also demonstrate that TGF-β-1 along with the angiogenic factors decreased significantly the production of progesterone, but not androstenedione. This effect on progesterone production was completely reversed by the pharmacological inhbitor.

6. The expression of several key steroidogenesis-related transcripts, including *CYP11A1*, *HSD3B1* and *LHR* was also downregulated in cells co-treated with TGF-β-1 and angiogenic factors; this effect was reversed by the pharmacological inhibitor.

7. BMP-6 dose-dependently inhibited endothelial cells network formation in VEGF/bFGF treated cells.

8. BMP-6 antagonist alone increased endothelial cells network formation under basal conditions, suggesting neutralization of an endogenous BMP-related ligand.

9. BMP-6 reduced P4 production but did not significantly affect A4 production in the present culture system whereas blocking endogenous ligand using a BMP-6 inhibitor significantly increased production of both A4 and P4.

10. Blocking of endogenous ligand using a BMP-6 inhibitor promoted significant increases in mRNA expression for all steroidogenesis-related transcripts except *CYP11A1*, consistent with the increase in A4 and P4 observed.

11. Despite the fact that *THSB1* expression was confirmed in various bovine endocrine tissues including the ovary, no evidence emerged to support a role for TSP-1 in endothelial tube formation.

7. General Discussion and Suggested Future Work

It is well known that, due to ethical considerations, the possibility of obtaining normal ovarian tissue from women in their reproductive years is extremely limited. This limitation does not apply to ovarian tissue from cattle. Bovine ovarian physiology has been studied extensively over the years and a great deal is known about endocrine and intra-ovarian control of bovine ovarian function and fertility. Similarities with the human ovary are considerable and have led various research groups to recognise the bovine ovary as a valuable, biomedically-relevant model. There are several advantages with using bovine ovarian models: fresh ovarian tissue for ex-vivo and in-vitro studies is easily available and cheap to obtain from the slaughterhouse on a regular basis. Cattle are large enough to allow detailed *in vivo* endocrinological and morphological studies (i.e. serial blood sampling, ovarian ultrasonography). Many characteristics of bovine reproductive biology are shared with women: they are both monovular, cycle continuously while not pregnant, have a 9-month gestation period, their ovaries are similar in size (~3cm x 2cm x 1.5cm) and ovarian, follicular and CL morphology are similar (Campbell et al., 2003; Sirard, 2017).

The experimental work reported in this thesis included *ex-vivo* analysis of gene expression in bovine ovarian tissue and use of *in vitro* models involving primary cultures of TC and GC recovered from bovine ovaries. The focus was on an examination of potential roles of several regulatory peptides (kisspeptin, NMB) that were shown to be expressed, along with their signaling receptors, in the bovine ovary. Modulatory effects on GC and TC function (steroidogenesis, cell proliferation/survival) were examined and effects on cell migration and follicular angiogenesis were also investigated.

In chapter 4, expression of *KiSS-1* gene and its receptor GPR54 were assessed at the level of mRNA abundance in different endocrine tissues including the adrenal gland, pituitary gland, testis and ovarian GC, TC and CL. Also, mRNA abundance in GC, and TC was measured at different stages of follicular development and in CL at different stages. The mRNA for kisspeptin and its receptor were detected in each of the previously mentioned tissues, with the adrenal gland showing the lowest level of expression of kiss-1 gene. Expression of *KiSS-1* receptor varied significantly, being by far the highest in pituitary and lowest in testis. More interestingly, our current data demonstrated for the first time distinct profiles of ovarian *KiSS-1* gene

and *GPR54* expression in theca and granulosa cells from bovine follicles at different stages of development. In the corpus luteum, the expression was also shown to vary in a stage-dependent manner. Since the present findings only related to detection of mRNA expression, useful follow-on work would be to obtain antibodies against bovine kisspeptin and GPR54 and use these to confirm expression of the translated proteins in bovine ovarian cells. Unfortunately this was not possible during the current research as no suitable antibodies were available. It has been reported previously that kisspeptin and its receptor are expressed in various tissues apart from hypothalamus (Ohtaki et al., 2001; Xu et al., 2012). With regard to the reproductive system, the kisspeptin/receptor system is expressed in the ovary, female genital tract, placenta and testis of several species including humans (Shahed and Young, 2009; Roman et al., 2012; Zhang et al., 2014).

The extra-hypothalamic functions of kisspeptins have attracted attention in areas relating to reproductive biology and clinical reproductive medicine. Several studies have documented that the kisspeptin/receptor system may contribute to physiological and pathological actions in the ovary (Hu et al., 2017). These actions are likely exerted in an autocrine/paracrine manner. The *in vitro* experiments reported in Chapter 4 examined potential intra-ovarian roles of kisspeptin/receptor system in regulating different aspects of ovarian function. Despite the presence of *KiSS-1* and *GPR54* mRNA in ovarian TC, GC and CL tissue, the present finding offer no evidence that kisspeptin has a direct intra-ovarian role to modulate follicular or luteal steroidogenesis in the bovine ovary. Neither did kisspeptin have any effect on cell migration assessed using wound-healing ('scratch') assays (Chapter 4). According to studies in chicken GC and rat luteal cells, kisspeptin directly induced the secretion of P4 in these species respectively (Xiao et al., 2011; Peng et al., 2013). Xiao et al (2011) findings showed that mRNA levels of P4 producing enzymes including StAR, P450scc and 3β-HSD were significantly increased with the treatment of kisspeptin-10 in chicken GC. Peng et al (2013) illustrated that kisspeptin alone exerted no effect on *HSD3B1* mRNA level of rat luteal cells, however this effects was stimulated when these cells co-treated with human chorionic gonadotropin (hCG). Thus, an indispensable function of ovarian kisspeptin has been suggested in regulating the production of P4. On the other hand, Peng et al (2013) studies showed no effect

on the production of E2 in rat luteal cells. Yet, the possibility of kisspeptin stimulating the synthesis of E2 when its expression peaked among GC of the growing follicle throughout the mid- and late- proliferative stage, has not been examined. The conclusive demonstration of a direct role(s) of kisspeptin signaling in ovarian physiological and/or pathophysiological is still pending, and further investigations are required to explore this, preferably in a range of species including human. Expanding our knowledge of the expression, functions, and molecular pathways of kisspeptin/receptor system in the human and other species including cattle ovary is fundamental for understanding whether therapeutic interventions targeting kisspeptin signaling can reduce reproductive pathology and/or infertility (Hu et al., 2017).

Experiments reported in chapter 5, were prompted by a theca cell microarray dataset generated in this laboratory (Glister et al., 2013) showing that NMB is expressed in cultured bovine TC and was amongst the most highly down-regulated transcripts in response to BMP-6 treatment. Consequently, to follow up this observation, *NMB* gene expression was assessed at the level of mRNA abundance in different endocrine tissues including the adrenal gland, pituitary gland, testis, GC, TC and CL. Also, mRNA abundance was measured for *NMB* and its receptor in TC and GC at different stages of follicular development, while NMB was measured at different corpus luteum stages. The mRNA for *NMB* was detected in all of the previously mentioned tissues. Testis showed the highest level of expression of NMB. More interestingly, our current data demonstrated that the profiles of ovarian NMB and its receptor expression in TC and GC from follicles at different stages of development are clearly distinct. However, a series of in vitro experiments carried out using cultured TC and GC offered no evidence that NMB has a direct intra-ovarian role to modulate follicular or luteal steroidogenesis.

According to our mRNA expression data, testis showed the highest level of expression, which may imply that NMB functions through autocrine and paracrine in the male gonad. Recently published studies on porcine Leydig cells report an effect of NMB on testosterone secretion, steroidogenesis, cell proliferation and apoptosis. It was confirmed that NMBR was expressed in leydig cells of porcine testis. Also, high testosterone secretion was found when these cells treated with a

specific dose of NMB (1nM); also NMB increased the proliferation of these cells. Moreover, the steroidogenic regulators including StAR, CYP11A1 and 3β-HSD, their mRNA and/or proteins were expressed at high level in the Leydig cell. Consequently, it has been suggested that the NMB/NMBR system might have a vital role in modulating the reproductive function that includes steroidogenesis and/or cell growth in porcine Leydig cells (Ma et al., 2018). Given the that testicular Leydig cell is the functional equivalent of the ovarian theca cell, it might have been expected that a modulatory action of NMB on thecal steroidogenesis or cell proliferation/survival would have been observed in the present studies; however, as documented in this thesis, no such effects were identified in the current bovine cell culture models.

The experiments reported in chapter 6 were prompted by a published study (Park et al., 2009) indicating that NMB is a novel angiogenic peptide. It is well established that angiogenesis is of great importance in the ovary due to the extensive tissue remodelling involved in cyclic growth/regression of follicles and luteal tissue (Reynolds and Redmer, 1999; Fraser and Lunn, 2000; Berisha et al., 2010; Berisha et al., 2013). A better understanding of ovarian angiogenesis that includes folliculogenesis and CL formation may lead to the elucidation of some causes of infertility/subfertility in mammals. Several key regulators of angiogenesis have been discovered to have important roles in the ovary including VEGF, FGF, angiopoietins (ANPT) and hypoxia-inducible factor (HIF) family members (Berisha et al., 2016). In this study, several different peptides, including kisspeptin and NMB) were tested for their role in modulating angiogenesis in a bovine theca interna angiogenesis culture model adapted from the method reported by Robinson et al (2009). It was confirmed that VEGF and FGF had a marked stimulatory effect on endothelial tube network formation. However, neither NMB nor kisspeptin had any discernible effect on basal or VEGF/FGF-induced network formation.

Given the considerable interest in the roles of TGF- β family members in regulating ovarian function, it was also decided to investigate potential effects of two ligands (TGF-β-1 and BMP-6) in the angiogenesis model. Interestingly, both TGF-β-1 and BMP6 induced a dose dependant inhibition of endothelial network

formation. Our funding is in agreement with (Jarad et al., 2017) who observed that TGF-β-1 (5ng/ml) had an inhibitory effect on bovine endothelial cord formation. However, BMP6 was previously reported to stimulate migration and tube formation of bovine aortic endothelial cells as well as the proliferation and migration of mouse embryonic endothelial cells (Valdimarsdottir et al., 2002; Ren et al., 2007; David et al., 2009).

The underlying reason for these discrepancies between different model systems is not yet known but obviously requires further experimentation and analysis. In future studies it would be useful to examine the effects of TGF-β-1 and BMP-6 on nuclear accumulation of phospho-Smad in the follicular angiogenesis model. This would help distinguish which cell type is responding directly to the ligand (i.e endothelial cells, steroidogenic cells). It would also be worthwhile carrying out more sophisticated image analysis than was undertaken for the present studies. This could involve use of proprietary software programs (e.g Image Pro-Plus) to quantify parameters such as tube length and number of network branch points in the histological sections. Unfortunately, there was insufficient time and funding available to carry out this analysis for the present project. Understanding the regulatory roles of TGF-β family members in follicular angiogenesis is important since these multiple ligands and receptors are expressed in the ovary and are known to modulate other aspects of follicle and luteal function including steroidogenesis (Knight and Glister, 2006). Future research into angiogenesis is likely to yield better understanding of interacting regulatory pathways involved and promote the discovery of novel therapeutic strategies for the treatment of diseases associated to disregulated angiogenesis, ischaemic disorders and vascular regression (Plendl, 2000, Ten Dijke and Arthur, 2007).

To conclude this thesis Figures 7.1 and 7.2 are schematic diagrams that attempt to summarise the experimental work undertaken and the principle findings obtained during the course of my research project.

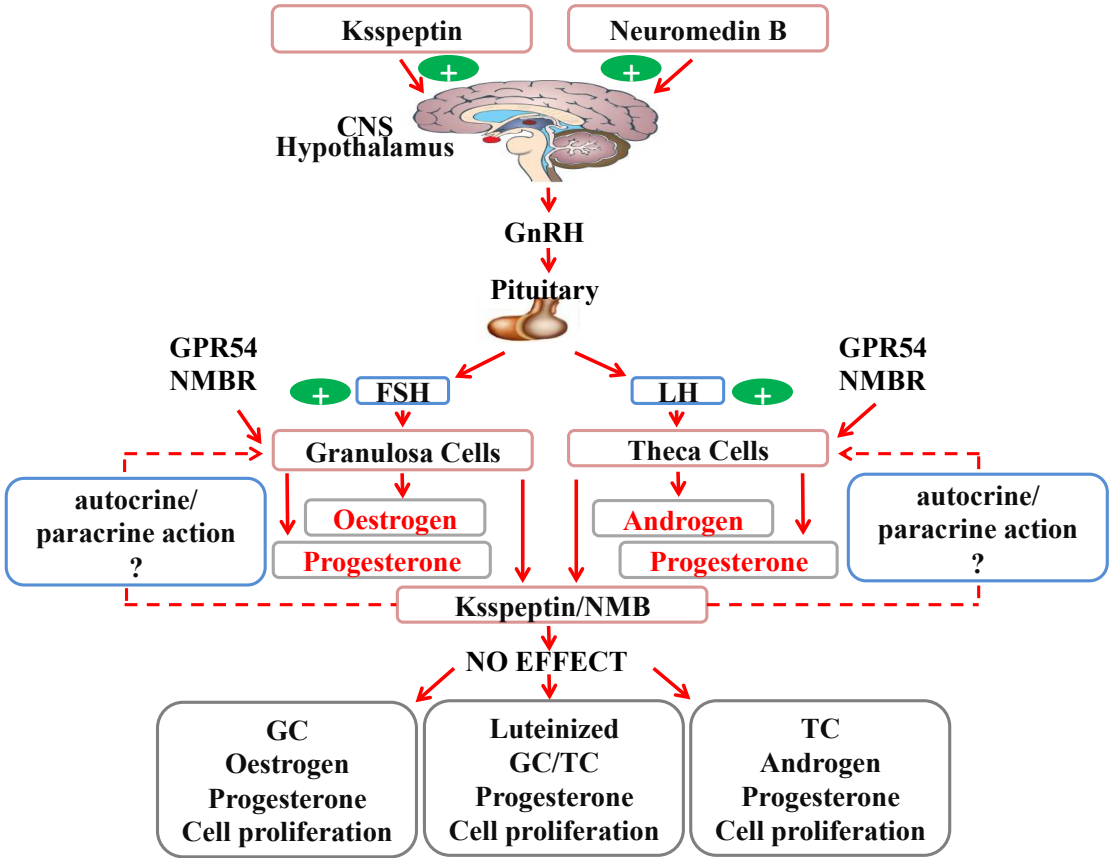


Figure 7. 1 Schematic diagram summarizing the potential involvement of hypothalamic and ovary-derived kisspeptin and NMB in regulating the H-P-O axis. The lower part of the figure indicates the lack of experimental evidence obtained to support direct actions at the ovarian level despite the finding that kisspeptin, NMB and their cognate receptors (GPR54, NMBR) are expressed by ovarian cells.

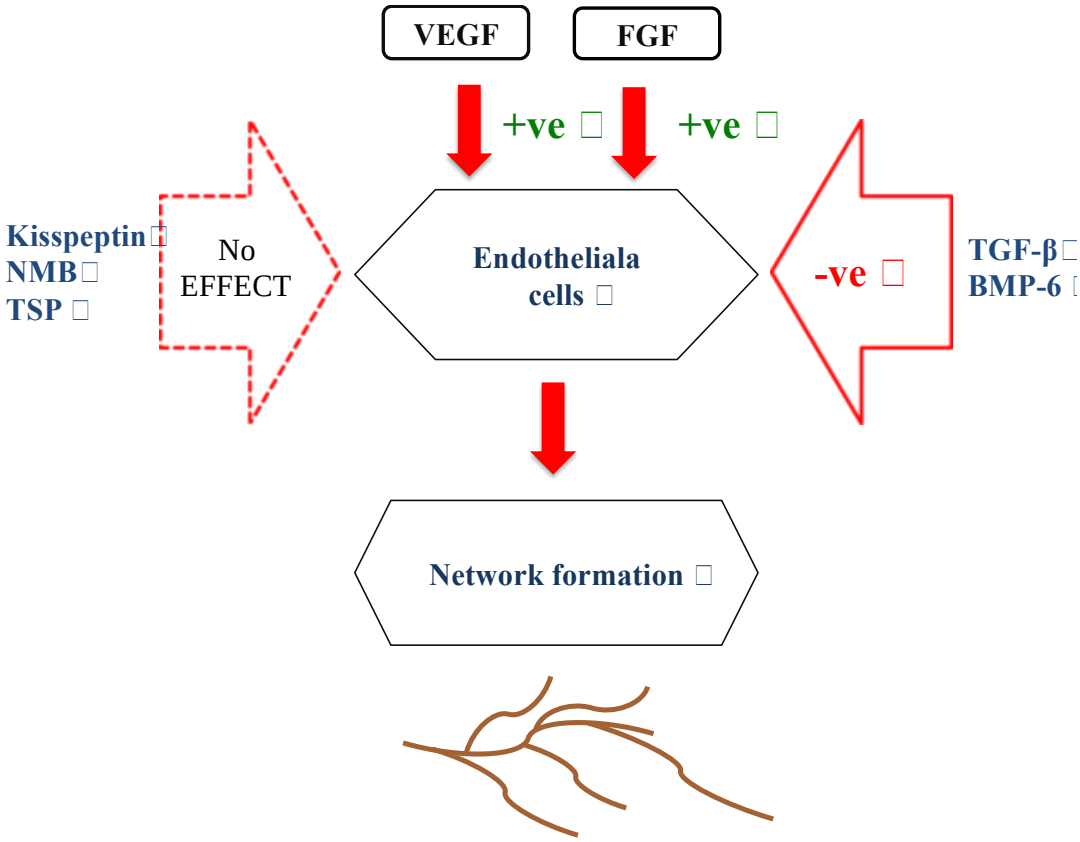


Figure 7. 2 Schematic diagram summarizing findings on the regulatory actions of VEGF, FGF, kisspeptin, NMB, BMP-6 and TGF-β-1 on angiogenesis in the bovine theca interna culture model. Note that BMP-6 and TGF-β-1 also had an inhibitor effect on steroidogenesis in this culture model.

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