Université de Montréal

THE ROLE OF TRANSFORMING GROWTH FACTOR-BETA 1 IN STEROIDOGENESIS, CELL PROLIFERATION, AND APOPTOSIS IN CULTURED BOVINE GRANULOSA CELLS

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présentée par

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RÉSUMÉ

L'infertilité bovine est une contrainte importante à la rentabilité de l'industrie laitière. La croissance folliculaire anormale, l'anoestrus et l'anovulation sont parmi les principales causes du déclin de la fertilité chez les vaches laitières hautes productrices. Afin de trouver des solutions à ces problèmes, une meilleure connaissance des mécanismes endocriniens qui régulent la croissance normale des follicules ovariens est nécessaire. La stimulation par les gonadotropines des follicules ovariens réceptifs est associée à une augmentation de la synthèse de l'oestradiol-17β (E_2) et de la progesterone (P_4). L'E_2 est un marqueur clé de la croissance et de la sélection du follicule dominant, alors que la P_4 est nécessaire au déclenchement de l'ovulation. Les cellules de la granulosa jouent un rôle important pour déterminer la destinée des follicules en développement soit vers l'ovulation, soit vers l'atrésie parce que ces cellules produisent de l'E_2 et de la P_4 et sont la cible des signaux impliqués dans la prolifération cellulaire et l'apoptose.

Le «transforming growth factor-β1» (TGF-β1) est un facteur important produit par les cellules folliculaires parce qu'il modifie la sécrétion de l'E_2 et de la P_4. Plusieurs études effectuées sur des cellules provenant de différents tissus ont rapporté des effets biologiques contradictoires du TGF-β1. Par exemple, le TGF-β1 stimule la sécrétion de l'E_2 et de la P_4 dans les cellules de la granulosa obtenues à partir de rates immatures traitées à l'E_2 alors que le TGF-β1 inhibe la sécrétion de l'E_2 et de la P_4 dans les cellules de la granulosa porcine et bovine. Le premier objectif de la présente étude était de déterminer le mécanisme

d'action du TGF-β1 sur les enzymes stéroïdogènes qui transforment les androgènes, l'oestrone (E_1) et le cholesterol en E_2 et en P_4 dans des cellules de granulosa bovines stimulées à la FSH et des cellules de granulosa quiescentes non-stimulées. Le deuxième objectif était de déterminer l'effet du TGF-β1 sur la différentiation et la prolifération cellulaire et sur l'apoptose. Les cellules de granulosa bovines obtenues à partir de follicules de 2-5 mm ont été mises en culture dans un milieu sans serum en présence de FSH ou en absence de FSH (cellules quiescentes). Les cellules de granulosa quiescentes se sont lutéinisées spontanément en augmentant la secretion de P_4 et l'expression de l'ARNm des enzymes impliquées dans la synthèse de la P_4: *StAR*, *CYP11A1*, *HSD3B* et *GSTA*. L'ajout de FSH a ralenti le processus de lutéinisation en augmentant la synthèse de l'E_2 et l'expression des enzymes *CYP19A1* et *HSD17B1* et en inhibant l'expression de *StAR*. Dans les cellules de la granulosa stimulées par la FSH, le TGF-β1 a inhibé l'augmentation d'E_2 et de P_4 en inhibant les enzymes stéroïdogènes correspondantes. TGF-β1 a inhibé l'expression du récepteur de FSH *(FSHr)* et a inhibé la stimulation par la FSH de l'expression de la *CYP19A1* et de la *HSD17B1* (mais pas de la *HSD17B7)* et a inhibé *StAR*, *CYP11A1*, *HSD3B* et *GSTA*. TGF-β1 a aussi inhibé la lutéinisation des cellules de granulosa quiescentes tout en préservant une certaine capacité oestrogénique. De plus, cette étude a démontré que l'activité réductionnelle de la HSD17B qui transforme l'E_1 en E_2 est très élevée dans les cellules de la granulosa bovine et que cette activité n'est pas affectée par la FSH ni par TGF-β1 et semble être corrélée avec la *HSD17B7* plutôt que la *HSD17B1*. Le traitement avec TGF-β1 a diminué de façon significative la proportion de cellules de granulosa dans la

phase proliférative du cycle cellulaire (S, G2 et M) et a augmenté la mortalité cellulaire par apoptose, tel que révélé par une augmentation de caspase-3 clivée. En résumé, ces résultats démontrent que le TGF-β1 inhibe la lutéinisation et maintient les cellules de granulosa dans un état plus immature en s'opposant à l'action de la FSH. En agissant de la sorte, TGF-β1 pourrait jouer un rôle physiologique pour limiter la prolifération et la différentiation des cellules de granulosa dans les follicules antraux en croissance et pourrait être impliqué dans le processus de sélection du follicule dominant.

Mots-clés : TGF-β1, bovin, ovaire, follicule, cellule de la granulosa, steroïdogenèse, FSH, stéroïdes, cycle cellulaire, apoptose, caspase-3

ABSTRACT

Infertility in dairy cattle restrains the economic success of this industry. Abnormal follicle growth, anoestrus and anovulation are among the principal causes of declining fertility in high-yielding dairy cattle. To resolve these problems, more basic knowledge is required to understand the endocrine mechanisms that control normal growth of the ovarian follicle. Gonadotropin stimulation of responsive follicles is associated with increased synthesis of estradiol-17β (E_2) and progesterone (P_4). E_2 is a key marker of growth and selection of the dominant follicle, and P_4 synthesis is necessary for the induction of ovulation. Granulosa cells play an important role in determining the fate of the developing follicle towards ovulation or atresia because these cells produce E_2 and P_4 and are the main target for cell proliferation and apoptosis signals.

Transforming growth factor-β1 (TGF-β1) is an important factor produced by the follicle cells and is known to modify E_2 and P_4 secretion, ovarian function and fertility. The physiological effects of TGF-β1 on granulosa cells is unclear. In rodent granulosa cells of E_2-treated immature rats, TGF-β1 stimulates E_2 and P_4 in vitro, but in cultured granulosa cells of domestic animals, TGF-β1 inhibits E_2 and P_4. The first objective of this study was to determine the mechanism of action of TGF-β1 on the steroidogenic enzymes that transform androgens, estrone (E_1) and cholesterol to E_2 and P_4 in FSH-stimulated and quiescent bovine granulosa cells. The second objective was to study the effect of TGF-β1 on granulosa cell differentiation, proliferation and apoptosis. Bovine granulosa cells were obtained from 2-5 mm follicles and were cultured in serum-free medium in the presence or

absence of FSH. Quiescent granulosa cells spontaneously luteinized by increasing P_4 secretion and the mRNA expression of P_4-related enzymes: *StAR*, *CYP11A1*, *HSD3B* and *GSTA*. The addition of FSH slowed down this process by increasing E_2 and E_2-synthetic enzymes *CYP19A1* and *HSD17B1* and by inhibiting *StAR* mRNA. In FSH-stimulated granulosa cells, TGF-β1 inhibited the rise in E_2 and P_4 secretion and inhibited the expression of the corresponding steroidogenic enzymes. TGF-β1 inhibited expression of FSH receptor (*FSHr*) mRNA and inhibited FSH-induced expression of *CYP19A1* and *HSD17B1* (but not *HSD17B7)* and *StAR*, *CYP11A1*, *HSD3B* and *GSTA*. In quiescent granulosa cells, TGF-β1 also inhibited luteinization of granulosa cells but preserved estrogenic capacity. Furthermore, this study shows that HSD17B reducing activity that transforms E_1 to E_2 is very high in bovine granulosa cells, is unaffected by FSH and TGF-β1 and appears to be correlated with *HSD17B7* but not *HSD17B1*. TGF-β1 treatment significantly decreased the proportion of granulosa cells in the proliferative phase of the cell cycle (S, G2 and M) and increased cell death by apoptosis as indicated by an increase of cleaved caspase-3. Overall, these results demonstrate that TGF-β1 inhibits luteinization and keeps granulosa cells in a more immature state by opposing FSH action. By acting in this manner, TGF-β1 may have a physiological role to limit proliferation and differentiation of granulosa cells in growing antral follicles and may be involved in the process of selection of the dominant follicle.

Keywords : TGF-β1, bovine, ovary, follicle, granulosa cell, steroidogenesis, FSH, steroids, cell cycle, apoptosis, caspase-3

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GENERAL DISCUSSION

LIST OF ABBREVIATIONS

AA	antral atresia
A4	androstenedione
ALK5	activin receptor-like kinase 5
AMH	anti-mullerian hormone
AR	androgen receptor
BA	basal atresia
bFGF	basic fibroblast growth factor
bp	base pair
BMP	bone morphogenetic protein
cAMP	cyclic andenosine monophosphate
cdk	cyclin-dependent kinase
cDNA	complementary DNA
CL	corpus luteum
CYP19A1	cytochrome P450 aromatase
CYP17	cytochrome P450 17 alpha-hydroxylase
CYP11A1	cytochrome P450 side chain cleavage
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
E_1	estrone
E_2	17β-estradiol
E2F	DNA-binding transcription factors
EGF	epidermal growth factor
FF	follicular fluid
Foxo3a	foxo transcription factor3a
FSH	follicle-stimulating hormone

FSHr	follicle-stimulating hormone receptor
G0	quiescence phase
G1	first gap phase
G2	second gap phase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF-9	growth differentiation factor-9
GnRH	gonadotropin-releasing hormone
GSTA	α glutathione S-transferase
hCG	human chorionic gonadotropin
HSD3B	3β-hydroxysteroid dehydrogenase
HSD17B	17β-hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
IP3K	inositol trisphosphate 3 kinase
kDa	kilodalton
KGF	keratinocyte growth factor
KL	kit ligand
LH	luteinizing hormone
LHr	luteinizing hormone receptor
LIF	leukaemia inhibitory factor
M	the mitotic phase
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase
mRNA	messenger RNA
P_4	progesterone
P_5	pregnenolone
PCR	polymerase chain reaction
PTEN	phosphatase and tensin homolog deleted on chromosome 10)
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

PKA	protein kinase A
PR	progesterone receptor
RB	retinoblastoma tumor suppressor proteins
RIA	radioimmunoassay
RNA	ribonucleic acid
RT	reverse transcriptase
S	DNA synthesis phase
StAR	steroidogenic acute regulatory protein
T	testosterone
TGF-β	transforming growth factor-β
TNFα	tumor necrosis factor α
VEGF	vascular endothelial growth factor

DEDICATION

To my great parents and my parents-in-law for their love, understanding and support.

To my wife Yang Li for the love and happiness she gives me

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INTRODUCTION

1. Overview of the bovine estrous cycle

The estrous cycle comprises the recurring physiologic changes that are induced by reproductive hormones in females. The estrous cycles start after puberty in sexually mature females. The four stages of the cycle (described bellow) succeed one another continuously until death and are interrupted only during pregancy and periods of anestrus. Typically the estrous cycle has been divided into four stages, 1. Proestrus: period of follicle development, occurring subsequent to luteal regression and ending at estrus; 2. Estrus: period of sexual receptivity; 3. Metestrus: period of initial development of the CL; 4. Diestrus: period of mature CL (Davidson 2007) (Figure 1). Based on the hormonal changes measured in blood (Figure 2), the estrous cycle has been further divided into a follicular phase (proestrus and estrus) which is characterized by the production of E2, and a luteal phase (metestrus and diestrus) which is characterized by the production of P4 (Davidson 2007).

1.1 Regulation of the ovarian cycle

The ovarian cycle corresponds to repetitive changes in development of follicles, formation of the CL along with the secretion of hormones. Successful manipulation of the ovarian cycle for induction of estrus requires a good understanding of the physiological changes that occur throughout the estrous cycle. Generally, the hypothalamo-pituitary-gonadal axis regulates the production of reproductive hormones that control the ovarian cycle through a feedback communication system (Figure 3). A major product of the

hypothalamus is GnRH, which induces the secretion of FSH and LH by the anterior pituitary. GnRH, FSH and LH are released in a pulsatile way. FSH and LH regulate follicular development (Armstrong & Dorrington 1977). The theca cells of the bovine follicle respond to LH and synthesize A_4 from cholesterol. The A_4 passes by diffusion into the granulosa cell, and under the influence of FSH, A_4 is converted to E_2 by CYP19A1 and HSD17B (Fortune 1986; Hillier *et al.* 1994). When secretion of E_2 reaches a certain threshold level, and P_4 from the regressing CL decreases, a positive feedback signal is generated in the hypothalamus and pituitary gland to increase the frequency of GnRH and LH pulses leading to generation of a GnRH surge and a LH surge which is responsible for ovulation induction. At the same time, estrous symptoms are triggered by the high concentration of E_2 in blood. The LH surge is responsible for the luteinization of granulosa cells which consists in a switch from E_2 to P_4 secretion. The increase in P_4 is necessary for ovulation induction and after ovulation, P_4 secretion by the CL is necessary to maintain pregnancy. During pregnancy, P_4 sends a negative feedback signal to decrease GnRH for inhibition of ovulation. If the ovum is not fertilized, the endometrium of the non-pregnant bovine uterus will release $PGF_{2\alpha}$ for regression of CL. Oxytocin produced in the CL is also known to play a role in luteolysis. When P_4 decreases in blood along with luteolysis, GnRH pulses start to increase again to promote a new ovarian cycle (Peters 1987).

1.2 Follicular development

Follicular development is a complex process involving communication among the oocyte, the granulosa cells, the thecal cells, and the inner cell environment of the ovary.

These communications occur through endocrine factors, the gonadotropic hormones, and ovarian cytokines which lead to expression of genes and proteins that determine the fate of the follicle (Fortune 1994; Hillier *et al.* 1994; Matzuk *et al.* 2002; Webb *et al.* 2007). The successful development of a follicle in single ovulating species includes activation of primordial follicles (Skinner 2005), growth of primary follicles to antral follicles (McNatty *et al.* 1999; Webb *et al.* 1999; Fortune 2003), dominant follicle selection (Fortune *et al.* 2001; Webb & Campbell 2007), and development of the preovulatory follicle toward ovulation (Richards *et al.* 2002). In cattle, the pattern of growth of antral follicles greater than 4 mm is called a follicular wave. Typically two or three successive waves of follicular development are observed during the estrous cycle and a large dominant follicle of 10-13 mm will emerge from each wave (Sirois & Fortune 1988). The follicular waves are distinct to the extent that regression of the large dominant follicle usually begins before the onset of growth of a new follicular wave. The dominant follicle that reaches maximium size during the luteal phase usually undergoes atresia, whereas the dominant follicle present at the time of luteal regression ovulates (Adams *et al.* 1992; Fortune 1994).

1.2.1 Primary follicle and antral follicle development

In the mammalian perinatal ovary, oocytes arrested in the diplotene stage of meiosis-I become surrounded by a single, squamous layer of somatic cells to form a finite population of non-growing primordial follicles which are progressively depleted during the reproductive life span (Peters 1969). Follicular development begins after the activation of primordial follicles. Although the mechanism triggering initiation of primordial follicle

growth remains largely unknown, studies suggest that activation of primordial follicles may be controlled by the balance between stimulatory and inhibitory factors. These stimulatory factors include KL, LIF, BMP's, KGF and bFGF which promote the primordial to primary follicle transition (Skinner 2005). Foxo3a and PTEN act as inhibitory factors because knock out of Foxo3a and PTEN proteins in mouse induced rapid activation of egg-containing primordial follicles (Castrillon *et al.* 2003; Reddy *et al.* 2008).

When the activation of primordial follicles occurs, primary follicles are formed and the surrounding single layer of somatic cells become cuboidal and proliferative and are thereafter called granulosa cells (Van Voorhis 1999). Ovarian transplant studies in both cattle and sheep demonstrated that it takes approximately 4 months for primordial follicles to reach the size of a dominant antral follicle (Webb & Campbell 2007). It appears that primary and preantral follicles require more time to grow than antral follicles. A study in cattle precisely estimated the time required for follicle growth, which took 27 days to grow from 0.13 to 0.67 mm, 6.8 days to grow from 0.68 to 3.67 mm and 7.8 days to grow from 3.68 to 8.56 mm (Lussier *et al.* 1987). The slowly growing phase of primary follicles to preantral follicles may be attributed to a lack of activation by FSH and some important factors at this period. In the FSHr knockout mouse, follicles continue to grow from the primary to the preantral follicle stage but folliculogenesis is blocked prior to antral follicle formation (Kumar *et al.* 1997). Hypophysectomy and GnRH-agonist-induced hypogonadotrophic hypogonadism, have also demonstrated that the growing bovine follicle

less than 2 mm is FSH-independent (Webb *et al.* 2003). Additionally, recent studies have shown that T and VEGF stimulate the primary to secondary follicle transition in bovine follicles in vitro (Yang & Fortune 2007, 2008).

Primary follicles lack an independent blood supply but antral follicles receive blood from arterioles with an anastomotic network of capillaries just outside the basal lamina. This blood supply allows the follicle to be exposed to factors circulating in the blood stream. In antral follicles, stromal cells near the basal lamina of the follicle become aligned parallel to each other and form a theca interna and a theca externa (Van Voorhis 1999). The cells of the theca layer play a critical role in steroidogenesis beyond the primary follicle stage. Once follicles enter the antral follicular growth phase, thecal cells express LHr and granulosa cells express FSHr and these follicles respond to gonadotropins to increase secretion of steroid hormones. Eventually, antral follicles enlarge both by the proliferation of granulosa cells and by an increase in the size of the oocyte (Hunter *et al.* 2004). During the antral follicle stage, the expression of steroidogenic enzymes in theca and granulosa cells are essential steps to ensure production of steroid hormones.

1.2.2 Dominant follicle selection and ovulation

In single ovulating species, like cattle, the process of dominant follicle selection assures growth of one or occasionally two antral follicles but eliminates the remaining antral follicles by atresia (Fortune *et al.* 2001; Ginther *et al.* 2001). The mechanism of dominant follicle selection is not well understood, but it is believed that LH is important in

this process. The knockout of LHr in female mice showed follicular maturation only until the antral stage, but never to the preovulatory stage, and these mice never ovulate (Lei *et al.* 2001; Zhang *et al.* 2001). LH appears to replace the effect of FSH in granulosa cells when the antral follicle is selected to be the dominant follicle (Hunter *et al.* 2004; Mihm *et al.* 2006). Granulosa cells in the selected antral follicle acquire LHr to allow them to increase aromatization in response to LH (Ginther *et al.* 1996). Although there was an increase in E_2 production by cultured bovine antral granulosa cells following stimulation with LH (Rouillier *et al.* 1996), LH does not appear to stimulate CYP19A1, and the role of LHr acquisition remains to be determined. Some studies also suggest a key role for changes in the intrafollicular IGF system in selection of the dominant follicle (Fortune *et al.* 2001; Mihm & Austin 2002). In the dominant follicle of the follicular phase, enhanced expression of the proliferative gene CCND2(cyclin D2) and the anti-apoptotic gene GADD45B (growth arrest and DNA damage-inducible45β) in granulosa cells may support further growth of the dominant follicle, while enhanced expression of CYP19A1, INHA(inhibin, β A) or OSAP(ovary-specific acidic protein) may support the increased E synthesizing capacity characteristic of the dominant follicle, and enhanced expression of the proliferative genes, anti-apoptotic gene and steroidogenic genes in granulosa cells may support further growth of the dominant follicle towards ovulation (Ndiaye *et al.* 2005; Mihm *et al.* 2006; Mihm *et al.* 2008).

1.2.3 Follicular atresia

Discussion of follicular development is not complete without considering the process of atresia. In mammals of single ovulating species, it has been recognized that >99% of follicles present at birth become atretic with <1% achieving ovulation (Tilly *et al.* 1991). Generally, atresia can occur at any stage of follicular development and is accompanied by a series of changes in steroidogenesis and morphology. However careful analysis has revealed that atresia is not equally prevalent across all stages. In human follicles, the highest rates of atresia occur in early antral follicles (Gougeon 1986). Similar findings have been revealed in cattle (Lussier *et al.* 1987).

During follicular atresia, the mechanism of cell loss is widely recognized as apoptotic cell death (Tilly *et al.* 1991). Within the follicle, the granulosa cell is the most susceptible cell type undergoing apoptosis associated with atresia of the maturing follicle. However, pig, chicken and rat exhibit theca cell apoptosis which occurs much later than granulosa cell apoptosis during the process of atresia (Tilly *et al.* 1991; Tilly *et al.* 1992; Palumbo & Yeh 1994; Foghi *et al.* 1998; Clark *et al.* 2004). The significance of this is not fully understood; however, it is believed that theca cells may perform an anti-apoptotic function for the adjacent granulosa cells (Johnson 2000). In support of this, bovine granulosa cells derived from small follicles were found to be less likely to undergo apoptosis in response to serum withdrawal when they were co-cultured with theca cells (Tajima *et al.* 2002).

Morphologically, atretic follicles are generally distinguished by oocyte involution, avascular theca and debris in the antrum. In addition, irregular shape of the follicle, increased intracellular spaces between granulosa cells, and nuclear pyknosis in the granulosa cell layer have been shown in the atretic follicle. Internucleosomal DNA cleavage of granulosa cells is also a hallmark of apoptosis (Guthrie *et al.* 1995). Based on the morphology of follicular atresia, a study described two basic phenotypes of AA and BA. In AA follicles, the cell death begins at the antrum and progresses towards the basal lamina, however, in BA follicles, the direction of cell death is reversed. Macrophages breach the follicular basal lamina of BA but not AA follicles (Irving-Rodgers *et al.* 2001).

In cattle, when follicle mature or become atretic, concentration of A_4, P_4 and E_2 in follicular fluid are usually related to a certain size or stage of follicular development (Ireland & Roche 1983). In the early studies, it has been recognized that atretic large follicles have a higher concentration of both androgens and P_4 than E_2 in follicular fluid, whereas healthy dominant follicles have a lower concentration of either androgens or P_4 than E_2 in their follicular fluid (Ireland & Roche 1982). The different concentration of E_2 in the healthy versus atretic follicle may be attributed to a high abundant aromatase activity in granulosa cells of healthy follicles compared to atretic follicles (Tilly *et al.* 1992; Fortune *et al.* 2001). Recently, a transcription level study also showed that CYP11A1 and HSD3B expression was uninterrupted in granulosa cells of early atretic follicle supporting the

production of P_4, however a decrease of E_2 secretion was due to a loss of the expression of CYP19A1 (Irving-Rodgers *et al.* 2003). Based on the different concentration of steroids observed in follicular fluid, early studies suggest that the ratio of concentration of E_2 to P_4 in FF is a reliable method of distinguishing healthy from atretic antral follicles in the cow (Ireland & Roche 1983). However, measurement of the concentration of E_2 and P_4 is not suited for determining atresia and health in small antral follicles due to insufficient amount of follicular fluid. (Irving-Rodgers *et al.* 2003).

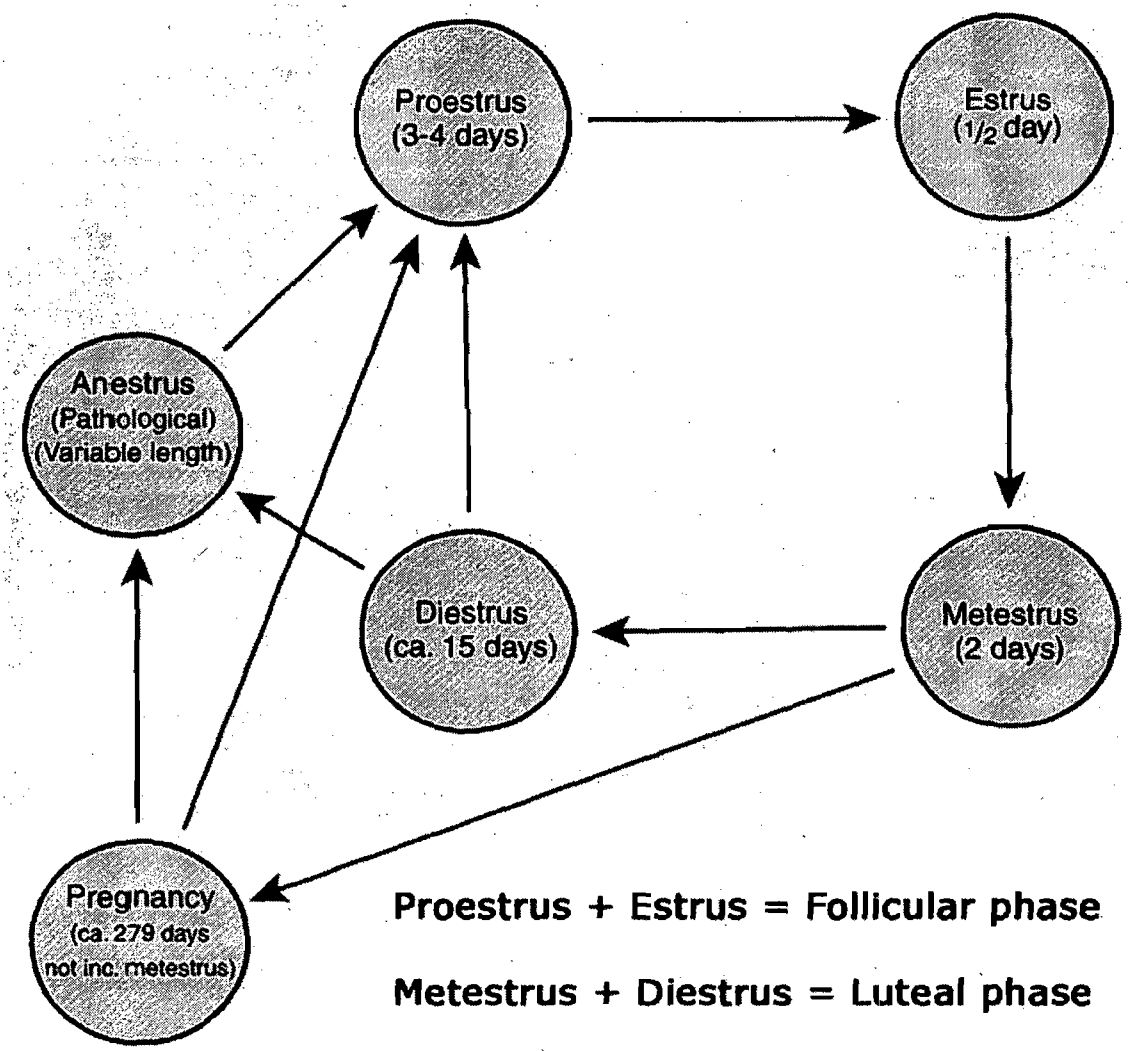


Figure 1. Various stages of the ovarian cycle of the cow. (Adapted from Davidson, 2007).

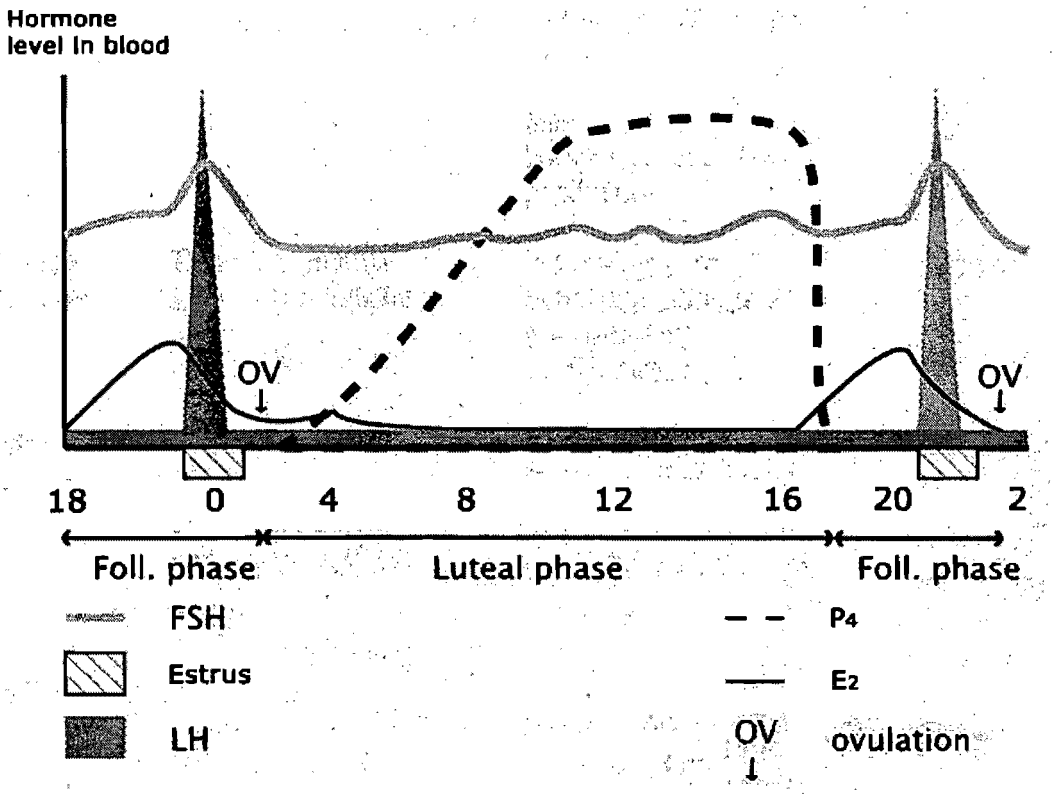


Figure 2. Hormone levels in blood during the estrous cycle of a cow. (Modified from Peters 1987).

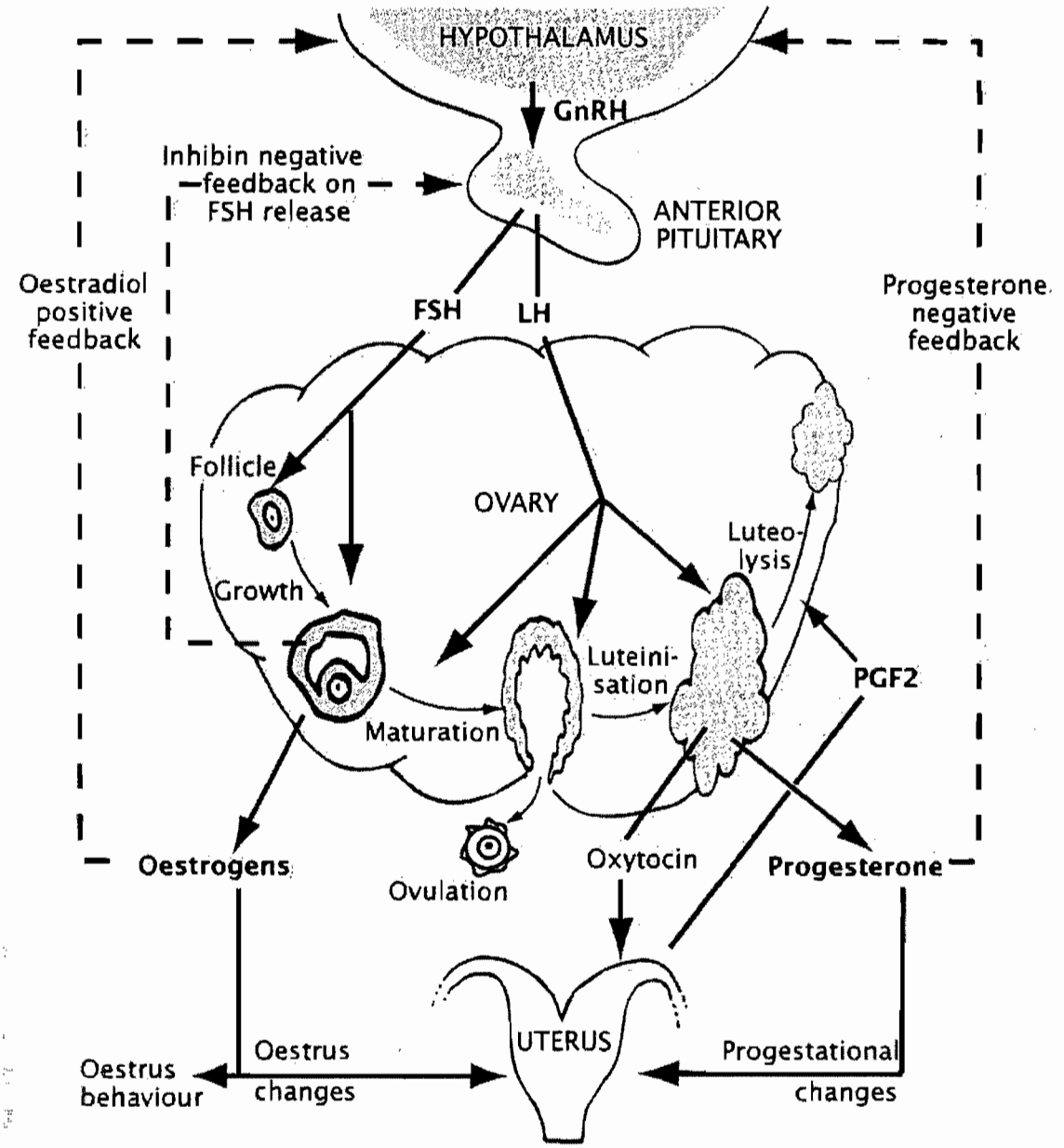


Figure 3. A summary of the hormonal control of the ovarian cycle (Adapted from Peters 1987).

2. Steroidogenesis in the ovary

All steroids are derived from cholesterol, which is a 27-carbon compound. It appears that circulating cholesterol is the most important precursor to ovarian follicular steroidogenesis. Cellular cholesterol includes free cholesterol, cholesterol stored in the cell membrane, and cholesterol stored in cytoplasmic lipid droplets. Once cholesterol has entered the cell, it undergoes a series of enzymatic conversions to either 21-carbon progestins, 19-carbon androgens, or 18-carbon estrogens (Gore-Langton & Armstrong 1994). The production of these steroid hormones is regulated largely by the relative levels and the tissue-specific array of steroidogenic enzymes expressed at the cellular level. The first step in the steroidogenic pathway is the transportation of cholesterol into the mitochondria from the outer mitochondrial membrane to the inner mitochondrial membrane. During the last few years two proteins were shown to be critical for this process: the mitochondrial translocator protein, previously known as peripheral-type benzodiazepine receptor, and the steroidogenic acute regulatory protein (StAR) (Stocco & Clark 1996). These two proteins functionally interact to facilitate cholesterol transport and may be part of a larger multimeric mitochondrial complex of proteins assembled to facilitate the hormone-induced cholesterol transfer into mitochondria. This complex might include proteins such as the mitochondrial voltage-dependent anion channel, the translocator protein-associated protein PAP7 which also functions as an A kinase anchor protein that binds and brings into the complex the regulatory subunit Iα of the cAMP-dependent protein kinase (Papadopoulos *et al.* 2007). In the mitochondria, the cholesterol

can be metabolized to P_5 via CYP11A1. P_5 can be further metabolized by two different enzymes. The first is HSD3B, which converts pregnenolone to P_4. The HSD3B is located in the endoplasmic reticulum and is responsible for the formation of P_4. The second pathway of metabolism of P_5 is via CYP17 which is located in the endoplasmic reticulum of thecal cells and is responsible for the conversion of P_5 to dehydroepiandrosterone which is then converted to A_4 by HSD3B. CYP17 can also convert P_4 to A_4. These two alternative pathways for the metabolism of pregnenolone are called the Δ4 and Δ5 pathways (Gore-Langton & Armstrong 1994; Conley & Bird 1997) (Figure 4), which refers to the number of the first carbon having a double bond in either the first (Δ4) or second (Δ5) ring of the steroid molecule. The Δ4 and Δ5 pathways are observed in human, rat, bovine species, and the Δ5 pathway is the preferred pathway in the human and bovine species (Zuber *et al.* 1986; Fortune 1986). Similar to the bovine species, follicular cells of the human ovary also appear to be unable to utilize the Δ4 pathway by lack of expression of the enzyme converting P_4 to A_4 (Fevold *et al.* 1989; Lin *et al.* 1991). However, the rat exhibits the ability to metabolize P_4 directly to A_4 by the Δ4 pathway (Namiki *et al.* 1988; Fevold *et al.* 1989). Once A_4 is formed within the ovarian theca cells, it can either be converted to T via a HSD17B or be converted to an estrogen by CYP19A1 in granulosa cells. Bovine granulosa cells prefer to metabolize A_4 to E_1 by the enzyme CYP19A1, and then the E_1 is metabolized to E_2 by HSD17B (Conley & Bird 1997). In the bovine species, HSD17B1 has been identified (Sahmi *et al.* 2004). In the rodent, HSD17B type 1, 7 and 12 which are known to convert E_1 to E_2 have been identified in the ovary. HSD17B type 7 and 12 are

found in the endoplasmic reticulum and type1 is cytoplasmic (Labrie *et al.* 1997; Luu-The *et al.* 2006). CYP19A1 is a cytochrome P450 enzyme located in the endoplasmic reticulum. This enzyme is responsible for the conversion of A_4 to E_1 or the conversion of T to E_2. CYP19A1 is a single enzyme encoded by a single gene (Fisher *et al.* 1998).

2.1 Steroidogenesis and the two-cell and two-gonadotropin hypothesis

The follicular somatic cell population is composed of two major cell types, the theca and granulosa cell. These two types of cells express steroidogenic enzymes to produce steroid hormones at different stages of follicular development. The two-cell, two-gonadotropin hypothesis has been confirmed in the bovine species (Armstrong & Dorrington 1977; Fortune 1986; Hillier *et al.* 1994; Adashi *et al.* 1995) (Figure 5). Under the effects of LH, the early steps of steroidogenesis take place in the theca, such that cholesterol is converted into P_5 and consequently to either P_4, or the androgens A_4 or T. These androgens pass by diffusion into the granulosa cell of the ovarian follicle, where, under the influence of FSH, they are converted into estrogens by CYP19A1 and HSD17B1. Granulosa cells also have the capability to produce P_4 when the follicle reaches the large antral and preovulatory stages through expression of the P_4-generating enzymes CYP11A1 and HSD3B (Bao & Garverick 1998).

2.1.1 Classes of follicular steroids

Ovarian follicles secrete three classes of steroids: androgens, estrogens and progestins. Steroids have autocrine and paracrine effects, and directly influence the

reproductive system via specific steroid nuclear receptors. In recent decades, development of knockout mouse models has provided new evidence to understand the function of steroids *in vivo*.

2.1.1.1 Estrogen

The three major naturally occurring estrogens are E_2, estrone and estriol. The active form of estrogen is E_2. During follicular development before the pre-antral stage, FSHr are expressed in granulosa cells, and FSH stimulation is linked to the production of E_2 (Erickson & Shimasaki 2001). Several functions of estrogen have been described in the follicle. Estrogen stimulates 1) granulosa cell proliferation (Richards 1980), 2) the synthesis of granulosa cell insulin-like growth factor 1 (Hernandez *et al.* 1989), 3) the level of estrogen receptor (Richards 1975), 4) the number and size of intracellular gap junctions (Burghardt & Anderson 1981), 5) the expression of CYP19A1 mRNA (Luo & Wiltbank 2006) and 6) induces LHr (Wang & Greenwald 1993). Conversely, estrogen attenuates granulosa cell apoptosis (Quirk *et al.* 2004) and inhibits androgen synthesis in theca cells (Leung & Armstrong 1979). CYP19A1 is a unique granulosa cell enzyme responsible for transformation of estrogen (Fisher *et al.* 1998). Estrogen affects the function of the ovary via granulosa cell estrogen receptor α and β (Sar & Welsch 1999; Sharma *et al.* 1999). Knockout of estrogen receptors and CYP19A1 have shown that estrogen is essential for folliculogenesis beyond the antral stage and is necessary to maintain the female phenotype of ovarian somatic cells (Drummond 2006).

2.1.1.2 Progestin

The active form of progestin is P_4. The production of P_4 and its metabolites is one of the major biosynthetic activities of the ovary. Although P_4 is secreted predominantly by the CL, P_4 is also secreted by theca cells and granulosa cells of developping follicles. In the female reproductive tract, P_4 plays key roles in ovulation, implantation and the maintenance of pregnancy (Graham & Clarke 1997). P_4 performs these functions through two forms of PR, PR-A and PR-B (Spelsberg *et al.* 1972; Kastner *et al.* 1990). In the mouse, knockout of PR-A, blocked ovulation supporting the role of P_4 in ovulation induction. PR-B knockout were ovulatory and produced viable offspring (Mulac-Jericevic *et al.* 2000; Mulac-Jericevic *et al.* 2003).

2.1.1.3 Androgen

Androgens, primarily A_4 and T, are produced by theca cells. These are the immediate biosynthetic precursors of the estrogenic steroids. High ratios of androgens to estrogens have been reported in FF of nonovulatory and atretic follicles (McNatty *et al.* 1979; Carson *et al.* 1981). Moreover, a single injection of DHT (non-aromatizable androgen) to cycling mice reduced the number of large follicles by 15% and left the mice subfertile (Nandedkar & Munshi 1981). Androgens also inhibit FSH-stimulated LHr expression by granulosa cells and enhance granulosa cell apoptosis (Jia *et al.* 1985; Billig *et al.* 1993). These studies have been conducted in the rat and indicate that androgens impede follicular development, especially at the antral follicle stage. However, in the early stages of folliculogenesis, androgens appear to promote follicular growth (Walters *et al.* 2008). In

support of this, administration of androgens to female rhesus monkeys and pregnant ewes (prenatally treated fetuses) stimulates primordial follicle initiation and increases the number of growing follicle and overall follicle survival (Vendola *et al.* 1998; Steckler *et al.* 2005; Forsdike *et al.* 2007). In primate and gilts, androgen stimulates follicle growth and increases *FSHr* mRNA expression in primary or preovulatory follicles (Weil *et al.* 1999; Cardenas *et al.* 2002). Treatment of androgens in cultured bovine granulosa cells also increased *FSHr* mRNA expression (Luo & Wiltbank 2006). However, these studies do not account for the possibility of androgen conversion and hence indirect androgen actions since androgens stimulate CYP19A1 activity and E_2 production (Hamel *et al.* 2005). Although, it is not clear if all the follicular effects observed can be attributed conclusively to direct androgen actions, the production of the AR conditional knockout model has provided a fundamental advance in unraveling the roles of AR-mediated androgen action in female reproductive physiology (Hu *et al.* 2004b). Female conditional AR knockout mice have longer estrous cycles and reduced fertility. Their ovaries contain normal numbers of follicles, although large antral follicles appeared to have fewer granulosa cells and there were reduced numbers of CL (Hu *et al.* 2004b).

2.1.2 Expression of steroidogenic enzymes and gonadotropin receptors during bovine follicular development

During follicular development, steroid hormone secretion and steroidogenic enzyme expression have been used as an index of follicular development. More recently, the bovine model has been used to study the relationship between steroidogenesis and follicular

development. In this review, bovine follicular development has been divided into six stages, primordial, primary, preantral, early antral, antral, and later antral follicle stages. The expression of steroidogenic enzymes and gonadotropin-receptors (FSHr and LHr) during the different stages of bovine follicle development is shown in Table.1.

The *primordial follicle* is formed during fetal life or soon after birth and is composed of a primary oocyte surrounded by a squamous layer of pre-granulosa cells. There is no evidence of active steroidogenesis in primordial follicles. Entry of primordial follicles into the growth phase occurs throughout the reproductive life span and is characterized by conversion of the flattened pre-granulosa cells surrounding the oocye into a single layer of cuboidal granulosa cells. This follicle is now termed a *primary follicle*. In the bovine species, expression of FSHr mRNA was first localized in granulosa cells at the primary follicle stage (Xu *et al.* 1995a; Bao *et al.* 1997b), but there was no expression of steroidogenic enzymes. In the mouse, knockout of FSHr has been shown to block antral follicle formation without affecting growth of primordial, primary and preantral follicles (Kumar *et al.* 1997). This suggests that FSH stimulation may not be a limiting step on primary follicle development. However, some studies suggest that FSH may improve the proliferation of bovine granulosa cells by acting at the primary follicle stage (Garverick *et al.* 2002; Hunter *et al.* 2004; Webb *et al.* 2007).

Following formation of theca cells around the granulosa cells in the *preantral follicle*, mRNA for LHr, CYP11A1, CYP17, and HSD3B are expressed in theca cells, and expression generally increases with growth of preantral and early antral follicles (Xu *et al.* 1995a,b; Bao *et al.* 1997a, b). In granulosa cells at these follicular stages, there is still expression of FSHr without expression of CYP19A1. Moreover, granulosa cells do not express P_4-generating enzymes, such as CYP11A1 and HSD3B. These results indicate that theca cells of follicles at preantral and early antral follicle stages are only able to synthesize androgens and progesterone, and granulosa cells are still steroid-inactive.

The follicular development to an *early antral follicle* (0.4 to 4mm in diameter) is believed to be gonadotropin-independent because growth of antral follicles continues up to 4mm but not above when endogenous concentrations of gonadotropins are suppressed by hypothalamic stalk transection or by removal or inhibition of gonadotropin secretion by a GnRH agonist (Awotwi *et al.* 1984; Campbell *et al.* 1995; Gong *et al.* 1996). The observation of FSHr and LHr knockout mouse models corroborates that development from primordial to early antral follicle stage is gonadotropin-independent (Kumar *et al.* 1997; Lei *et al.* 2001; Zhang *et al.* 2001).

During the *antral follicle* stage (4 to 8 mm in diameter), theca cells keep expressing mRNA of LHr, CYP11A1, CYP17, and HSD3B to promote the secretion of androgens and progesterone (Xu *et al.* 1995a, b; Bao *et al.* 1997a, b). At this stage, expression of

CYP19A1 appears in granulosa cells (Xu *et al.* 1995b) which respond to FSH in vitro (Gutierrez *et al.* 1997a), suggesting that these follicles are able to convert androgens to E_2. Granulosa cells do not express mRNA of LHr, CYP11A1, HSD3B, which suggests that granulosa cells at the antral follicle stage are not yet capable of producing P_4. In the antral and later antral follicular stages, the granulosa and theca cells express FSHr and LHr. The steroid hormone production is increased by gonadotropin stimulation (Campbell *et al.* 1995). These stages can be considered gonadotropin-dependent.

The *later antral follicle* stage is defined as growth of the dominant follicle to the preovulatory follicle stage. At this stage, mRNA of LHr, StAR, CYP11A1, CYP17 and HSD3B are expressed in the theca cells. In granulosa cells, LHr, CYP11A1 and HSD3B mRNA expression are detected for the first time in addition to FSHr and CYP19A1. Furthermore, GSTA is expressed at the later antral follicle stage and codes for a protein with high HSD3B activity (Rabahi *et al.* 1999; Raffalli-Mathieu *et al.* 2007). These results indicate granulosa cells are able to convert androgen to E_2, and also secrete P_4. P_5 production by granulosa cells could be utilized by thecal cells to produce androgen as well (Fortune 1986).

2.1.3 Steroidogenesis in the atretic follicle

The depletion of primordial follicles occurs as the result of one of two processes: atresia or entry into the growth phase. Atresia occurs at any stage of follicular development (Lussier *et al.* 1987). In granulosa cells of preantral and early antral follicles, morphological

signs, such as pyknotic nuclei, are more useful to determine follicular atresia because granulosa cell steroidogenesis is not very active in these follicles. However, changes in steroidogenesis are earlier signs of atresia than changes in morphology in antral and later antral follicles (Xu *et al.* 1995a, b).

Several studies have shown that healthy antral follicles have higher concentrations of E_2 than either P_4 or androgen, while atretic antral follicles have elevated concentrations of P_4 or androgens, resulting in a low ratio of the concentration of E_2 to P_4 in follicular fluid of atretic larger antral follicles (Ireland & Roche 1982; McNatty *et al.* 1984; Spicer *et al.* 1987). In agreement with the decreased secretion of estradiol in atretic antral follicles, expression of mRNA for CYP19A1 and CYP19A1 activity in granulosa cells is the most affected in the early atretic follicle, while expression of mRNA for LHr, CYP11A1, P450C17, HSD3B and StAR in theca cells and FSHr in granulosa cells is less sensitive to atresia, which preserves the secretion of progesterone or androgens in theca cells during the early atretic process (McNatty *et al.* 1984; Price *et al.* 1995; Bao *et al.* 1997a, b). In a few follicles at the advanced or late stages of atresia, the remaining few layers of granulosa cells express CYP11A1, HSD3B, and StAR mRNA (Xu *et al.* 1995b, a; Bao *et al.* 1997b, a; Braw-Tal & Roth 2005), and these follicles have been confirmed as BA which means granulosa cell death can commence from the basal lamina and progresses toward the antrum (Irving-Rodgers *et al.* 2003). Some layers of granulosa cells in basal atretic follicles are morphologically healthy and spontaneously luteinize with CYP11A1, HSD3B, and

StAR mRNA expression similar to that found in granulosa cells during luteinization caused by the preovulatory LH surge (Tilly *et al.* 1992; Irving-Rodgers *et al.* 2003).

2.2 Regulation of steroidogenesis in cultured bovine granulosa cells

In vivo, the complexities of hormonal interactions with the cytokines produced by ovarian cells limit our investigations of steroidogenesis in follicle development. To examine regulation of steroidogenesis, many studies focused on culturing granulosa cells *in vitro* to mimic the changes of steroidogenesis during follicle development.

Granulosa cells in culture luteinize spontaneously, as demonstrated by the shift in the principal secretory product from E_2 to P_4 (Luck *et al.* 1990; Meidan *et al.* 1990). However, in bovine granulosa cells harvested from small antral follicles and cultured in serum-free medium in the presence of low doses of FSH or IGF, E_2 secretion increases gradually over 6 days of culture (Rouillier *et al.* 1996; Gutierrez *et al.* 1997a; Gutierrez *et al.* 1997b; Rouillier *et al.* 1998; Sahmi *et al.* 2004). The E_2-secreting potential of cultured granulosa cells may be associated with the maturation or differentiation stage of the follicle. Although granulosa cells from large antral follicles could secrete E_2, these follicles rapidly loose the potential to produce estradiol with time of culture. Conversely, granulosa cells from small follicles consistently increase E_2 secretion with time, which suggests that granulosa cells from these follicles are less mature and retain the potential to secrete E_2 (Gutierrez *et al.* 1997a).

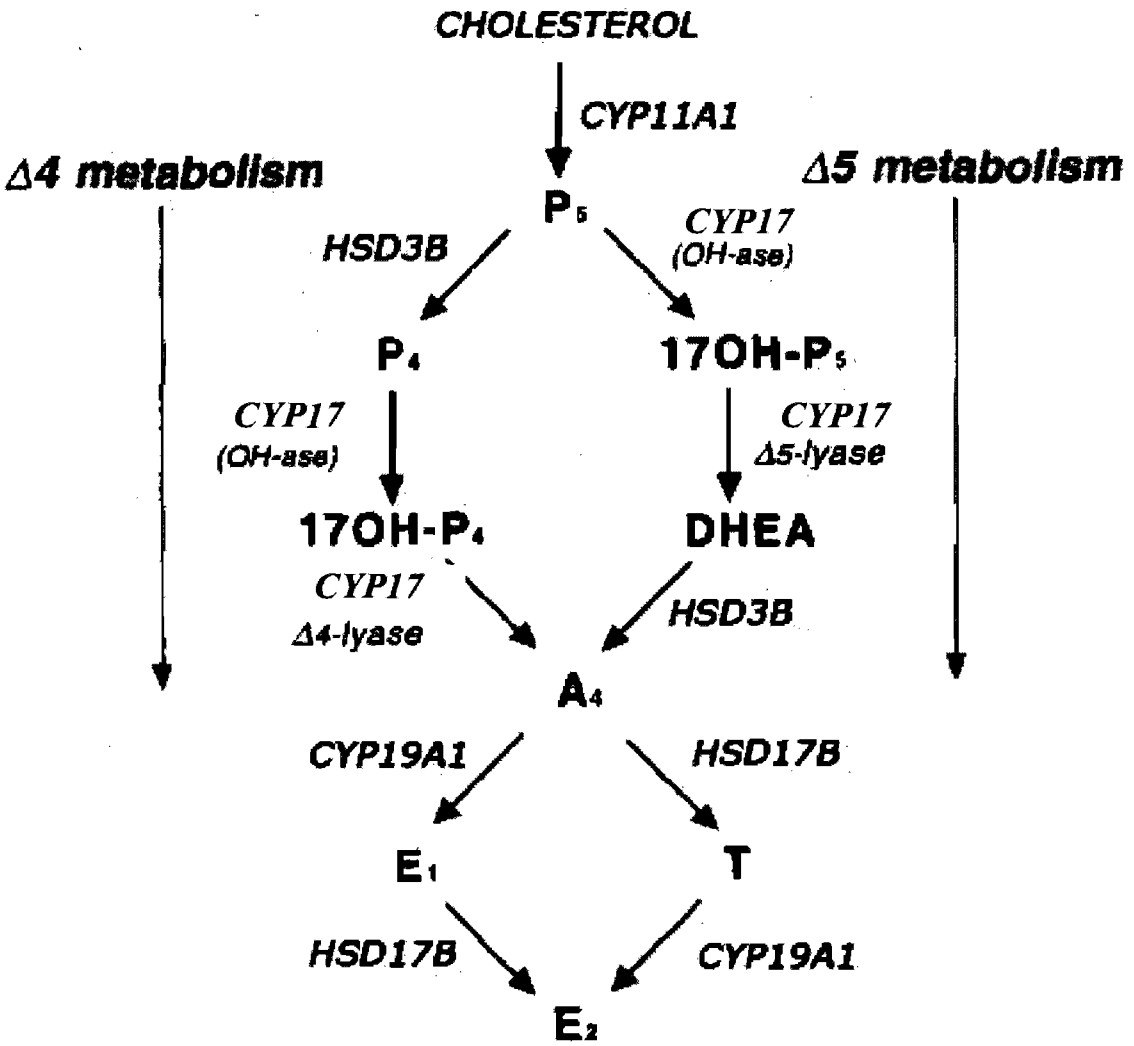


Figure 4. The Δ4 and Δ5 pathways of gonadal steroid synthesis. (Modified from Conley 1997)

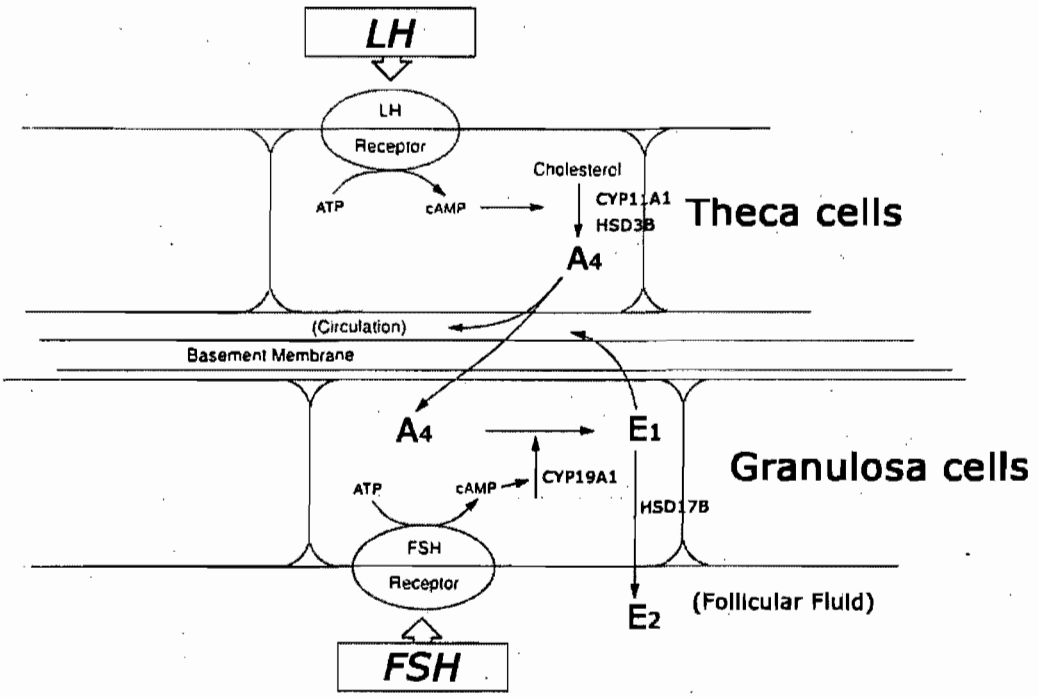


Figure 5. The two-cell, two-gonadotropin theory of follicular steroidogenesis. (Modified from Adashi 1995)

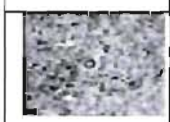
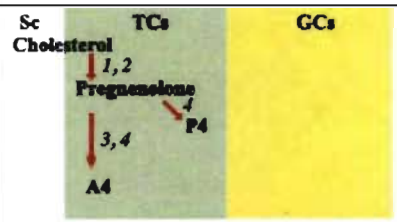
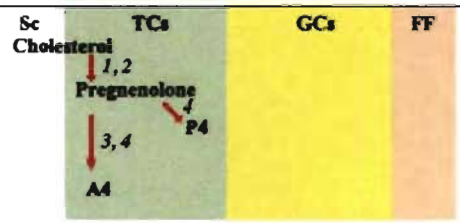
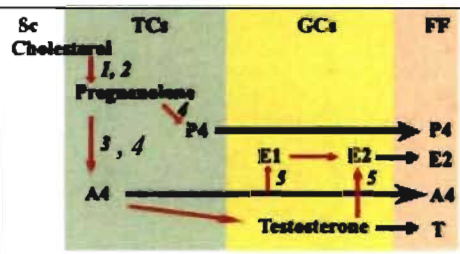
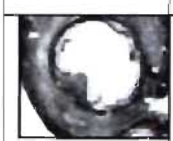
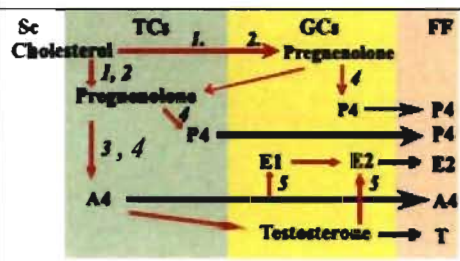
	Healthy Bovine Follicle	Steroidogenic Enzymes & Gonadotropin Receptors	Steroidogenic pathway (Index: Sc: stromal cells, TCs: thecal cells GCs: granulosa cells, FF: follicular fluid, A4: Androstenedione, P4: Progesterone, E1: Estrone, E2: Estradiol)	Ref :
	Primordial Follicle			
	Primary Follicle	FSHr(GC)		(Bao *et al.* 1997b) (Xu *et al.* 1995a)
	Preantral Follicle <0.4mm	FSHr (GC) LHr (TC) *1.* StAR *2.* CYP11A1 *3.* CYP17 *4.* HSD3B		(Xu *et al.* 1995a) (Xu *et al.* 1995b) (Bao *et al.* 1997b)
	Early Antral Follicle 0.4mm-4mm	FSHr (GC) LHr (TC) *1.* StAR *2.* CYP11A1 *3.* CYP17 *4.* HSD3B		(Xu *et al.* 1995b) (Xu *et al.* 1995a) (Bao *et al.* 1998)
	Antral Follicle (4mm-8mm)	FSHr (GC) LHr (TC) *1.* StAR *2.* CYP11A1 *3.* CYP17 *4.* HSD3B *5.* CYP19A1		(Xu *et al.* 1995a) (Xu *et al.* 1995b) (Bao *et al.* 1998)
	Later Antral Follicle >8mm	FSHr (GC) LHr (TC, GC) *1.* StAR *2.* CYP11A1 *3.* CYP17 *4.* HSD3B *5.* CYP19A1		(Xu *et al.* 1995a) (Xu *et al.* 1995b) (Bao *et al.* 1998) (Bao *et al.* 1997a)

Table 1. In vivo expression of steroidogenic enzymes and gonadotropin receptors during bovine follicular development shown by in situ hybridization.

3. Cell proliferation and apoptosis in granulosa cells

In any developing tissue, cell proliferation must be controlled in such a manner that allows some cells to reach terminal differentiation while others are eliminated by programmed cell death or apoptosis. Primordial follicles enter into the growing phase with two potential destinies: ovulation or atresia. Successful follicle development for ovulation requires granulosa cell differentiation accompanied by proliferation (van Wezel & Rodgers 1996; Robker & Richards 1998). Interruption of either proliferation or differentiation will cause exclusion of granulosa cells by apoptosis. Follicular development will depend on the intricate effects of growth factors, gonadotropins (LH and FSH), and steroid hormones on proliferation or apoptosis of granulosa cells (Asselin *et al.* 2000; Jiang J. Y. *et al.* 2003; Quirk *et al.* 2004).

3.1 Cell cycle

Although information on the mechanisms regulating cell cycle progression in granulosa cells is insufficient, much progress in understanding the regulation of the cell cycle has been made in other cell models, such as cancer cells (Sherr & Roberts 1999; Lundberg & Weinberg 1999). The cell cycle consists of four distinct phases: G1, S, G2 and M. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are considered to have entered a state of quiescence called G0 phase. Mechanisms regulating the cell cycle are briefly described here based on previous studies (Sherr & Roberts 1999; Lundberg & Weinberg 1999; Quirk *et al.* 2004). Cell cycle progression is mediated by a protein family

of cdk that are sequentially activated by binding to specific cyclin proteins synthesized periodically during the cell cycle. The mitotic decision in eukaryotic cells is made at the G1 phase of the cell cycle, when mitogens initiate the division process. During early- to mid-G1 phase, progression requires the formation of complexes of cdk4 or cdk6 with cyclin D and cdk2 with cyclin E. The cdk4/6-cyclinD complex combined with the cdk2-cyclin E will initiate hyperphosphorylation of the RB, resulting in loss of its ability to inhibit cell cycle progression during late-G1 phase. Hyperphosphorylation of RB permits liberation of the transcription factors of the E2F family that upregulate expression of the cyclins, kinases, and other proteins necessary for continuation through the S, G2 and M phases of the cell cycle. Formation of additional complexes between various cdks and their cyclin partners are also required for progression through S, G2 and M phases.

3.1.1 Proliferation in granulosa cells

Growth rates vary with the size of the follicle; in the bovine species, a follicle takes 27 days to grow from 0.13 to 0.67 mm, 6.8 days from 0.68 to 3.67 mm and 7.8 days from 3.68 to 8.56 mm (Lussier *et al.* 1987). Growth rate of bovine follicles was estimated based on the mitotic index of granulosa cells (Lussier *et al.* 1987). In primordial follicles, the oocyte is surrounded by a single layer of nondividing granulosa cells arrested in G0 phase of the cell cycle. Once primordial follicles leave this quiescent state, the granulosa cells have entered into the cell cycle but proliferation is exceedingly slow (Hirshfield 1991). Under the influence of gonadotropins, growth factors and steroids, granulosa cells of antral follicles enter into cell cycle progression which results in a rapid burst of proliferation and

the formation of large preovulatory follicles (Rao *et al.* 1978). The rapid phase of proliferation in granulosa cells was measured by a marked increase in the labelling of granulosa cells by tritiated thymidine (Rao *et al.* 1978), as well as by 5-bromodeoxyuridine (BrdU) incorporation or calculation of the mitotic index (Lussier *et al.* 1987; Gaytan *et al.* 1996). Treatment with IGF or estradiol in cultured bovine granulosa cells also induced an increase in G0/G1 to S phase progression (Quirk *et al.* 2004; Hu *et al.* 2004a). When selected follicles reach the preovulatory stage, the LH surge terminates follicular growth by causing granulosa cells to exit the cell cycle and remain in the G0 stage (Rao *et al.* 1978; Stocco *et al.* 2007).

3.2 Apoptosis

Apoptosis is programmed cell death and provides a means for multicellular organisms to eliminate unwanted cells in response to developmental signals or toxic stimuli. The histological features of apoptosis in tissue include cytoplasmic vacuolization, chromatin condensation as well as the appearance of eosinophilic apoptotic bodies as round cytoplasmic masses or as masses of pyknotic chromatin surrounded by a narrow rim of the cytoplasm. In culture, the features of apoptosis include cell shrinkage, surface convolutions, formation of protuberances with buddings and subsequent formation of the apoptotic bodies (Wyllie 1993; Hussein 2005). The process of apoptosis is controlled by a diverse range of cell signals, such as toxins, hormones, growth factors, nitric oxide or cytokines (Asselin *et al.* 2000; Popov *et al.* 2002; Brune 2003; Jiang *et al.* 2003). It is well established that apoptotic signals must be connected to the actual death pathway. In vertebrates, current

evidence from studies of a number of cell types indicates several apoptotic signal pathways leading to cell death (Tilly *et al.* 2004). Two general apoptotic signal pathways have been identified. The intrinsic pathway is generated by internal signals targeting mitochondria functionality, while the extrinsic pathway is directly triggered by the binding of death molecules (TNF and Fas) to cell surface receptors (Amsterdam *et al.* 2003; Hussein *et al.* 2003) (Figure 6). The intrinsic and extrinsic pathways activate the death factors in the cell, such as Bid, cytC, Apaf-1 to generate the death signals. At the end of any death signal transduction, apoptotic signal requires activation of the caspases to accomplish transduction of death signal in mammalian cells (Tilly *et al.* 2004). Caspases include both 'initiator' caspases (8 and 9) and 'effector' caspases (3, 6 and 7). Once caspases 3, 6 and 7 activated, this executioner enzyme is irreversible, and results in the cleavage of numerous structural and functional proteins (such as PARP which is DNA repair protein) and the internucleosomal cleavage of genomic DNA. (Matikainen *et al.* 2001; Johnson & Bridgham 2002; Tilly *et al.* 2004). Cell death pathways are opposed by anti-apoptotic proteins that protect mitochondrial membrane integrity (Bcl-x, Bcl-2), block caspase activity (inhibitor of apoptosis factors :IAPs) or prevent signaling via death receptors (the adaptor protein, FLIP). Expression of these anti-apoptotic proteins is regulated by gonadotropins (FSH and LH) and locally produced growth factors (IGF-I and EGF family ligands) via cell survival signaling pathways.

3.2.1 Apoptosis in granulosa cells

During the postnatal development of primordial through to preantral mammalian

follicles, atresia is initiated mainly by the death of the oocyte, and percentage of atresia at these stages is lower than in antral follicles (Lussier *et al.* 1987; Morita & Tilly 1999). It is established that degeneration of atretic antral follicles in mammalian ovaries can be explained by apoptosis of granulosa cells and theca cells (Tilly 1996). Within the follicle, the most obvious cell type undergoing apoptosis in association with maturing follicle atresia is the granulosa cell (Tilly *et al.* 1991; Tilly *et al.* 1992; Palumbo & Yeh 1994). The apoptosis of granulosa cells has been observed on the basis of the changes in morphology and also detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique, DNA agarose gel electrophoresis with the formation of a DNA ladder, and flow cytometry that measures degraded DNA which appears as cells with hypodiploid DNA content and are represented in so-called "sub-G1" peaks on DNA histograms. (Nicoletti *et al.* 1991; Wyllie 1993; Rouillier *et al.* 1998; Yang & Rajamahendran 2000; Irving-Rodgers *et al.* 2001). Moreover, the signals, receptors and intracellular signalling pathways leading to apoptosis within granulosa cells have been obtained from studies of genetic mouse and in vitro cultured cells. Although studies in terms of apoptosis are still ongoing, it appears that multiple molecules are involved. These molecules include survival factors, such as gonadotropins, IGF-1, E_2, interleukin-1ß, EGF, bFGF, and TGF-α; and also include apoptotic factors, such as caspase, Fas antigens, reactive oxygen species, interleukin-6, androgens, bax, p53, and TNF-α (Asselin *et al.* 2000; Jiang *et al.* 2003; Quirk *et al.* 2004; Hussein 2005). Caspase-3 is a caspase common to several different apoptosis pathways (Matikainen *et al.* 2001; Johnson & Bridgham

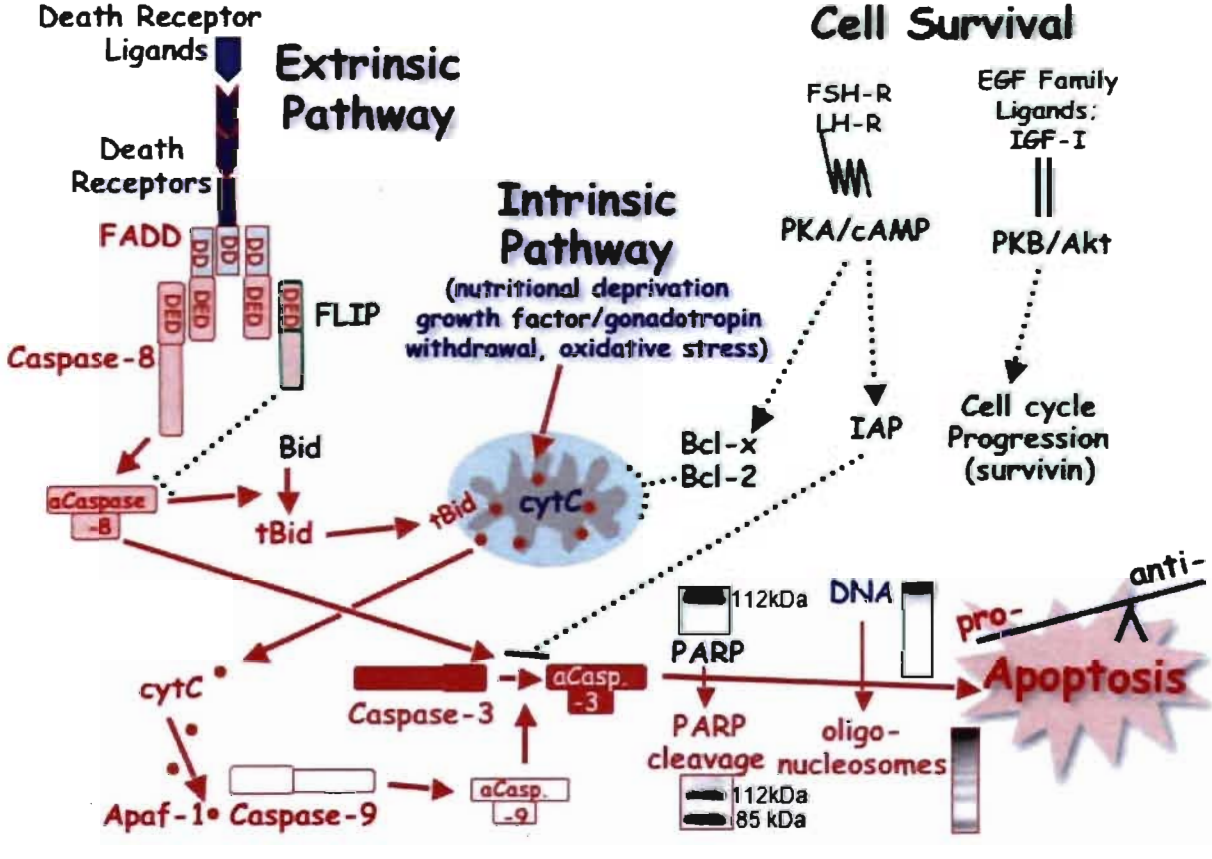
2002). Caspase-3 is actively involved in follicular atresia, and present in granulosa cells of atretic, but not healthy follicles (Boone & Tsang 1998; Nicholas *et al.* 2005). Furthermore, several intracellular signalling pathways have been linked directly to granulosa cell survival as well, including gonadotropin- and vasoactive intestinal peptide-induced cAMP formation, MAPK-ErK and IP3K-AKt (Flaws *et al.* 1995; Johnson *et al.* 1999; Gebauer *et al.* 1999; Asselin *et al.* 2001; Johnson *et al.* 2001).

3.2.2 Cell cycle and apoptosis

The transition from preantral to antral follicles occurs after exposure of the granulosa cells to FSH. Then, rapid proliferation and differentiation of the granulosa cells is initiated during antral follicular development. At this period, the proportion of bovine follicles undergoing atresia increases dramatically with follicular diameter and the rate of atresia doubles between 2 and 8 mm (Lussier *et al.* 1987). In the rat, the highest frequency of atresia occurs in antral follicles with relatively rapid granulosa cell proliferation in comparison to smaller follicles (Hirshfield & Midgley 1978). These observations provide evidence that granulosa cells that are dividing rapidly are most susceptible to apoptosis. In agreement with this finding, in vitro studies have demonstrated that cell survival is linked to maintenance or stimulation of G1-to-S phase transition whereas blocking this transition increases susceptibility to apoptosis (Quirk *et al.* 2004). In addition, the LH surge terminates follicular growth by causing granulosa cells to exit the cell cycle and remain in the G0 stage (Rao *et al.* 1978; Hirshfield 1991). When the granulosa cell remains in the G0 stage of the cell cycle, postmitotic differentiation happens and prevents granulosa cell

susceptibility to apoptosis, and a sign of differentiation of granulosa cells at this period is an increase in secretion of P_4 (Lundberg & Weinberg 1999; Peluso 2003). Based on the expression pattern of the PR, it is predictable that P_4 inhibits apoptosis in cultured granulosa cells isolated during the gonadotropin (LH) surge or periovulatory period (Peluso & Pappalardo 1998; Svensson *et al.* 2000).

Figure 6. A summary of apoptotic and survival signalling pathways. The absence of sufficient anti-apoptotic protein expression (dotted green lines) is proposed to tip the balance in favor of activating pro-apoptotic pathways (solid red lines). Abbreviations: aCasp.-3, -7, -8, activated caspases; Apaf-1, Apoptosis Protease Activating Factor-1; Bid and tBid, intact or truncated BH3 Interacting Domain death agonist; cytC, cytochrome C; DD, death domain; DED, death effector domain; EGF, epidermal growth factor; FADD, Fas-Associated Death Domain; FLIP, Flice-like inhibitory protein; FSH, follicle-stimulating hormone; Inhibitor of Apoptosis Protein, IAP; IGF-I, insulin-like growth factor I; LH, luteinizing hormone; PARP, Poly-(ADP-ribose)-polymerase; PKA/cAMP, protein kinase A/cyclic AMP signaling pathway; PKB/Akt, protein kinase B/Akt signaling pathway. Each of the proteins and cell signaling pathways depicted has been characterized in hen granulosa cells, in vitro. (adapted from http://www.nd.edu/~avianova/Apoptosis.html)


Death Receptor Ligands
Extrinsic Pathway
Death Receptors
FADD
DD
DED
FLIP
Caspase-8
Intrinsic Pathway
(nutritional deprivation growth factor/gonadotropin withdrawal, oxidative stress)
Cell Survival
FSH-R
LH-R
PKA/cAMP
EGF Family Ligands; IGF-I
PKB/Akt
Cell cycle Progression (survivin)
IAP
Bcl-x
Bcl-2
Bid
aCaspase -8
tBid
cytC
112kDa
PARP
DNA
pro-
anti-
Apoptosis
cytC
Caspase-3
aCasp. -3
PARP cleavage
oligo-nucleosomes
Apaf-1
Caspase-9
aCasp. -9
112kDa
85 kDa

4. Characterization of TGF-β

The TGF-β superfamily is a structurally conserved but functionally diverse group of proteins comprised of at least 35 members in vertebrates. These proteins include TGF-β proteins (TGF-β1, TGF-β2, TGF-β3), AMH, inhibins (types A and B), activins (types A, B and AB), BMP (1 to 20), GDF (1to 9) and nodal (Chang *et al.* 2002). Many members in the TGF-β superfamily are widely distributed throughout the body of mammals and control cellular growth and differentiation during both pre and postnatal life (Massague & Wotton 2000).

The TGF-β isoforms are synthesized as dimeric precursor proproteins consisting of a signal peptide, a large proregion, and a smaller biologically active mature region. The proproteins are cleaved during secretion to yield mature cytokines (Massague 1990). Mature TGF-β also associates with its propeptide through noncovalent interactions, creating a latent complex from which TGF-β must be released to elicit its biological activity (Lawrence 1991; Gleizes *et al.* 1997). The role of the proregion of the TGF-β superfamily members is thought to be important for the correct folding and dimerization of the molecule as dimerization occurs before proteolytic processing to release the biologically active mature region (Shimasaki *et al.* 2004). The proregion also appears to be important for regulating the activity of the mature region through non-covalent association with the mature dimer to either inhibit or enhance biological activity (Massague 1990;

Jiang *et al.* 2004). Another characteristic of TGF-β proteins is that the mature bioactive forms are homo or hetero-dimers corresponding to the cleaved carboxyterminal regions of larger pre-proteins. These dimers are usually covalently bound through a conserved cysteine residue (Chang *et al.* 2002). However, some members, such as GDF9 and BMP15 can form homo-dimers as well as hetero-dimers through non-covalent interactions in vitro (Liao *et al.* 2003; Liao *et al.* 2004).

4.1 Signalling cascade of TGF-β in the cell

The active TGF-β ligand binds with two types of Ser/Thr kinase receptor named TGF-β type-I and type-II receptor, forming hetero-tetrameric complexes. In mammals, this basic receptor complex consists of five type-II receptor subunits and seven TGF-β type-I subunits (Chang *et al.* 2002; Knight & Glister 2003). Generally, different TGF-β superfamily ligands can interact with the TGF-β type II receptor, with subsequent recruitment of a TGF-β type I receptor into a heterotetrameric receptor complex (Juengel & McNatty 2005). In addition to TGF-β type-I and TGF-β type-II receptors, there is also TGF-β type-III receptor (known as β-glycan). Although the function of TGF-β type-III receptor is not fully understood, a study showed this receptor can act as a non-signalling co-receptor to bind TGF-β isoforms or inhibin and enhance the presentation of these ligands to type-II receptors (Lewis *et al.* 2000). Binding of ligand to the TGF-β type-II receptor and type I receptor complex through phosphorylation of their intracellular kinase domains results in phosphorylation of downstream-signalling molecules called the receptor type

Smads (rSmad), including Smad 1, 2, 3, 5, and 8. The phosphorylated receptor Smad then forms a complex with a common Smad (cSmad, known as Smad4) and translocates to the nucleus to modulate target gene expression through interaction with various transcription factors, co-activators and co-repressors (Derynck & Zhang 2003; Fuller & Chu 2004) (Figure 7).

Inhibition of TGF-β superfamily signalling can occur by preventing the ligand from binding to active receptor complexes and preventing activation of rSmads. Binding of the ligand to a soluble binding protein (bp) (such as follistatin) or a soluble type-I receptor or binding to a "decoy receptor" (such as BAMBI) prevents ligand binding to the TGF-β receptors and thus prevents activation of rSmads (Gumienny & Padgett 2002). Inhibition can also occur at the level of activation of the Smad as the inhibitory Smads (iSmad, such as Smad 6 & 7) can interact with the activated type I receptor, thereby preventing phosphorylation and subsequent activation of the rSmads (Derynck & Zhang 2003).

4.2 Role of TGF-β in the ovary

Expression of TGF-β superfamily members is present in the ovary of different species and these molecules play a important role during follicular development (Juengel & McNatty 2005). Studies on knockout or transgenic mouse models provide some evidence in support of a physiologic role for TGF-β superfamily members during follicular development. For instance, GDF-9-deficient female mice were found to be infertile and

follicular development was blocked at the primary one-layer follicle stage (Dong *et al.* 1996). BMP-15 knockout female mice exhibited normal folliculogenesis but were subfertile due to defects in ovulation and early embryonic development (Yan *et al.* 2001). Recently, overexpression of BMP-15 in transgenic mice led to accelerated folliculogenesis by promoting follicle growth, but preventing follicle maturation, resulting in an early decline of the ovarian reserve (McMahon *et al.* 2008). However, inherent species-specific differences exist in the ovulation quota, follicular waves, duration of the ovarian cycle between the traditional animal model (polyovulatory mouse) versus monoovulatory species such as cattle, sheep and primates. Compared with mice, different physiological roles of BMP-15 and GDF-9 were found on follicular development in sheep, where heterozygous point mutations in either BMP15 or GDF9 genes resulted in increases in ovulation quota and litter size (Galloway *et al.* 2000; Hanrahan *et al.* 2004).

However, TGF-β1, TGF-β2, TGF-β3 knockout mice could not successfully explain the role of TGF-β in vivo. Limited information about the role of TGF-β1, 2, 3 in follicle development can be gained from knockout mouse models because these mice die within hours, days, or weeks of birth (Dickson *et al.* 1995; Kaartinen *et al.* 1995; Sanford *et al.* 1997). Alternatively, studies of TGF-β in cultured ovarian somatic cells provide data for understanding the role of TGF-β1, 2, 3 in the ovary.

4.2.1 Expression and regulation of TGF-β1, 2, 3 and TGF-β type I, II receptors in the ovary

TGF-βs exist in five structurally related isoforms, three of which are expressed in mammals and designated as TGF-β1, TGF-β2, and TGF-β3 (Lawrence 1996). Signals from all three isoforms appear to be mediated by a single type-II receptor called TβR-II and one type-I receptor referred to as TβR-I or ALK-5 (Roberts 1999). Expression of TGF-βs and their receptors at certain periods of the estrous cycle may be critical to successfully carrying out the function of TGF-βs in the ovary. Studies provide evidence that mRNA/proteins of TGF-βs and their receptors are present in granulosa and thecal cells as well as in the oocyte in human, sheep, cow, rat, mouse, pig, quail, hamster (reviewed in Nilsson *et al.* 2003; Juengel & McNatty 2005). The expression patterns of TGF-βs and their receptors are known to vary among these species, and the reasons for these discrepancies are uncertain. However, some in vitro studies examining the regulation of expression of TGF-βs and their receptors in the ovary provide possible clues related to the expression patterns of TGF-βs and theirs receptors in vivo.

For instance, FSH treatment decreased TGF-β1 mRNA expression in bovine granulosa cells, but did not affect TGF-β2 and TGF-β3 mRNA (Nilsson *et al.* 2003). Similarly, FSH treatment of rat granulosa cells resulted in decreased TGF-β2 mRNA expression, and treatment of rat theca cells with LH decreased TGF-β2 expression as well

(Mulheron & Schomberg 1990; Mulheron *et al.* 1991; Magoffin *et al.* 1995). FSH also decreased ALK-5 mRNA expression in cultured bovine and ovine granulosa cells (Jayawardana *et al.* 2006; Chen *et al.* 2008). Conversely, in humans, TGF-β1 protein level in follicular fluid was increased by treatment of FSH (Fried *et al.* 1998). Gonadotropins are not the only factors regulating expression of TGF-βs and their receptors. Steroid hormones such as E_2 also have the potential to regulate the expression of TGF-βs and their receptors. In bovine follicular fluid of 6.5 mm follicles, E_2 was negatively correlated with protein levels of TGF-β1 (Ouellette *et al.* 2005). This is in agreement with results in the rat where E_2 inhibited both active and total TGF-β secretion (Magoffin *et al.* 1995). Conversely, E_2 increased ALK5 mRNA expression in cultured bovine and ovine granulosa cells (Jayawardana *et al.* 2006; Chen *et al.* 2008).

4.2.2 Effects of TGF-β on function of granulosa cells

When studying the action of TGF-βs on granulosa cell function, TGF-β1 or TGF-β2 are usually selected as representative of the TGF-β superfamily. TGF-β1 stimulated P_4 synthesis in cultured rodent granulosa cells (Dodson & Schomberg 1987; Hutchinson *et al.* 1987; Knecht *et al.* 1987). In addition, TGF-β1 augmented FSH-stimulated E_2 production from rodent granulosa cells (Adashi *et al.* 1989; Zachow *et al.* 1999). This effect was attributed to stimulatory effects of TGF-β1 on CYP19A1 activity and FSHr (Hutchinson *et al.* 1987; Inoue *et al.* 2003). TGF-β1 also brings about dose-dependent increases in FSH-induced LHr mRNA by increasing the half-life of LHr mRNA (Inoue *et al.* 2002).

Conversely, TGF-β1 inhibited granulosa cell secretion of E_2 and P_4 from pigs and ruminants (Wandji *et al.* 1996; Chang *et al.* 1996; Ford & Howard 1997; Juengel *et al.* 2004; Ouellette *et al.* 2005). In vivo, a previous study showed a negative relationship between protein level of TGF-β1 and follicular fluid E_2 concentration and between protein level of TGF-β1 and follicle size (Ouellette *et al.* 2005). Moreover, species differences were also observed in the regulation of cell proliferation where TGF-β1 inhibited EGF- or FSH-stimulated DNA synthesis and mitosis in cultured bovine, porcine or ovine granulosa cells (Skinner *et al.* 1987; Gangrade & May 1990; Juengel *et al.* 2004), but it increased FSH-stimulated DNA synthesis and mitosis in rat granulosa cells (Dorrington *et al.* 1993). The contradictory results of TGF-β1 function in ovarian physiology between animal species confirm the multifunctional role of TGF-β1 that has been reported in many other tissues (Herrmann *et al.* 2002) and would appear to be related to the stage of differentiation and the state of stimulation of the tissues examined in vitro.

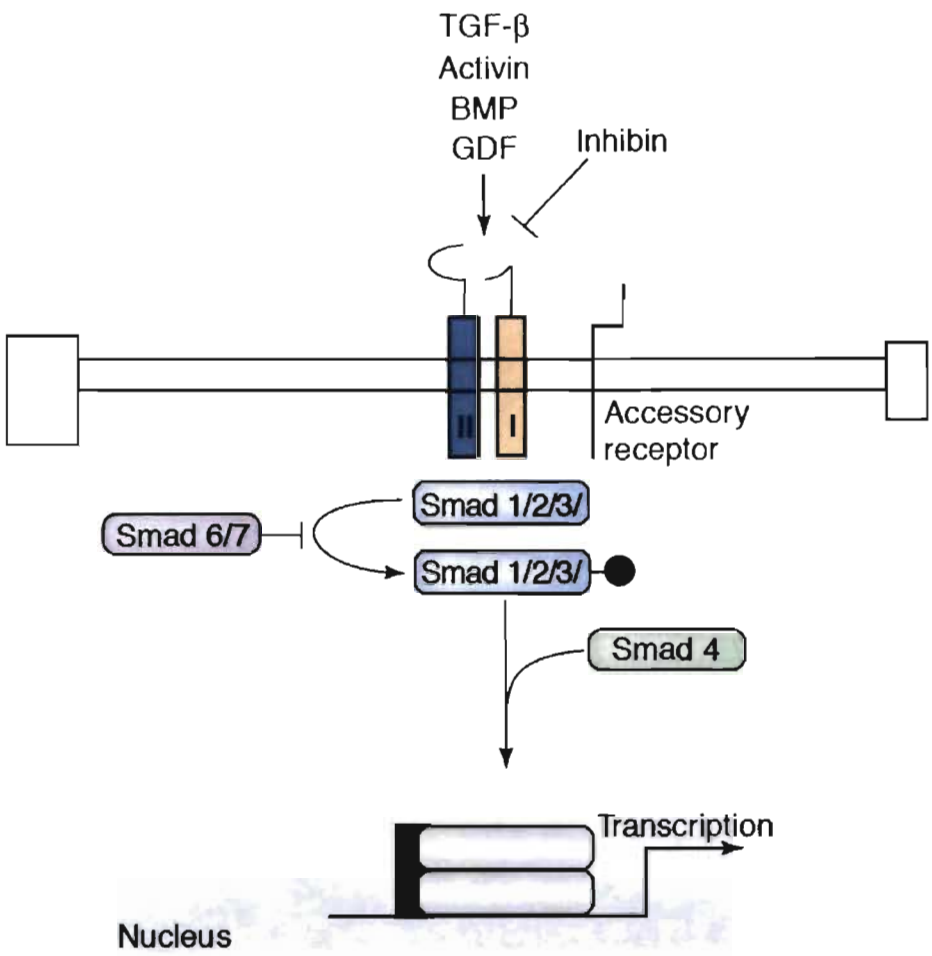


Figure 7. TGF-β family signalling (adapted from Fuller 2004).

HYPOTHESIS AND OBJECTIVES

Synthesis of E_2 and P_4 in granulosa cells, granulosa cell proliferation and apoptosis are important to follicular development. In the normally developing follicle, circulating molecules and locally produced factors are involved to control the rate and the time of secretion of these two key steroid hormones, as well as cell proliferation and apoptosis. Locally produced growth factors, such as the transforming growth factor-β superfamily, affect steroidogenesis, cell proliferation and apoptosis and eventually control follicle development. The hypothesis of this study is that the change in steroid concentrations observed during treatment with TGF-β1 is a result of the modulation of the expression and activity of specific steroidogenic enzymes responsible for the synthesis of E_2 and P_4. The overall objective was to assess the effects of TGF-β1 on steroidogenesis, cell proliferation and apoptosis.

The specific objectives of this study were :

1. To determine the effect of TGF-β1 on E_2 and P_4 synthesis and the corresponding expression and activity of key steroidogenic enzymes in FSH-stimulated bovine granulosa cells.

2. To assess the effects of TGF-β1 on E_2 and P_4 synthesis and the corresponding expression and activity of key steroidogenic enzymes in quiescent bovine granulosa cells.

3. To determine the effects of TGF-β1 on cell proliferation and apoptosis in quiescent and FSH-stimulated granulosa cells.

ARTICLE ONE

Title: Role of transforming growth factor-β1 in gene expression and activity of estradiol and progesterone-generating enzymes in FSH-stimulated bovine granulosa cells.

Thesis author's contribution to this work: As the primary author, I was responsible for all aspects of this article. Second, third and fourth authorship credits reflect technical support. Fifth authorship credit represents directorship.

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Role of transforming growth factor-β1 in gene expression and activity of estradiol and progesterone-generating enzymes in FSH-stimulated bovine granulosa cells.

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Short title: TGF-β1 inhibition of stimulated granulosa cell steroidogenesis

ABSTRACT

Survival factors and inhibitory factors regulate steroidogenesis and determine the fate of developing follicles. The objective of this study was to determine the role of Transforming growth factor-beta 1 (TGF-β1) in the regulation of oestradiol (E_2) and progesterone (P_4) secretion in FSH-stimulated bovine granulosa cells. Granulosa cells were obtained from 2-5 mm follicles and were cultured for 6 days in the absence of serum. FSH dose and time in culture increased E_2 secretion and mRNA expression of E_2-related enzymes *CYP19A1*, *HSD17B1*, but not *HSD17B7*. TGF-β1 inhibited E_2 secretion, and decreased mRNA expression of *FSHr*, *CYP19A1* and *HSD17B1*, but not *HSD17B7*. FSH dose did not affect P_4 secretion and mRNA expression of *HSD3B* and *GSTA*, but inhibited the amount of *StAR* mRNA, and stimulated *CYP11A1* mRNA only at the highest dose. Conversely, P_4 and the P_4-related enzyme mRNA for *StAR*, *CYP11A1*, *HSD3B*, *GSTA* increased with time in culture. TGF-β1 inhibited P_4 secretion and decreased mRNA expression of *StAR, CYP11A1, HSD3B* and *GSTA*. TGF-β1 modified the formation of granulosa cell clumps and caused a small reduction in total cell protein. Finally, TGF-β1 decreased conversion of androgens to E_2 but did not decrease the conversion of oestrone (E_1) to E_2 and pregnenolone to P_4. Overall these results indicate that TGF-β1 counteracts stimulation of E_2 and P_4 synthesis in granulosa cells by inhibiting key enzymes involved in the conversion of androgens to E_2 and cholesterol to P_4 without shutting down the reducing HSD17B and HSD3B activites.

INTRODUCTION

In cattle, follicle development occurs in waves consisting of rhythmic emergence and growth of a new cohort of antral follicles (Ireland et al. 2000). During this process the proportion of follicles undergoing atresia increases dramatically with follicular diameter and the rate of atresia doubles between 2 and 8 mm (Lussier et al. 1987). At the final growth phase, most follicles are atretic and only one follicle (exceptionally two) is selected to become dominant and continue to grow until ovulation (Fortune et al. 2001). Many factors are involved in follicular development, including gonadotropins, steroid hormones, cytokines and other endocrine, paracrine and autocrine factors. These factors induce cell death or survival and thus determine the fate of the growing follicle.

Gonadotrophin stimulation of responsive follicles is associated with increased synthesis of oestradiol-17β (E2) and progesterone (P4), two key steroid hormones associated with the development of the ovulatory follicle (Price et al. 1999; Kolibianakis et al. 2005). E2 is produced by granulosa cells and is important for granulosa cell growth, attenuation of granulosa cell apoptosis and positive and negative feedback regulation of the hypothalamic-pituitary-ovarian axis (Kolibianakis et al. 2005). In ruminants and humans, gonadotrophin stimulation of E2 requires LH stimulation of theca cells to produce androgens, mostly as androstenedione (A4), and FSH-mediated conversion of theca-derived A4 to E2 in granulosa cells (Fortune 1986; Hillier et al. 1994). A4 is converted to oestrone (E1) by

cytochrome P450 aromatase (CYP19A1) then into E2 by 17β-hydroxysteroid dehydrogenase (HSD17B) reducing enzymes (Hillier et al. 1994; Mindnich et al. 2004). Granulosa cells also produce P4 which is necessary for the induction of ovulation (Drummond 2006). The production of P4 involves transformation of cholesterol to pregnenolone (P5) by the cytochrome P450 side-chain cleavage (CYP11A1) followed by the conversion of P5 to P4 by 3β-hydroxysteroid dehydrogenase (HSD3B). In addition, α glutathione S-transferase (GSTA) is expressed in bovine granulosa cells and codes for a protein with high HSD3B activity (Rabahi et al. 1999; Raffalli-Mathieu et al. 2007). In vivo, most of the steroidogenic enzyme genes are not expressed in granulosa cells of small bovine antral follicles (0.4-4 mm) (Bao & Garverick 1998). In granulosa cells, expression of CYP19A1 and CYP11A1 gradually increases during follicle growth, and HSD3B and HSD17B are first observed in non-atretic follicles at approximately 8 mm (Xu et al. 1995; Bao et al. 1997; Sahmi et al. 2004). These data indicate that the timely expression of steroidogenic enzymes at distinct periods of follicular development are important for the selection of the dominant follicle and successful ovulation. A better characterization of the regulation of these two steroid hormones in granulosa cells will improve our understanding of follicle development.

In recent years, much attention has focused on the role of members of the transforming growth factor-β (TGF-β) superfamily acting as paracrine and autocrine factors to modulate ovarian function and fertility (Knight & Glister 2006). TGF-β1 is present in granulosa and

theca cells and in the vascular system of the ovary in many species (Nilsson et al. 2003; Juengel & McNatty 2005), and has been shown to either stimulate or inhibit E2 and P4 synthesis. For instance, TGF-β1 stimulated E2 and P4 secretion from rodent granulosa cells (Zachow et al. 1999; Knight & Glister 2006), whereas TGF-β1 inhibited granulosa cell secretion of E2 and P4 from pigs and ruminants (Wandji & Fortune 1996; Chang et al. 1996; Ford & Howard 1997; Juengel et al. 2004; Ouellette et al. 2005). These reports detailing the effect of TGF-b1 on ovarian cells appear to be highly dependent on the species studied, stage of follicle differentiation and the presence of different growth factors as cotreatments, and the mechanism of TGF-β1 action in granulosa cells is not clear. In vivo, the complexities of hormonal interactions with the cytokines produced by ovarian cells limit our understanding of steroidogenesis in follicle development. Fortunately, E2 secretion can be maintained for several days in bovine granulosa cells cultured in vitro with FSH in serum-free conditions (Gutierrez et al. 1997). This in vitro model mimics the gradual increase in E2 secretion seen in growing follicles and can be used to identify the key factors regulating steroidogenesis in granulosa cells. Therefore, the objective of the present study was to determine the effect of TGF-β1 on E2 and P4 synthesis and the corresponding expression and activity of key steroidogenic enzymes in FSH-stimulated bovine granulosa cells.

MATERIALS AND METHODS

Experimental design

As it has not been established if FSH stimulates all the steroidogenic enzyme genes in granulosa cells, we first determined the effect of FSH by culturing cells as described below with graded doses of FSH (0, 1 or 10 ng/ml) for 6 days. Based on these data, the development of gene expression during culture was assessed by culturing cells with 1 ng/ml FSH for 2, 4 or 6 days, and cells were recovered at each time-point for the extraction of RNA. Expression of the estrogenic enzyme genes was highest on day 6, therefore all subsequent experiments with TGF-β1 were performed for 6 days in the presence of 1 ng/ml FSH.

Cell Culture

Cell culture was performed essentially as described by Gutierrez *et al.* (1997). Briefly, ovaries were collected at a local abattoir from adult cows irrespective of stage of the oestrous cycle and transported to the laboratory at 37°C in phosphate buffered saline (PBS) containing penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone (1 μg/ml). Follicles of 2-5 mm in diameter were dissected from the ovaries, and granulosa cells were isolated mechanically by rinsing sections of follicle walls repeatedly through a disposable

pipette. The granulosa cell suspension was filtered through a 150 mesh steel sieve (Sigma, St-Louis, MO, USA) to remove oocytes.

Cells were seeded into 24-well tissue culture plates (Corning Glass Works, Corning, NY, USA) at a density of 10^6 viable cells (tested by Trypan blue exclusion) in 1 ml of α-MEM with L-glutamine containing sodium bicarbonate (10 mM), Hepes (20 mM), nonessential amino acid mix (1.1 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml), protease-free BSA (0.1 %), sodium selenite (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (A_4) (100 nM) (all from Sigma, St-Louis, MO, USA), ovine FSH (1 ng/ml) (oFSH, AFP-5332B; NIDDKD, Torrance, CA, USA), insulin (10 ng/ml) (Invitrogen, USA) and graded doses of recombinant active human TGF-β1 (R & D Systems, Minneapolis, MN, USA) starting on the first day of culture. Cultures were maintained at 37°C in 5% CO_2 for 6 days, with 700 µl of medium being replaced every 2 days.

At the end of culture, medium was collected and frozen for subsequent steroid assay and granulosa cells were collected for RNA extraction or lysed for total protein measurement. Total cell protein was extracted by the addition of 200 µl of 1M NaOH to each well for 2 h at room temperature, followed by neutralization with 200 µl of 1 M HCl. Protein concentrations were measured using the Bio-Rad micro-assay (Bio-Rad, Mississauga, ON, Canada). All experiments were performed with at least three independent cell cultures.

Steroid Assays

Culture medium samples were assayed for E_2 as previously reported (Bélanger *et al.* 1990) but without C-18 column extraction. Cross-reaction of A_4 and oestrone (E_1) with the E_2 assay was less than 0.1% (Bélanger *et al.* 1980). P_4 was measured in duplicate as described (Lafrance & Goff 1985). Intra- and inter-assay coefficients of variation were less than 15% for both assays. The sensitivity of the E_2, and P_4 assays were 8 and 32 pg per tube respectively. The steroid hormone concentrations were corrected for cell number by normalization to total cell protein.

RT-PCR

Total RNA was extracted using the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions and treated with DNAse (QIAGEN, Valencia, CA). The reverse transcription reaction was performed on 1 μg total RNA with Omniscript enzyme (QIAGEN, Valencia, CA). Gene expression was measured by semi-quantitative PCR. The primers used were those described previously for *Cyclophilin* (*1B15*) (Bettegowda *et al.* 2006), *GAPDH* (Sahmi *et al.* 2004), *CYP11A1* (Vanselow *et al.* 2004), *HSD3B* (Vanselow *et al.* 2004), *CYP19A1* (Sahmi *et al.* 2004), and *FSHr* (Ndiaye *et al.* 2005). Sense (5'-TTGTGCGAGAGTCTGGCGATTCT-3') and antisense (5'-AGGAATCGCTCGGTGGTGAAGTA-3') primers for *HSD17B1* were designed based on the bovine sequence (NM_001102365) with a product size of 287 bp. Sense (5'-CGGTTGCTGGAAGAAGATGATG-3') and antisense (5'-

TCACCAGAGGATTGAGAGACTCG-3') primers for *HSD17B7* were designed based on the bovine sequence (XM_581467) with a product size of 789 bp. Sense (5'-GACACGGTCATCACTCACGAGTT-3') and antisense (5'-ATGCTGAGCAGCCAGGTGAGTT-3') primers for *StAR* were designed based on the bovine sequence (XM_001250261) with a product size of 248 bp. Sense (5'-GAATGGAGTGCATTCGGTGGCTC-3') and antisense (5'-GACTGCTGACTCTGGCTTTTAGG-3') primers for *GSTA* were designed based on the bovine sequence (BTU49179) with a product size of 528 bp. Forward and reverse primers used in the PCR reaction were located in different exons to avoid amplification of any residual genomic DNA. The PCR products for each gene were sequenced to confirm the identity of the gene. PCR was performed under the following conditions: 1) initial denaturation at 94°C for 3 min; 2) amplification cycles of denaturation at 94°C for 45 sec, annealing for 45 sec at 60°C (*CYP11A1*, *HSD3B*, *FSHr* and *GAPDH*) or 64°C (*HSD17B1*, *HSD17B7*, *GSTA*), or for 30 sec at 62°C (*StAR*, *1B15* and *CYP19A1*); 3) elongation at 72°C for 1 min; 4) final elongation at 72°C for 5 min. Optimal cycle number for amplification during the exponential phase was determined for each gene. The reactions were performed for 31 cycles for *StAR*, 35 cycles for *CYP11A1*, 34 cycles for *HSD3B*, 25 cycles for *GSTA*, 29 cycles for *CYP19A1*, 36 cycles for *HSD17B1* and *HSD17B7*, 37 cycles for *FSHr*, 25 cycles for *GAPDH* and 27 cycles for *1B15*. The PCR products were separated on 2% agarose gels containing 0.001% ethidium bromide and visualized under UV light.

Quantification of band intensity was performed with NIH Image J software. Target gene mRNA abundance was expressed relative to *1B15* mRNA abundance.

Enzyme activity assays

Short-term incubation of tritiated steroid hormone precursors was conducted at the end of day 6 of culture to measure specific enzyme activities. On day 6, all the medium of each well was removed and replaced with fresh medium without A_4 but containing 6-11 nM (1.6 $x10^6$ DPM/ml medium) of either [^{3}H]-E_1, [^{3}H]-A_4, [^{3}H]-testosterone ([^{3}H]-T) or [^{3}H]-P_5 and 0, 0.1 or 0.5 ng/ml of TGF-β1. Cells were incubated for 1.5 or 3 h at 37°C. At the end of the incubation, medium was recovered and frozen at -20°C until analysis of steroid metabolism by thin layer chromatography (TLC) as previously described (Godin *et al.* 1999). Briefly, steroids were extracted from the medium using diethyl ether and resolved on DC-Alufolien neutral (Type E) paper plates (Whatman, Maidstone, Kent, England) in toluene: acetone (4:1). Each TLC plate contained [^{3}H]-E_1, [^{3}H]-E_2, [^{3}H]-A_4, [^{3}H]-T and [^{3}H]-P_5 as standards. A culture medium control was performed by incubating tracer in culture medium without cells, and was used to obtain background radioactivity that was subtracted from product counts. After migration of samples, TLC plates were exposed to phosphor screens designed for tritium detection, and tritiated steroid metabolites were localized with a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, California, USA). The rate of production of specific steroid metabolites was quantified by scraping the

corresponding sample and background spots from the TLC plate and counting radioactivity using PCS scintillation fluid (Amersham, UK).

Activity assays were validated by determining the conditions required to maintain excess substrate. In preliminary time course experiments, 1.6 x10^6 DPM of [^{3}H]-precursor (6-11 nM) were incubated with granulosa cells for 3 and 6 h. The amount of conversion of [^{3}H]-A_4 and [^{3}H]-T precursors to [^{3}H]-E_2 was similar with 29 and 58 % conversion after 3 and 6 h respectively and thus an incubation time of 3 h was selected to measure the rate of conversion of [^{3}H]-A_4 and [^{3}H]-T to [^{3}H]-E_2. For HSD17B reducing activity, the amount of conversion of [^{3}H]-E_1 precursor to [^{3}H]-E_2 was 92% and 98% after 3 and 6 h respectively. Therefore, to obtain experimental conditions of excess precursor, increasing concentrations of unlabeled E_1 (10^{-7}M, 10^{-6}M, 10^{-5}M) were added to the 8.8 pmoles/ml of [^{3}H]-E_1, and incubation time was reduced to 1.5 h. This resulted in 6%, 20% and 30% conversion of [^{3}H]-E_1 to [^{3}H]-E_2 and therefore 10^{-6}M unlabeled E_1 was added to all [^{3}H]-E_1 incubations to quantify HSD17B reducing activity. For HSD3B activity, the amount of conversion of [^{3}H]-P_5 precursor to [^{3}H]-P_4 was 37%, 69%, 79% and 90% conversion after 1.5, 3, 4.5 and 6 h respectively and thus an incubation time of 1.5 h was selected to measure the rate of conversion of [^{3}H]-P_5 to [^{3}H]-P_4.

Statistical analysis

Statistical analysis was performed using the JMP® software (SAS Institute, Cary, NC, USA). The data are presented as means ± SEM. Data was normally distributed as verified using the Shapiro-Wilk test. One-way ANOVA was used to test the main effect of FSH, time and TGF-β1 on the measured parameters. Differences between treatment dose of FSH or TGF-β1 with the 0 dose control; or between 4 and 6 day cultures with the 2 day culture group were identified with Dunnett's test.

RESULTS

Effect of FSH dose and Time in culture

In granulosa cells cultured for 6 days, FSH at 1 and 10 ng/ml had no effect on P_4 synthesis, but significantly increased E_2 secretion (Fig.1 A, F). FSH decreased *StAR* mRNA level; increased *CYP11A1* (only at the 10 ng/ml dose); and did not affect *HSD3B* and *GSTA* mRNA levels (Fig.1 B-E). For E_2 secretion, FSH had no effect on *HSD17B* mRNA level but increased *CYP19A1* and *HSD17B* mRNA levels (Fig.1 H-J). In the presence of FSH at 1 ng/ml, both P_4 and E_2 accumulation increased from Day 2 to Day 6 (Fig. 2 A, F). In agreement with increased secretion of P_4 with time in culture, mRNA expression of the P_4-related enzymes *StAR*, *CYP11A1*, *HSD3B* and *GSTA* also increased with time (Fig. 2 B-E). For E_2-related enzymes, time in culture had no effect on *HSD17B7* mRNA level but *CYP19A1* and *HSD17B1* mRNA levels increased with time (Fig.2 H-J).

Effects of TGF-β1 on secretion of steroid hormones and mRNA expression and activity of steroidogenic enzymes

Since P_4 and E_2 secretion was stimulated in granulosa cells cultured for 6 days with 1 ng/ml FSH (see below), we examined the effect of TGF-β1 on steroid hormones and mRNA expression of steroidogenic enzymes under this condition. The addition of TGF-β1 caused a significant dose-dependent inhibition of P_4 and E_2 secretion (Fig. 3). To determine

enzymatic activities, the tritiated steroid hormone precursors of A_4, T, E_1 and P_5 were converted to the corresponding radio-labelled product (E_2 and P_4) confirming that the CYP19A1, reducing HSD17B and HSD3B activities measured were specific. When granulosa cells were cultured with 0.5 ng/ml of TGF-β1, there was a significant inhibition of combined CYP19A1 and HSD17B activity as measured by the conversion of [^{3}H]-A_4 to [^{3}H]-E_2 (Fig.4 A), although the intermediary product [^{3}H]-E_1 was not detected. Both doses of TGF-β1 caused a significant inhibition in CYP19A1 activity measured by the conversion of [^{3}H]-T to [^{3}H]-E_2 (Fig. 4 B). TGF-β1 did not alter HSD17B or HSD3B reducing activity (Fig. 5). Corresponding to the decreased secretion of P_4 and E_2, TGF-β1 caused a significant inhibition in mRNA expression of *StAR*, *CYP11A1*, *HSD3B*, *GSTA*, *CYP19A1*, *HSD17B1* and *FSHr* (Fig. 6). However, *HSD17B7* mRNA was not changed by treatment of TGF-β1 (Fig. 6 I).

Cell morphology and total cell protein

Granulosa cells were seeded into wells and cultured for 6 days in serum-free medium. The granulosa cells initially formed tightly packed aggregates from Day 2, which enlarged with time in culture (data not shown). When TGF-β1 was added to FSH-stimulated cells, granulosa cell clumps were smaller and appeared more spherical than control cells (Fig. 7 D). Total cell protein was 30% and 38% higher in the 1 and 10 ng/ml FSH-treated groups compared to the control group without FSH (Fig. 7 A). In contrast, in the presence of 1 ng/ml of FSH, the highest dose of TGF-β1 (0.5 ng/ml) decreased total cell protein by 23%

compared to the control group without TGF-β1 (Fig. 7 B). Furthermore, in the absence of FSH, 6 days of treatment with 0.1, 0.5 or 1 ng/ml of TGF-β1 had no significant effect on total cell protein (data not shown).

DISCUSSION

The regulation of E_2 and P_4 production in granulosa cells is critical for ovarian follicle growth. The synthesis of E_2 from androgens requires aromatization by *CYP19A1*. In agreement with previous studies (Gutierrez *et al.* 1997; Silva & Price 2000; Sahmi *et al.* 2004), the physiological dose of FSH used in the present study (1 ng/ml) stimulated E_2 secretion and abundance of *CYP19A1* and *HSD17B1* mRNA. Under this stimulatory condition, TGF-β1 caused a marked inhibition of E_2 secretion and CYP19A1 activity, due to decreased expression of *CYP19A1* mRNA. These findings concur with those obtained in extra-gonadal tissues where TGF-β1 was shown to inhibit CYP19A1 activity, *CYP19A1* mRNA and CYP19A1 protein levels in cultured human fetal hepatocytes, trophobast cells and adipose stroma cells (Simpson *et al.* 1989; Rainey *et al.* 1992; Luo *et al.* 2002). The conversion of A_4 to E_2 also requires HSD17B reducing activity, however TGF-β1 did not affect HSD17B activity in the present study. TGF-β1 did inhibit *HSD17B1* expression, suggesting that another enzyme may be contributing to total HSD17B activity. Another enzyme known to convert E_1 to E_2 is *HSD17B7* (Krazeisen *et al.* 1999; Krusche *et al.* 2001) and in the present study this isoform was detected in bovine granulosa cells and was not inhibited by TGF-β1. In rodent and rabbit ovaries, this isoform is present only in the corpus luteum and was first reported as the prolactin receptor associated protein (Nokelainen *et al.* 1998; Krusche *et al.* 2001; Risk *et al.* 2005). In humans, *HSD17B7* transcript was found in

ovaries of non-pregnant, but not pregnant women (Krazeisen *et al.* 1999). The mRNA expression of *HSD17B7* was not affected by TGF-β1 in agreement with a lack of effect of TGF-β1 on HSD17B activity. FSH dose and time in culture also did not affect expression of *HSD17B7* mRNA in the present study. For HSD17B activity, a thousand fold-excess of unlabeled E_1 had to be added and incubation time had to be shortened to obtain comparable conditions of substrate excess, indicating that HSD17B reducing activity is very high in cultured bovine granulosa cells. The high HSD17B activity could explain why the CYP19A1 product [^{3}H]-E_1 was undetectable in the presence or absence of TGF-β1.

In developing antral follicles, a positive E_2/P_4 ratio must be maintained and it is critical to limit P_4 secretion until the time of ovulation induction, because premature increase in P_4 is associated with follicular atresia (Ireland & Roche 1982; Irving-Rodgers *et al.* 2003). In agreement with the above, low dose of FSH stimulated E_2 but not P_4 in the present study and *CYP11A1* was only stimulated by the highest dose of FSH (10 ng/ml). Additionally, the present study is the first report showing that FSH down-regulates expression of *StAR* in vitro. This finding may explain why *StAR* is undetectable in granulosa of healthy antral follicles at any size in vivo (Soumano & Price 1997; Bao *et al.* 1998). In our time-course experiments, in the presence of low dose of FSH, the production of P_4 increased as well as the expression of *StAR, CYP11A1, HSD3B and GSTA*. These findings are in agreement with a previous study (Sahmi *et al.* 2004) who observed an increase with time in *HSD3B*, and *CYP11A1*. Overall these results indicate that P_4 increases spontaneously in cultured

granulosa cells and that readily available stores of cholesterol are present in granulosa cells which can be transformed to P_4 by *CYP11A1*, *HSD3B* and *GSTA*. Similar to the effect of TGF-β1 on E_2 synthesis, TGF-β1 also inhibited the progestin synthetic pathway. TGF-β1 inhibited P_4 secretion and abundance of mRNA encoding *StAR*, *CYP11A1*, *HSD3B* and *GSTA*, which are required for sustained production of P_4 from cholesterol. These data are consistent with the effects of TGF-β1 on *StAR*, *CYP11A1* and *HSD3B* in thecal cell, adrenocortical cell and adrenal tumor cell among different species (Rainey *et al.* 1991; Cherradi *et al.* 1995; Naaman-Reperant *et al.* 1996; Attia *et al.* 2000; Herrmann *et al.* 2002), and this is the first time that TGF-β1 has been shown to inhibit mRNA encoding *GSTA*. Surprisingly, despite the reduced abundance of *HSD3B* and *GSTA* mRNA, TGF-β1 did not affect total HSD3B activity. Similarly, TGF-β1 did not significantly alter HSD3B activity in the rat fetal testis (Gautier *et al.* 1997). TGF-β1 may have stabilized the corresponding enzyme proteins of *HSD3B* and *GSTA* or, alternatively, other unknown gene products with HSD3B activity may be involved.

In agreement with previous morphological studies (Gutierrez *et al.* 1997; Marsters *et al.* 2003), FSH increased cell number. In this model, it has been suggested that after dispersion, granulosa cells revert to a less mature phenotype, re-establish cell-cell communications and proliferate in the presence of FSH (Gutierrez *et al.* 1997; Marsters *et al.* 2003). The moderate increase in total cell protein observed in the presence of FSH could indicate that FSH stimulated proliferation, or alternatively that FSH prevented apoptosis

and increased cell survival. FSH and E_2 are known to act as survival factors to induce granulosa cell proliferation and prevent apoptosis (Gutierrez *et al.* 1997; Yang & Rajamahendran 2000; Jiang *et al.* 2003; Quirk *et al.* 2004). In the present study, TGF-β1 caused visible differences in the morphology of granulosa cell clumps, which appeared smaller and more spherical than in FSH-treated controls. TGF-β1 at the highest dose also caused a slight decrease in total granulosa cell protein. It is unclear at the present time whether the TGF-induced reduction in steroidogenesis is a result or cause of the change in cell proliferation or survival. But, in the absence of FSH, TGF-β1 did not significantly affect total granulosa cell protein. Similarly, TGF-β1 alone had no effect on DNA synthesis in cultured bovine granulosa cells (Lerner *et al.* 1995). Therefore, the effects of TGF-β1 on granulosa cells could be due to inhibition of the cell-surviving activity of FSH, and/or mediated through a loss of E_2-stimulated cell survival (Yang & Rajamahendran 2000; Quirk *et al.* 2004).

We conclude that TGF-β1 plays an inhibitory role in E_2 and P_4 steroidogenesis in granulosa cells cultured in conditions where E_2 and P_4 secretion is being stimulated, and that TGF-β1 counteracts the stimulation of mRNA encoding steroidogenic enzymes. As TGF-β1 inhibited *FSHr* mRNA levels and inhibited FSH-induced *CYP19A1* and *HSD17B1* but not *HSD17B7*, we propose that the inhibitory effects of TGF-β1 on FSH-stimulated E_2 secretion may be due at least in part to inhibition of *FSHr* (Fig 8). These selective inhibitory effects suggest that TGF-β1 may be acting in a physiological manner to limit the

amount of E_2 and P_4 produced by granulosa cells without totally shuting down the steroidogenic potential. By acting in this manner, the physiological role of TGF-β1 may be to limit FSH-stimulated growth and differentiation of granulosa cells and play an active role in determining the fate of the developing follicle towards ovulation or atresia.

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Fig. 1. Effect of FSH dose (0, 1, 10 ng/ml) on secretion of oestradiol (E_2) and progesterone (P_4) and mRNA expression of steroidogenic enzymes in bovine granulosa cells. Cells were cultured for 6 days without serum in the presence of 0, 1, 10 ng/ml of FSH starting at day 0. The culture medium and cellular RNA were collected on day 6. The data represent secretion of E2 and P4 during the last 48 h of culture (A and F). Levels of *StAR*, *CYP11A1*, *HSD3B*, *GSTA*, *HSD17B7*, *CYP19A1*, and *HSD17B1* mRNA expression were measured by semiquantitative RT-PCR. Data are presented as the amount of steroidogenic enzyme expressed relative to the amount of expression of the constitutively expressed gene *1B15* (B-E, H-J) . Data are means ± SEM of 3 separate culture replicates. Asterisk (*) indicates that the mean is significantly different from 0 dose control ($p<0.05$, one-way ANOVA, with Dunnett's test). Panel (G) is a representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and *1B15* constitutively expressed house-keeping genes.

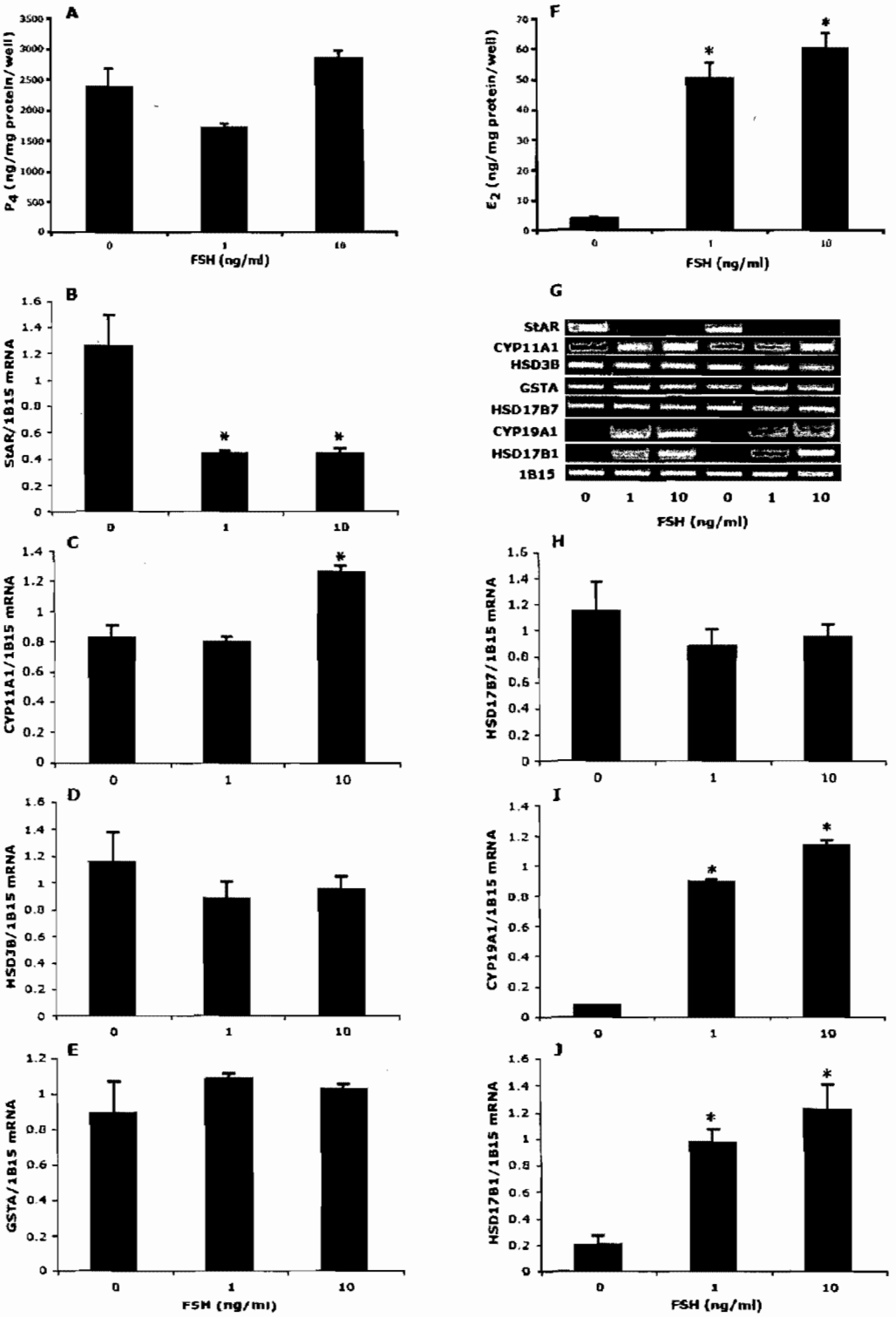

A
P_4 (ng/mg protein/well)
FSH (ng/ml)
B
StAR/1B15 mRNA
C
CYP11A1/1B15 mRNA
D
HSD3B/1B15 mRNA
E
GSTA/1B15 mRNA
FSH (ng/ml)
F
E_2 (ng/mg protein/well)
FSH (ng/ml)
G
StAR
CYP11A1
HSD3B
GSTA
HSD17B7
CYP19A1
HSD17B1
1B15
0 1 10 0 1 10
FSH (ng/ml)
H
HSD17B7/1B15 mRNA
I
CYP19A1/1B15 mRNA
J
HSD17B1/1B15 mRNA
FSH (ng/ml)

Fig. 2. Effect of time in culture (2, 4, 6 days) on secretion of E_2 and P_4 and mRNA expression of steroidogenic enzymes in bovine granulosa cells. Granulosa cells were cultured in serum-free medium with 1 ng/ml FSH starting at day 0. The data represent secretion of E_2 and P_4 during the last 48 h of culture (A and F). Abundance of *StAR*, *CYP11A1*, *HSD3B*, *GSTA*, *HSD17B7*, *CYP19A1*, and *HSD17B1* mRNA (B-E, H-J) was measured by semiquantitative RT-PCR and normalized to the house keeping gene *1B15*. Data are means ± SEM of 3 separate culture replicates. Asterisk (*) indicates that the mean is significantly different from control at day 2 ($p<0.05$, one-way ANOVA, with Dunnett's test). Panel (G) is a representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and *1B15* constitutively expressed house-keeping genes.

Figure 2

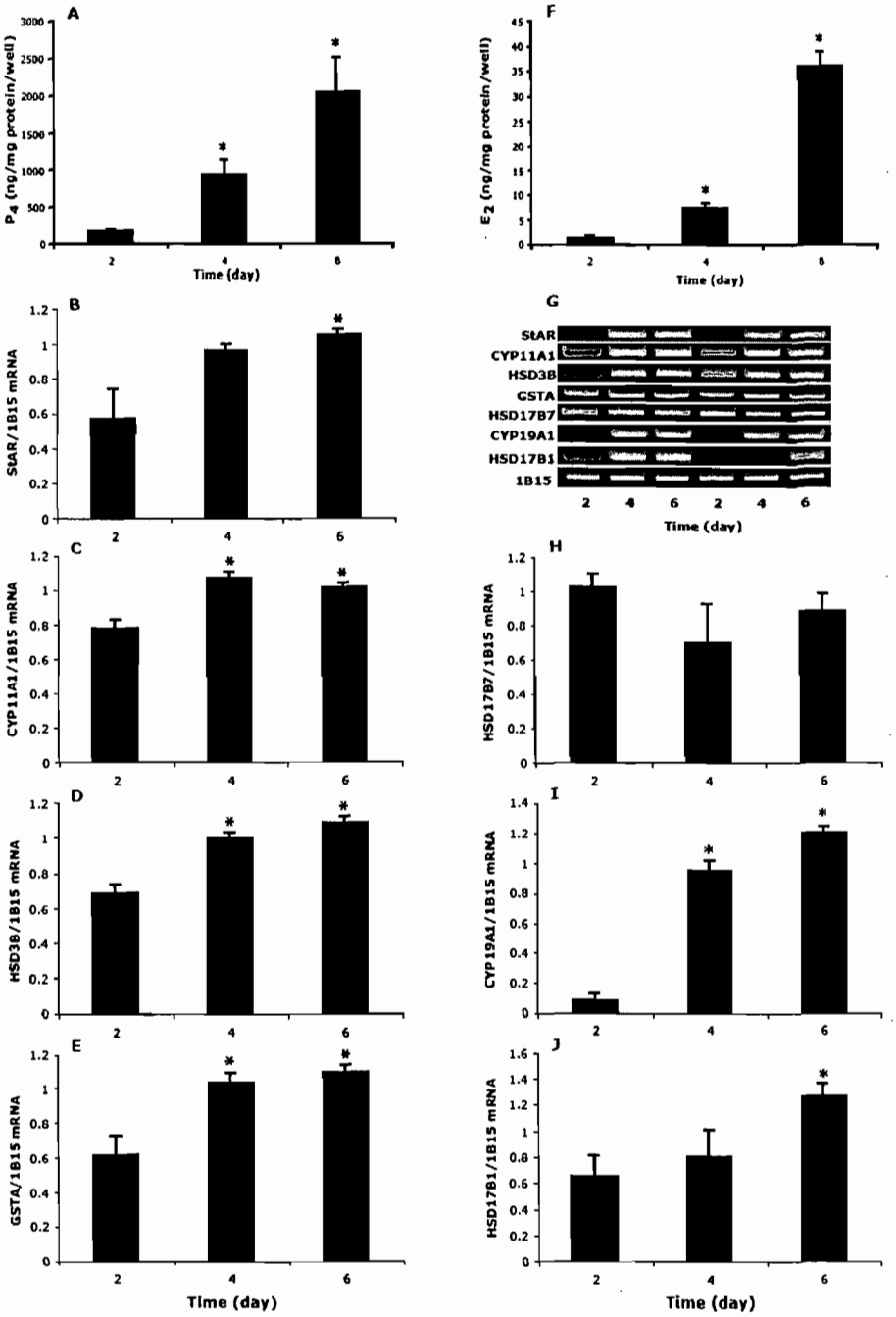

A
P_4 (ng/mg protein/well)
Time (day)
F
E_2 (ng/mg protein/well)
Time (day)
B
StAR/1B15 mRNA
G
StAR
CYP11A1
HSD3B
GSTA
HSD17B7
CYP19A1
HSD17B1
1B15
Time (day)
C
CYP11A1/1B15 mRNA
H
HSD17B7/1B15 mRNA
D
HSD3B/1B15 mRNA
I
CYP19A1/1B15 mRNA
E
GSTA/1B15 mRNA
J
HSD17B1/1B15 mRNA
Time (day)

Fig. 3. Effect of TGF-β1 on E_2 and P_4 secretion from cultured bovine granulosa cells. Cells were cultured for 6 days without serum in the presence of 1 ng/ml of FSH and were treated with TGF-β1 beginning on the first day of culture. The medium was collected on day 6 and the data represent steroid produced during the last 48 h of culture. Data were corrected for total cell protein and represent means ± SEM derived from 4 different pools of ovaries. The asterisk (*) indicates that steroid hormone secretion was significantly different from the 0 dose control group ($p<0.05$, $n=4$, one-way ANOVA, with Dunnett's test).

Figure 3

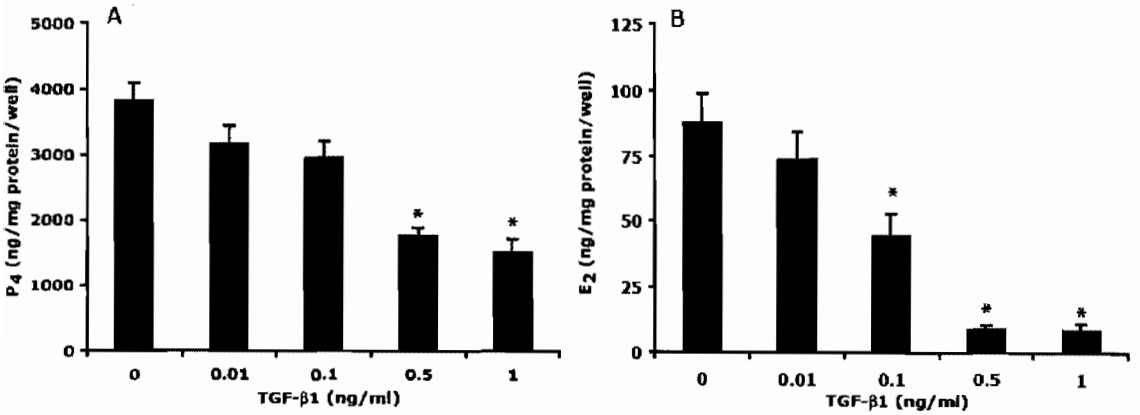

A
5000
4000
3000
2000
1000
0
P_4 (ng/mg protein/well)
0
0.01
0.1
0.5
1
TGF-β1 (ng/ml)
*
*
B
125
100
75
50
25
0
E_2 (ng/mg protein/well)
0
0.01
0.1
0.5
1
TGF-β1 (ng/ml)
*
*

Fig. 4. Effect of TGF-β1 on conversion of androgens to E_2. Bovine granulosa cells were cultured for 6 days in serum-free medium. Labelled steroid precursor was added on day 6 and incubated for 3 h and enzymatic activity assays were conducted as described in Materials and Methods. Data are means ± SEM of 3 separate culture replicates. Panel A shows conversion of [^{3}H]A_4 to [^{3}H]E_2 (CYP19A1 + HSD17B reducing activity). Panel B shows [^{3}H]T conversion to [^{3}H]E_2 (CYP19A1 activity). The asterisk (*) indicates that enzyme activity is significantly different from the 0 dose control group ($P<0.05$, n=3, one-way ANOVA, with Dunnett's test).

Figure 4

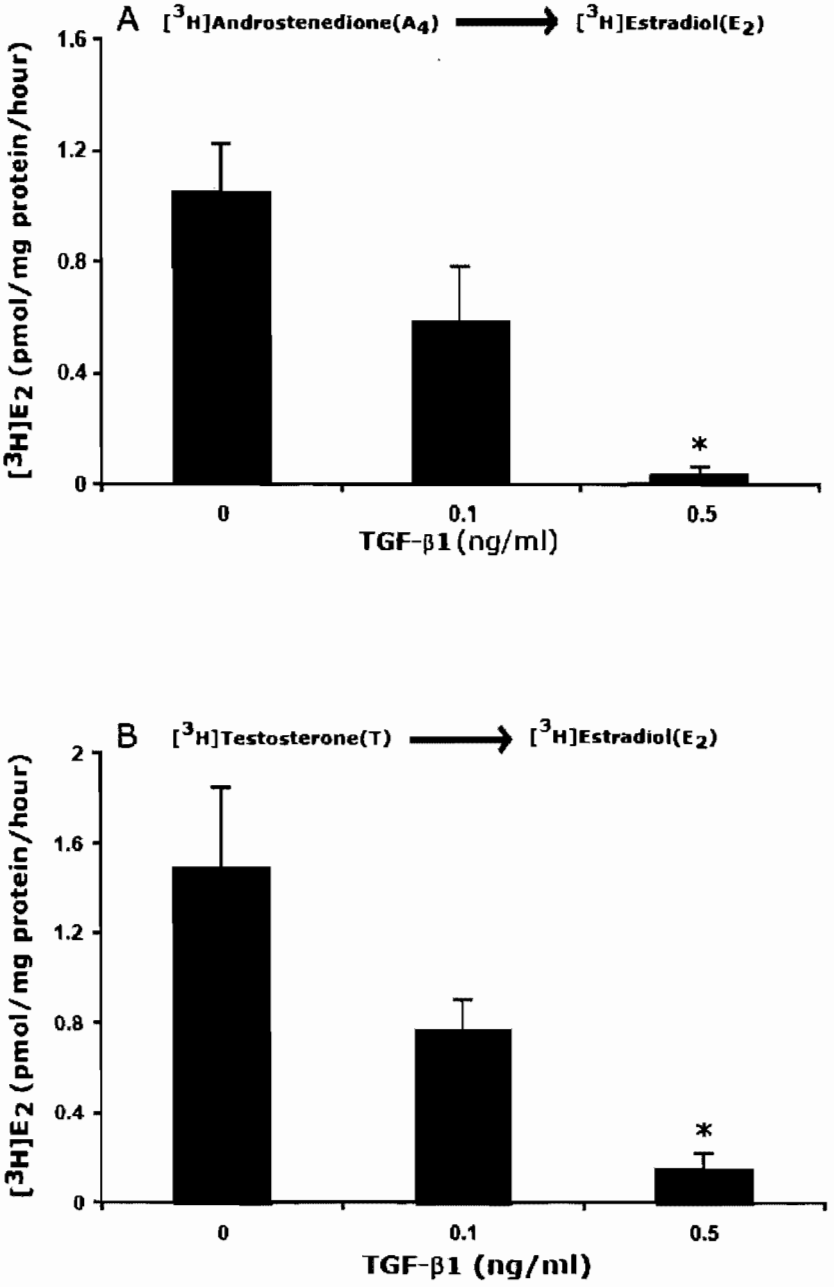

A
[3H]Androstenedione(A4)
[3H]Estradiol(E2)
[3H]E2 (pmol/mg protein/hour)
1.6
1.2
0.8
0.4
0
*
0
0.1
0.5
TGF-β1 (ng/ml)
B
[3H]Testosterone(T)
[3H]Estradiol(E2)
[3H]E2 (pmol/mg protein/hour)
2
1.6
1.2
0.8
0.4
0
*
0
0.1
0.5
TGF-β1 (ng/ml)

Fig. 5. Effect of TGF-β1 on HSD17B reducing activity (A) and HSD3B dehydrogenase/isomerase activity (B) in cultured bovine granulosa cells. Cells were cultured for 6 days in serum-free medium with TGF-β1. Labelled steroid precursor ([^{3}H]E_1 or [^{3}H]P_5) was added on day 6 and enzymatic activity assays were conducted as described in Materials and Methods. The [^{3}H]E_1 precursor was incubated with 10^{-6}M (1000 fold excess) unlabeled E_1 for 1.5 h and [^{3}H]P_5 was incubated for 1.5 h without unlabeled P_5. Data are means ± SEM of 3 separate culture replicates. The group of TGF-β1 treatment did not significantly different from the 0 dose control group ($p>0.05$, n=3, one-way ANOVA, with Dunnett's test).

Figure 5

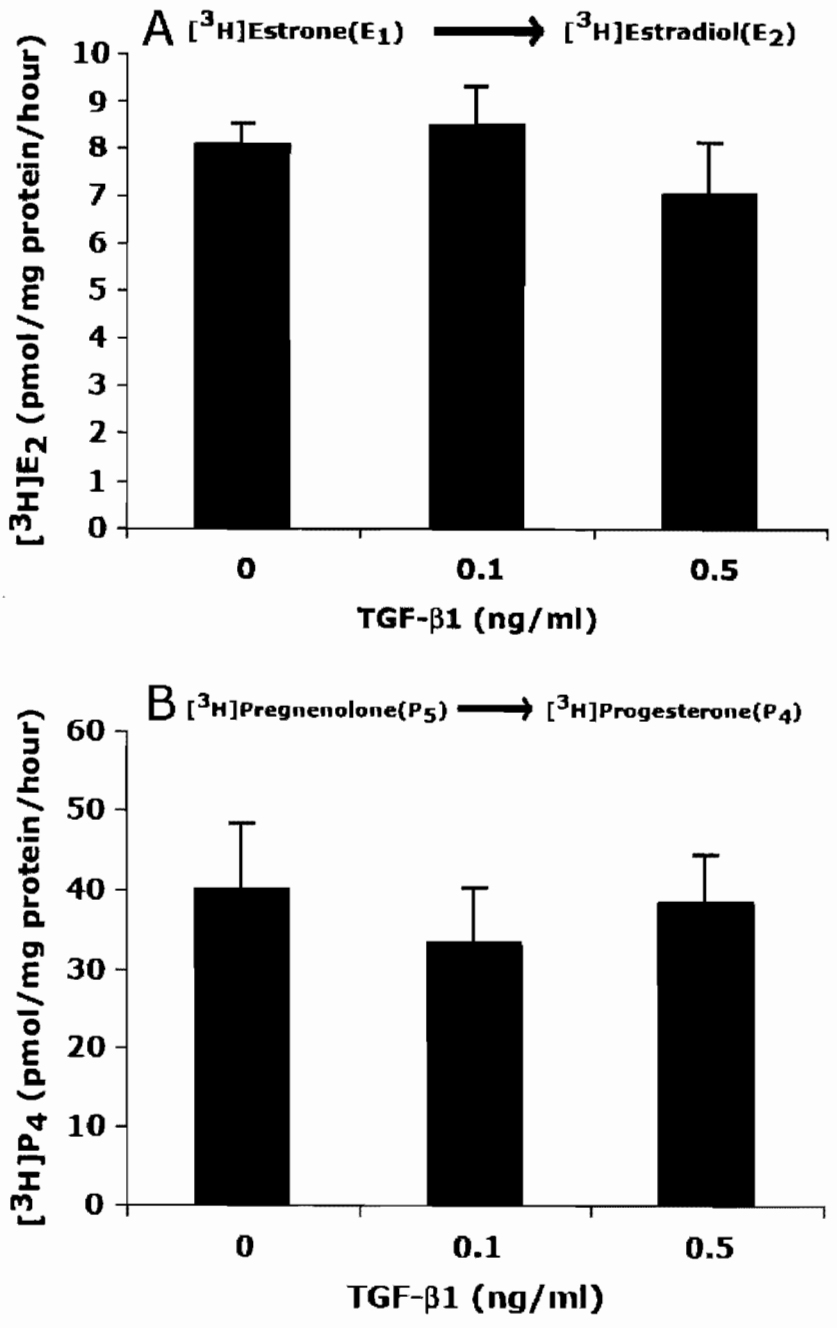

A [3H]Estrone(E1) → [3H]Estradiol(E2)
[3H]E2 (pmol/mg protein/hour)
10
9
8
7
6
5
4
3
2
1
0
0
0.1
0.5
TGF-β1 (ng/ml)
B [3H]Pregnenolone(P5) → [3H]Progesterone(P4)
[3H]P4 (pmol/mg protein/hour)
60
50
40
30
20
10
0
0
0.1
0.5
TGF-β1 (ng/ml)

Fig. 6. Effect of TGF-β1 on mRNA expression of steroidogenic enzymes and *FSHr* in bovine granulosa cells. Cells were cultured in serum-free medium for 6 days in the presence of 1 ng/ml of FSH with stated doses of TGF-β1. Abundance of *StAR*, *CYP11A1*, *HSD3B*, *GSTA*, *CYP19A1*, *HSD17B1*, *HSD17B7*, and *FSHr* mRNA (B-E, G-J) was measured by semiquantitative RT-PCR and normalized to the house keeping gene *1B15*. Data are means ± SEM of 4 separate culture replicates. Asterisk (*) indicates that mRNA content is significantly different from 0 dose control ($p<0.05$, one-way ANOVA, with Dunnett's test). Panels (A) and (F) are representative agarose gels from two replicates showing PCR products for each steroidogenic enzyme, *FSHr* and *1B15*constitutively expressed house-keeping genes.

Figure 6

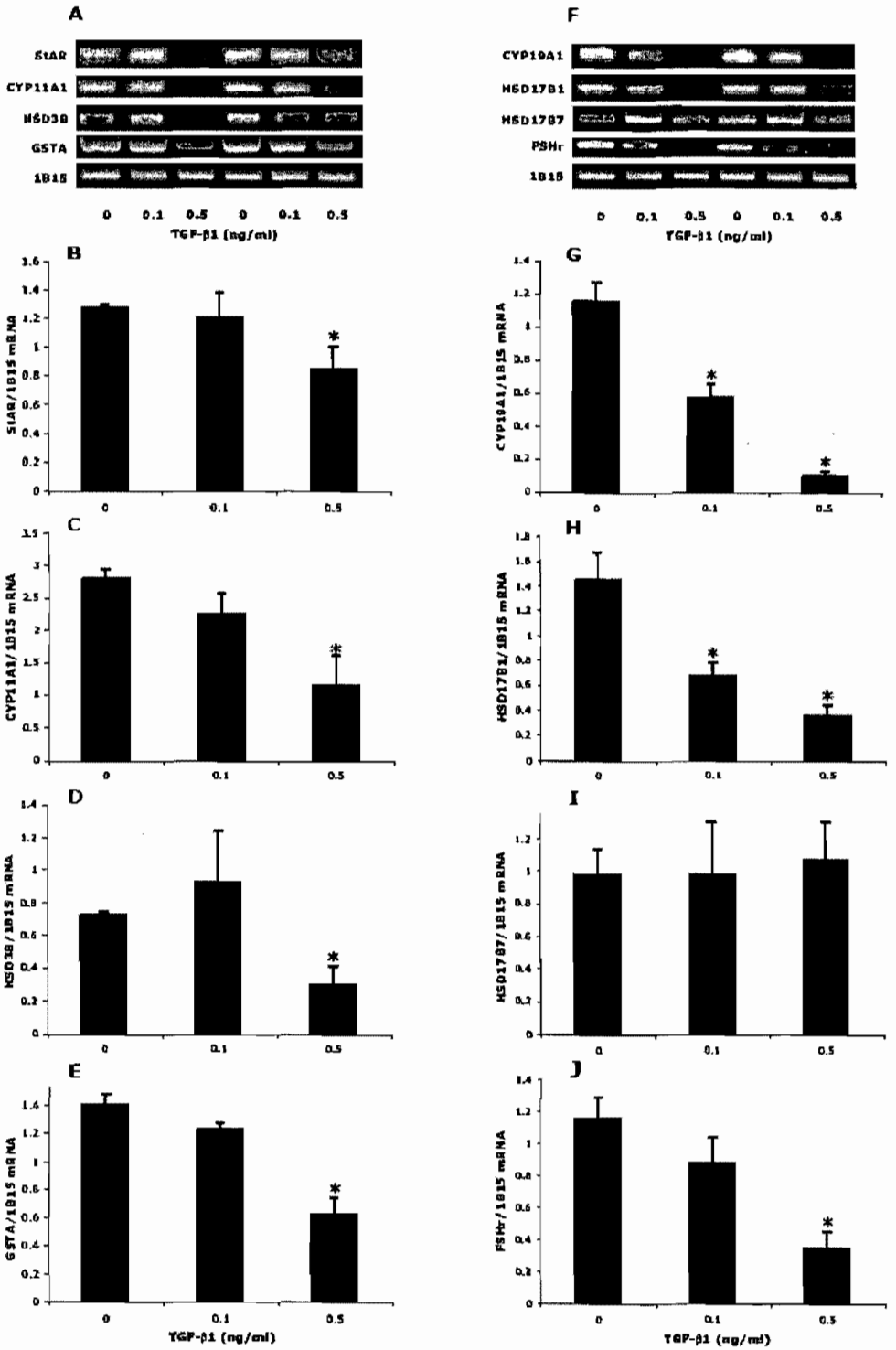

A
StAR
CYP11A1
HSD3B
GSTA
18S
0 0.1 0.5 0 0.1 0.5
TGF-β1 (ng/ml)
F
CYP19A1
HSD17B1
HSD17B7
PSHr
18S
0 0.1 0.5 0 0.1 0.5
TGF-β1 (ng/ml)
B
StAR/18S mRNA
C
CYP11A1/18S mRNA
D
HSD3B/18S mRNA
E
GSTA/18S mRNA
0 0.1 0.5
TGF-β1 (ng/ml)
G
CYP19A1/18S mRNA
H
HSD17B1/18S mRNA
I
HSD17B7/18S mRNA
J
PSHr/18S mRNA
0 0.1 0.5
TGF-β1 (ng/ml)

Fig. 7. Effect of FSH and TGF-β1 on total cell protein and morphology of granulosa cells. Cells were cultured for 6 days in serum-free medium with different doses of FSH (A and C) and with different doses of TGF-β1 in the presence of 1 ng/ml of FSH (B and D) as described in materials and methods. Data for total protein content (A and B) are means ± SEM of 4 and 6 separate culture replicates respectively. Asterisk (*) indicates significant differences compared to the 0 dose control ($p<0.05$, one-way ANOVA, with Dunnett's test). White bar=500μm.

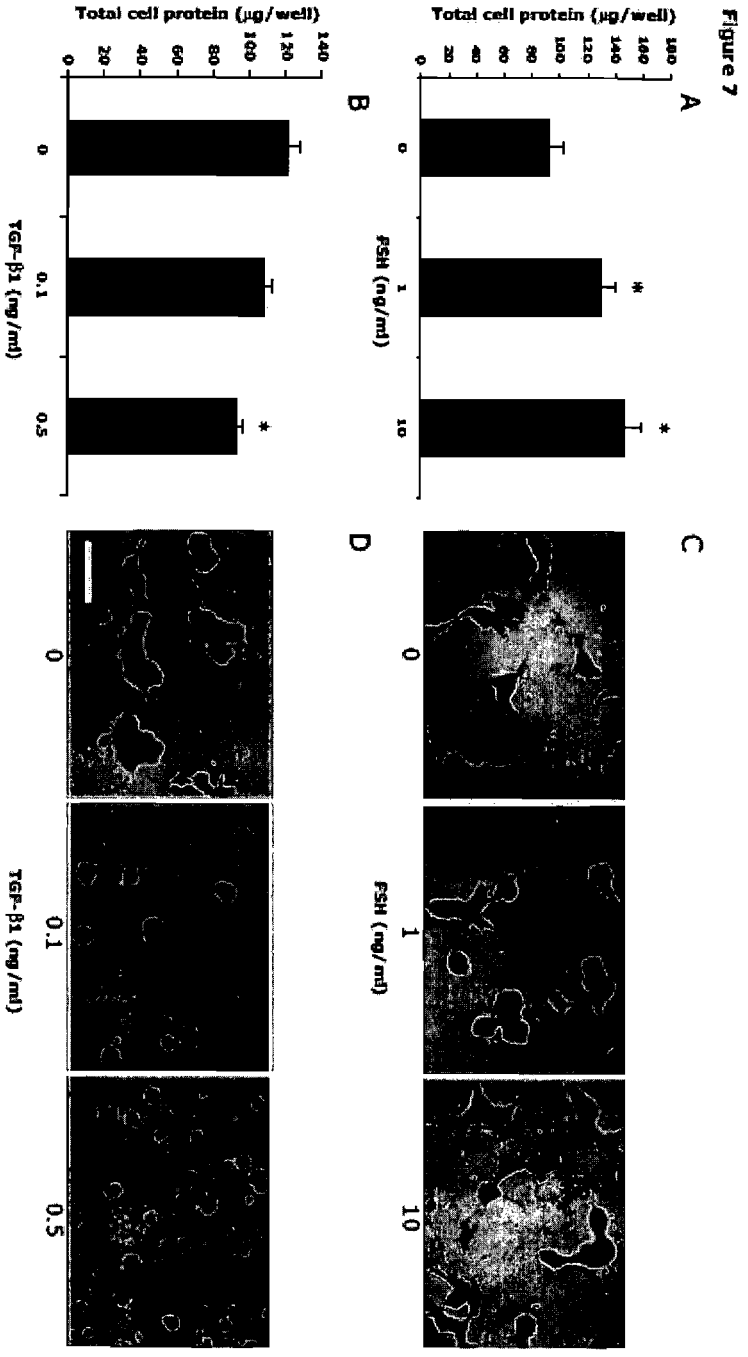
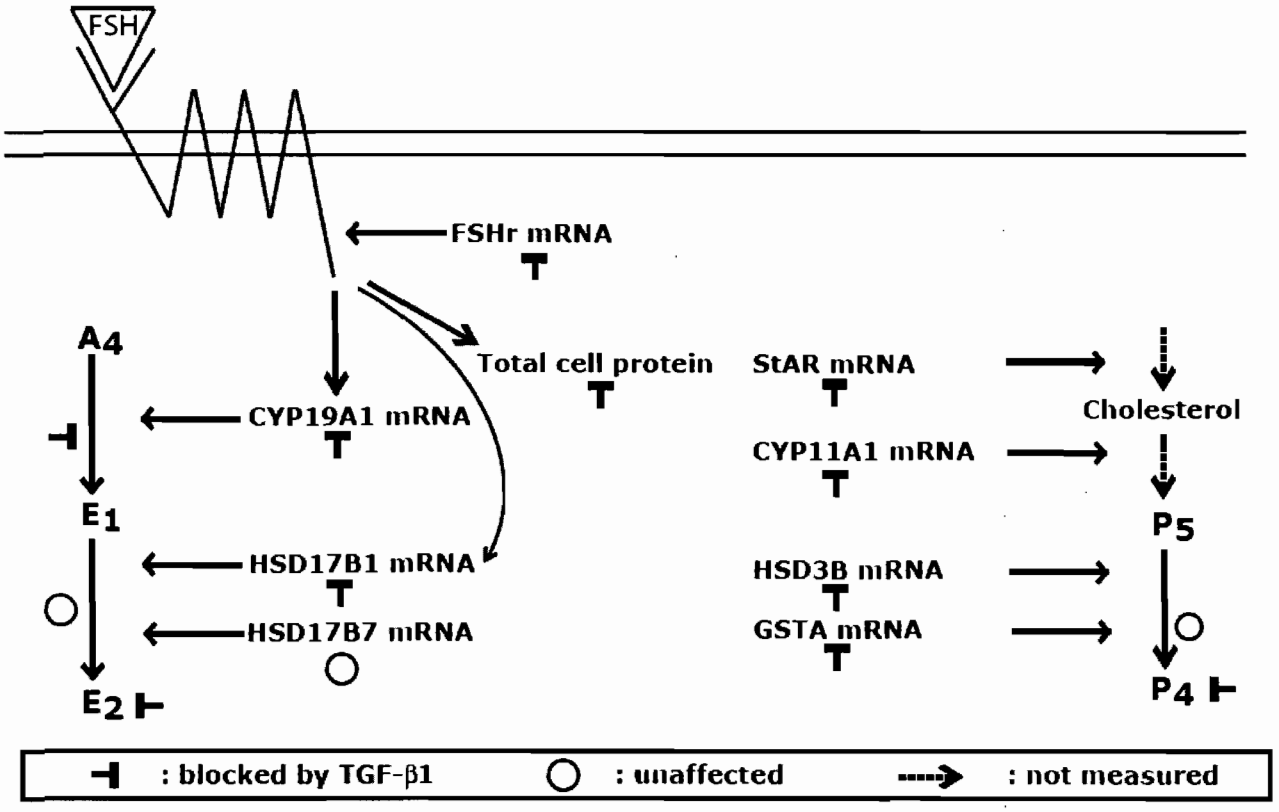


Figure 7
A
Total cell protein (μg/well)
0
20
40
60
80
100
120
140
160
180
0
1
10
FSH (ng/ml)
*
*
B
Total cell protein (μg/well)
0
20
40
60
80
100
120
140
0
0.1
0.5
TGF-β1 (ng/ml)
*
C
D
0
1
10
FSH (ng/ml)
0
0.1
0.5
TGF-β1 (ng/ml)

Fig. 8. Schematic summary diagram showing the effect of TGF-β1 in the presence of FSH (1ng/ml) on mRNA expression of steroidogenic enzymes and *FSHr*, total cell protein, and steroidogenic enzyme activity in cultured bovine granulosa cells. The meaning of the signs is shown in the black square.


FSH
FSHr mRNA
A4
CYP19A1 mRNA
Total cell protein
StAR mRNA
Cholesterol
CYP11A1 mRNA
E1
P5
HSD17B1 mRNA
HSD3B mRNA
HSD17B7 mRNA
GSTA mRNA
E2
P4
: blocked by TGF-β1
: unaffected
: not measured

ARTICLE TWO

Title: Transforming growth factor-β1 inhibits luteinization and promotes apoptosis in bovine granulosa cells

Thesis author's contribution to this work: As the primary author, I was responsible for all aspects of this article. Second authorship credits reflect technical support. Third author credits directorship. (Journal: Reproduction, submitted, 2008)

Transforming growth factor-β1 inhibits luteinization and promotes apoptosis in bovine granulosa cells

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Short title: TGF-β1 regulation of steroidogenesis and apoptosis

ABSTRACT

Although TGF-β1 has been shown to inhibit estradiol (E_2) and progesterone (P_4) biosynthesis in ruminant FSH-stimulated granulosa cells, its effects in the absence of FSH remain unknown. The objective of this study was to assess the effects of TGF-β1 on E_2 and P_4 steroidogenesis in quiescent bovine granulosa cells and to determine the effects of TGF-β1 on cell proliferation and apoptosis in quiescent and FSH-stimulated granulosa cells. Bovine granulosa cells were cultured in serum-free medium without FSH for 2-6 days. E_2 secretion decreased as a function of time, whereas P_4 secretion increased ($P < 0.05$). Addition of TGF-β1 for 6 days decreased P_4 secretion and partially reversed the decline of E_2 biosynthesis observed in control cells ($P < 0.05$). The effect of TGF-β1 on E_2 was attributed to increased *CYP19A1* and *HSD17B1* mRNA levels relative to controls, with an accompanying increase in CYP19A1 activity ($P < 0.05$). Conversely, *HSD17B7* expression and HSD17B activity were unaffected by TGF-β1. The effect of TGF-β1 on P_4 secretion was associated with decreases in mRNA levels of the P_4 synthesis-associated genes *StAR*, *CYP11A1*, *HSD3B* and *GSTA* ($P < 0.05$). On the other hand, TGF-β1 decreased the proportion of cells in the proliferative phase of the cell cycle (S, G2 and M) and increased cells in the G0 and G1 phases in the presence ($P < 0.05$) or absence ($P < 0.1$) of FSH. Furthermore, in both quiescent and FSH-stimulated granulosa cells, TGF-β1 increased the proportion of dead cells and increased levels of cleaved caspase-3 ($P < 0.05$), indicating that cell death was due to apoptosis. Our results therefore indicate that TGF-β1 contributes

to maintaining the estrogenic capacity of granulosa cells while inhibiting luteinization, and this effect was associated with increased apoptosis.

INTRODUCTION

The secretion of estradiol (E_2) and progesterone (P_4) and the autocrine or paracrine effects of these steroids are important functions of granulosa cells during follicular development (Drummond 2006). E_2 is a key marker of growth and selection of the dominant follicle (Kolibianakis *et al.* 2005). Increased production of E_2 in granulosa cells requires transformation of androstenedione to estrone (E_1) by cytochrome P450 aromatase (CYP19A1) activity and conversion of E_1 to E_2 by 17β-hydroxysteroid dehydrogenase (HSD17B) reducing enzyme activity (Mindnich *et al.* 2004; Zheng *et al.* 2008). In large antral and preovulatory follicles, granulosa cells develop the capacity to transform cholesterol into P_4 through the expression of P_4-synthetic enzymes, including cytochrome P450 side-chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B) and α glutathione S-transferase (GSTA) (Bao & Garverick 1998; Rabahi *et al.* 1999; Raffalli-Mathieu *et al.* 2007).

In any developing tissue, cell proliferation must be controlled in such a manner that allows some cells to reach terminal differentiation while others are eliminated by programmed cell death or apoptosis. In the ovary, the destiny of the majority of developing follicles is

atresia, and only a limited number of follicles will develop to the ovulatory stage. Apoptosis of granulosa cells is a hallmark of follicular atresia (Tilly *et al.* 1991). Many signaling processes are involved in the initiation of apoptosis, one of which is the proteolytic activation of caspase-3, which is a caspase common to several different apoptosis pathways (Matikainen *et al.* 2001; Johnson & Bridgham 2002). The proportion of bovine follicles undergoing atresia increases dramatically with follicular diameter, and the rate of atresia doubles between 2 and 8 mm (Lussier *et al.* 1987). This increased rate of atresia is associated with an increase in the mitotic index and a decrease in the doubling time of granulosa cells. Thus granulosa cells that are dividing rapidly are also more susceptible to apoptosis. Research on the mechanisms that regulate cell cycle progression have identified specific phases in which cells are more susceptible to apoptosis. During the first gap phase or G1, cells are quiescent but potentially receptive to mitogenic stimuli, which stimulate cells to embark into the proliferative phases of the cell cycle consisting of the DNA synthesis phase (S), the second gap phase (G2) and the mitotic phase (M). In granulosa cells of the developing follicle, the G1/S transition phase is a critical check-point, as progression from the quiescent stage (G0/G1) to the S phase requires the action of cell proliferation and survival factors such as insulin-like growth 1factor (IGF-1) and E_2 (Quirk *et al.* 2004; Hu *et al.* 2004). If these survival factors are not present or remain at a low concentration, granulosa cells of developing follicles will undergo apoptosis and follicular atresia will ensue. When selected follicles reach the preovulatory stage, the LH surge terminates follicular growth by causing granulosa cells to exit the cell cycle and remain in

the G0 stage (Rao *et al.* 1978; Stocco *et al.* 2007). At this time, the luteinization process, which is characterized by increased progesterone secretion by granulosa/lutein cells, is believed to play a protective role by preventing apoptosis of granulosa cells (Peluso & Pappalardo 1998; Svensson *et al.* 2000).

To understand ovarian follicle development, studies have focused on the regulation of granulosa cell steroidogenesis, proliferation and apoptosis. In the last decade, many autocrine/paracrine growth factors, including transforming growth factor-β (TGF-β1) have been identified in the ovary and shown to regulate physiologic processes in granulosa cells. TGF-β1 is expressed in the ovary of human, rodent and sheep (Juengel & McNatty 2005). In earlier studies, TGF-β1 knockout mice did not survive past weaning age, which prevented researchers from defining the ovarian role of TGF-β1 *in vivo* (Shull *et al.* 1992; Kulkarni *et al.* 1993). More recently, female TGF-β1 knockout mice on the immunocompromised *scid* background provided evidence that follicular estrogen synthesis is less sensitive to TGF-β1 deficiency than luteal cell P_4 synthesis, as TGF-β1 null mutant mice had low serum P_4 levels, but estrogen was unaffected (Ingman *et al.* 2006). In agreement with this *in vivo* result, TGF-β1 stimulated P_4 synthesis in cultured rodent granulosa cells (Dodson & Schomberg 1987), however TGF-β1 also stimulated E_2 production (Adashi *et al.* 1989). Conversely, TGF-β1 inhibited granulosa cell secretion of E_2 and P_4 from pigs and ruminants (Chang *et al.* 1996; Wandji *et al.* 1996; Ford & Howard 1997; Juengel *et al.* 2004; Ouellette *et al.* 2005; Zheng *et al.* 2008). In the bovine species,

TGF-β1 expression was observed in granulosa cells of small but not of large antral follicles (Nilsson *et al.* 2003). In agreement with this finding, a previous study showed a negative relationship between follicular fluid TGF-β1 and E_2 concentrations and between TGF-β1 levels and follicle size (Ouellette *et al.* 2005). Concerning cell proliferation, TGF-β1 inhibited EGF- or FSH-stimulated DNA synthesis and mitosis in cultured bovine, porcine or ovine granulosa cells (Skinner *et al.* 1987; Gangrade & May 1990; Juengel *et al.* 2004), but it increased FSH-stimulated DNA synthesis and mitosis in rat granulosa cells (Dorrington *et al.* 1993). The conflicting results of TGF-β1 function in ovarian physiology among animal species may reflect the multifunctional roles of TGF-β1 that have been reported in many other tissues (Herrmann *et al.* 2002). The effects of TGF-β1 also appear to be dependant on the stage of differentiation and the state of stimulation of the tissues examined *in vitro*.

In bovine granulosa cells, it is well established that TGF-β1 negatively affects secretion of E_2 and P_4 (Wandji *et al.* 1996; Ouellette *et al.* 2005; Zheng *et al.* 2008). Our previous study showed that TGF-β1 inhibits E_2 and P_4 synthesis without totally shutting down steroidogenesis in FSH-stimulated granulosa cells. However, the effects of TGF-β1 on steroidogenesis in quiescent granulosa cells (i.e., without FSH treatment) and its effects on cell proliferation and apoptosis in quiescent and FSH-stimulated granulosa remain unknown. The objective of this study was therefore to assess the effects of TGF-β1 on steroidogenesis, cell proliferation and apoptosis in cultured bovine granulosa cell.

MATERIALS AND METHODS

Cell Culture

Cell culture was performed essentially as described by (Gutierrez *et al.* 1997). Briefly, ovaries were collected at a local abattoir from adult cows irrespective of stage of the estrous cycle and transported to the laboratory at 37°C in phosphate buffered saline (PBS) containing penicillin (100 IU/ml), streptomycin (100 µg/ml) and fungizone (1 µg/ml). Follicles of 2-5 mm in diameter were dissected from the ovaries, and granulosa cells were isolated mechanically by rinsing sections of follicle walls repeatedly through a disposable pipette. The granulosa cell suspension was filtered through a 150 mesh steel sieve (Sigma, St-Louis, MO, USA) to remove oocytes.

Cells were seeded into 24-well tissue culture plates (Corning Glass Works, Corning, NY, USA) at a density of 10^6 viable cells (tested by Trypan blue exclusion) in 1 ml of α-MEM with L-glutamine containing sodium bicarbonate (10 mM), Hepes (20 mM), nonessential amino acid mix (1.1 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml), protease-free BSA (0.1 %), sodium selenite (4 ng/ml), transferrin (2.5 µg/ml), androstenedione (A_4) (100 nM) (all from Sigma, St-Louis, MO, USA), insulin (10 ng/ml) (Invitrogen, USA) and graded doses of recombinant active human TGF-β1 (R & D Systems, Minneapolis, MN, USA), with or without ovine FSH (1 ng/ml) (oFSH, AFP-5332B; NIDDKD, Torrance, CA,

USA) starting on the first day of culture. Cultures were maintained at 37°C in 5% CO_2 for 2-6 days, with 700 µl of medium being replaced every 2 days.

At the end of culture, medium was collected and frozen for subsequent steroid assays, and granulosa cells were collected for RNA or protein extraction. Total cell protein was extracted by the addition of 200 µl of 1M NaOH to each well for 2 h at room temperature, followed by neutralization with 200 µl of 1 M HCl. Protein concentrations were measured using the Bio-Rad micro-assay (Bio-Rad, Mississauga, ON, Canada). All experiments were performed with at least three independent cell cultures.

Steroid Assays

Culture medium samples were assayed for E_2 as previously reported (Belanger *et al.* 1990) but without C-18 column extraction. Cross-reaction of A_4 and estrone (E_1) with the E_2 assay was less than 0.1% (Belanger *et al.* 1980). P_4 was measured in duplicate as described (Lafrance & Goff 1985). Intra- and inter-assay coefficients of variation were less than 15% for both assays. The sensitivity of the E_2, and P_4 assays were 5 and 18 pg per tube, respectively. The steroid hormone concentrations were corrected for cell number by normalization to total cell protein.

RT-PCR

Total RNA was extracted using the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions and treated with DNAse (QIAGEN, Valencia, CA). Reverse transcription was performed on 1 μg total RNA with Omniscript enzyme (QIAGEN, Valencia, CA). Gene expression was measured by semi-quantitative PCR. The primers used were those described previously (Zheng *et al.* 2008). PCR was performed under the following conditions: 1) initial denaturation at 94°C for 3 min; 2) amplification cycles of denaturation at 94°C for 45 sec, annealing for 45 sec at 60°C (*CYP11A1* and *HSD3B*) or 64°C (*HSD17B1*, *HSD17B7*, *GSTA*), or for 30 sec at 62°C (*StAR*, *1B15* and *CYP19A1*); 3) elongation at 72°C for 1 min; 4) final elongation at 72°C for 5 min. Optimal cycle number for amplification during the exponential phase was determined for each gene. The reactions were performed for 27 cycles for *1B15,* 31 cycles for *StAR*, 35 cycles for *CYP11A1*, 34 cycles for *HSD3B*, 25 cycles for *GSTA*, 38 cycles for *CYP19A1*, 36 cycles for *HSD17B1* and *HSD17B7*. The PCR products were separated on 2% agarose gels containing 0.001% ethidium bromide and visualized under UV light. Quantification of band intensity was performed with NIH Image J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Specific gene mRNA abundance was normalized to 1B15 mRNA abundance.

Enzyme activity assays

Short-term incubation of tritiated steroid hormone precursors was conducted at the end of day 6 of culture to measure specific enzyme activities. On day 6, all the medium of each

well was removed and replaced with fresh medium without A_4 but containing 6-11 nM (1.6 x10^6 DPM/ml medium) of either [^{3}H]-E_1, [^{3}H]-A_4, or [^{3}H]-testosterone ([^{3}H]-T) and 0, 0.5 or 1 ng/ml of TGF-β1. Cells were incubated for 1.5 or 3 h at 37°C. At the end of the incubation, medium was recovered and frozen at -20°C until analysis of steroid metabolism by thin layer chromatography (TLC) as previously described (Godin *et al.* 1999). Briefly, steroids were extracted from the medium using diethyl ether and resolved on DC-Alufolien neutral (Type E) paper plates (Whatman, Maidstone, Kent, England) in toluene: acetone (4:1). Each TLC plate contained [^{3}H]-E_1, [^{3}H]-E_2, [^{3}H]-A_4, and [^{3}H]-T as standards. A culture medium control was performed by incubating tracer in culture medium without cells, and was used to obtain background radioactivity that was subtracted from product counts. After migration of samples, TLC plates were exposed to phosphor screens designed for tritium detection, and tritiated steroid metabolites were localized with a Storm 840 phosphorimager (Molecular Dynamics, Sunnyvale, California, USA). The rate of production of specific steroid metabolites was quantified by scraping the corresponding sample and background spots from the TLC plate and counting radioactivity using PCS scintillation fluid (Amersham, UK).

Activity assays were validated in quiescent bovine granulosa cells by determining the conditions required to maintain excess substrate. In preliminary time course experiments without FSH treatment, 1.6 x10^6 DPM of [^{3}H]-A_4 and [^{3}H]-T (6-11 nM) were incubated with granulosa cells for 12, 24 and 48h. In 12 and 24 h incubations, the conversion of [^{3}H]-

A_4 and [^{3}H]-T precursors to [^{3}H]-E_2 was too low to be detected. After 48 h of incubation, the amount of conversion of [^{3}H]-A_4 and [^{3}H]-T precursors to [^{3}H]-E_2 was 13.8% and 5.7% respectively, and thus an incubation time of 48 h was selected. For HSD17B reducing activity, unlabeled E_1 (10^{-6}M) was added to the 8.8 pmoles/ml of [^{3}H]-E_1, and incubation time was 1.5 h. This resulted in 13.7% conversion of [^{3}H]-E_1 to [^{3}H]-E_2 and therefore 10^{-6} M unlabeled E_1 was added to all [^{3}H]-E_1 incubations.

Cell cycle and apoptosis analysis by flow cytometry

Flow cytometry using a fluorescence-activated cell sorter (FACS) has been used to determine the proportion of granulosa cells in the different phases of the cycle, and is recognized method to quantify the rate of apoptosis (Nicoletti *et al.* 1991; Rouillier *et al.* 1998). Isolated granulosa cells were centrifuged (500 X g for 5 min at 4 °C) and washed two times in ice-cold 1X PBS. Cells were fixed by drop-wise addition of 1 ml ice-cold 70% ethanol while vortexing. Ethanol-fixed cells were stored at 4°C for at least 24 h before propidium iodide (PI) staining. Granulosa cells were centrifuged 500G for 5 min at 4°C, ethanol removed, and then treated with 0.5 ml of PI staining solution (1mg/ml PI, 0.1% Tritonx-100 and 0.2 mg/ml RNase A in 1X PBS). Stained cells were held at room temperature in the dark for at least 1 h before FACS analysis. Immediately before analysis, cells were passed through a 70 μm nylon mesh sieve to obtain a single-cell suspension and to remove aggregated cells. Flow cytometry analysis was performed with a BD Bioscience FACSVantage SE. Apoptotic cells or their isolated nuclei show a low DNA stainability

resulting in a distinct, quantifiable region below the G0/G1 peak. The proportion of apoptotic cells and the proportion of cells in G0/G1, S and G2/M phases of the cell cycle were calculated using Cell Quest Pro software (BD Bioscience, Oakville, ON, Canada).

Western Blotting

Granulosa cells were lysed using Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific) and 25 μg protein samples were separated electrophoretically on a 18% sodium dodecyl sulfate-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, blocked in 5% non-fat dried milk in tris-buffered saline with Tween (TBST), and incubated overnight (4°C) with the primary antibody, anti-cleaved caspase-3 (Asp175, Cell Signaling Technology, Danvers, MA, USA). After washing with TBST three times, secondary antibody was added and incubated 1 h at room temperature and protein bands were visualized using ECL Plus (Amersham, Arlington Heights, IL, USA). The membranes were then stripped and re-probed with a mouse anti-β-actin antibody (Santa Cruz biotechnology). The molecular size of immunoreactive bands was determined by comigration of a ladder of low-range rainbow molecular weight markers (Amersham, UK) applied to a lane in each gel. Protein expression was calculated as the ratio of each specific band to corresponding β-actin signals. Experiments were repeated three times.

Statistical analysis

All statistical analyses were performed using JMP® software (SAS Institute, Cary, NC, USA). The data are presented as means ± SEM of duplicate to triplicate measurements in at least three separate cultures. Data were transformed to logarithms if they were not normally distributed as verified using the Shapiro-Wilk test. One-way ANOVA was used to test the main effect of time and TGF-β1 on estradiol secretion, progesterone secretion, steroidogenic enzyme activities and protein expression of cleaved caspase-3. Differences between treatment dose of TGF-β1 with the 0 dose control; or between 4 and 6 day cultures and the 2 day culture group were identified with Dunnett's test. Two sets of analyses were performed in flow cytometry experiments. First, the effect of time in culture on % apoptosis cell, % G0/G1 and % S+G2/M were analyzed from 0 h to 144 h in the presence or absence of FSH and TGF-β1. Differences at each time point were identified with Tukey HSD's all pairs comparison. Second, one-way ANOVA was used to test the effect of TGF-β1 on the measured parameters of each time point. Differences between treatment doses of TGF-β1 with the 0 dose control were identified with Dunnett's test. A probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

In granulosa cells cultured without FSH treatment, E_2 accumulation in culture medium decreased from Day 2 to Day 6 (Fig. 1 A). P_4 secretion increased from Day 2 to Day 6 (Fig. 1 B). In agreement with increased secretion of P_4 with time in culture, mRNA expression of the P_4-related enzymes *StAR*, *CYP11A1*, *HSD3B* and *GSTA* also increased with time (Fig. 1 D-G).

The addition of TGF-β1 during the 6-day culture period caused a significant ($P < 0.05$) dose-dependent inhibition of P_4, and increased E_2 secretion (Fig. 2 A and B). To determine if the increase in E_2 secretion was due to an increase in CYP19A1 activity and HSD17B reducing activity, conversion of the tritiated steroid hormone precursors of A_4, T, and E_1 to E_2 was measured. When quiescent granulosa cells were cultured with 1 ng/ml of TGF-β1, there was a significant increase ($P < 0.05$) of combined CYP19A1 activity and HSD17B reducing activity relative to untreated cells, as measured by the conversion of [^{3}H]-A_4 to [^{3}H]-E_2 (Fig. 3 A). The intermediate product [^{3}H]-E_1 was not detected. TGF-β1 at a dose of 1 ng/ml caused a significant increase ($P < 0.05$) in CYP19A1 activity, as measured by the conversion of [^{3}H]-T to [^{3}H]-E_2 (Fig. 3 B). TGF-β1 did not alter HSD17B reducing activity, as measured by the conversion of [^{3}H]-E_1 to [^{3}H]-E_2 (Fig. 3 C). The effect of TGF-β1 on E_2 was attributed to an increase in *CYP19A1* and *HSD17B* mRNA levels ($P < 0.05$),

but no differences in *HSD17B* mRNA levels were detected (Fig. 4 A, C and D). Corresponding to the decreased secretion of P_4, TGF-β1 caused a significant inhibition ($P < 0.05$) in mRNA expression of *StAR*, *CYP11A1*, *HSD3B*, and *GSTA* (Fig. 4 E-H).

Most of the cells (between 88%-97%) were in the G0/G1 phase and apoptotic phase during 144 h of culture. The percentage of apoptotic granulosa cells increased with time in culture in all four groups after 24 to 48 h (Fig. 5 A&B, *P<0.05*). The lowest increase in apoptosis (2-fold) was seen in FSH-stimulated granulosa cells compared to more than 3-fold increase in quiescent granulosa cells cultured without FSH. At 48h, 96h and 144 h, TGF-β1 significantly increased the percentage of apoptotic cells in both FSH-treated and quiescent granulosa cells resulting in a proportion of more than 60% of apoptotic cells at 144 h (Fig. 5 A&B, *P<0.05).* Conversely, the percentage of cells in G0/G1 phase decreased with time in culture in all four groups and TGF-β1 caused a significant decrease in the percentage of cells in G0/G1 in the presence or absence of FSH (Fig. 5 C&D, *P<0.05*). In all four groups, the proportion of cells in the proliferative phase of the cell cycle (S, G2 and M) was low and remained under 12% of the total cell population. The proportion of S+G2/M cells did not change significantly with time in culture in FSH-stimulated cells, but TGF-β1 treatment decreased the amount of proliferating cells at 144 h. In quiescent cells, the proportion of S+G2/M cells decreased with time in culture and TGF-β1 caused a marked decrease in the proportion of cells in S, G2 and M phase at 144 h (Fig. 5, E&F, *P<0.05*). The TGF-β1-

mediated increases in the proportion of apoptotic cells were associated with increased levels of cleaved caspase-3 ($P < 0.05$) in the presence or absence of FSH (Fig. 6 A and B).

DISCUSSION

It has been confirmed in a number of studies that TGF-β1 inhibits steroidogenesis in the granulosa cells of domestic animals (Wandji *et al.* 1996; Juengel *et al.* 2004; Ouellette *et al.* 2005), but no study had previously described the effects of TGF-β1 on steroidogenesis in quiescent granulosa cells. In the presence of FSH, our previous study showed that TGF-β1 inhibits E_2 and P_4 synthesis in bovine granulosa cells (Zheng *et al.* 2008). In the present study, we tested if TGF-β1 affects steroidogenesis in quiescent granulosa cells cultured without FSH. The results showed that TGF-β1 still inhibits P_4 secretion but stimulates low levels of E_2 production. This is the first study that provides evidence for a positive effect of TGF-β1 on estrogen production in domestic animals. Furthermore, we tested the effects of TGF-β1 on cell proliferation and apoptosis in both quiescent and FSH-stimulated granulosa cells. We found that TGF-β1 induces apoptosis and inhibits cell proliferation. TGF-β1 also increased protein expression of cleaved caspase 3, providing additional evidence that TGF-β1 induces apoptosis of granulosa cells.

In vitro, granulosa cells harvested from follicles always spontaneously luteinize (Meidan *et al.* 1990). However, in cultured bovine granulosa cells harvested from small bovine antral follicles, the addition of low dose of FSH maintains follicle characteristics by stimulating

E_2 and CYP19 for several days (Gutierrez *et al.* 1997; Silva & Price 2002; Sahmi *et al.* 2004) and appears to counteract the process of luteinization by inhibiting StAR mRNA expression (Zheng *et al.* 2008). Our previous study showed that TGF-β1 inhibits stimulation of E_2 and P_4 synthesis in bovine granulosa cells cultured in the presence of FSH (Zheng *et al.* 2008). In quiescent bovine granulosa cells, the present study also shows that TGF-β1 inhibits P_4 secretion and the expression of P_4-related enzymes *StAR*, *CYP11A1*, *HSD3B*, *GSTA*, indicating an inhibition of luteinization. Similarly, inhibition of P_4 secretion by TGF-β1 has also been observed in bovine luteal cells (Hou *et al.* 2008). Conversely, in the absence of FSH, this is the first study to show that TGF-β1 increases E_2 secretion and the expression of E_2-synthetic enzyme *CYP19A1* and its activity in bovine granulosa cells cultured for 6 days. However, since the actual concentration of E_2 produced at Day 6 did not exceed the amount of E_2 secreted on Day 2 and that E_2 production decreases with time in cultured quiescent granulosa cells, the effect of TGF-β1 may simply be to prevent the decline in E_2 secretion rather than stimulate E_2 production. Therefore in quiescent granulosa cells, TGF-β1 inhibits luteinization while preserving estrogenic capacity. Furthermore, in the present study, TGF-β1 stimulated expression of *HSD17B1* mRNA but did not affect expression of *HSD17B7* mRNA and HSD17B-reducing activity. In our previous study, using FSH-stimulated bovine granulosa cells, TGF-β1 inhibited expression of *HSD17B1* mRNA but similar to the present study, TGF-β1 did not affect expression of *HSD17B7* and HSD17B-reducing activity (Zheng *et al.* 2008). These results confirmed that the expression of *HSD17B1* mRNA does not consistently correlate with overall cellular

HSD17B activity. Other candidates such as *HSD17B7* may therefore contribute to HSD17B reducing enzyme activity in granulosa cells.

In a previous in vitro study using cultured bovine granulosa cells, less than 15% of total cells were in the proliferation phase during 3 days of culture with a minimal dose of FSH (Rouillier *et al.* 1998). Our study shows a similar percentage of cells in the proliferation phase (12%), with most of the cells remaining in the G0/G1 phase and apoptotic phase. In the present study, spontaneous apoptosis of GCs with time in culture is in agreement with a previous study using cultured bovine granulosa cells obtained from different size follicles (Yang & Rajamahendran 2000). In the same study, FSH was also shown to prevent apoptosis of GCs (Yang & Rajamahendran 2000). Our results show that TGF-β1 increased apoptosis in both FSH-treated and quiescent cells. TGF-β1 not only sharply induced apoptosis of GCs during time in culture but also inhibited proliferation. However, the proportion of cells in the proliferative phase was very low to start with which makes it unlikely that the previously observed decrease in protein and DNA content by TGF-β1 is due to decrease in cell proliferation (Juengel *et al.* 2004; Zheng *et al.* 2008). The present study clearly shows that induction of cell death by apoptosis is the main effect of TGF-β1 in cultured bovine granulosa cells.

The data presented herein indicate that, in the presence or absence of FSH stimulation, TGF-β1 promotes apoptosis and reduces entry into the proliferative phase of the cell cycle.

Whether these effects of TGF-β1 are somehow related to its effects on luteinization and steroidogenesis or if they are wholly independent remains unclear. Recent work in bovine granulosa cells has demonstrated a link between their differentiation state/proliferative capacity and their susceptibility to undergo apoptosis (Quirk *et al.* 2004). Specifically, granulosa cells are thought to become resistant to apoptosis through luteinization, whereas highly proliferative but less differentiated cells are more susceptible. In this regard, the increased rate of apoptosis that we observed in TGF-β1-treated cells may simply be a consequence of their less luteinized state. The reduced proportion of cells in the proliferative (i.e., S+G2/M) phase of the cell cycle that we observed in response to TGF-β1 was somewhat paradoxical, in that one might expect a higher rate of proliferation to accompany the resultant lesser state of luteinization. We speculate that this may in fact be due to the higher rate of apoptosis cause by TGF-β1. Indeed, the G1/S transition phase seems to act as a critical checkpoint, at which granulosa cells may either progress through the cell cycle or undergo apoptosis, according to the relative abundance of growth and pro- or anti-apoptotic factors that may be present (Quirk *et al.* 2004). The presence of TGF-β1 may commit a higher proportion of cells to apoptosis at the G1/S transition, resulting in the presence of fewer cells in the S and G2/M phases of the cycle and therefore a lesser rate of proliferation. Finally, we cannot exclude the possibility that TGF-β1 also affects cell proliferation more directly by negatively regulating cell cycle progression.

Although it is often difficult to relate observations in cultured granulosa cells to follicle development *in vivo*, our findings may provide some insight into mechanisms of follicle selection. The bovine ovarian cycle is characterized by the recruitment of waves of small follicles, which proceed to grow until a poorly understood selection process designates a dominant follicle to continue development, while the remaining recruited follicles undergo atresia (Ireland 1987; Fortune *et al.* 2001). TGF-β1 is expressed in the granulosa cells of bovine small antral follicles (Nilsson *et al.* 2003). Given the pro-apoptotic properties of TGF-β1 that we have observed in cultured granulosa cells, it seems reasonable to speculate that its relative abundance in a given small follicle may be a determinant if it will undergo atresia (i.e. apoptosis) or achieve dominant status. In surviving follicles, the anti-luteinization properties of TGF-β1 may serve to prevent the premature luteinization of follicular granulosa cells. For continued development of these surviving follicles, FSH must overcome TGF-β1 inhibition of FSH-induced conversion of androgens to E_2. One possible way to achieve this effect could be down-regulation of TGF-β1 action. Indeed, some studies provide evidence that FSH treatment decreases *TGF-β1* mRNA and *ALK-5* (activin receptor-like kinase 5) mRNA expression in cultured bovine granulosa cells (Nilsson *et al.* 2003; Jayawardana *et al.* 2006). Further studies, including experiments involving manipulation of TGF-β1 activity *in vivo* and *in vitro*, will be required explore these possibilities.

In conclusion, TGF-β1 negatively regulates luteinization of granulosa cells, as evidenced by inhibition of P_4 secretion and P_4-generating enzymes. Granulosa cell secretion of E_2 and expression of E_2-generating enzymes are regulated differently by TGF-β1 depending on the presence or absence of FSH. TGF-β1 also inhibited granulosa cell proliferation and induced apoptosis. By acting in this manner, TGF-β1 may have a physiological role to limit growth and differentiation of granulosa cells in follicles and may be involved in dominant follicle selection.

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Fig. 1. Effect of time in culture (2, 4, 6 days) on secretion of E_2 and P_4 with P_4 synthesis-associated gene expression in untreated quiescent bovine granulosa cells. Granulosa cells were cultured in serum-free medium without stimulation starting at day 0. The data represent secretion of E_2 and P_4 during the previous 48 h of culture (A and B). Transcript abundance of P_4 synthesis-associated genes *StAR*, *CYP11A1*, *3β-HSD*, and *GSTA* mRNA (D-G) was measured by semiquantitative RT-PCR and normalized to the housekeeping gene *1B15*. Data are means ± SEM of 3 separate culture replicates. Asterisk (*) indicates that the mean is significantly different from control at day 2 ($p<0.05$, one-way ANOVA, with Dunnett's test). Panel (C) is a representative agarose gel from two replicates showing PCR products for each steroidogenic enzyme and housekeeping gene *1B15*.

Figure 1

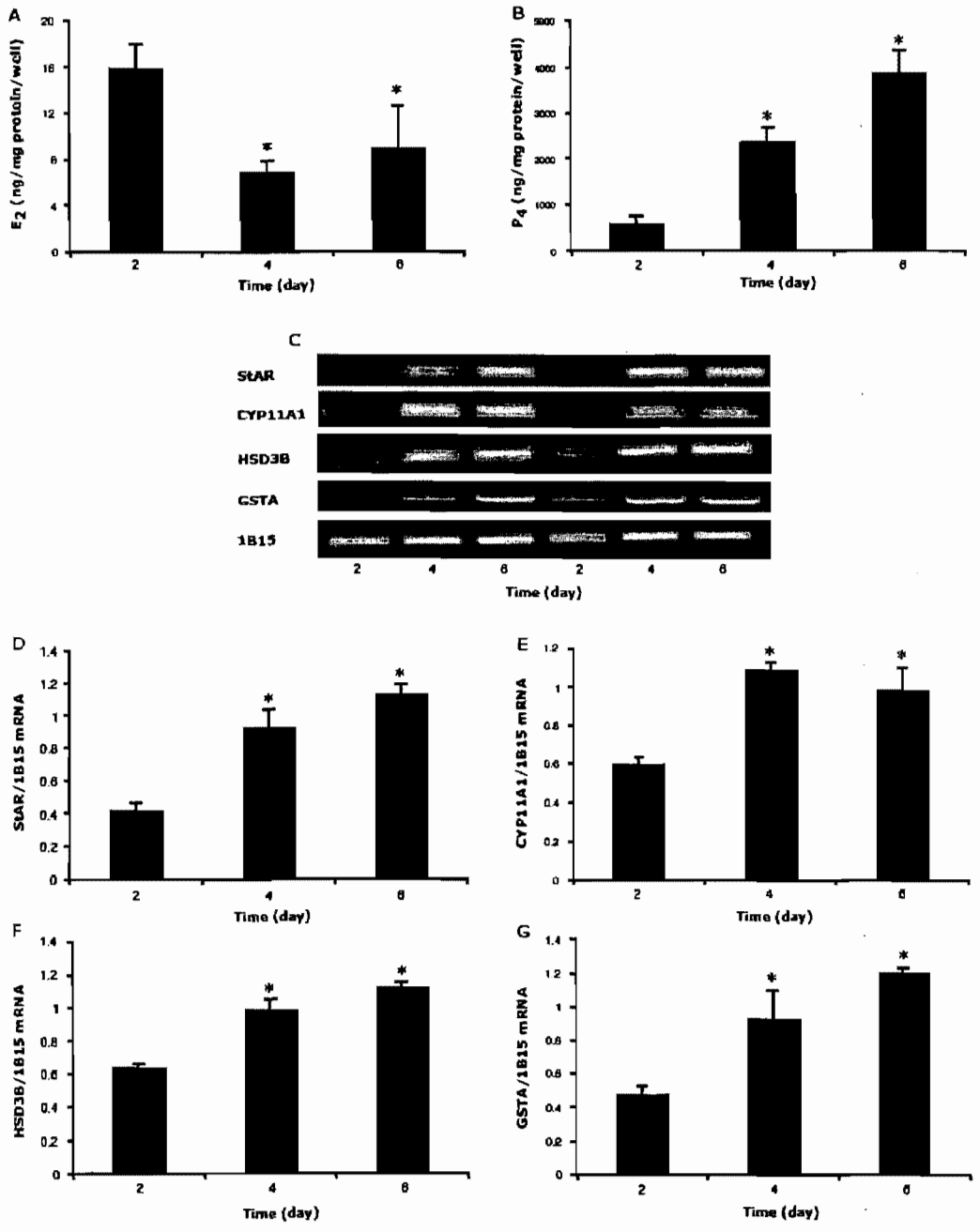

A
E_2 (ng/mg protein/well)
Time (day)
B
P_4 (ng/mg protein/well)
Time (day)
C
StAR
CYP11A1
HSD3B
GSTA
1B15
Time (day)
D
StAR/1B15 mRNA
Time (day)
E
CYP11A1/1B15 mRNA
Time (day)
F
HSD3B/1B15 mRNA
Time (day)
G
GSTA/1B15 mRNA
Time (day)

Fig. 2. Effect of TGF-β1 on E_2 (A) and P_4 (B) secretion from cultured quiescent bovine granulosa cells. Cells were cultured for 6 days without serum and treated with TGF-β1 beginning on the first day of culture. The medium was collected on day 6 and the data represent steroids produced during the final 48 h of culture. Data were corrected for total cell protein and represent means ± SEM derived from 4 different pools of ovaries. The asterisk (*) indicates that steroid hormone secretion was significantly different from the 0 dose control group ($p<0.05$, n=4, one-way ANOVA, with Dunnett's test).

Figure 2

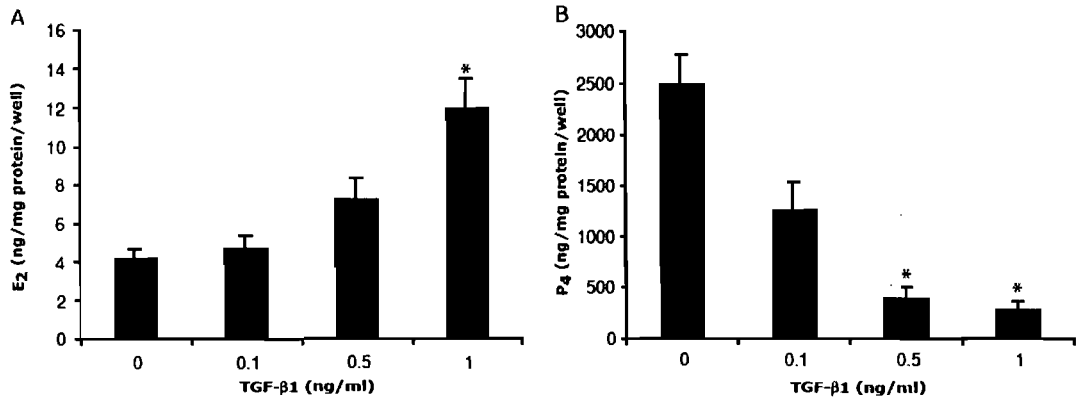

A
E_2 (ng/mg protein/well)
16
14
12
10
8
6
4
2
0
*
0
0.1
0.5
1
TGF-β1 (ng/ml)
B
P_4 (ng/mg protein/well)
3000
2500
2000
1500
1000
500
0
*
*
0
0.1
0.5
1
TGF-β1 (ng/ml)

Fig. 3. Effect of TGF-β1 on conversion of androgens to E_2 and HSD17B reducing activity. Quiescent bovine granulosa cells were cultured for 6 days in serum-free medium with TGF-β1 treatment. The culture medium was replaced on day 6 with fresh medium containing labelled steroid precursor, and enzymatic activity assays were conducted as described in Materials and Methods. [^{3}H]A_4 and [^{3}H]T were incubated for 48 h and [^{3}H]E_1 precursor was incubated with 10^{-6}M (1000 fold excess) unlabeled E_1 for 1.5 h. Data are means ± SEM of 3 separate culture replicates. Panel A shows conversion of [^{3}H]A_4 to [^{3}H]E_2 (CYP19A1 + HSD17B reducing activity). Panel B shows [^{3}H]T conversion to [^{3}H]E_2 (CYP19A1 activity). Panel C shows [^{3}H]E_1 conversion to [^{3}H]E_2 (HSD17B reducing activity). The asterisk (*) indicates that enzyme activity is significantly different from the 0 dose control group ($P<0.05$, n=3, one-way ANOVA, with Dunnett's test).

Figure 3

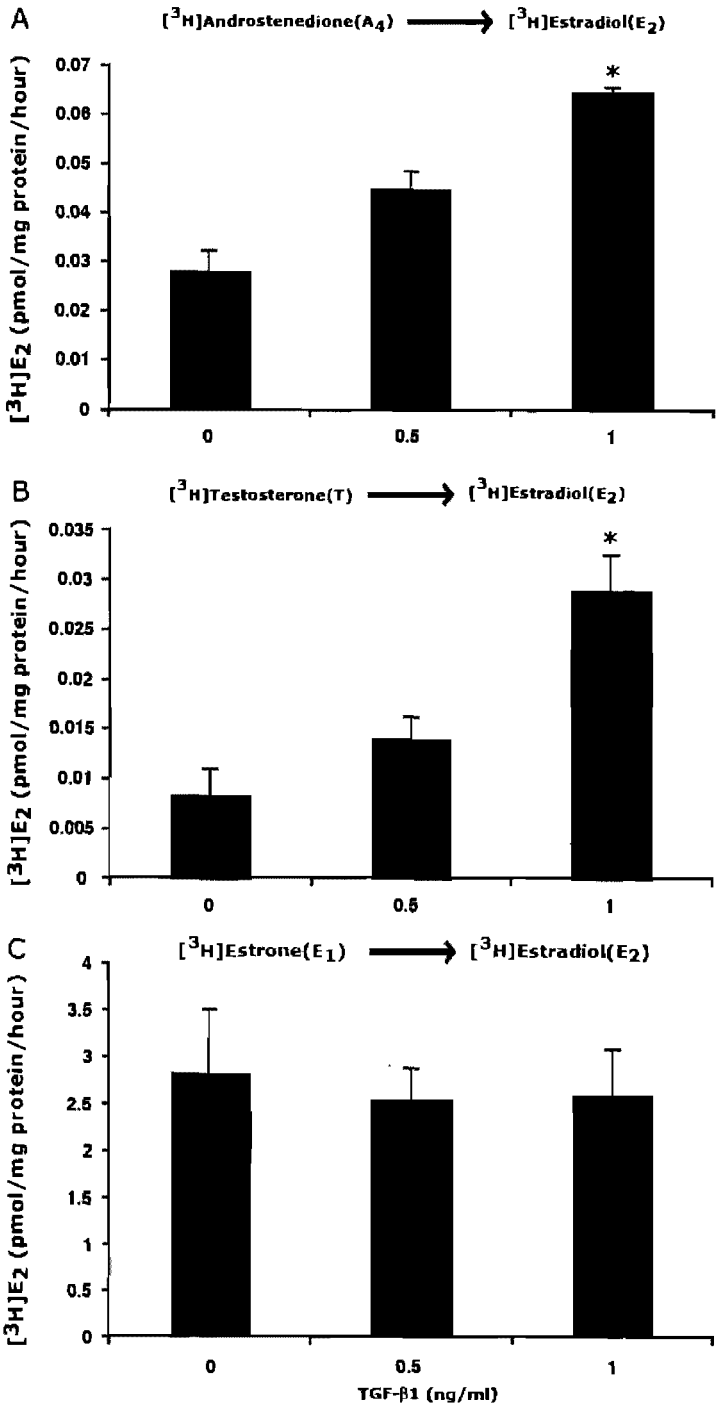

A
[3H]Androstenedione(A4) → [3H]Estradiol(E2)
[3H]E2 (pmol/mg protein/hour)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
0.5
1
*
B
[3H]Testosterone(T) → [3H]Estradiol(E2)
[3H]E2 (pmol/mg protein/hour)
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
0.5
1
*
C
[3H]Estrone(E1) → [3H]Estradiol(E2)
[3H]E2 (pmol/mg protein/hour)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
0.5
1
TGF-β1 (ng/ml)

Fig. 4. Effect of TGF-β1 on mRNA expression of steroidogenic enzymes in quiescent bovine granulosa cells. Cells were cultured in serum-free medium for 6 days in the presence of the stated doses of TGF-β1. Abundance of *CYP19A1*, *HSD17B1*, *HSD17B*, *StAR*, *CYP11A1*, *HSD3B*, and *GSTA* mRNA (A, C-H) was measured by semiquantitative RT-PCR and normalized to the housekeeping gene *1B15*. Data are means ± SEM of 4 separate culture replicates. Asterisk (*) indicates that mRNA content is significantly different from 0 dose control ($p<0.05$, one-way ANOVA, with Dunnett's test). Panels (B) are representative agarose gels from two replicates showing PCR products for each steroidogenic enzyme and the housekeeping gene *1B15*.

Figure 4

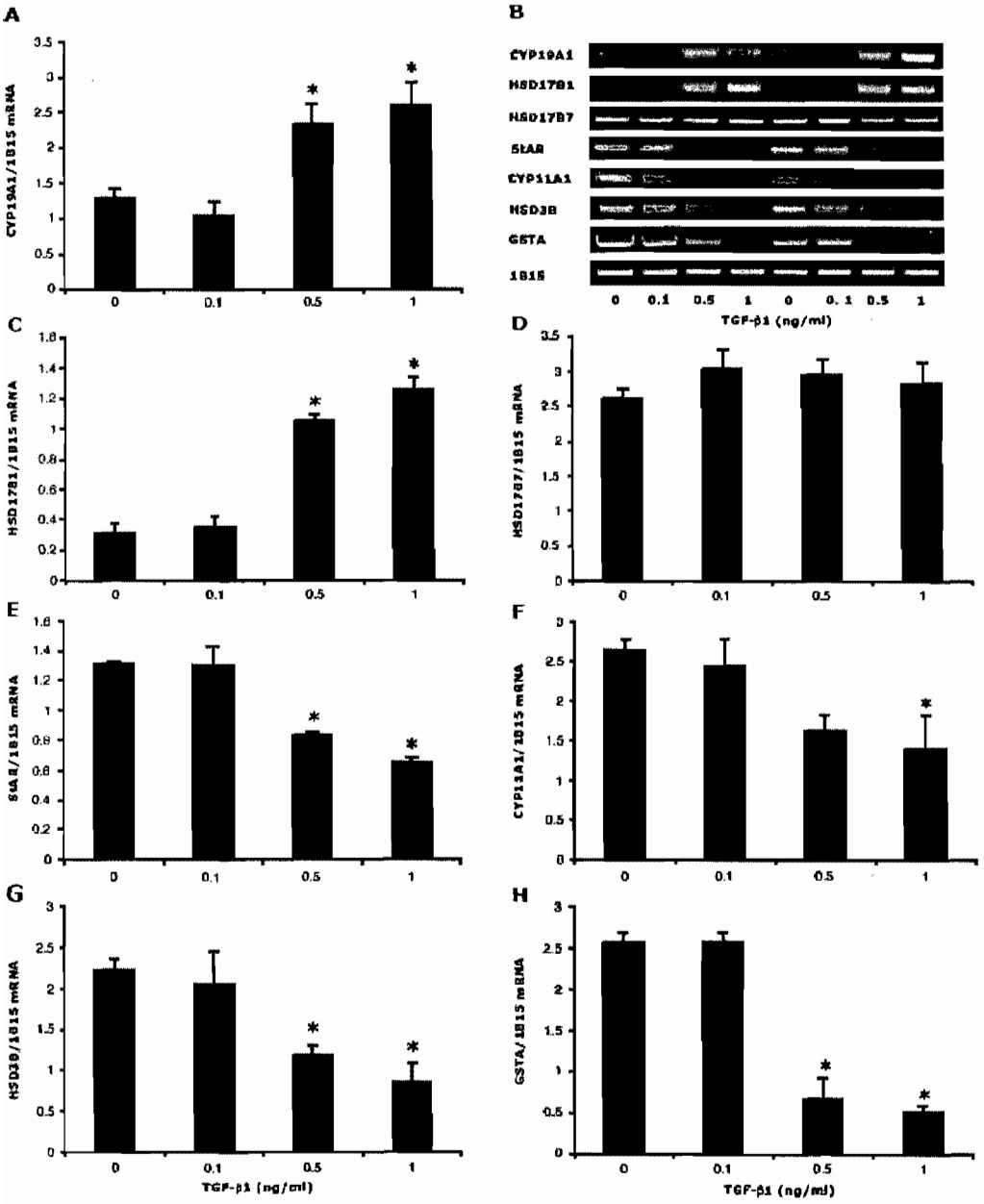

A
B
C
D
E
F
G
H
CYP19A1/18S mRNA
HSD17B1/18S mRNA
HSD17B7/18S mRNA
StAR/18S mRNA
CYP11A1/18S mRNA
HSD3B/18S mRNA
GSTA/18S mRNA
CYP19A1
HSD17B1
HSD17B7
StAR
CYP11A1
HSD3B
GSTA
18S
0 0.1 0.5 1 0 0.1 0.5 1
TGF-β1 (ng/ml)

Fig. 5. Effects of TGF-β1 treatment on apoptosis and cell cycle phase of granulosa cells in the presence and absence of FSH. Cells were treated with TGF-β1 for 6 days in the presence (pannels A, C and E) or absence (pannels B, D and F) of FSH, stained with propidium iodide and analyzed by FACS. A and B display the proportion of apoptotic cells in relation to total cell counts. C and D display the proportion of G0/G1; E and F show the proportion of cells in the proliferative phase of the cell cycle (S+G2/M). Asterisk (**) indicates significant differences ($p<0.05$) from granulosa cells cultured without TGF-β1 at individual time points. Different letters of (a, b, c, d) indicate significant differences ($p<0.05$) between different time points in the presence of TGF-β1. Different letters of (x, y) indicate significant differences ($p<0.05$) between different time points in the absence of TGF-β1. Data are means ± SEM of 3 separate culture replicates.

Figure 5

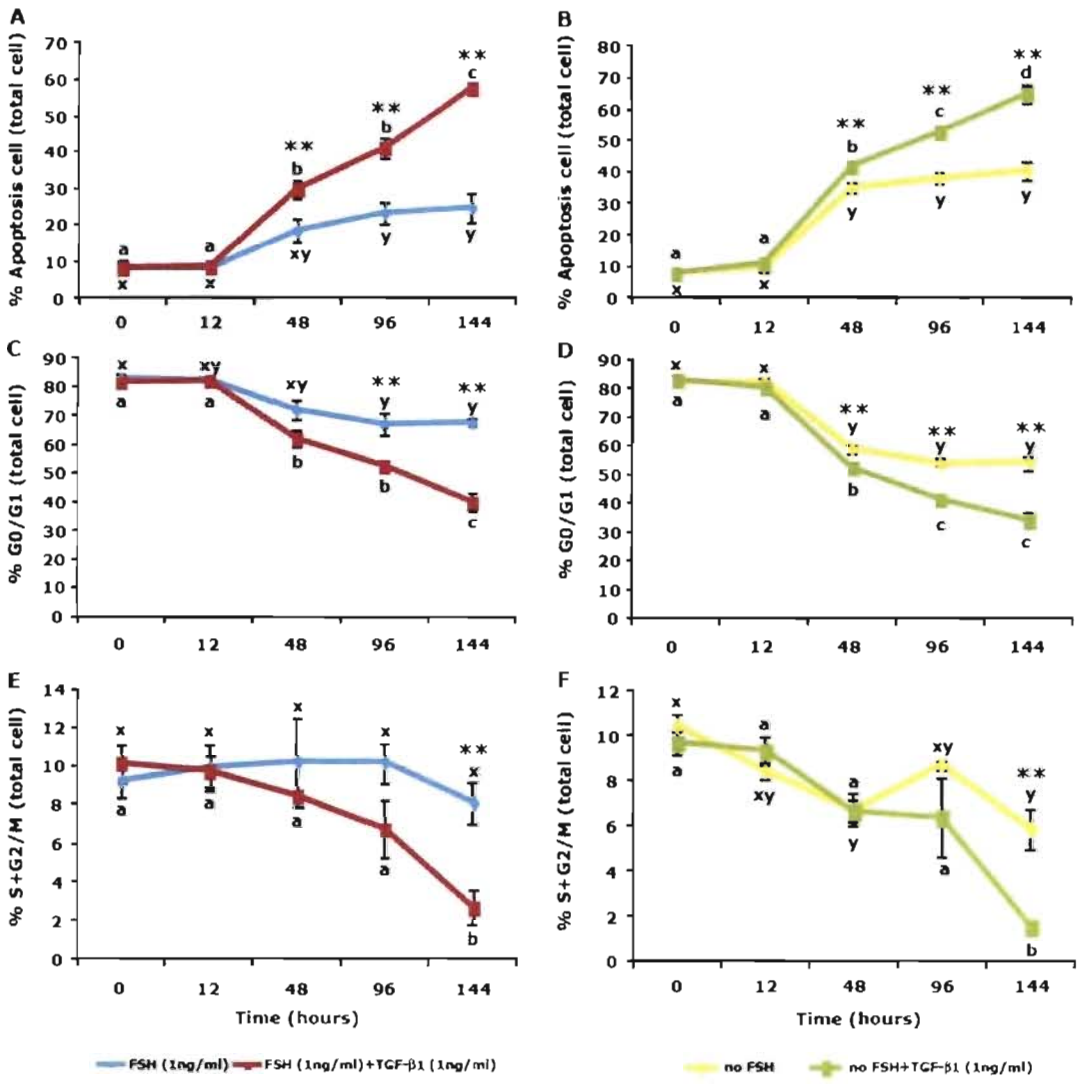
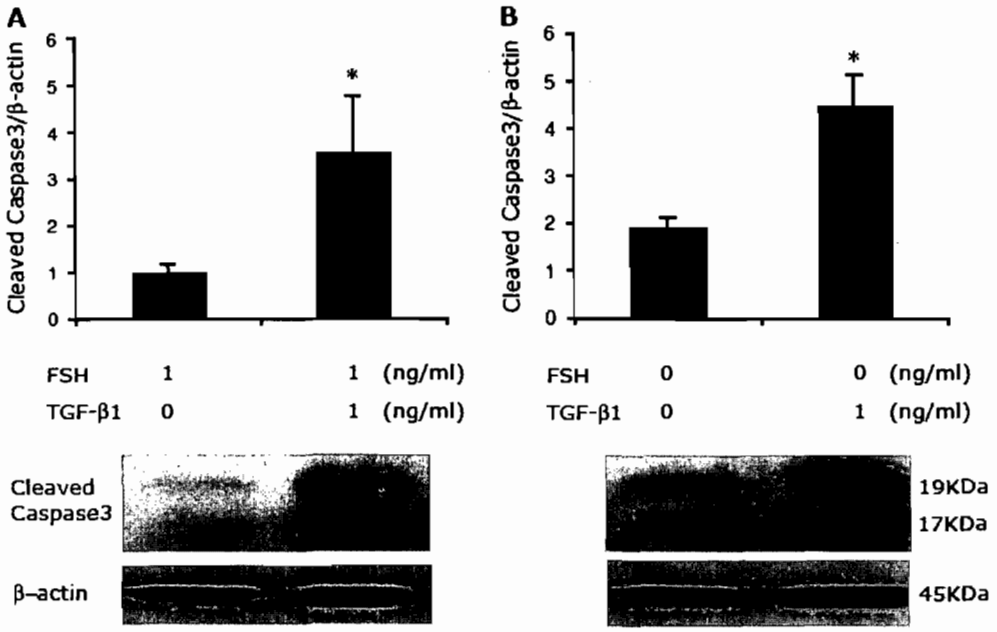

A
% Apoptosis cell (total cell)
B
% Apoptosis cell (total cell)
C
% G0/G1 (total cell)
D
% G0/G1 (total cell)
E
% S+G2/M (total cell)
F
% S+G2/M (total cell)
0
12
48
96
144
Time (hours)
FSH (1ng/ml)
FSH (1ng/ml)+TGF-β1 (1ng/ml)
no FSH
no FSH+TGF-β1 (1ng/ml)

Fig. 6. Western blot analysis of cleaved caspase-3 in granulosa cells treated with TGF-β1 in the presence or absence of FSH. The 17KDa and 19KDa cleaved caspase-3 bands were normalized to the housekeeping protein β-actin. Asterisk (*) indicate that cleaved caspase3 is significantly different from 0 dose treatment of TGF-β1. Data for are means ± SEM of 3 separate culture replicates. ($p<0.05$, one-way ANOVA, with Dunnett's test).

Figure 6


A
B
Cleaved Caspase3/β-actin
0
1
2
3
4
5
6
*
FSH 1 1 (ng/ml)
TGF-β1 0 1 (ng/ml)
FSH 0 0 (ng/ml)
TGF-β1 0 1 (ng/ml)
Cleaved Caspase3
β–actin
19KDa
17KDa
45KDa

GENERAL DISCUSSION

1. TGF-β1 influence on steroidogenesis in cultured bovine granulosa cells

Maturation of bovine granulosa cells in developing follicles is accompanied by a shift from a predominantly E_2-producing to a P_4-producing cell (Luck *et al.* 1990; Meidan *et al.* 1990). CYP19A1 expression and resultant estrogen production by granulosa cells begins during early antral follicle development. During growth to the preovulatory stage, P_4 related-enzymes CYP11A1 and HSD3B are gradually induced in granulosa cells and P_4 synthesis begins to increase, accompanied by a progressive loss of estrogen-producing capacity (Bao & Garverick 1998). Estrogenic features are eventually replaced in fully luteinized granulosa cells by expression of StAR and a shift to P_4 secretion (Murphy 2004). In vitro, granulosa cells harvested from follicles always spontaneously luteinize (Meidan *et al.* 1990). However, the process of luteinization in vitro is wholly dependent on factors present in the culture medium (Figure 8). When serum is present in culture medium, luteinization of granuosa cells is accelarated compared to serum-free conditions (Gutierrez *et al.* 1997b). In serum-free cultured bovine granulosa cells harvested from small bovine antral follicles, the addition of a low dose of FSH can slow down the process of luteinization by stimulating E_2 and CYP19A1 for several days (Gutierrez *et al.* 1997a; Silva & Price 2002; Sahmi *et al.* 2004) and by inhibiting StAR mRNA expression (present study). Furthermore, as shown in this thesis, TGF-β1 consistently inhibited luteinization in

both quiescent and FSH-stimulated bovine granulosa cells by inhibiting P_4 secretion and the expression of P_4-related enzymes StAR, CYP11A1, HSD3B and GSTA.

In contrast, granulosa cell secretion of E_2 and expression of E_2-generating enzymes are regulated differently by TGF-β1 depending on the presence or absence of FSH. TGF-β1 counteracts stimulation of E_2 synthesis and the expression and activity of E_2-synthetic enzyme CYP19A1 in FSH-stimulated bovine granulosa cells. In contrast, TGF-β1 increased E_2 secretion and CYP19A1 in quiescent bovine granulosa cells cultured with a low dose of insulin but without FSH. In quiescent granulosa cells, E_2 synthesis was low and declined with time in culture so that the effect of TGF-β1 after 6 days of culture was to sustain E_2 synthesis rather than enhance stimulation. The role of different factors present in the culture medium on the maturation process of bovine granulosa cells is summarized in Figure 6.

In addition, the conversion of A_4 to E_2 also requires HSD17B reducing activity (Mindnich *et al.* 2004), however TGF-β1 did not affect HSD17B activity in quiescent or FSH-stimulated bovine granulosa cells. TGF-β1 inhibited HSD17B1 expression in FSH-stimulated bovine granulosa cells but stimulated HSD17B1 expression in quiescent bovine granulosa cells, without affecting HSD17B reducing activity. This suggests that another HSD17B isoenzyme, which is not regulated by TGF-β1 may be contributing in a major way to the HSD17B reducing activity. In the present study, HSD17B7, another enzyme

known to convert E_1 to E_2 (Krazeisen *et al.* 1999); (Krusche *et al.* 2001) was detected for the first time in bovine granulosa cells and was not inhibited by TGF-β1 in quiescent or FSH-stimulated bovine granulosa cells. FSH dose and time in culture was also without any effect on expression of HSD17B7 mRNA. For HSD17B activity, a thousand fold-excess of unlabeled E_1 had to be added and incubation time had to be shortened to obtain comparable conditions of substrate excess, indicating that HSD17B reducing activity is very high in cultured quiescent or FSH-stimulated bovine granulosa cells. The physiological significance of this finding remains unclear but it suggests that the capacity of granulosa cells to transform estrone to estradiol is an essential feature of developing follicles. Any estrone produced by the granulosa cell would be quickly transformed to estradiol. This finding also raises the possibility that supply of estrone from the outside and its delivery to the ovary by the peripheral circulation may be a contributing factor to the production of follicular estradiol. In this respect, androgens which are also produced by the adrenal gland and peripheral tissues, can be converted to estrone in the adipose tissue (Simpson *et al.* 1989). Therefore in a physiological context, during small antral follicle development, minimal estradiol synthesis necessary to maintain follicle viability for a certain time might depend on adequate supply of peripheral blood estrone to the ovary. Nevertheless, TGF-β1 would still prevent marked secretion of estradiol from theca androgens by inhibiting CYP19A1 activity. Sustained HSD17B reducing activity with adequate estrone delivery may be a mechanism responsible for recruitment of antral follicles. This mechanism may also be involved in preserving estrogenic potential of subordinate follicles during follicle

selection since it has been shown that the F2 follicle can replace the F1 when the latter has been destroyed by ultrasound-guided transvaginal aspiration (Ginther *et al.* 1996).

2. TGF-β1 influence on survival, death and morphology of granulosa cells

Granulosa cell survival must proceed in such a manner that allows some cells to enter into the proliferation phase, while other cells reach the postmitotic differentiation stage for luteinization. If proliferation and differentiation are prevented, then granulosa cells must be eliminated by apoptosis (Figure 9). The presence of serum in culture medium promotes differentiation and prevents apoptosis by inducing luteinization of granulosa cells, whereas granulosa cells undergo apoptosis in response to serum withdrawal (Gutierrez *et al.* 1997b; Hu C. L. *et al.* 2001; Tajima *et al.* 2002). IGF-I and E_2 attenuate apoptotic cell death by stimulating progression from the quiescent stage (G0/G1) to the S phase during culture (Quirk *et al.* 2004). IGF and FSH also increase the number of bovine granulosa cells after 6 days in culture and prevent apoptosis (Gutierrez *et al.* 1997a; Rouillier *et al.* 1998; Yang & Rajamahendran 2000). Conversely, in the present study, TGF-β1 treatment significantly decreased the proportion of cells in the proliferative phase of the cell cycle and increased cell apoptosis, in the presence or absence of FSH. These effects of TGF-β1 in terms of apoptosis may contribute to either inhibit luteinization of granulosa cell or block the effects of FSH. Figure 9 briefly indicates the factors in culture medium that determine the survival or death of granulosa cells.

Granulosa cell interactions are mediated by gap-junctions and the extracellular matrix (McClellan *et al.* 1975; Rodgers *et al.* 2003) which are necessary for metabolic exchange, transport of molecules and regulation of steroid hormone production (Schultz 1985; Rodgers *et al.* 2003). Mechanical dispersion of granulosa cells disrupts gap junctions and the extracellular matrix environment of the cells and when cells are put into serum-free culture in the presence of FSH they gradually re-associate and form clumps with abundant gap-junctions (Gutierrez *et al.* 1997b). TGF-β1 caused a visible change in the pattern of re-association and the morphology of the granulosa cell clumps that appeared smaller and more spherical than in FSH-treated controls. A similar observation has also been found in cultured quiescent granulosa cells (data not shown). The results of the current study suggest that addition of TGF-β1 modifies the pattern of cellular aggregations, perhaps by causing a change in the formation of extracellular matrix or by modifying cell-cell communication through inhibition of gap junction formation or by inducing apoiptosis and cell shrinkage.

3. Role of TGF-β1 during follicle development and future directions

Although it is often difficult to relate observations in cultured granulosa cells to follicle development *in vivo*, our findings may provide some insights into mechanisms of follicle selection. The present study has shown that TGF-β1 affects steroidogenesis, survival, death and morphology of granulosa cells. However, whether these in vitro effects of TGF-β1 happen or not during follicle development, is wholly dependent on the successful expression of TGF-β1 and its receptors at certain periods of follicle

development. TGF-β1 is expressed in the granulosa cells of bovine small antral follicles but not in large antral follicles (Nilsson *et al.* 2003). A previous study also showed a negative relationship between follicular fluid TGF-β1 and E_2 concentrations before dominant follicle selection (Ouellette *et al.* 2005), suggesting an inhibitory role of granulosa cell TGF-β1 on small antral and antral follicle development. Given the pro-apoptotic properties of TGF-β1 that we have observed in cultured granulosa cells, it seems reasonable to speculate that the relative abundance of TGF-β1 in a given small follicle may determine if the follicle will undergo atresia (i.e. apoptosis) or achieve dominant status. Figure 10 illustrates the possible stages of follicle development where TGF-β1 may be playing a physiological role. When FSH begins to recruit a follicle wave, it may be inhibiting TGF-β action since FSH treatment has been shown to decrease TGF-β1 mRNA and ALK-5 mRNA expression in cultured bovine granulosa cells (Nilsson *et al.* 2003; Jayawardana *et al.* 2006). If TGF-β action is decreased by FSH, based on the present study, then E_2 secretion should begin to increase. Interestingly, recent studies have shown that E_2 increases ALK5 mRNA expression in cultured bovine and ovine granulosa cells (Jayawardana *et al.* 2006; Chen *et al.* 2008). Together with the present study, this finding raises the intriguing possibility that E_2 may down regulate its own secretion by enhancing TGF-β1 action through activation of the ALK5 receptor. E_2 induced enhancement of TGF-β action would then result in FSHr blockade, decreased E_2 synthesis from androgens, inhibition in progression into the cell cycle, granulosa cell apoptosis and eventually follicle atresia. Selection of the dominant follicle would therefore require the action of other factors to overcome E_2 induced

activation of TGF-β action. These factors could include LH, IGF or androgens. Factors such as androgens might overcome TGFβ inhibition by enhancing FSH action, since androgens have been shown to increase FSHr (Luo & Wiltbank 2006). Another factor could be LH acting either directly on granulosa cells that express LH receptors or indirectly by stimulating theca production of androgens. Therefore opposing effects of FSH and E_2 on the regulation of TGF-β action could play a determining role on continued growth or atresia of the ovarian follicle. However, it is not clear how this happens. Further studies, including experiments involving manipulation of TGF-β1 activity (TGFβ receptors) *in vivo* and *in vitro*, will be required to explore these possibilities.

Figure 8. Factors influencing steroidogenesis in cultured bovine granulosa cells.

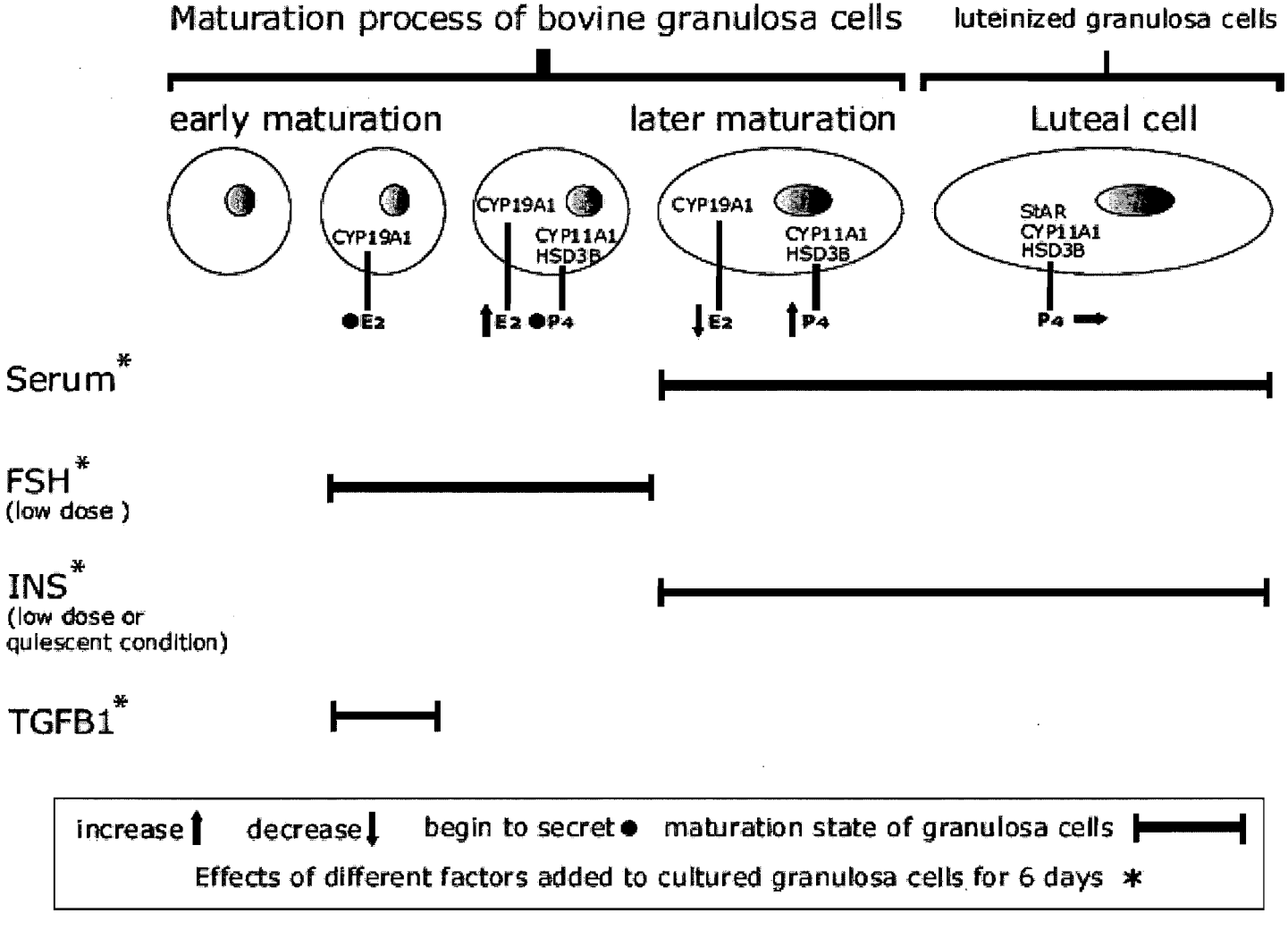

Maturation process of bovine granulosa cells
luteinized granulosa cells
early maturation
later maturation
Luteal cell
CYP19A1
CYP19A1
CYP11A1
HSD3B
CYP19A1
CYP11A1
HSD3B
StAR
CYP11A1
HSD3B
E2
E2 P4
E2
P4
P4
Serum*
FSH*
(low dose)
INS*
(low dose or
quiescent condition)
TGFB1*
increase
decrease
begin to secret
maturation state of granulosa cells
Effects of different factors added to cultured granulosa cells for 6 days *

Figure 9. Factors influencing survival and death of granulosa cells.

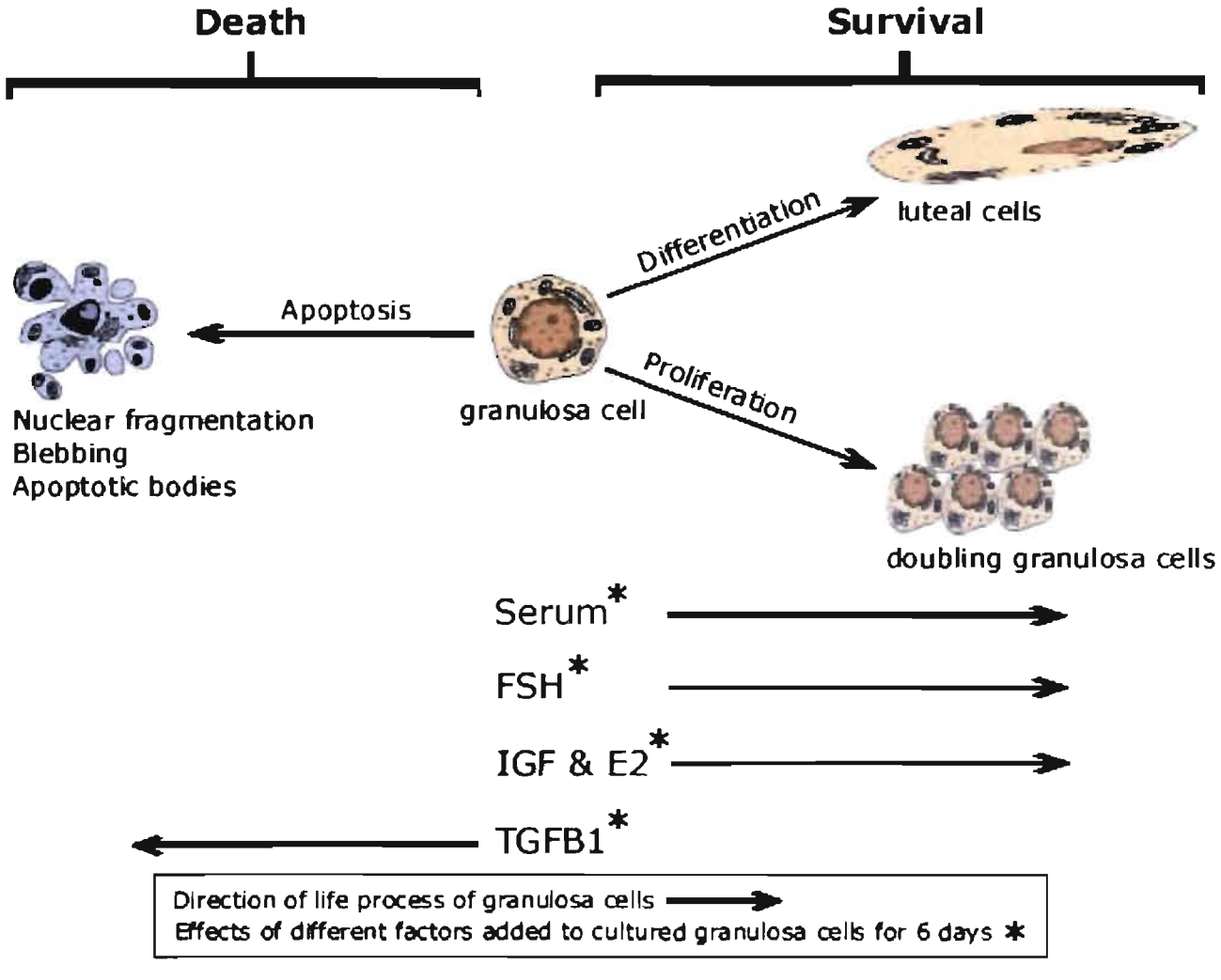
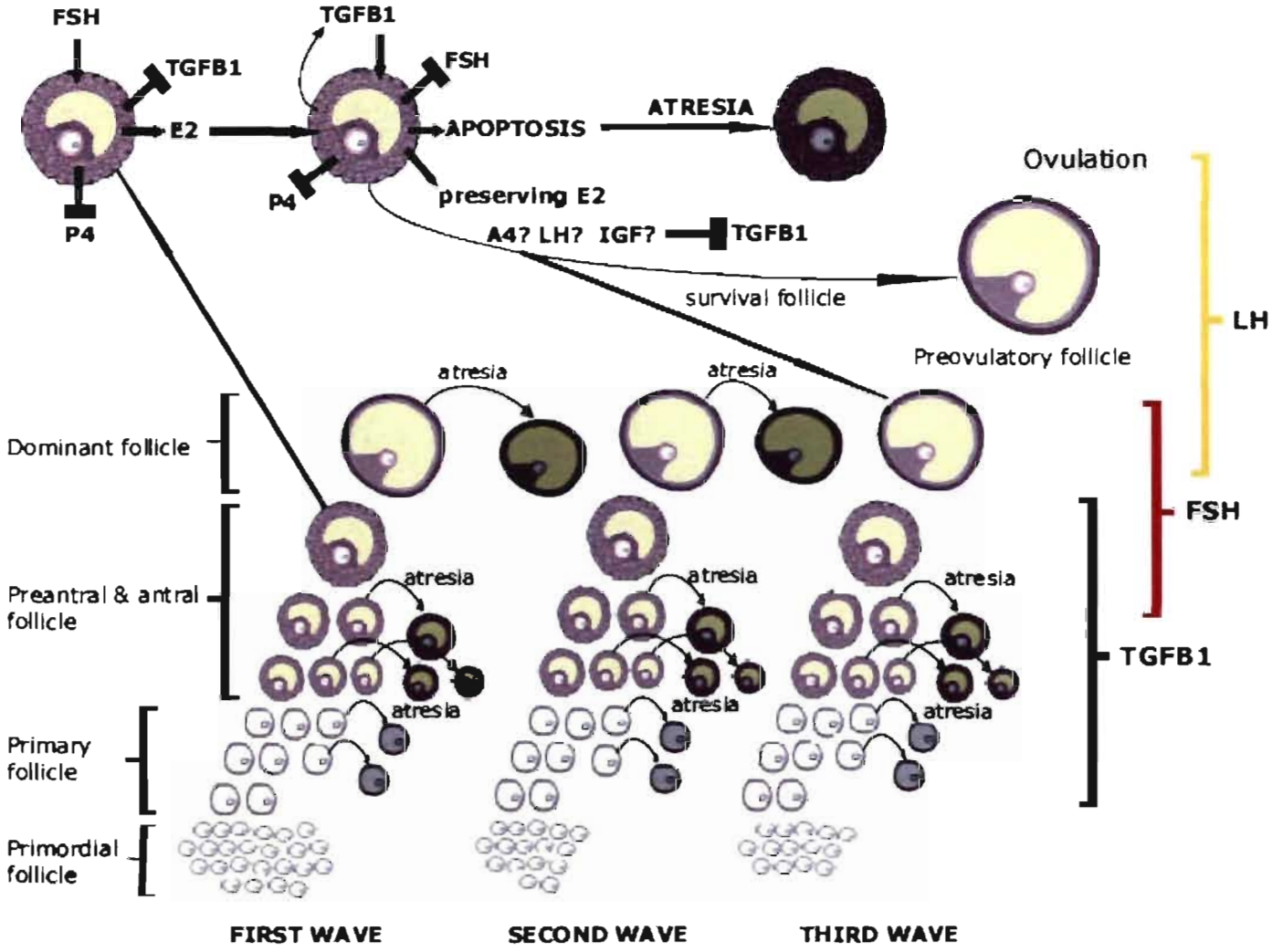

Death
Survival
luteal cells
Differentiation
Apoptosis
Proliferation
granulosa cell
Nuclear fragmentation
Blebbing
Apoptotic bodies
doubling granulosa cells
Serum*
FSH*
IGF & E2*
TGFB1*
Direction of life process of granulosa cells
Effects of different factors added to cultured granulosa cells for 6 days *

Figure 10. Possible role of TGF-β1 during follicle development.


FSH
TGFB1
E2
P4
TGFB1
FSH
APOPTOSIS
ATRESIA
P4
preserving E2
A4? LH? IGF?
TGFB1
Ovulation
survival follicle
Preovulatory follicle
LH
FSH
TGFB1
atresia
atresia
Dominant follicle
Preantral & antral follicle
Primary follicle
Primordial follicle
atresia
atresia
atresia
atresia
atresia
atresia
FIRST WAVE
SECOND WAVE
THIRD WAVE

CONCLUSION

The living cell has one of two destinies. Either it is in a young proliferating phase aimed at becoming a mature differentiated cell or else it is already a mature cell accomplishing its task to keep the body alive. In our in vitro granulosa cell culture system, TGF-β1 seems to inhibit both destinies. It inhibits P_4 differentiation (which is necessary to prevent premature ovulation induction) and it also inhibits proliferation by limiting entry into the S+G2/M phase of the cell cycle (an effect that may depend on sufficient estrogenic stimulation and/or the presence of other survival factors). Overall if TGF-β1 effects are left unopposed, the cell has lost its reason to be and apoptosis is triggered.

In the present study, the inhibition of P_4 was absolute and observed in quiescent as well as FSH-stimulated granulosa cells. Conversly, the inhibition of E_2 secretion is not absolute. In quiescent cells, TGF-β1 seems to maintain some estrogenic capacity but in the absence of FSH, the amount of E_2 produced and/or the presence of other survival factors is insufficient to prevent apoptosis. In FSH-treated cells, TGF-β1 clearly opposes the main drive of E_2 secretion by FSH.

In a physiological context, it is postulated that the follicle that produces the most TGF-β1 in the early stage of follicle development and that subsequently is the first to repress TGF-β inhibition of FSH action will be selected to become the ovulatory follicle.

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