ABSTRACT

SRIPERUMBUDUR, RAJAGOPAL. The Role of TGF-β in Luteinization in the Pig. (Under the direction of Dr. John E. Gadsby).

The purpose of this research was to investigate the role of TGF-β in the luteinizing follicle and to examine the expression and localization of TGF-β and components of its signaling pathway in the peri-ovulatory porcine follicle. It is well known that the LH-surge causes ovulation of follicles and initiates luteinization. There is also evidence from our own studies as well from the literature suggesting that TGF-β may also play a role in luteinization. In the first study we proposed to examine the expression of TGF-β ligands (i.e. TGF-β1, 2 and 3) and components of its signaling pathway {the TGF-β receptors, TβRI and II, the Smad [the vertebrate homologue of Drosophila Mad (Mad =Mothers against decapentaplegic) and the related Caenorhabditis elegans gene Sma] proteins, Smad 2, 3, 4, 6 and 7, and the Smad Anchor for Receptor Activation, (SARA)} in porcine follicles induced to luteinize in vivo with hCG treatment. Pre-pubertal pigs were injected with a half dose of PG-600 (200 I.U. eCG and 100 I.U. hCG) to induce ovarian follicular development and then injected with hCG 3 days later to induce ovulation. Ovaries were collected surgically at 0, 1, 12, 24, 48 and 96 hours following hCG treatment, and used for RNA and protein extraction, and for immunohistochemistry. Semi-quantitative RT-PCR analysis was used to assess mRNA expression of components of the TGF-β signaling pathway. Our data revealed that hCG caused up-regulation of mRNA expression of TGF-β3 at 12h and TβRII at 96h versus 0h control. However, protein expression in these samples, though mirroring mRNA expression for TβRII**,** was not significantly different compared to the 0h control. Using immunohistochemistry, we also localized TGF-β1 expression to granulosa cells (GC), TGF-

β2 to theca cells (TC) in the pre-ovulatory follicle, TGF-β1 and TGF-β2 expression in developing luteal cells (LC) in the post-ovulatory follicle, and TβRI and TβRII expression in GC, TC and LC of the pre- and post-ovulatory follicle. Since hCG, whose actions simulate those of naturally-occurring LH, upregulates some components of the TGF-β signaling pathway and induces luteinization, we suggest that TGF-β signaling may play a role in mediating luteinization that occurs during the normal cycle.

In the second study, we proposed to examine the expression of TGF-β ligands (i.e. TGF-β1, 2 and 3) and components of its signaling pathway (the TGF-β receptors, TβRI and II, the Smad proteins, Smad 2, 3, 4, 6, 7 and SARA) in porcine granulosa cells induced to luteinize in vitro with either LH/IGF-1, various doses of TGF-β1 or a combination of the two treatments. Granulosa cells were obtained from slaughterhouse porcine follicles and were placed into culture in M199 containing 10% fetal bovine serum on collagen I coated culture dishes. Granulosa cell cultures were treated with either LH (250 ng/ml) / IGF-I (10 ng/ml), varying doses of TGFβ-1 (10 and 100 ng/ml) or with a combination of the two treatments (LH/IGF-1 + TGF-β1 10 ng/ml or LH/IGF-1 + TGF-β1 100ng/ml). Progesterone levels were measured using radioimmunoassay, and semi-quantitative RT-PCR analysis was used to assess mRNA expression of components of the TGF-β signaling pathway in granulosa cells. Our data revealed that the combination treatment of LH/IGF-1 and TGF-β1 (10 ng/ml) increased progesterone production in the granulosa cells, compared to the individual treatments themselves, suggesting a possible synergistic effect of LH and TGF-β1 in luteinization of the cells. It also appeared that LH/IGF-I treatment may upregulate most components of the TGF-β signaling pathway, indicating the possibility that TGF-β may at least in part mediate the luteinizing action of LH/IGF-I. Though we cannot draw definite

conclusions because of the limited number of viable cultures, the preliminary results are very interesting, nonetheless. Because TGF-β1 synergizes with LH/IGF-1 in increasing progesterone production and LH/IGF-1 may upregulate components of the TGF-β signaling pathway in luteinizing granulosa cells, we suggest that TGF-β signaling may play a role in mediating the LH-induced luteinization.

THE **ROLE OF TGF-β IN LUTEINIZATION IN THE PIG**

By

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DEDICATION

This work is dedicated to my family and friends - my parents, Narasimhan and Vanjula Sriperumbudur, who have encouraged me from my childhood to always follow my dreams and to do what I enjoy doing; to my wife, Priya Narayanan, who has been completely supportive of me and has endured so many sacrifices, and without whose encouragement, I would not have been able to complete my Ph.D.; to my daughter Sumitra, whose birth has brought me immense joy and the motivation to move ahead with my work; to my late brother Madhusudanan Sriperumbudur, who will forever remain a source of inspiration to me; to all my friends who believed in me. Finally, I would be remiss not to acknowledge the countless pigs that made my research possible.

BIOGRAPHY

The author grew up in Chennai (formerly Madras), India. He received his DVM degree in 2000 from the Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA. He started his Ph.D. at the North Carolina State University College of Veterinary Medicine, Raleigh in August 2000. In August 2001, he transferred to Dr. Gadsby's lab where he has worked since then.

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LITERATURE REVIEW

The first part of this section takes one through all the stages of folliculogenesis, from the primordial germ cell stage to ovulation and luteinization. The second part discusses the role of TGF-β superfamily members in folliculogenesis and luteinization.

Follicular development

Development of the follicle begins soon after conception in the female fetus. After the the primordial germ cells migrate to the gonadal ridge, they are called oogonia [1].A layer of mesenchymal cells then surround the oogonia to form oogonial clusters or syncytia [2]. Depending on the species, the oogonia undergo a predetermined number of mitotic divisions until they enter meiosis to give rise to oocytes [1]. This occurs usually around mid to late gestation, depending on the species [1]. Follicle formation starts when the mesenchymal cells surrounding the oogonia transform into flattened granulosa cells [3], to give rise to primordial follicles. In rodents, follicular assembly begins immediately after birth, while in primates and domestic animals, it occurs around mid to late gestation [4]. Meiosis marks the end of replication of female germ cells; thus, all the oocytes that a female will have in her lifetime are set at this stage [2]. After initiation of meiosis, the primary oocyte progresses through leptotene, zygotene and pachytene stages of the first prophase, before arresting in the diplotene/dictyate stage until it receives further signals to resume growth [1]. In the pig, there is evidence that the oogonial to oocyte transformation continues after birth [5]

Primordial follicle activation to resume growth is marked by the transformation of the granulosa cells from flattened into cuboidal shape and is also accompanied by granulosa cell proliferation [6]. The primordial to primary follicle transition might take a few days for some follicles, or much longer, up to a few years, for other follicles, depending on the order

in which they were formed [207]. It is evident from the vast literature available on follicle development that there is a myriad of endocrine and paracrine factors that can signal the follicle to resume development, although how they act singly or together to induce the resumption of follicular growth remains to be worked out. Among these signals are growth factors like insulin and insulin-like growth factors (IGFs), transforming growth factor (TGF)-α, members of the transforming growth factor-beta superfamily such as TGF-β, bone morphogenic proteins (BMPs) inhibin, activin, anti Müllerian hormone (AMH), and growth differentiation factors (GDFs), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), cytokines like tumor necrosis factor-α (TNF-α), and intra-ovarian steroid hormones like estrogen (E_2) and progesterone (P_4) [3, 6-10]. These signals initiate a multitude of signaling cascades to bring about changes in gene expression, which in turn cause follicle selection, and growth and differentiation of the follicular components.

As the primordial follicles resume growth, they leave the pool of resting follicles and become primary follicles; this transition is irreversible, and the follicle is now commited to growth, ending in either atresia or ovulation [3]. The mechanism of selection of certain follicles to proceed to ovulation, while the majority undergoes atresia is poorly understood. However, recent research suggests that local factors produced by the oocyte and the cells surrounding it influence the growth and development of one another (e.g. c-kit/Kit ligand) [11]. The oocyte has been shown to be the source of some of the growth factors and cytokines mentioned earlier (e.g. GDF-9, BMP-15) that may play a role in follicle growth and development [9]. The initial follicle growth is thought to be gonadotropin-independent [11, 12] because granulosa cells at this stage do not express LH [13] or FSH [14] receptors. In the hamster, however, there is evidence for the presence of FSH receptors in small primary

and secondary follicles [15]. The accumulation of mitochondria, and smooth and rough endoplasmic reticulum in the bovine oocyte at the primary follicle stage provides a clue to the increasing energy and synthetic requirements of the oocyte for growth [16]

When the follicle develops two or more layers of granulosa cells, it is called a secondary or preantral follicle [17]; at this stage, the granulosa cells proliferate, and the theca cells which arise from interstitial stromal cells, form a layer around the granulosa layer. Also at this stage, oocyte RNA synthesis is first detectable [18]. Also during this stage, the oocyte secretes a glycoprotein structure around it, called the zona pellucida. The granulosa cells form gap junctions with each other and with the oocyte via connexin proteins [19, 20]. These gap junctions enable critical communication between the oocyte and the granulosa cells. Thus, the oocyte can promote the growth and differentiation of the granulosa cells surrounding it, while the granulosa cells can signal the growth and differentiation of the oocyte [9, 21]. The cells surrounding the oocyte continue to proliferate and differentiate into two layers of theca cells, theca interna and externa, which surround the granulosa cells [22]. At this stage, the follicle may become receptive to FSH [23, 24] and LH [23, 24], as indicated by the appearance of their receptors on granulosa and theca cells [25]. Estrogen is also thought to play a role at this stage of follicle development; in the rat, estrogen receptors ERα and ERβ have been found in theca and granulosa cells respectively of primary follicles [26]. Hypophysectomized rats, and mutant mice lacking FSHR, LHR or ERα exhibit follicles arrested at the preantral stage [27]. Thus, it is clear that gonadotropins and estrogen are necessary for normal follicular growth beyond this stage, and especially the final, rapid phase of preovulatory follicle development. Environmental factors such as nutrition also influence follicle development at this stage [28]. In the pig, it is known that starting at the preantral

stage, when theca interna cells appear [29], the granulosa cells with their FSH and estradiol receptors also proliferate. Under the influence of FSH and estradiol, theca interna cells acquire LH receptors [30]. Based on rodent models, it is clear that theca cells, under LH influence, convert cholesterol to androstenedione, which then diffuses across the basement membrane to granulosa cells [31]. The granulosa cells possess P450aromatase, used in converting the androstenedione to estradiol [30]. The difference between the rodent and the pig is that the porcine theca interna cells also possess aromatase activity and hence are capable of producing estradiol [32].

The rapid increase in steroid biosynthesis is thought to result in the increase and accumulation of follicular fluid, forming the antral cavity, and consequently, the follicle is now called an antral follicle. Antral fluid production increases with increasing follicular vascularization and permeability of blood vessels [17]. The factors responsible for the formation of the antrum are not clear, but in vitro studies in rodent, porcine and bovine follicles suggest that FSH [33, 34], LH [35], activin [36], Kit ligand [37], EGF [38] may all be involved. The antral follicle has a layer of granulosa cells closely associated with the oocyte (together called the cumulus-oocyte complex or COC), with the antral fluid separating the COC from the other granulosa cells (called mural granulosa cells) along the inside of the follicular wall [22]. The oocyte acquires the ability to resume nuclear maturation, and the competency to undergo fertilization and cell cleavage [17].

As the antral follicles continue to develop, one or more of these follicles, depending on the species and breed of animal, is (are) selected to ovulate and these are called dominant follicles. Again, factors responsible for this selection step are not clearly understood, but it has been shown that dominant follicles have increased estradiol and inhibin activity [39],

which, via a negative feedback on the hypothalamo-pituitary axis, control FSH secretion (FSH levels decline to levels below what is necessary for further follicular selection) and thus inhibit the growth of subordinate follicles [40, 41]. At the same time, granulosa cells acquire LH receptors that are necessary for continued development [42]. The dominant follicle(s) with LH receptors continue their growth and development, while the subordinate follicles undergo atresia [42, 43]. Whereas FSH promotes rapid growth of GCs in the preantral follicles by the regulation and/or induction of genes involved in cell cycle control, LH promotes the exit from cell cycle and terminal differentiation of the GCs in the mature follicle, and induces genes related to ovulation and luteinization [25]. A very interesting fact is that both of these hormones act via seemingly identical cAMP signaling pathways, and through structurally related receptors, but yet produce completely different outcomes.

Ovulation

The process of mature ovarian follicle rupture to release a fertilizable oocyte is termed ovulation [44]. The main stimulus for ovulation is a surge in LH, which brings about change in expression of multiple genes, some leading to follicle rupture and oocyte release and some leading to subsequent luteinization of the follicle remnant [44]. The timing of the expression of these genes which include A Disintegrin-like And Metalloprotease domain with ThromboSpondin type I (ADAMTS) members, Metallothionein (MT)-I, epidermal growth factor (EGF)-like factors and CGMP-dependent protein kinase II, is absolutely crucial; if the luteinization genes are turned on before the ovulation genes, then the granulosa and theca cells start to luteinize before the oocyte can be released, thus resulting in a failure of ovulation ([45-50]. The genes responsible for ovulation are thought to bring about (i) locally increased flow of blood to the follicle, causing inflammatory cells like leukocytes and

macrophages to invade the follicle [51]; (ii) increased expression of proteolytic enzymes such as ADAMTS-1 and cathepsin L [44], plasminogen activator [52], the matrix metalloproteinases MMP2, MMP9 and MMP 13 [53], all of which digest the extracellular matrix of the follicle, thus weakening the follicular wall and facilitating rupture [54, 55]; and (iii) activation of adenylyl cyclase/protein kinase A pathways, which in turn cause the expression of genes involved in ovulation, like progesterone receptor, cyclooxygenase (COX)-2, the CAAT enhancer binding protein, prostaglandin endoperoxidase synthase-2 etc. [44, 53]; the targets of progesterone receptor, in turn, include plasminogen activators, matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of MMPs; TIMPs) [53, 56-60]), all of which help to degrade the extracellular matrix around the follicle, while the TIMPs are thought to protect other growing follicles from the degrading actions of the MMPs [52]. With the help of all these factors, the COC is released from the follicle.

Luteinization and the corpus luteum

The corpus luteum (CL), an endocrine gland, develops from the follicle tissue remnants after ovulation, by a process called luteinization. The CL is the source of progesterone, the steroid hormone responsible for creating a uterine environment conducive to maintaining pregnancy. If pregnancy does not occur, the CL undergoes a process of regression, at which time progesterone production declines (functional luteolysis), and ultimately the CL itself degenerates (structural luteolysis), a process mediated by prostaglandin $F_{2\alpha}$ [61]. Luteolysis brings an end to the lifespan of the CL, and initiates the process of follicular maturation, ovulation and formation of a new CL of the next cycle. In pregnant animals, the CL is maintained throughout the length of the gestation and is the major source of progesterone in several species [61]. In the pig, the CL is required for the

entire duration of pregnancy to ensure progesterone availability and pregnancy maintenance. The role of progesterone for maintenance of pregnancy is underlined by the fact that the hormone, by itself, can support gestation in ovariectomized gilts [62]. In the pig, ovulation occurs 36-48 hours after the LH surge [63]. In sows, this time of ovulation after the LH surge is extremely variable, reported anywhere from 26 to 34 hours [64]. After ovulation, the follicle undergoes hyperemic and hypertrophic changes [62, 65]; the granulosa cell layer is thrown into extensive folds around the follicular antrum; there is breakdown of the basement membrane and the theca cells are carried by the vascular and connective tissue into the core of the developing CL along these folds. The development of vascular supply proceeds in centripetal as well as lateral directions [66]. The hypertrophic changes in the granulosa cells and the proliferation of endothelial cells in the CL is phenomenally rapid, even exceeding similar changes in some of the most malignant tumors [65]. All these changes transform the fluid-filled follicle into a solid mass of cells that comprise the CL, within a few days (4-6) days after ovulation. Analyses of the cell types in the CL in the guinea pig [67], sheep [68, 69] and the cow [70] have yielded information about the composition of the CL during various stages of its development. Based on the classification described by others [61, 69, 70], it is accepted that the cell types of the CL can be broadly divided into steroidogenic and non-steroidogenic components. The steroidogenic cells can be further subdivided into small luteal cells (SLC) and large luteal cells (LLC). Endothelial cells, fibroblasts, pericytes and macrophages form the non steroidogenic component [68, 70].

It is generally believed that in the pig, the theca cells are the precursors to the SLC, whereas the granulosa cells give rise to the LLC [62, 71]. Studies in the sheep and cow indicate that the SLCs may transform into the LLCs later during the luteal phase [61].

The LLCs are characterized by their larger size (>26-31 μm in sheep) and round shape, and by the presence of abundant rough and smooth endoplasmic reticulum, numerous mitochondria, lipid droplets and secretory granules in the cytoplasm, all characteristics of a steroidogenically active cell type [70]. Although the LLCs make up about a third of the CL volume, they account for over 80 % of progesterone secretion at peak production (mid luteal phase) [68, 70]. The SLC constitute around 20 % of the CL volume; together, the LLCs and the SLCs roughly make up about 35 % of the total cell number in the CL [68, 72]. During CL development, the LLCs increase in diameter by 3 to 4 fold, though the total cell number of LLCs remains fairly constant [68]. The LLCs, but not the SLCs, also possess $PGF_{2\alpha}$ receptors, and hence appear to mediate the luteolytic actions of $PGF_{2\alpha}$ [73]

The SLCs are about a third of the size of the LLCs in domestic ruminants, and are irregularly shaped, and have a lot of smooth endoplasmic reticulum, lipid droplets, a lot less rough endoplasmic reticulum and secretory granules compared to the LLCs [68, 70]. The SLCs maintain a constant size throughout the luteal phase, but increase in cell number by up to five fold [68]. The SLCs, but not the LLCs, possess LH receptors; therefore, they are able to respond to LH and increase their otherwise low basal P4 production [74].

In the pig, it is thought that endothelial cells, usually found lining the capillary lumina, migrate from capillaries into the developing CL in apparent response to signals like VEGF produced by granulosa and theca cells [75]. But the factors that signal the other non-steroidogenic cell types to migrate into the developing CL are unknown. The numbers of endothelial cells and pericytes increase almost 4-fold during CL development, and they also contribute to around 50 % of the total cell number, and around 10-12 % of the total luteal volume [68, 70].

Luteinization is also generally associated with the loss of cytochrome P450aromatase expression in most species, and with the onset of synthesis of progesterone in significant quantities [62]. In the pig, however, aromatase activity is never completely lost and the porcine CL continues to synthesize small quantities of estradiol [76-78]. Steroid production switches from predominantly estradiol production by the follicle, to predominantly progesterone production by the CL. There is also a concomitant upregulation in the amounts of other critical components involved in progesterone synthetic pathway – low density lipoprotein (LDL) receptor, the cholesterol side chain cleavage enzyme P450scc, and 3β-HSD, another enzyme involved in progesterone synthesis, as well as the cholesterol transport protein StAR (Steroidogenic Acute Regulatory protein) [79, 80].

Progesterone production by the CL

Cholesterol, in the form of low-density and high-density lipoproteins (LDL and HDL), is used by the CL as the substrate for steroid production [81]. Of the two kinds of lipoproteins, LDL is the major substrate used by the porcine CL [80].

Steroidogenic cells take up LDL or HDL when it binds to its receptor or biding protein on the cell membrane [82]. After being transported to the cytosol, the LDL or HDL can be broken down to yield free cholesterol to be used in the next step of steroid synthesis, incorporated into the lipid bilayered cell membrane or be stored in the cytosol as lipid droplets [83]. The cholesterol to be used for steroid synthesis is transported to the outer membrane of the mitochondria with the help of sterol carrier protein 2 (SCP2)[80]. Another protein called the steroidogenic acute regulatory protein, StAR, transports the cholesterol from the outer to the inner mitochondrial membrane, and this transport step is considered to be a rate-limiting step in progesterone synthesis [84]. The enzyme P450scc, present in the

inner mitochondrial membrane, cleaves the cholesterol side chain to yield pregnenolone, which is then transported to the smooth endoplasmic reticulum; here, it is converted to progesterone by the enzyme 3β-HSD [61].

In the pig, as the CL continues to develop, progesterone production increases exponentially, peaking between days 8-12 after ovulation [63]. Progesterone levels decrease thereafter, and by day 16, they are reduced to approximately one twelfth of that measured on day 8 as CL undergoes regression [63].

Regulation of CL formation and function

The initial stimulus for luteinization is believed to be the LH surge, and LH initiates a number of downstream events that result in successful ovulation and the formation and maintenance of the CL. There have been a number of models employed to study the process of luteinization, both in-vivo and in-vitro [85]. Although these models have increased our understanding of the process of luteinization, the regulatory mechanisms inducing luteinization still remain unclear. It is clear, however, that the process begins before ovulation, at the cellular level [86]. At the genetic level, a number of genes have been identified, mostly from rodent studies [44, 45, 47, 48, 86-91], and a recent study in primates [92], as being involved in luteinization. Based on these studies, the genes can be classified into major classes such as

(i) genes involved in steroidogenesis – P450scc, StAR, LDLr, 3β-HSD, P450aromatase, 17β-HSD etc. The genes involved in progesterone synthesis such as P450scc, StAR, LDLr, and 3β-HSD and estrogen degradation such as 17β-HSD are upregulated, and the ones involved in estrogen synthesis such as P450aromatase are downregulated, as luteinization proceeds [80, 88, 93, 94].

(ii) genes involved in extra cellular matrix modeling – ADAMTS-1, Metallothionein (MT)-1, Tissue Inhibitor of Metallo Proteinase (TIMP-1) etc. ADAMTS-1, a metalloproteinase, is upregulated around ovulation and early luteinization, possibly contributing to the rupture of the follicle and tissue remodeling that transforms the follicle into the CL; TIMP-1, an inhibitor of metalloproteinases like ADAMTS-1, and MT-1, are both upregulated after ovulation and possibly help to moderate the degradative actions of metalloproteinases, mitigate the local inflammatory reaction caused by ovulation, help in healing and promote angiogenesis [45, 90]

(iii) genes involved in inflammatory processes and angiogenesis: for example, there is evidence that the expression of the enzyme cycloxygenase-2 (COX-2) is induced by the LH surge [95]. Along with lipoxygenase enzymes, COX-2, catalyzes the arachidonic acid pathway, the interruption of which has been shown to reduce the rate of ovulation and luteinization [96, 97]. Message levels of VEGF, a potent angiogenic factor, has been shown to remain constant during the luteal phase and decline only during luteal regression in the mare [98]. In the porcine CL, VEGF receptor 1 (VEGFR-1) mRNA levels increase from low levels after ovulation, to highest levels in mid to late luteal phase [99].

(iv) genes that help in maintaining CL function: during the luteal phase, insulin and IGF-I, which are present in the circulating blood, are believed to help in the luteinization process and the support of the CL [62]. In particular, increased IGF-1 expression/blood levels, IGF-type I receptor and the steroidogenic response to

IGF-1, correlates with the early stages of CL development (days 4-10), strongly suggesting a role of IGF-1 in this process in the pig [100-104].

(v) genes involved in cell cycle regulation: luteinization marks the exit of granulosa and theca cells from the cell cycle [62]. In this respect, luteinization can be defined as the terminal differentiation of the follicular cells and marks the transition from a proliferative to a differentiated state. The differentiation of the follicle into CL is also marked by the phenomenal rate of tissue growth and cellular proliferation [61]. The proliferation is evident from mitotic markers such as the proliferating cell nuclear antigen (PCNA) [62] during early CL development, but the labeling index gradually decreases and eventually is undetectable in the LLC over the lifespan of the CL [105]. Thus, it is thought that terminal differentiation occurs in the porcine CL, with increasing restriction in the proliferating capacity over time. Recent evidence [53, 106] shows that in the early CL in rodents, mitosis is initiated by members of the retinoblastoma gene family such as pRb, p107 and p130 [107] but in the late CL, these proteins are inactivated, thereby inactivating positive cell cycle regulators such as cyclins D1, D2 and E . Another factor known to be involved in regulation of the cell cycle is $p27^{Kip1}$, a member of the family of cyclin-dependent kinases. It is an inhibitor of the cell cycle progression and in knockout studies in mice, it has been shown that p27 levels decrease after the ovulatory surge of LH, thereby preventing granulosa cells from proliferating and facilitate their differentiation into luteal cells [108]

(vi) genes that regulate the transcription of other genes: gene transcription plays an important role in CL formation [62]. This process involves chromatin

modification, coactivator recruitment, and synthesis and activation of transcription factors. It is well known that the LH surge increases cAMP levels, which in turn activate the protein kinase A pathway and phosphorylation of the cAMP response binding protein (CREB) [106]. Among other genes, the ones for P450scc and StAR transcription, are activated by CREB. The transcription of several genes is also stimulated by VEGF, including its own receptors [109].

Members of the TGF-β superfamily in the ovary

Members of the TGF-β superfamily are involved in every stage of follicular development, ovulation and luteinization [110, 111]. Because of their pleiotropic roles in these processes, they are described in this section separately. These members include bone morphogenic proteins (BMPs), TGF-βs, growth differentiation factors (GDFs), anti-Mullerian hormone (AMH), activins, inhibin, etc. Most data on the role of TGF-β family members in the ovary come from studies in rodent species, but recently, a number of studies point to their role as intraovarian factors in other species such as the sheep, cat, cow, primates etc. A review of the existing literature shows that TGF-β superfamily ligands and components of their signaling pathways are expressed both by oocytes and granulosa and theca cells, and that this varies not only between species, but also with the stage of follicle, making the task of determining the precise role of each member extremely difficult. But the clear message that emerges from all of this evidence is that these proteins play key roles in all aspects of follicle development, from the development of the primordial follicle to formation of the CL. They are involved in granulosa and theca cell proliferation and atresia, steroidogenesis, gonadotropin receptor expression, oocyte maturation, ovulation and

luteinization (as reviewed in [111]). The following few sections illustrate the patterns of stage-dependent expression of these proteins during folliculogenesis.

TGF-β members in primordial and primary follicles

In rats, it has been shown that intra bursal administration of BMP-7 decreases the number of primordial follicles, while increasing the number of follicles from later stages such as primary, secondary and antral follicles [112]. In another rat study [113], BMP-4 produced similar results in a whole ovary-organ culture system; primary follicles increased while primordial follicles decreased. In the same study, it was also found that when ovaries were exposed to a neutralizing antibody to BMP-4, they were much smaller than controls, with accompanying changes such as progressive loss of oocytes and primordial follicles and also increased cellular apoptosis, eventually resulting in loss of ovarian tissue morphology.

Other TGF-β superfamily members have been shown to be expressed specifically in oocytes in the primordial and primary stage follicles. In rodents and ruminants, GDF-9, BMP-15 and BMP-6 have been identified in oocytes [114-118]. The receptors for these ligands have been identified in the pre-granulosa and granulosa cells, indicating that these cells are targets of signaling by these ligands. Mice null for *gdf-9* gene are infertile and have follicles arrested at the primary stage [119, 120]. In another study [121], GDF-9 treatment of rat follicles in vitro caused progression of follicle growth from early to late primary stage. Sheep with homozygous mutations in the *bmp-15* or *gdf-9* genes are infertile [122, 123].

It has been shown that AMH inhibits the initiation of primordial follicle growth in the mouse, and is expressed by granulosa cells of primary stage follicles onward [124]. In another study, mice with targeted deletion of the *amh* gene exhibited abnormally increased rate of primordial follicle recruitment [125]. These findings support the idea that AMH

secreted by growing primary stage follicles and all the way up to pre-ovulatory stage follicles suppress the premature recruitment of primordial follicles.

TGF-β members in primary to antral stage follicles

Several members of the TGF-β superfamily are thought to be involved in the growth of primary follicles to secondary and antral follicles. In vitro studies in rodents [121, 126-128] have shown that exposing ovaries to GDF-9 promotes primary follicle progression. In other studies, in mice null for GDF-9 [119], and in sheep with homozygous mutations in the *gdf-9* gene [129], follicle development did not proceed beyond the primary follicle stage. Sheep with homozygous mutations in the *bmp-15* gene are also completely infertile with follicle development not proceeding beyond the primordial follicle stage [129, 130]. Thus, GDF-9 and BMP-15 seem to promote transition of primary follicles to later stages. In the rat, thecal cells from the primary stage onward express BMP-4 and BMP-7 [131]. Taken together with the study mentioned above, where intrabursal administration of BMP-7 caused an increase in the transition of primordial to primary stage follicles and up, these data suggest that thecal cell-derived BMP-7 likely causes growth of granulosa cells. As the follicle reaches the antral stage, more BMP types are expressed in addition to the ones mentioned above, and the expression varies within different compartments of the follicle: it has been shown from studies in the rodent [117, 131], human [132], sheep [133] and cow [134], that BMP-6, BMP-15 and GDF-9 are expressed in the oocyte, BMP-2, BMP-5 and BMP-6 are expressed by granulosa cells, and BMP-2, BMP-3b, BMP-4 and BMP-7 are expressed by theca cells. Rat studies have shown that BMP-4, BMP-6, BMP-7, BMP-15 and GDF-9 attenuate the effects of FSH on granulosa cells from antral follicles through unknown mechanisms [112, 135, 136]. In sheep [123], GDF-9 and BMP-15, alone and in combination,

suppressed FSH-stimulated progesterone production and caused granulosa cells to proliferate. In the pig, BMP-2 reduced FSH-stimulated progesterone production by granulosa cells [137]. In the cow, BMP-4, BMP-6 and BMP-7 also suppressed basal as well as LH-induced androgen production by theca cells [138]

Activin βA and βB subunits have also been detected in primary stage follicles onward in many species including human [139, 140]. Though the exact role of activin in follicle development is still unclear, there is some evidence that it may promote granulosa cell proliferation and differentiation (as reviewed in [141]). In the antral follicle however, activin A has been shown to accelerate oocyte maturation and improve oocyte developmental competence [142, 143]. On the other hand, AMH is thought to have a negative effect on primordial follicle recruitment, as seen earlier. It is also known that AMH inhibits FSH-dependent growth of late preantral follicles in cultured mouse ovaries [144].

Message and protein from TGF-β ligands (TGF-β1, TGF-β2, TGF-β3) and receptors (type-I and type-II) have been detected first at the preantral follicle stage of several species, including the rat [145], cow [146], sheep [147] and human [148], but there is species to species as well as spatio-temporal expression variation and the role of these proteins is still unclear. In the antral follicle of the rat [149], TGF-β and in the human [150], TGF-β1 and 2 have been shown to be produced by both theca and granulosa cells. In sheep, cows and pigs [11], TGF-β1 is mainly produced by thecal cells of antral follicles. In the rat study, TGF-β (the specific ligand unknown) was shown to be expressed in theca cells and exhibited proliferative and cytodifferentiative actions on granulosa cells [151]. In another rat study, TGF-β was shown to increase FSH receptor mRNA and protein [152]. In pig studies, it was established that TGF-β is produced by thecal cells [153] and inhibits proliferation of

granulosa cells, in contrast to the rat study [154, 155]. The actions of TGF-β on granulosa cells has been further substantiated by the discovery of TGF-β receptors on porcine granulosa cells [156]. More evidence that pig granulosa cells may not produce TGF-β comes the studies of Mulheron and coworkers [157], who showed that TGF-β2 mRNA was not expressed by these cells.

TGF-β members in ovulation and luteinization

In most species, inhibin/activin subunit expression is down-regulated after ovulation and during CL formation [111]. Follicular expression of BMP-2, BMP-3b, BMP-4, BMP-6, BMP-7, BMP-15 and GDF-9 is also downregulated in the rat after ovulation [131, 135]. It is thought that these factors inhibit luteinization in the follicle until ovulation has occurred.

Mouse luteal cells express both TGF-β1 and TGF-β2 [158], and so does the rat luteal macrophage [159]. In another rat study, prolactin, a progesterone production enhancing peptide, and TGF-β had similar effects on cultured luteal cells; they both suppressed 20α-HSD, whose activity decreases progesterone [160]. In this study, it was shown that prolactin also stimulates TGF-β2 expression in luteal macrophages. In another study, human granulosa-lutein cells treated with TGF-β1 showed decreased apoptosis [161]. Taken together, it appears that TGF-β, in concert with prolactin, supports CL function by increasing progesterone and decreasing apoptosis.

Although there is evidence of expression of TGF-β ligands and its signaling pathway components during follicular development, there is very little information on the exact role of these proteins on ovulation and luteinization. The following studies suggest the possible role of TGF-β in ovulation and luteinization:

(i) When immature mice were sequentially treated with FSH for 3 consecutive days and hCG on the fourth day[162], it was shown that the receptors TβRI and TβRII were immunolocalized to both theca and granulosa cells, the oocyte stained for TβRI only while Smads were present in all cell types, with Smad 4 staining the strongest. Protein and mRNA expression analysis showed that TGF-β2, TβRI, Smad 2 and Smad 4 were upregulated, whereas that of Smad 6 was downregulated, in response to the gonadotropins (FSH and LH). This study thus shows that components of the TGF-β pathway are upregulated in the peri-ovulatory follicle in response to LH and FSH. We used a similar model for our study with pre-pubertal pigs treated sequentially with gonadotropins (P.G.600, a combination of hCG and eCG, followed by hCG 3 days later). Our findings appear in the next chapter.

(ii) In the marmoset, TGF-β1 and TβRII were co-immunolocalized with the luteinization marker 3β-HSD in granulosa and theca cells of periovulatory follicles; on the other hand, the expression of TGF-β1 and TβRII was absent in atretic follicles [163]. In another marmoset study, when large, non-luteinized follicles were cultured and induced to luteinize with treatments known to induce luteinization, such as LH, TGF-β and cAMP, increased expression of TβRII was observed, along with other expected changes such as breakdown of basement membrane of the follicle, increased expression of 3β-HSD, and decreased expression of connexin-43 (an ovarian gap junction protein that decreases during luteinization) and TβRII [164]. The consistent, increased expression of TβRII in luteinizing cells only prompted the authors to start using TβRII as a marker of luteinization in subsequent studies.

(iii) In a follow up study to the studies described in (ii) above, [165], the authors monitored the effects of Tumor Necrosis Factor (TNF)-α (which is known to have an

inhibitory effect on luteinization), on porcine granulosa cells in vitro and monitored the cells for luteinization. TNF-α downregulated TβRII along with other luteinization-inhibition parameters such as decreased progesterone receptor expression, and influenced the balance between proliferation and apoptosis.

(iv) In the chicken, TGF-β1, on its own or in combination with FSH, increased the expression of FSH receptor and LH receptor in undifferentiated granulosa cells; the increase in LH receptor sensitizes the granulosa cells to LH action by increasing P450scc and StAR expression, thus increasing the cells' ability to produce more progesterone [166].

(v) The presence of TGF-β1 and 2 in murine luteal cells [158] and rat luteal macrophages [159] has been reported. A few studies have also indicated the possible role of TGF-β in the CL: Prolactin, a luteotropic hormone, and TGF-β increased progesterone production in cultured rat luteal cells by suppressing 20α-HSD expression, and in rat macrophage cells, prolactin stimulated TGF-β expression [160]. Also, in the same study, when the luteal cells were treated with an anti-TGF-β antibody, the suppressive action of prolactin on 20α-HSD was decreased, thus indicating that the luteotropic actions of prolactin may partly be mediated by TGF-β.

(vi) More evidence about the role of TGF-β in CL formation and maintenance comes from two studies in the rhesus monkey. In the first study, when CLs at various stages of pregnancy were examined for the presence of StAR, TGF-β1 and TβRII mRNA and protein, it was found that their expression progressively increased during early pregnancy, when the CL was being formed and was functional, while in the regressing CL, their expression decreased [167]. Also, in this study, Interferon (IFN)-γ, a luteolytic cytokine decreased the expression of all three luteotropic proteins mentioned above. In a follow-up study, CL

development was induced in the rhesus monkey using eCG/hCG treatment, and the expression of TGF-β1, TβRI, TβRII and StAR was examined in CLs at various stages of development using immunohistochemistry and in-situ hybridization [168]. It was found that their expression increased to reach peak levels when the CLs were functional, and decreased when the CL regressed, and IFN-γ reduced their expression. These studies strongly suggest that TGF-β and its receptors may play a role in CL formation and maintenance of its function.

All of the above data support the view that TGF-β working via TβRI and TβRII plays a role in maintaining luteal function in these species. In the sheep however, a study shows that TGF-β1 and TGF-β2 inhibit progesterone production in cultured granulosa cells [147], suggesting that TGF-β may be suppressing luteinization in this species.

Before describing our studies to investigate the role of TGF-β in luteinization in the pig, a review of TGF-β and its signaling pathways is provided below.

TGF-β superfamily

Members of the transforming growth factor (TGF)-β superfamily are important regulators of cellular processes in many cell types [169]. The members of this superfamily are structurally and functionally similar, and influence such crucial processes as cell growth and differentiation, early embryonic development, extracellular matrix deposition, immunoregulation, cell motility and apoptosis [170]. Some of the prominent members of the superfamily are TGF-β1-3, bone morphogenic proteins (BMPs), growth and differentiation factor (GDF), Müllerian inhibiting substance (MIS), activins and inhibins.

For the purposes of describing the signal transduction mechanisms of members of the TGF-β superfamily, the prototype member, TGF-β, will be considered here, especially as it is the focus of the studies described in this thesis.

TGF-β structure and activation

TGF-β has three structurally similar isoforms, TGF-β1-3, which are encoded by three distinct genes in mammals [171]. They all have similar bioactivity in vitro [169], with TGF-β1 being the most prevalent isoform [172]. When initially synthesized, the precursor TGF-β protein is large, consisting of around 390-412 amino acids [169]. The precursor protein is comprised of three domains: 1) an N-terminal signal domain called the latency associated peptide (LAP, 249 amino acids), 2) a signal peptide (SP, 29 amino acids) and 3) a C-terminal domain (112 amino acids) [173]. The C-terminal fragment is small and forms the mature portion of the protein, which is involved in triggering intracellular signal transduction. The signal domain functions to target the precursor to the secretory pathways, while the pro-domain is thought to help in folding, dimerization and regulation of the biological activity of the C-terminal fragment. The mature TGF-β molecule usually has 7-9 cysteine residues, and exists as a homodimer formed by disulfide bonds [174].

The TGF-β precursor is thought to be activated through different mechanisms [173, 175], as described below. (i) The LAP can be cleaved by proteases, or (ii) a conformational change in the LAP can be induced by interaction with integrins or (iii) the non-covalent bonds between the LAP and the mature peptide can be broken [173]. Once the mature peptide is released, it can interact with its receptor to induce signaling.

Overview of TGF-β signaling

Members of the TGF-β superfamily use common mechanisms to signal to the nucleus [170]. They bind to membrane receptors possessing a cytoplasmic serine/threonine kinase domain. Once the ligand binds to its receptor, the ligand-receptor complex phosphorylates members of the Smad (the vertebrate homologue of Drosophila Mad (Mad =Mothers against decapentaplegic) and the related Caenorhabditis elegans gene Sma) or MAPK (Mitogen Activate Protein Kinase) protein families. These protein families can in turn translocate to the nucleus themselves, or phosphorylate downstream targets which can move to the nucleus and control target gene expression [169, 170]. There is now evidence to suggest that the Smad and the MAPK pathways intersect [169]. A simplified schematic of TGF-β signaling is given in figure 1. The components of the TGF-β signaling pathway are discussed in detail in the following paragraphs.

TGF-β receptors

Signaling by TGF−β is initiated by its binding to its receptor complex. All members of the TGF-β superfamily transduce their signals through two serine/threonine transmembrane receptors, called Type I and Type II receptors and their downstream effectors, the Smad proteins [173, 176].

To date, five type II receptors and seven type I receptors have been discovered in mammals, each binding to different ligands [177]. The type II receptor TGF−β RII (TβRII) binds only to TGF−β isoforms, with a higher affinity for TGF−β1 and TGF−β3, and a lower affinity for TGF−β2. The kinase domain of the type II receptor is the most conserved domain [177]. Of the type I receptors, ALK5, ALK1 and ALK2 can transmit TGF-β signaling, but ALK5 (also known as TβRI) is the major type I receptor and like TβRII, is a single

transmembrane serine/threonine kinase [177]. The receptors TβRI and TβRII receptors are structurally similar, although TβRI does have a shorter extracellular domain, a much shorter C-terminal tail, and a highly conserved glycine and serine (GS)-rich region immediately preceding the kinase domain. The SGSGLP amino acid sequence is considered to be a signature of TβRI. The phosphorylation of serines and threonines in the GS domain by TβRII is required for the activation of TβRI and TGF−β signaling [177].

There are also type III or accessory receptors for TGF-β signaling, and include betaglycan and endoglin [177]. The biological functions of both betaglycan and endoglin are not clear. It was thought that betaglycan may function to present the TGF−βs, especially TGF−β2, to TβRII [178]. More recently, it has been shown that betaglycan is responsible for endocardial cell transformation (Brown et al., 1999). In addition, some cell lines can secrete soluble betaglycan, corresponding to the extracellular domain of betaglycan, which can inhibit the binding of TGF−β to the TβRI-TβRII complex [179]. Supporting evidence comes from studies showing that this soluble betaglycan can inhibit tumorigenesis and metastasis of MDA-MB-231 human breast cancer cells by reducing the activity of TGF−β1 and TGF−β2 secreted by these cells [180].

Mechanisms of transforming growth factor-β receptor activation

All Type I and Type II TGF−β receptors exist as homodimers in the absence of ligand [177, 181]. For TGF−β receptors, TGF−β binds poorly TβRI to alone [182]; TGF−β must first bind to the TβRII homodimer, which then recruits TβRI to form a heterotetrameric complex [183, 184]. TβRII is constitutively hyperphosphorylated at several serine residues [183, 185]. The formation of TGF−β receptor heterotetrameric complex results in the

phosphorylation of serine and threonine residues in the TβRI GS domain by TβRII [183]. The phosphorylated TβRI then becomes activated, and signals are transduced to cytoplasmic targets [186, 187]. Since TGF−β2 has only low affinity for TβRII, the binding of TGF−β2 to Type III receptors can facilitate the binding of TGF−β2 to TβRII, which, in turn, can recruit TβRI to form an active receptor complex [188, 189]. However, TGF−β2 can also directly bind to TGF−β receptor complexes in the absence of RIII in some cell types [190-192].

Structural requirements for transforming growth factor-β receptor activation

Specific domains in TβRI and TβRII are important for TGF−β signaling [170]. The extracellular domains of both receptors are required for ligand binding [193, 194]. The cytoplasmic domains of both TβRI and TβRII are required for the dimerization of the receptors, which is essential for TGF−β signaling [187, 194, 195]. In addition, the kinase domain of TβRII is required for TβRII to phosphorylate TβRI, while the kinase domain of TβRI is required for TβRI to phosphorylate downstream targets [183, 194, 196, 197]. It is also possible that TβRII can directly phosphorylate other downstream targets. The glycine and serine-rich GS domain is essential for the activation of TβRI by TβRII [183, 196]

Some of the serine and threonine residues in the Type I and Type II receptors are important for TGF−β receptor signaling as well [177]. TβRII primarily autophosphorylates threonines in vitro, but serines in vivo [183]. TβRII autophosphorylates at least three serine residues: Ser409 and Ser416 in the protein kinase domain and Ser213 in the juxtamembrane domain [187]. Phosphorylation of Ser213 and Ser409 is essential for TβRII kinase signaling, while phosphorylation of Ser416 has been shown to inhibit TβRII function [187].

Phosphorylation of TβRII has also been observed on Ser551 and Ser553 of the C-terminus and Ser225, Ser228, and Ser229 in the juxtamembrane domain [183, 198]. Since C-terminal tail deletion did not affect TβRII actions [194, 197], the phosphorylation of the C-terminal tail is not thought to play a critical role in TβRII signaling. However, it is still not known whether the phosphorylation of the serine residues in the juxtamembrane domain plays a role in TβRII signaling. In vitro, TβRII can autophosphorylate on Tyr259 in the kinase domain, Tyr336 in subdomain V, and Try424 in subdomain VIII [199]. The role of TβRII tyrosine autophosphorylation in TGF−β signaling is not clear. TβRII also phosphorylates TβRI at four sites in the GS domain (Thr186, Ser187, Ser189, and Ser191) and at Ser165 in the juxtamembrane domain. The phosphorylation of Ser165 was thought to be the direct effect of TβRII, and may modulate the signaling specificity of TGF−β receptors [198].

Smads

Smads can be divided into three groups based upon their structure and function. The first group, referred to as receptor-activated Smads (RSmads), includes Smads 1-3, 5, and 8. The RSmads specific for TGF-β signaling are Smad2 and Smad3. The second group consists of the single member Smad 4, and is referred to as common-partner Smad (Co-Smad). The last group is referred to as the inhibitory Smads, including Smads 6 and 7 [170].

Structure of Smads

Smads share extensive sequence homology in two distinct regions, an N-terminal domain (called MH1 for MAD-homology 1) and a C-terminal domain (called MH2), while the proline-rich linker regions are poorly conserved. The MH1 domain is highly conserved among RSmads and Co-Smad, but not in the inhibitory Smads. The RSmads, but not the Co-

Smad, also have a characteristic serine-rich SXS motif in their MH2 domain. The phosphorylation of the two serine residues in the SXS motif of the MH2 domain activates the RSmad [200, 201].

Smad signaling

The general mechanisms mediating TGF−β receptor activation of Smads are as follows: binding of the ligand to the TGF−β receptor complex induces the phosphorylation of RSmads. Generally, Smads 1, 5, and 8 can be phosphorylated by BMPs, while TGF−β and activin can phosphorylate Smad2 and Smad3. It is known that TGF−β can also phosphorylate Smad1 in Hs578T human breast cancer cells and IEC 4-1 cells [169]. In addition, it has been shown that TGF−β can phosphorylate Smad5 in human hematopoietic cells [202].

The active TβRI phosphorylates the RSmad at the serine-rich sequence SXS in the C-terminal tail of the MH2 domain, thus activating it. Smad4 does not have this SXS motif, and hence is not phosphorylated by TβRI [169]. Structural analysis suggests that the inability of Smad4 to bind to TβRI is due to a lack of a basic, positively-charged surface patch at the MH2 domain of Smad4 [203]. The phosphorylation of RSmad causes conformational changes and results in its dissociation from TβR1, as well as increases its affinity for the CoSmad, and thus, facilitating the assembly of the RSmad-CoSmad complex [201]. There is evidence that the RSmad-CoSmad complex is a heterotrimer, made of two RSmads and the CoSmad [204]. It is thought that after activation and trimerization, the Smad complex can translocate to the nucleus. Once in the nucleus, the Smad complex either can directly bind to DNA or can associate with other transcription factors to induce target gene transcription [170, 192, 197]. Moreover, Smad signaling can be modulated by many Smad-associated proteins and by other signaling pathways, including the Ras/MAPK pathways [199]. In the

linker region of RSmads, there are several consensus MAPK phosphorylation sites that are responsible for crosstalk between the Smad and MAPK pathways [169, 205].The inhibitory Smads, 6 and 7, appear to block the activation of RSmads by TGF−β receptors [183].

The function of the Smad anchor for receptor activation

After ligand binding, RSmads can transiently associate with Type I receptors with the help of the auxiliary protein, Smad anchor for receptor activation (SARA) [196]. The SARA protein contains a FYVE (a double zinc finger) domain, which can bind to phosphatidylinositol-3-phosphate in the lipid layer endosomal membrane. There is also a peptide sequence of SARA that can interact with the RSmads [206]. TβRI-mediated phosphorylation of SARA-bound RSmad is more efficient in SARA-rich endosomes ([201]. The role of SARA as a regulator of TGF-β signaling has been demonstrated in studies where a mutation in SARA in Mv1Lu or HepG2 cell cultures caused disruption of TGF-β signaling [206]

Summary of literature review

The control of follicle development involves the interaction of multiple hormone and peptide signaling cascades, and the differential expression of various genes, the details of which are just beginning to be understood but are still unclear. The literature review illustrates the complexity of these processes and lists the majority of factors known to date that are thought to be involved in the control of folliculogenesis, through various stages of follicle development, ovulation and luteinization. Of particular interest in our laboratory is the control of the follicular to luteal transition.

Members of the TGF-β superfamily have been shown to be involved at all stages of follicle development. There is evidence that the prototypical member of this superfamily, TGF-β, is involved in pre-antral to antral follicle development and ovulation. But there are only a few studies that have examined the role of this cytokine in the post-ovulatory follicle. Even among these studies, data for the pig is limited. Preliminary studies from our laboratory indicated that TGF-β2 precursor as being one of the genes that was upregulated very early in response to the pre-ovulatory surge of LH. This observation led us to believe that TGF-β may be involved in the process of luteinization.

To test our hypothesis, the following research was performed. In the first study, the message and protein expression of TGF-β and components of its signaling pathway was examined in peri-ovulatory porcine follicles at various time points after hCG administration. In the second study, the expression of TGF-β and components of its signaling pathway were examined in granulosa cells induced to luteinize in vitro with LH/IGF-1. The effects of TGF-β alone or in combination with LH/IGF-1 on luteinization were also examined in by monitoring progesterone production and mRNA expression of key enzymes and proteins involved in progesterone production.

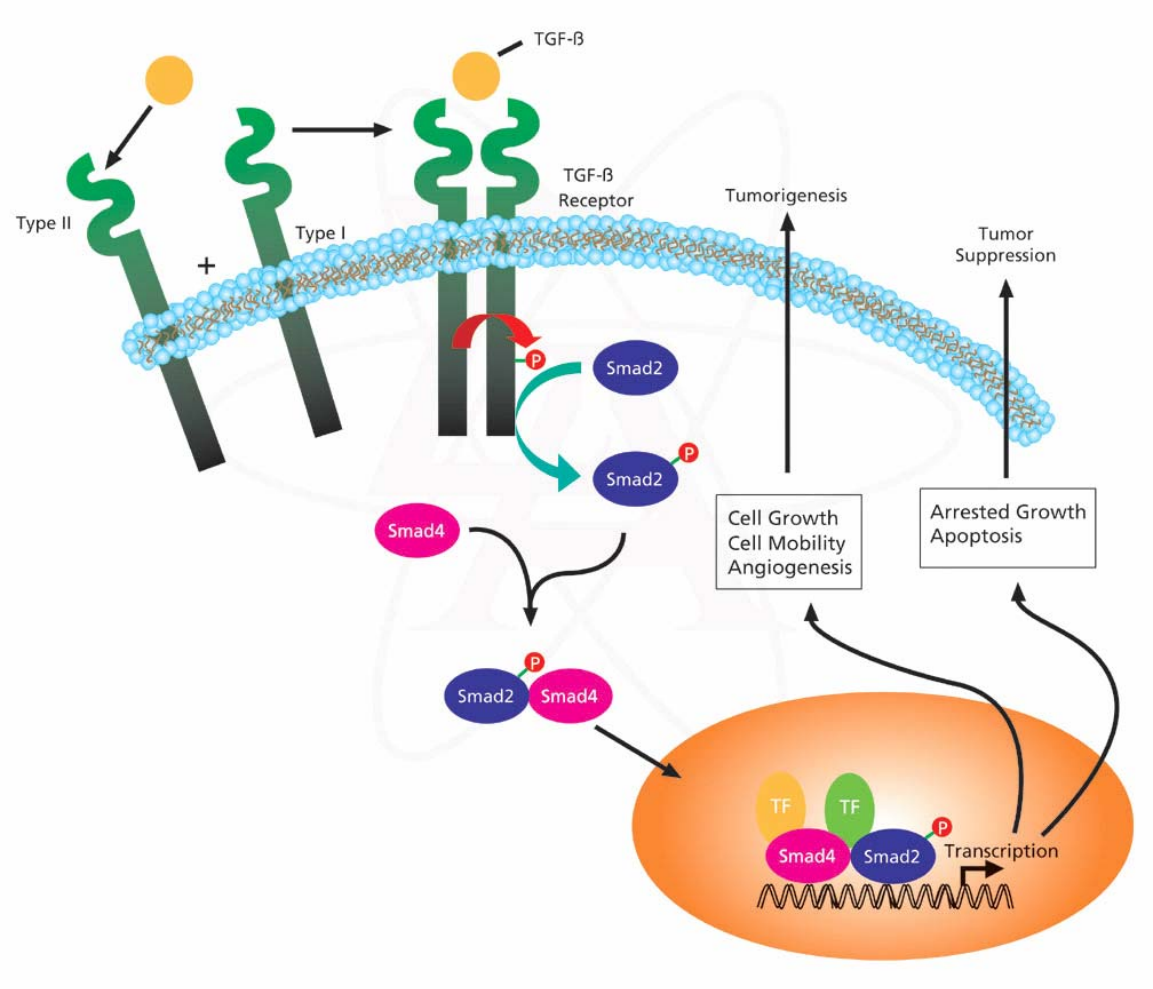

Figure 1. An overview of TGF-β signaling (Courtesy: Sigma-Aldrich)

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TGF-β ligands, receptors and Smad expression in peri-ovulatory porcine follicles

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ABSTRACT

It is well known that the LH-surge causes ovulation of follicles and initiates luteinization. There is also evidence from our own studies as well from the literature suggesting that TGF-β may also play a role in luteinization. In this study we proposed to examine the expression of TGF-β ligands (i.e. TGF-β1, 2 and 3) and components of its signaling pathway (the TGF-β receptors, TβRI and II, the Smad proteins, Smad 2, 3, 4, 6 and 7, and the Smad Anchor for Receptor Activation, SARA) in porcine follicles induced to luteinize in vivo with hCG treatment. Pre-pubertal pigs were injected with PG-600 to induce ovarian follicular development and hCG 3 days later to induce ovulation. Ovulation consistently occurred between 24 and 48h post-hCG in all of the treated animals. Ovaries were collected surgically at 0, 1, 12, 24, 48 and 96 hours following hCG treatment, and used for RNA and protein extraction, and for immunohistochemistry. Expression of mRNA for the components of the TGF-β signaling pathway was measured using semi-quantitative RT-PCR. Our data revealed that hCG caused up-regulation of mRNA expression for TGF-β3 at 12h, and TβRII at 96h, (versus 0h control) while there was not a significant change in expression versus the 0h control in these and the other components of the TGF-β signaling pathway at all other times. However, protein expression of TGF-β1, TGF-β2, TβRI and TβRII in these samples was not significantly different compared to the 0 h control. Using immunohistochemistry, we also localized TGF-β1 expression to granulosa cells (GC), TGF-β2 to theca cells (TC) in the pre-ovulatory follicle, TGF-β1 and TGF-β2 expression in luteal cells (LC) in the post-ovulatory follicle and TβRI and TβRII expression in GC, TC and LC of the pre- and post-ovulatory follicle. Since hCG, whose actions simulate those of naturally-occurring LH, up-regulates some components of the TGF-β signaling pathway, and

induces luteinization, we suggest that TGF-β signaling may play a role in mediating the LH-surge induced luteinization that occurs during the normal cycle.

INTRODUCTION

The processes of ovulation and luteinization are initiated by a surge of luteinizing hormone (LH). The LH surge sets off a cascade of events, including rapid changes in the expression of genes within the follicle that are involved in these processes, such as those for pro-inflammatory cytokines like interleukin-1B and its receptor, interleukin-1BR [1], prostaglandins (i.e. endoperoxidase synthase-2) [2], angiogenic factors such as vascular endothelial growth factor [3], metalloproteinases that degrade follicular connective tissue [4, 5], and components of the steroidogenic pathway facilitating increased progesterone production, such as steroidogenic acute regulatory protein [6]. In response to the LH surge following ovulation, the steroidogenic cells of the follicle, the granulosa and theca cells, undergo luteinization and differentiate into the large and small (respectively) luteal cells of the corpus luteum (CL) [7] through a process of terminal differentiation [8, 9]. Grossly, ovulation is marked by rupture of the follicle and release of the oocyte (ovulation), and this is followed by a transient hyperemia, (i.e., in which the follicle fills with blood), and a gradual replacement of blood with the developing CL tissue via extensive hypertrophy of the granulosa and theca cells, and remodeling of extracellular matrix [9-12]. In addition, new blood vessels grow into the ruptured follicle (neovascularization), which involves endothelial cell invasion and growth, and invasion by macrophages [9]. In rodents, the LH surge also causes granulosa and theca cells to immediately exit from the cell cycle [8, 9]. This might not be entirely true in the pig, where there is evidence of continued mitosis into the luteal phase, with gradual loss of proliferation [13]. During the post-ovulatory period, follicular steroidogenesis switches from producing predominantly estrogen to producing predominantly

progesterone, and this change is accomplished by changes in expression of enzymes that regulate steroidogenesis. These changes include decreased protein and message expression of aromatase, and increased protein and message expression of P450scc, 3β-HSD and StAR [14-17].

Although the LH surge is known to cause ovulation and luteinization, complete details of the pathways and mechanisms by which these processes occur remain largely unidentified. From preliminary studies carried out in our laboratory using cDNA array analysis (Sriperumbudur and Gadsby, Appendix 1), we had detected elevated transforming growth factor β-2 (TGF-β2) precursor mRNA expression in porcine follicles treated with hCG (in vivo), suggesting a possible role for TGF-β in ovulation or luteinization in the pig.

Although there is considerable evidence for the expression and actions of TGF-β and members of the TGF-β superfamily in follicle development, much less is known about the possible roles for TGF-β in follicle ovulation and luteinization, or in the corpus luteum itself. Clues to the possible involvement of TGF-β in luteinization come from but a handful of studies:

(i) FSH treatment followed by hCG caused the increase in mRNA and protein for TGF-β2, TβRI, the stimulatory Smads, Smad 2 and Smad 4 and decrease in mRNA and protein for the inhibitory Smad 6 in immature mice [18], suggesting that components of the TGF-β pathway are upregulated in the peri-ovulatory follicle in response to FSH and LH.

(ii) In a marmoset study, TβRII immunolocalization was consistently observed only in luteinizing cells, and the expression increased as luteinization progressed [19], prompting the authors to start using TβRII as a marker of luteinization in subsequent studies.

(iii) In a follow up study to the studies described in (ii) above, [20], luteolytic TNF-α downregulated TβRII and decreased progesterone receptor expression in porcine granulosa cells in vitro.

(iv) In the chicken, TGF-β1, on its own or in combination with FSH, increased the expression of FSH receptor and LH receptor in undifferentiated granulosa cells; the increase in LH receptor sensitized the granulosa cells to LH action leading to increased P450scc and StAR expression, thus increasing the cells' ability to produce more progesterone [21].

(v) Prolactin, a luteotropic hormone, and TGF-β increased progesterone production in cultured rat luteal cells by suppressing 20α-HSD expression, and in rat macrophage cells, prolactin stimulated TGF-β expression [22]. Also, in the same study, when the luteal cells were treated with an anti-TGF-β antibody, the suppressive action of prolactin on 20α-HSD was decreased, thus indicating that the luteotropic actions of prolactin may partly be mediated by TGF-β.

(vi) More evidence about the role of TGF-β in CL formation and maintenance comes from two studies in the rhesus monkey. In the first study, when CLs at various stages of pregnancy were examined for the presence of StAR, TGF-β1 and TβRII mRNA and protein, it was found that their expression progressively increased during early pregnancy, when the CL was being formed and was functional, while in the regressing CL, their expression decreased [23]. In a follow-up study, CL development was induced in the rhesus monkey using eCG/hCG treatment, and the expression of StAR , TGF-β1, TβRI and TβRII was examined in CLs at various stages of development using immunohistochemistry and in-situ hybridization [24]. It was found that their expression increased to reach peak levels when the CLs were functional, and decreased when the CL regressed. Treating the animals with the luteolytic cytokines IFN-γ and TNF-α also reduced their expression. These studies strongly suggest that TGF-β and its receptors may play a role in CL formation and maintenance of its function.

All of the above data support the view that TGF-β working via TβRI and TβRII plays a role in maintaining luteal function in these species.

Thus, based on the aforementioned literature indicating a possible role for TGF-β in luteinization and the corpus luteum, we proposed to examine the role of TGF-β in ovulation and luteinization in the pig by characterizing the temporal and spatial patterns of expression of TGF-β ligands, receptors and Smad proteins involved in the TGF-β signaling pathway in porcine follicles in response to hCG (given to mimic the naturally occurring pre-ovulatory surge of LH), in vivo.

METHODS AND MATERIALS

Reagents

All buffers and reagents, unless otherwise specified, were purchased from Sigma Chemical Co (St. Louis, MO) or Fisher Scientific (Clackamas, OR).

Animals and Tissue collection

All animals were housed at the NCSU Swine Educational Unit, Lake Wheeler Rd, Raleigh, NC. All animal care, handling and treatments, as well as surgery and post-surgical recovery, were approved according by the NCSU Institutional Animal Care and Use Committee. 150-160 day old prepubertal (Landrace/Yorkshire X Duroc/Hampshire hybrid) gilts were treated with a half dose of PG-600 (200 I.U. eCG plus 100 I.U. hCG, i.m.; Intervet, UK) per animal to promote follicle growth and maturation. Seventy two hours later, the animals were given 300 IU hCG (i.m.; Sigma) to induce ovulation and luteinization. Ovaries were surgically harvested at different times (n=7 animals for 0, 1, 24 and 48h time points; n=5 animals for the 12h time point; n=4 for the 96h time point) after hCG administration. The study was performed over a two

month period in the Spring. On any given day, surgeries were performed on animals at random and different time points post-hCG, with a maximum of four surgeries per day. Vascular, peri-ovulatory follicles were dissected from the ovaries and were flash-frozen in liquid nitrogen for subsequent mRNA and protein analysis. Follicles to be used for immunohistochemistry (n=2 follicles from different animals per time point) were fixed in 10% neutral-buffered formalin for 48h, then stored in 70% ethanol until further processing. Two follicles per time point for 0h, 12h, 24h and 48h were also used for separation into enriched granulosa and theca cell compartments by using a modification of the procedure described in [25]. Briefly, follicles were bisected, the follicle lining grasped at its base with sterile forceps and removed from the follicle by everting; granulosa cells were scraped for 2 min using a sterile inoculation loop into 250 μl sterile M-199 (Cambrex Bioscience, Baltimore, MD). The granulosa cell suspension was labeled GC and flash frozen; the remaining follicle lining, consisting mostly of theca cells, was labeled TC and flash frozen.

Semi-quantitative analysis of mRNA expression patterns of TGF-β ligands, receptors and Smad proteins:

Total RNA was extracted from follicle tissue and the separated GC and TC fractions with TRI-Reagent® (MRC, Inc., Cincinnati, OH) and the RNAqueous kit (Ambion, Inc., Austin, TX) and 100 μg of total RNA was subsequently DNAse-treated with Turbo-DNA free enzyme (Ambion) to minimize genomic DNA contamination. Total RNA was reverse transcribed to generate cDNA using Omniscript RT kit (Qiagen, Valencia, CA). To ensure that there was no genomic contamination, -RT controls for all samples were also included in the reverse transcription reaction. To determine the linear phase of amplification, a pooled RNA sample across all samples was reverse transcribed and PCR was performed with each primer pair. The

pool was constructed by using 3 μg of total RNA from each sample and reverse transcribing to obtain the pool cDNA. Amplification by PCR was performed on the pool cDNA using porcine-specific primers (Sigma-Genosys, Woodlands, TX) for TGF-β ligands, receptors and Smad proteins (See Table 1 for primer sequences and amplicon sizes) for a total of 40 cycles, and 2 tubes per sample were removed every 5 cycles beginning with cycle 20. Amplicons were visualized on ethidium bromide-stained 2 % agarose gels and band densities were determined using the Alpha Imager gel documentation system (Alpha Innotech, San Leandro, CA). The densities were then graphed to determine the number of cycles which corresponded to the linear phase of amplification for each primer pair. Nucleotide sequencing of all PCR products was performed at the Gene Technologies Labarotories, Texas A & M University (College Station, TX) and compared with existing porcine sequences from the GenBank database. All gene products were confirmed as authentic based on published nucleotide sequences. Thus, for individual samples, PCR was performed under the following conditions: all samples were placed in AB biosystem's 9600 thermocycler for 3 min at 94ºC (initial denaturation), denaturation at 94ºC for 30 seconds. The standard PCR program included annealing at 50ºC for 30 seconds, extension at 72ºC for 60 seconds for appropriate numbers of cycles (specified in Table 1) for each primer, and a final extension at 72ºC for 5 minutes. Semi-quantitative analysis was performed on PCR products obtained during the linear phase of PCR amplification, after normalizing levels of gene expression to expression levels of Histone (H)2A, as a housekeeping gene. Absence of amplicons bands for the –RT samples confirmed the absence of genomic DNA contamination (data not shown). The intra-assay CV for PCR assays ranged from 2.6% to 13.7%. To prove that our animal model resulted in luteinization, we also examined several other genes (e.g. Cyclin D2, Aromatase, and StAR), which are known to be responsive to LH.

Western blotting

Protein expression of some of the components of the TGF-β signaling pathway in hCG treated follicles was determined using Western blot analysis. Briefly, follicles were homogenized in RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology Inc., Santa Cruz, CA; 30 mM TrisHCl, pH 7.4, 0.1% SDS, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 2 mM EDTA with sodium orthovanadate, PMSF and protease inhibitor cocktail) and centrifuged to remove cell debris. The protein concentration of the lysates was determined using micro BCA protein assay (Pierce, Rockford, IL). Thirty μg of total protein (per sample) was separated on mini 4-12 % Bis-Tris gels (Invitrogen, Carlsbad, CA) at 200V for 30 minutes at room temperature under reducing conditions. Following electrophoresis, the gel-resolved proteins were (semi-dry) transferred onto PVDF membranes using the XCell II blot module (Invitrogen, Carlsbad, CA) at 30V for 1h at room temperature. Nonspecific binding sites were then blocked in tris-buffered saline with 0.2% Tween in PBS with 5% milk and probed overnight at 4ºC with primary antibody (1:200 for TGF-β pathway components, 1:5000 for β-actin). Primary antibodies used were TGF-β1 (sc-146), TGF-β2 (sc-90), TβRI (sc-398), TβRII (sc-400), Smad3 (sc-8332), p-Smad3 (sc-11769) (all polyclonal, SantaCruz) and the housekeeping protein β-actin (ab8224, polyclonal, Abcam, Amherst, MA). Specificity of the antibodies was verified using blocking peptides (Santa Cruz); the primary antibody was incubated with its blocking peptide (1:5) for 1 h at room temperature immediately before probing the membrane. Porcine specific antibodies were not available for TGF-β3 and Smads 2, 4, 6 and 7. Following incubation with the primary antibody, the membrane was washed with wash solution (0.2% Tween in PBS) and incubated at room temperature for 1 h with the secondary antibody. Appropriate (anti-rabbit for sc-146, sc-90, sc-398, sc-400, sc-8332, sc-11769, Santa Cruz, 1:20,000 dilution and anti-mouse for ab8224,

Sigma, 1:20,000 dilution) secondary antibodies were used to bind to the primary antibody. The membranes were washed four times for 10 minutes each in was solution. Protein bands were visualized by chemiluminescence using Luminol reagent (sc-2048, SantaCruz) in the Lumi Imager system (Roche) and quantified after normalizing to β-actin, using the Alpha Imager gel documentation system (Alpha Innotech, San Leandro, CA).

Immunohistochemistry:

Fixed tissue stored in 70% EtOH was embedded in paraffin and cut into 5 μm sections. Sections on slides were deparaffinized in xylene and hydrated in alcohol series in the following sequence:

1. Incubation of sections in Xylene: 2 to 3 changes, 5 min. each.
2. 100% absolute ethanol: 2 changes, 3 min. each
3. 95% ethanol: 2 changes, 3 min. each
4. 80% ethanol: 3 min.
5. 50% ethanol: 3 min.
6. Rinsing with distilled water: 2 changes, 3 min. each.

To unmask the antigen, slides were immersed in 10 mM sodium citrate buffer for 20 minutes at 95ºC. Sections were cooled in the sodium citrate buffer for 10 min at RT, then rinsed in dI H_2O. Endogenous peroxidase was neutralized by incubating sections in 3% H_2O_2 for 10 min at RT. Sections were rinsed in running water for 2min, then placed in PBS briefly. Sections were blocked in 5% goat serum (Biogenex, San Ramon, CA) for 20 min at RT and then incubated with primary antibody (Santa Cruz, conc. 1:50 for TGF-β1 and TGF-β2, and 1:500 for TβRI and TβRII) overnight at 4ºC. For negative controls, the primary antibody was replaced

with rabbit IgG at the same dilution. Sections were washed with PBS (3X5min) at RT and then incubated with biotinylated secondary Ab (Biogenex, San Ramon, CA) for 2 h at RT at a concentration of 1:40. After washing with PBS (3X5min) at RT, sections were labeled with avidin HRP (Biogenex) for 20 min at RT at a concentration of 1:40. They were then washed with PBS, 3X5min, and incubated with diaminobenzidine until color developed (30 sec-2min). After rinsing with dI H_2O, nuclei were counterstained with hematoxylin blue mix for 15 sec-1min. Sections were finally washed in dI H_2O for 2min, quickly dehydrated in alcohol series and xylene and coverslipped with mounting medium. Photographs of the sections were taken using the Leica DM5000 compound fluorescent/DIC microscope and Simple PCI software (Compix Inc., Sewickley, PA).

Statistical Analysis

All data for expression of mRNA were expressed as the ratio of mRNA of interest to H2A mRNA. Expression of protein was expressed as the ratio of protein of interest to β-actin. The normalized data for message and protein expression are presented as least squares means ± SEM (except for data for the separated GC and TC fractions, which are expressed as raw means ± SEM) for each time point examined. Comparison of message and protein levels was performed using one-way analysis of variance using the GLM procedure, followed by Scheffe multiple comparison test (SAS, Cary, NC). Differences were considered statistically significant at the 95 % confidence level ($p<0.05$). For the protein data, statistical analyses showed that there was a significant replicate effect for TβRII and Smad3, most likely due to one outlying replicate. Statistical analyses were not performed on data from the separated fractions.

RESULTS

mRNA expression in whole follicles:

Luteinization-associated genes:

The levels of mRNA expression for the cell cycle regulatory protein, Cyclin D2, and two critical regulators of steroidogenesis, P450aromatase and StAR, are shown in Figure 1. No significant changes in Cyclin D2 were observed. Aromatase expression showed some dramatic changes in response to hCG, and declined significantly to reach lowest levels at 48h and 96h post-hCG. Finally, StAR expression showed a biphasic response, with highest levels at 0h, significantly declining at 1h, 12h and 24h post-hCG, increasing again at 48h and 96h, post-hCG, as luteinization proceeded.

TGF-β ligands:

As shown in the top panel of Figure 2, there were no statistically significant changes in the expression of either TGF-β1 or TGF-β2 mRNAs over time post-hCG treatment. However, TGF-β3 mRNA expression did show significant increase at 12h post-hCG, compared with all other time points (Fig.2, top panel)

TGF-β receptors:

The levels of expression of mRNA for the TGF-β receptors in porcine follicles over time post-hCG are shown in the bottom panel of Figure 2. There were no statistically significant changes in TGF-β receptor type I (TβRI) expression, but the type II receptor (TβRII) did display significantly higher expression at 96h post-hCG; at this time point, the follicles are post-ovulatory and luteinization is progressing (Figure 2, bottom panel).

Smads and regulatory protein SARA:

There were no significant changes in the expression of mRNAs for the stimulatory Smads 2, 3, 4 and the Smad anchor for receptor activation, SARA (Fig. 3, top panel), or the inhibitory Smads 6, 7 (Fig. 3, bottom panel) in response to hCG. A representative gel demonstrating Smad3 amplification products is shown in figure 4.

mRNA expression in separated granulosa and theca cells:

TGF-β ligands:

As shown in the top and middle panels of Figure 5, mRNA expression of TGF-β1 and TGF-β2 appeared to be greater in the TC fraction compared to the GC fraction. No definitive patterns of expression were apparent in the mRNA expression pattern of TGF-β3, as seen in the bottom panel of Figure 5.

TGF-β receptors:

The levels of expression of mRNA for the type I TGF-β receptor in the separated fractions over time post-hCG is shown in the top panel of Figure 6. There was no apparent pattern of expression for TβRI mRNA in the two cell types. TβRII levels, shown in the bottom panel of Figure 6, appeared to increase progressively with time post-hCG in both GC and TC fractions starting with the 12h time point. Also, TβRII levels appear to be greater in the TC fraction compared to the GC fraction.

Protein expression:

TGF-β ligands:

The levels of protein expression from Western Blot analyses of TGF-β1 and TGF-β2 (Fig. 7, top panel), are shown. No significant differences (p value = 0.3855 for TGF-β1, 0.0859 for TGF-β2) in the protein levels of either TGF-β ligand were observed.

TGF-β receptors:

The protein expression levels of TβRI and TβRII (Fig. 7, bottom panel) also did not vary significantly over time after hCG treatment, but there was a numerical increase in expression of TβRII at 96h, again when the follicles are undergoing luteinization (p value = 0.1751). The numerical increase in TβRI was not significant because of a high p value (0.6746)

Smad3, phospho-Smad3:

There were no significant changes in the expression of protein for Smad 3 and phosphorylated Smad3, although pSmad3 appeared to increase at 12h post-hCG (Fig. 8) (p value = 0.2228 for Smad3, 0.1551 for pSmad3).

Representative Western blots for all the proteins examined are shown in figure 9. For all proteins examined, pre-incubation of each antibody with its respective blocking peptide eliminated the antibody signal (data not shown).

Immunohistochemistry:

In pre-ovulatory follicles (0h post-hCG), TGF-β1 was most highly expressed in granulosa cells (GC) while TGF-β2 was localized mainly to theca cells (TC); (Fig. 10). TβRI and TβRII were shown to be expressed to the same degree in both granulosa and theca cells in pre-ovulatory follicles (0h post-hCG); (Fig. 10). At both 48h and 96h post-hCG (i.e., post-ovulation), it was hard to distinguish the theca cells from the granulosa cells, and thus the cells were labeled as LC (luteinizing cells). Both TGF-β1 and TGF-β2 continue to show expression in luteinizing cells at 48h and 96h post-hCG. Similarly, both TβRI and TβRII were immunolocalized to luteinizing cells of post-ovulatory follicles (48h and 96h post-hCG; Figure 10).

DISCUSSION

In this study, we explored the expression patterns of TGF-β ligands, TGF-β receptors and post-receptor signaling mediators (Smads and SARA) in porcine follicles which had been stimulated to develop in immature (pre-pubertal) gilts with PG-600 and then induced to ovulate and luteinize with hCG. PG-600 has been shown by others [26] to reliably produce a normal (i.e. not superovulated) number of ovulated follicles which go on to luteinize and develop into corpora lutea (Table 2). The data presented in Table 2 also indicated that hCG-treated follicles consistently ovulated between 24h and 48h, which is close to the approximately 40h time frame for ovulation post LH-surge in cycling gilts [27]. In addition, the expression patterns of luteinization associated-genes presented in Fig. 1 confirmed that in this model, porcine follicles did indeed undergo ovulation and luteinization. We examined these genes to provide a positive control for the other genes being examined in this study. These data support the view that hCG-treated follicles obtained from our model underwent critical changes in steroidogenic enzymes and other proteins consistent with those previously described in the pig, rodent and cow [14, 15, 28, 29]. These data also reflect the critical change in steroidogenic potential of peri-ovulatory follicles from predominantly estrogen secreting structures before the LH-surge, to the predominantly progesterone secreting luteinizing follicle/developing corpus luteum. The lack of change in the mRNA expression patterns of CyclinD2 is in agreement that in the pig, unlike in rodents, follicular granulosa and theca cells continue to undergo cell division following the LH-surge [30].

In this study, we described some important changes in TGF-β ligand and TGF-β receptor expression in porcine follicles following hCG treatment. Specifically, we showed a significant elevation of TGF-β3 mRNA expression at 12h post-hCG. Furthermore, we showed a significant

increase in the type II TGF-β receptor mRNA at 96h post-hCG. These data suggest that hCG-induced ovulation and luteinization is associated with increased expression of TGF-β3 and its receptor TβRII, leading us to speculate that TGF-β may play a role in mediating the luteinizing actions of LH (or hCG). In addition, our observation that levels of TGF-β3 mRNA increased significantly following hCG exposure in the follicle, might suggest a specific role for this isoform early in the peri-ovulatory period in the pig. However, monitoring protein expression of TβRII did not show the change reflected in its mRNA expression. Since a porcine-specific antibody for TGF-β3 was not available, its protein expression pattern could not be examined; should one become available in the near future, it can be used to verify if the protein expression pattern follows the mRNA expression pattern, only after which the possible role of TGF-β3 in ovulation and luteinization will be clearer. However, the protein expression for the active form of Smad3, the phosphorylated Smad3, showed a tendency to increase at 12h post-hCG, suggesting that the TGF-β pathway may be active at this time point.

To our knowledge, this is the first report of variable expression of TGF-β3 mRNA in the luteinizing follicle in the pig; only one other study reports expression of this isoform in the cow in the pre-antral and antral stage follicle [31].

The expression patterns of mRNA for the TGF-β ligands and receptors from the separated GC and TC compartments did not show any changes over time post-hCG. But there appeared to be differences in the levels of expression of TGF-β1, TGF-β2 and TβRII in the two compartments; all three components seemed to be expressed greater in the TC compartment. Though not significant, these data provide a clue that TGF-β pathway components may be differentially expressed in these compartments. A possible explanation as to why changes in

mRNA expression seen in whole follicles are not reflected in the separated components can be due to the small sample number.

With regard to the possible functions of TGF-β in the periovulatory porcine follicle, it was surprising that we could not detect any statistical differences in the expression of any of the Smads or SARA, at either mRNA or protein levels. Perhaps the most sensitive test of Smad activation is protein phosphorylation, and yet even here we could not observe any differences post-hCG that might haven been anticipated with TGF-β actions, especially at 48h and 96 h. It is possible that despite an increase in ligand (TGF-β3) and receptor (TβRII) mRNA levels, it is neither reflected in the protein levels of TβRII, nor the activation of the downstream components of the TGF-β pathway. There is the possibility of post-transcriptional regulation where the increase in TβRII mRNA levels does translate into increased protein levels. Future studies may be designed to test this hypothesis.

Finally, we observed some interesting patterns in the immunolocalization of TGF-β ligands and the receptors in peri-ovulatory porcine follicles. It was very interesting to observe the contrasting expression patterns of TGF-β1 and TGF-β2 in the steroidogenic components of the follicle. TGF-β1 was strongly expressed by granulosa cells but not theca cells of the pre-ovulatory follicle. In contrast, TGF-β2 expression was just the opposite with strong expression in the theca cells but not in the granulosa cells. After ovulation, the distinction between granulosa and theca cells could not be made and the cells were labeled “luteinizing cells” (LC). At 48 and 96h, the LCs expressed both ligands. The receptors TβRI and II were expressed in both theca and granulosa, as well as in luteinizing cells in the post-ovulatory follicle. It was interesting to note that these observations were different from the mRNA expression observed in the separated theca and granulosa compartments, where TGF-β1 and TGF-β2 mRNA expression appeared to

be higher in the theca compartment. Previous studies have shown that theca cells from immature pigs are the source of TGF-β in culture [32, 33], but these studies do not mention the specific TGF-β isoform examined. Another study showed that porcine granulosa cells do not express TGF-β2 mRNA [34]. This observation may explain the low levels of TGF-β2 protein expression in granulosa cells in our immunohistochemical studies. Thus, our findings demonstrate for the first time differential protein expression of TGF-β ligands within the peri-ovulatory porcine follicle.

In summary, we have presented evidence that TGF-β3 and its receptor TβRII are upregulated at the mRNA level particularly in post-ovulatory, luteinizing porcine follicles. This is not reflected in the protein levels of TβRII, but there was a tendency for pSmad3 protein levels to increase at 12h post-hCG. This leads us to suggest that TGF-β3 and its receptor, TβRII are regulated at the post-transcriptional level, and hCG may not stimulate the TGF-β pathway. On the other hand, TGF-β has been shown to increase the expression of LH receptors in immature granulosa cells in the chicken [21]and promote their differentiation and increase progesterone production. Thus, TGF-β may promote luteinization indirectly by enhancing the effects of LH. In addition, based on the immunohistochemical localization of TGF-β ligands and their receptors, we suggest that TGF-β may have both autocrine as well as paracrine actions within peri-ovulatory follicles, that may account its proposed luteinizing actions.

FIGURE LEGENDS

Figure 1. mRNA expression of some LH-responsive genes in hCG-treated follicles at 0 h (control), 1 h, 12 h, 24 h, 48 h and 96 h post-hCG

Figure 2. mRNA expression of TGF-β ligands (top panel) and receptors (bottom panel).

Figure 3. mRNA expression of stimulatory Smads and SARA (top panel) and inhibitory Smads (bottom panel).

For figures 1-3, quantification of mRNA was performed using semi-quantitative PCR, with band densities expressed as the least squares mean ± SEM (n = 4 - 7 animals per time) of ratios relative to H2A. Data were analyzed using PROC GLM ANOVA, followed by Scheffe Multiple Comparison Test using SAS. Means for each gene with asterisks indicate significant differences ($p<0.05$; versus 0h post-hCG) over time post-hCG.

Figure 4. Representative ethidium bromide-stained agarose gel of Smad3 amplification product from hCG-treated follicles. Lane 1, base pair marker (100 bp ladder); Lane 2 and 3, 0h sample; Lane 4, 1h sample; Lane 5, 12h sample; Lane 6, 24h sample; Lane 7, 48h sample; Lane 8, 96h sample; Lane 9, -RT control. The expected amplicon size for Smad3 is 541 bp.

Figures 5 and 6. mRNA expression of TGF-β1 (Fig.5, top panel), TGF-β2 (Fig. 5, middle panel) and TGF-β3 (Fig.5, bottom panel), TβRI (Fig.6, top panel) and TβRII (Fig. 7, bottom panel) in separated granulosa and theca cells from hCG treated follicles at 0 h (control), 12 h, 24 h, and 48

h post-hCG. Quantification of mRNA was performed using semi-quantitative PCR, with band densities expressed as the mean ± SEM (n = 2 animals per time) of ratios relative to H2A.

Figures 7 and 8. Protein expression of TGF-β ligands (Fig.7, top panel), receptors (Fig. 7, bottom panel), Smad 3 and phosphorylated Smad 3 (Fig. 8) in hCG-treated follicles at 0 h (control), 1 h, 12 h, 24 h, 48 h and 96 h post-hCG. Quantification of protein was performed using Western blot analyses, with band densities expressed as the least squares mean ± SEM (n = 4 - 7 animals per time) of ratios relative to β-actin. Data were analyzed using PROC GLM ANOVA, followed by Scheffe Multiple Comparison Test using SAS. None of the means were significantly (based on $p<0.05$; versus 0h post-hCG) different over time post- hCG.

Figure 9. Representative Western blots from hCG-treated follicles. Top panel, TGF-β ligands TGF-β1 and TGF-β2. Middle panel, TGF-β receptors TβRI and TβRII. Bottom panel, Smad3 and pSmad3. The expected size of each product is given for each protein adjacent to its blot.

Figure 10. Immunohistochemical localization of TGF-β1 (1^{st} row), TGF-β2 (2^{nd} row), TβRI (3^{rd} row) and TβRII (4^{th} row) in representative sections from hCG treated follicles at 0 h (control), 48 h and 96 h post-hCG. For the negative control (last row), the primary antibody was substituted with normal goat serum. The antrum (A), granulosa cells (GC), theca cells (TC) and luteinizing cells (LC) are labeled. Magnification 200 x.

Table 1. Primers used for semi-q RT-PCR

Gene	Primer sequence (5'-3')	Extension cycles	Product size (bp)
TGF-β1	F:CTGTGTCTGTCCACCATTCATTTG R:CAACTTTGCTATGTCTGTCTCCCC	33	496
TGF-β2	F:CATCTCCTGCTAATGTTGTTGCC R:CGGTTCTAAATCCTGGGACACG	30	324
TGF-β3	F:CAGACTGGAAGATGCTTGGAGC R:CTTAGAGGGGTCAGGAGAGGAAAC	33	477
TβRI	F:GCAGTATGTCTTCTAGCCTGCCT R:TTCGCAGGCAGCTAACTGTATCC	33	524
TβRII	F:CTGAGGCAGAACACATCTGAGC R:TCGCTGTGCAGGTGTGCAATGC	30	305
Smad2	F:GAAGAGAAGTGGTGTGAGAAAGCAG R:AATACTGGAGGCAAAACTGGTGTC	30	428
Smad3	F:TGGAGGAGGTGGAGAAATCAGAAC R:CACACTCGCTTGCTCACTGTAATC	33	541
Smad4	F:CCTGAGTATTGGTGTTCCATTGC R:TGATGCTCTGCCTTGGGTAATC	33	598
Smad6	F:CGTCAGCATCTTCTACGACCTACC R:TATCTGGGGAGGGTTCTTCCG	33	500
Smad7	F:TACTGGGAGGAGAAGACGAGAGTG R:TGGCTGACTTGATGAAGATGGG	33	241
SARA	F:TCCACATTTCTTCTCTCCCACTTC R:CAGTTTCTGAGTCACAGCCACAAC	33	230
H2A	F:AGGACGACTAGCCATGGACGTGTG R:CCACCACCAGCAATTGTAGCCTTG	28	210
Cyclin-D2	F:CCCCTACCCCCTTCAAAAACTC R:TGTTGTTGACACTTCTGCTCCTCAC	33	351
Aromatase	F:CATCACCAAGCACCTGGACAAG R:TAGTTCCTCTTCAACCTGGGGG	33	472
StAR	F: GAGTGGTTCCATTCTTCCAGAGC R: CAACTATCCCTTCCATCCGTCTC	25	564

Table 2. Summary table of ovarian characteristics at ovariectomy across all animals

	Time post-hCG					
	0h	1h	12h	24h	48h	96h
Number of unovulated follicles	29	41	31	27	19	0
Number of Corpora Lutea	0	0	0	0	45	18

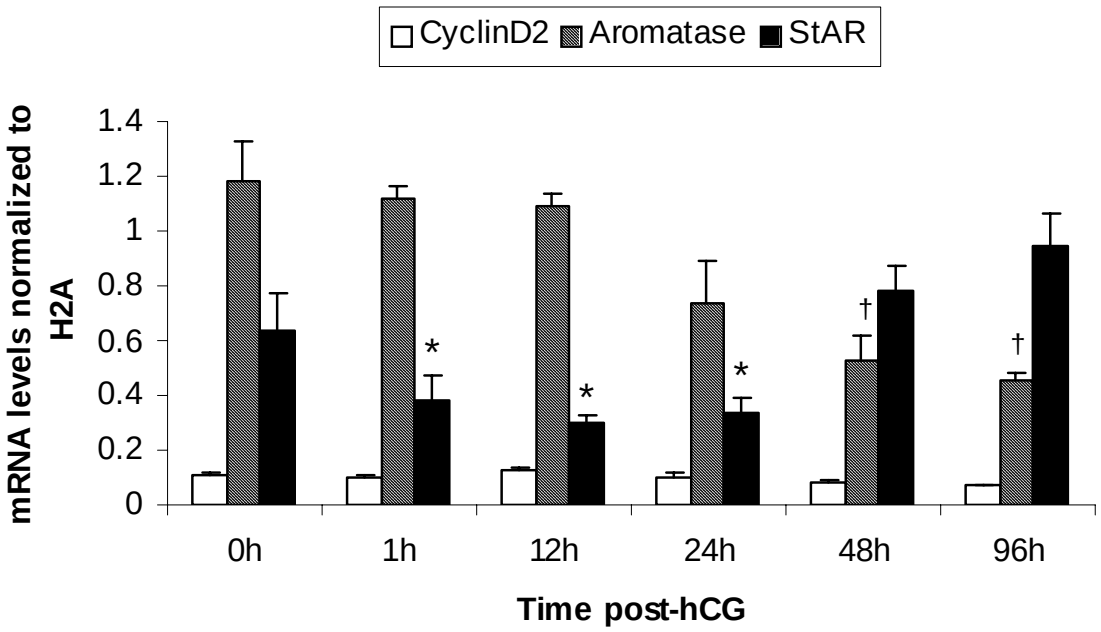

Figure 1. mRNA expression patterns of some LH-responsive genes in hCG-treated follicles

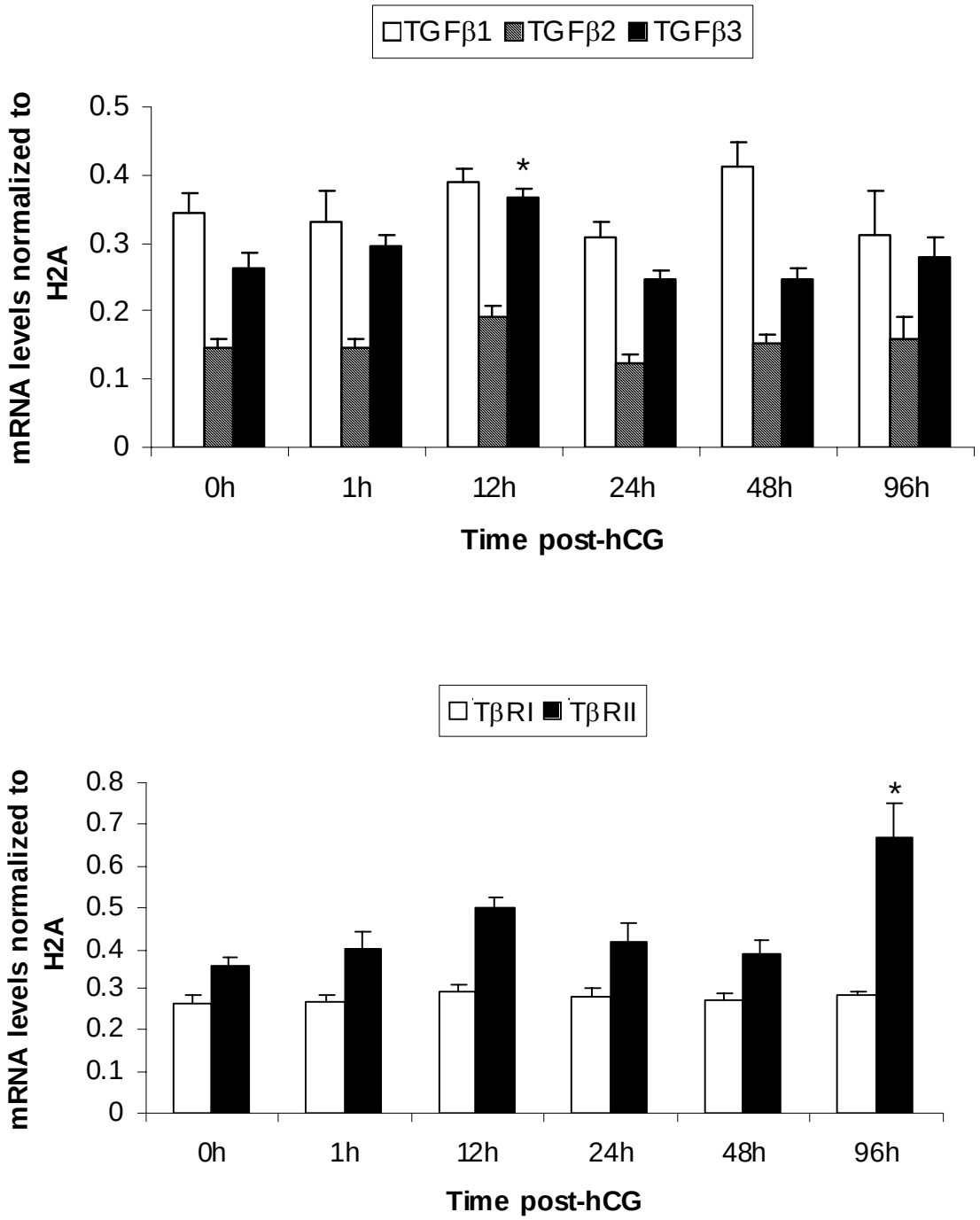

Figure 2. mRNA expression patterns of TGF-β ligands and receptors in hCG-treated follicles

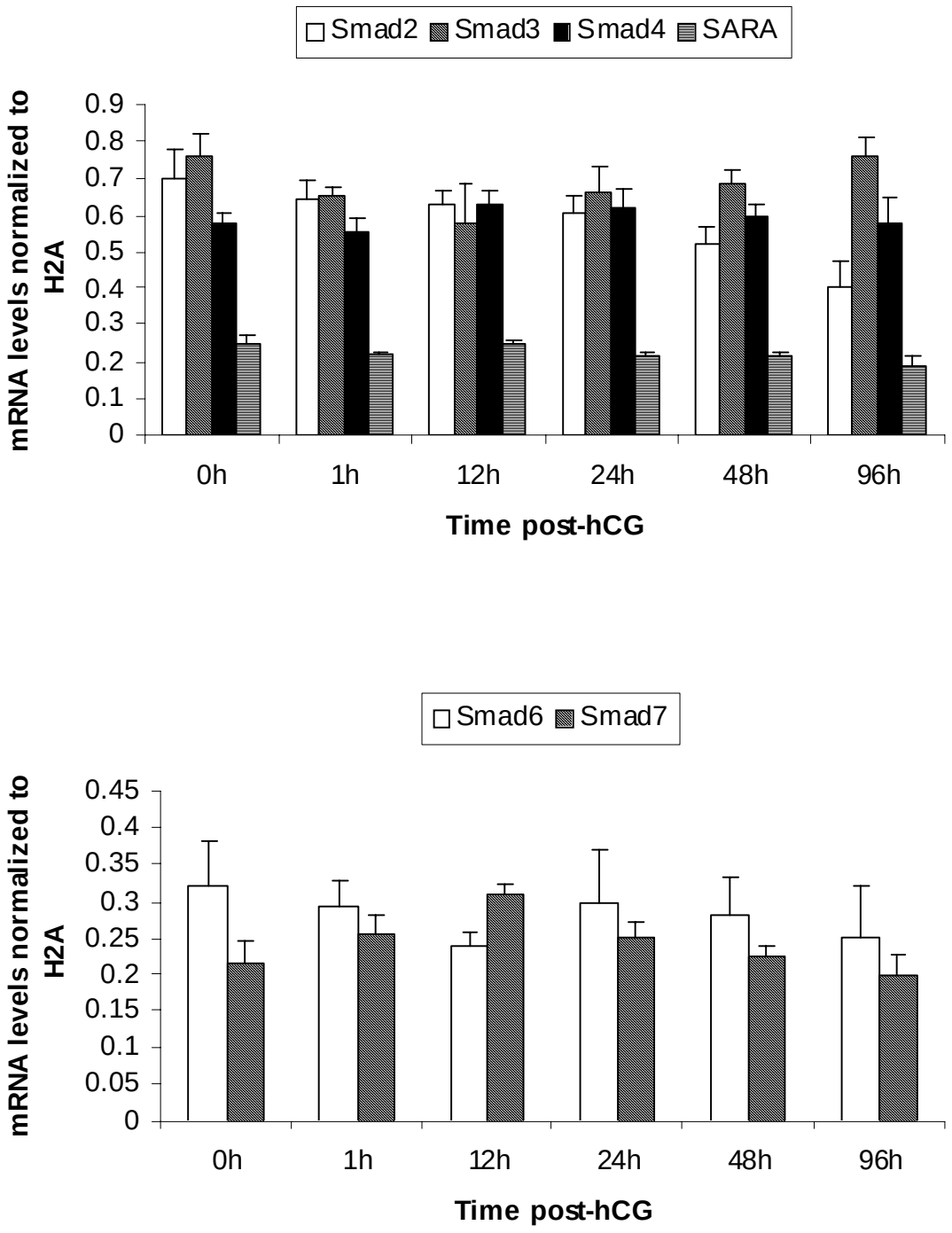

Figure 3. mRNA expression patterns of stimulatory Smads, SARA and inhibitory Smads in hCG-treated follicles

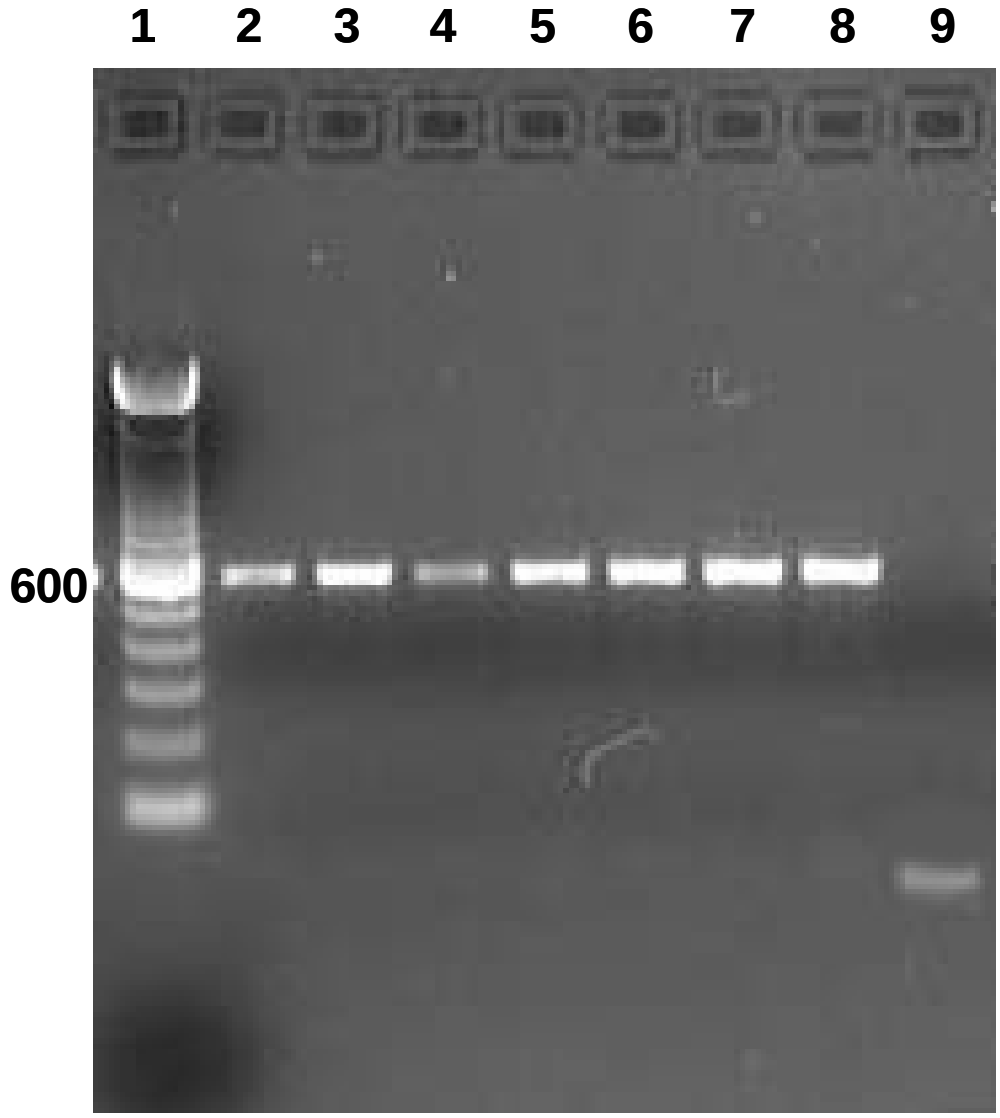

Figure 4. Example of an ethidium bromide-stained agarose gel of Smad3 amplification products from hCG-treated follicles

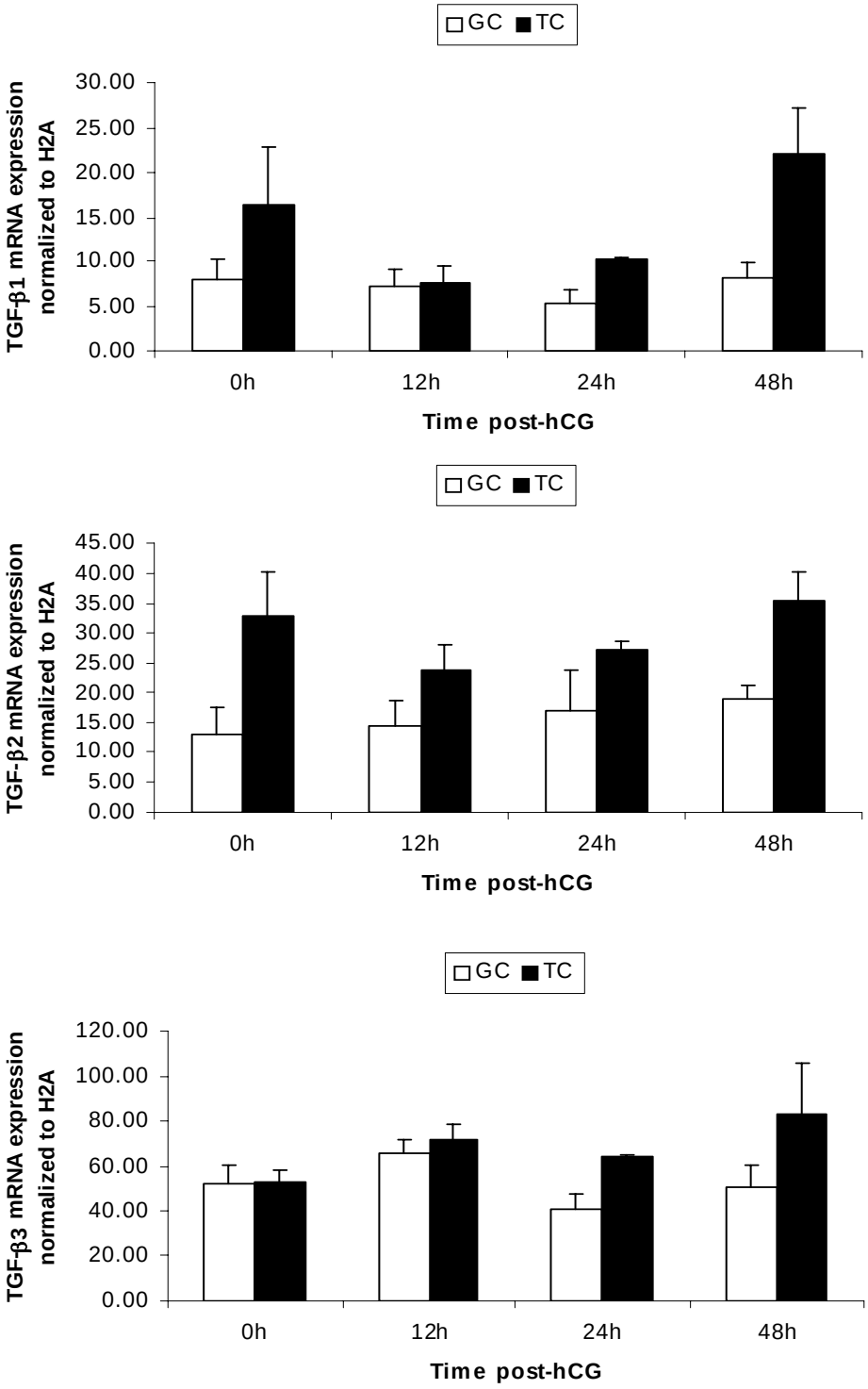

Figure 5. mRNA expression patterns of TGF-β ligands in granulosa and theca compartments separated from hCG-treated follicles

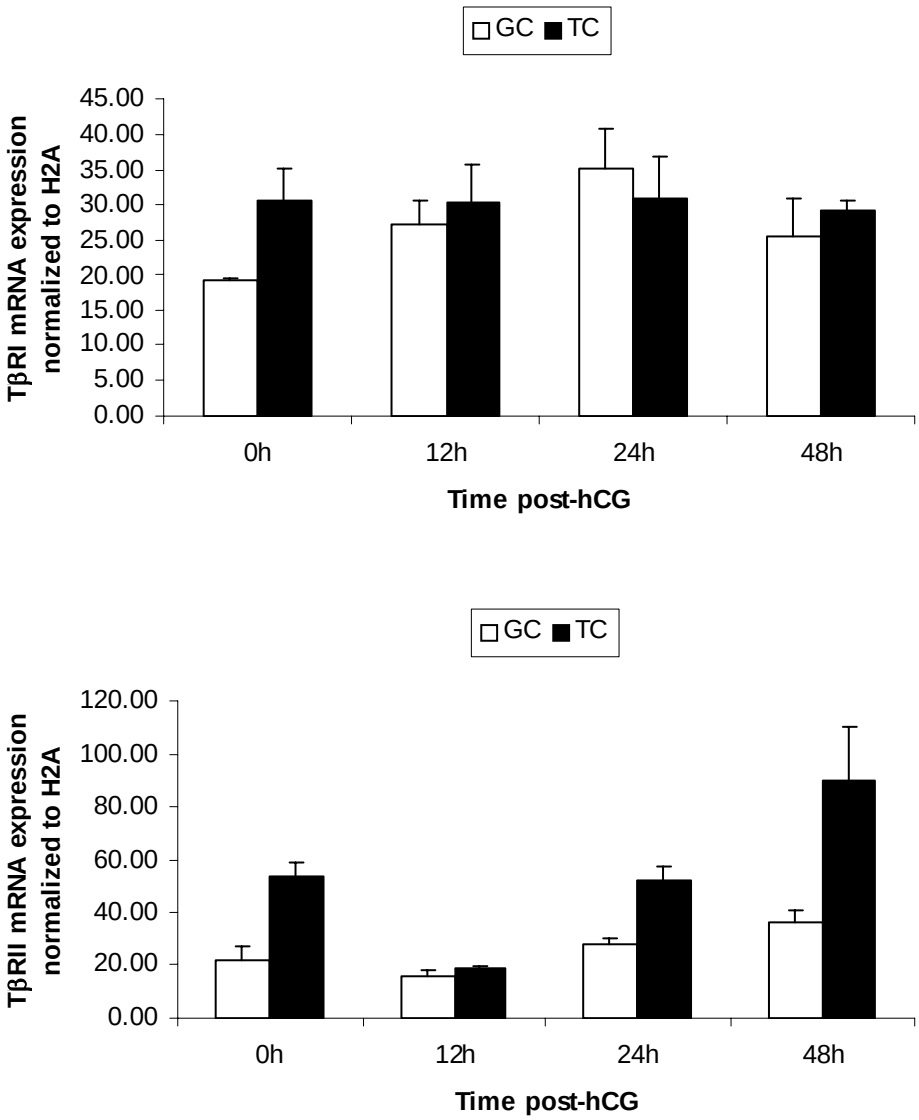

Figure 6. mRNA expression patterns of TGF-β receptors in granulosa and theca compartments separated from hCG-treated follicles

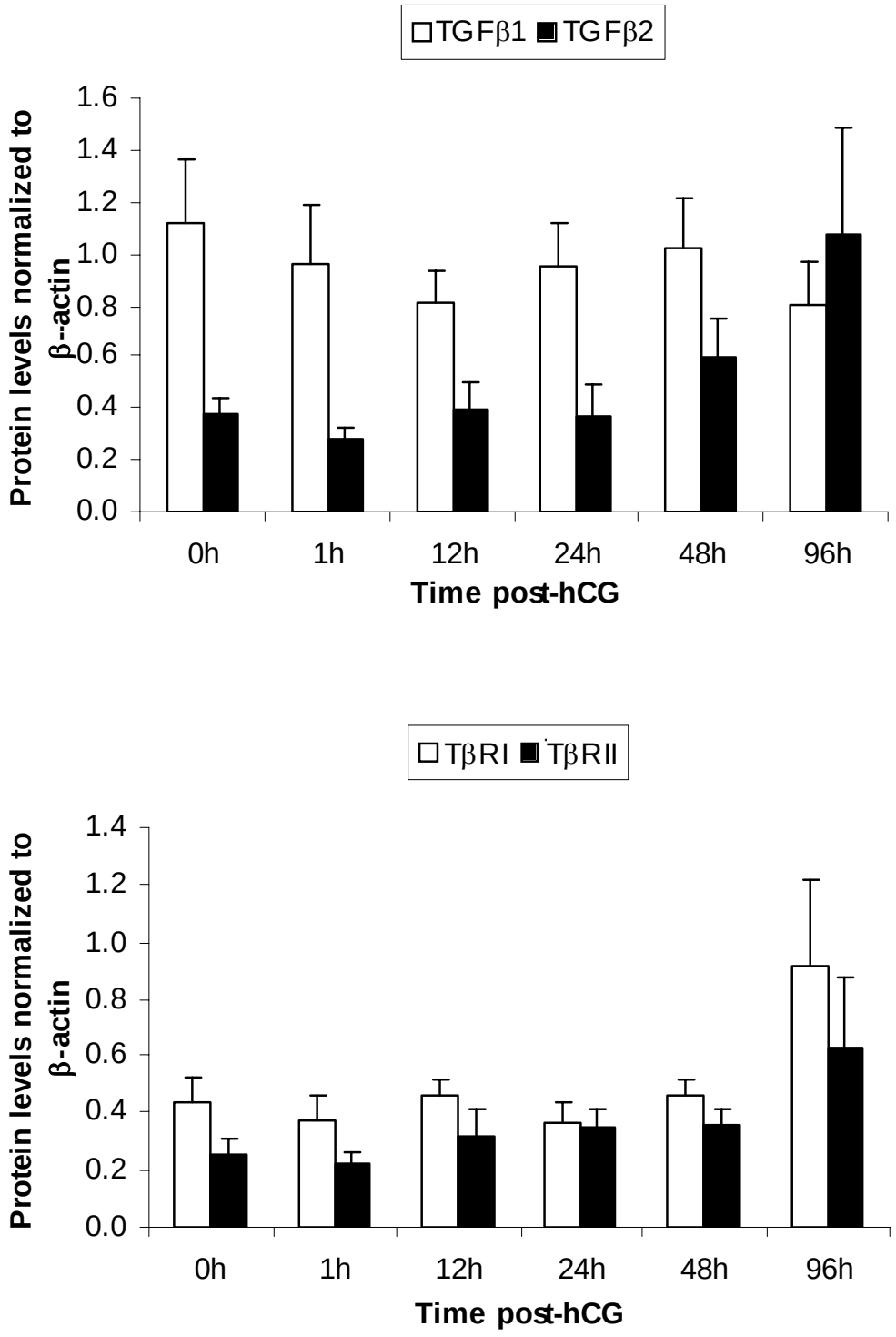

Figure 7. Protein expression of TGF-β ligands and receptors in hCG-treated follicles

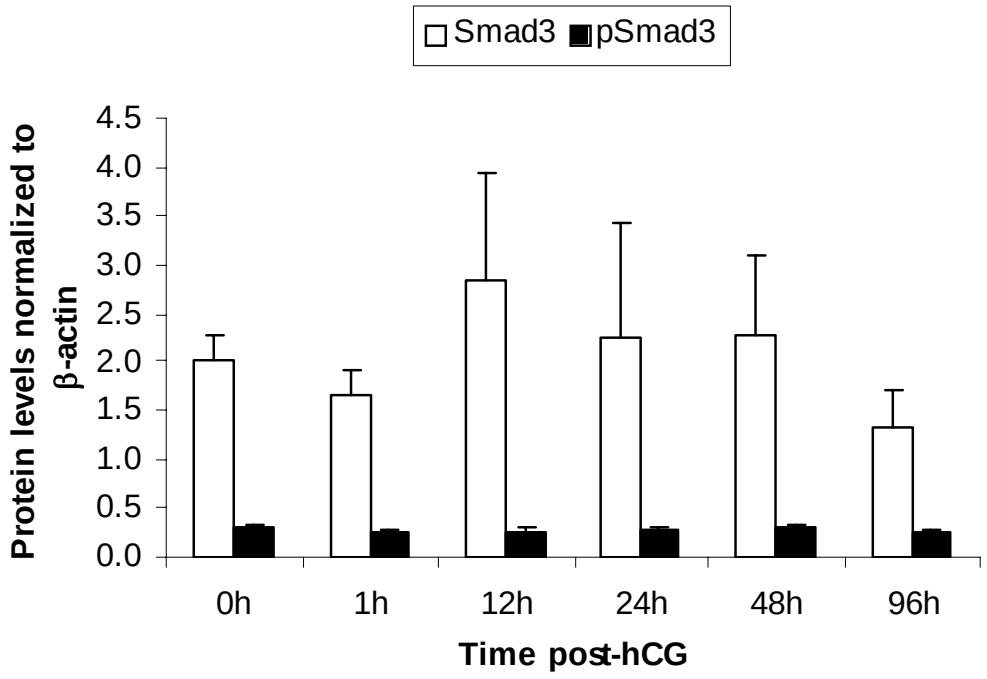

Figure 8. Protein expression of Smad3 and p-Smad3 in hCG-treated follicles

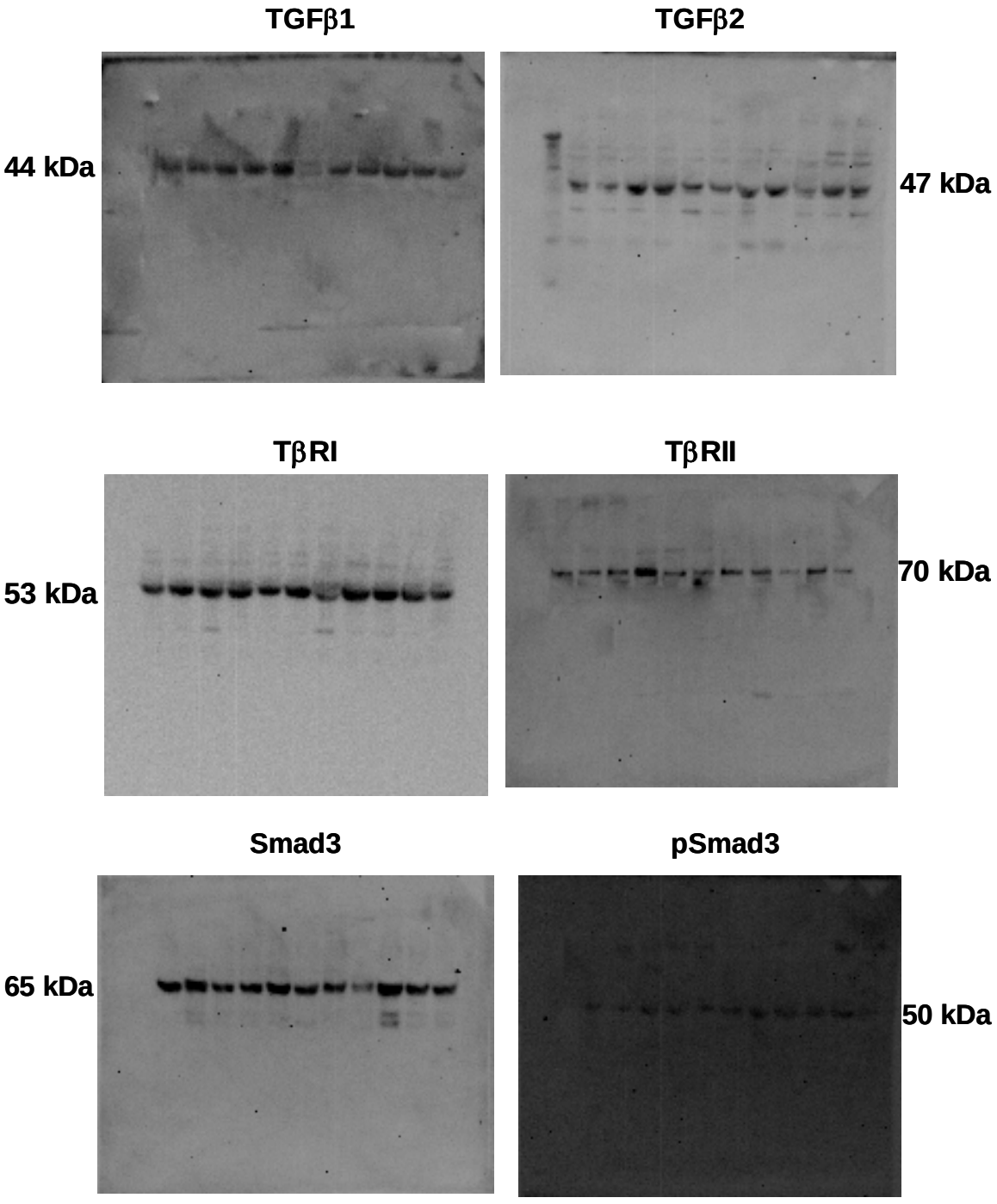

Figure 9. Representative Western blots of TGF-β ligands, receptors, Smad3 and pSmad3

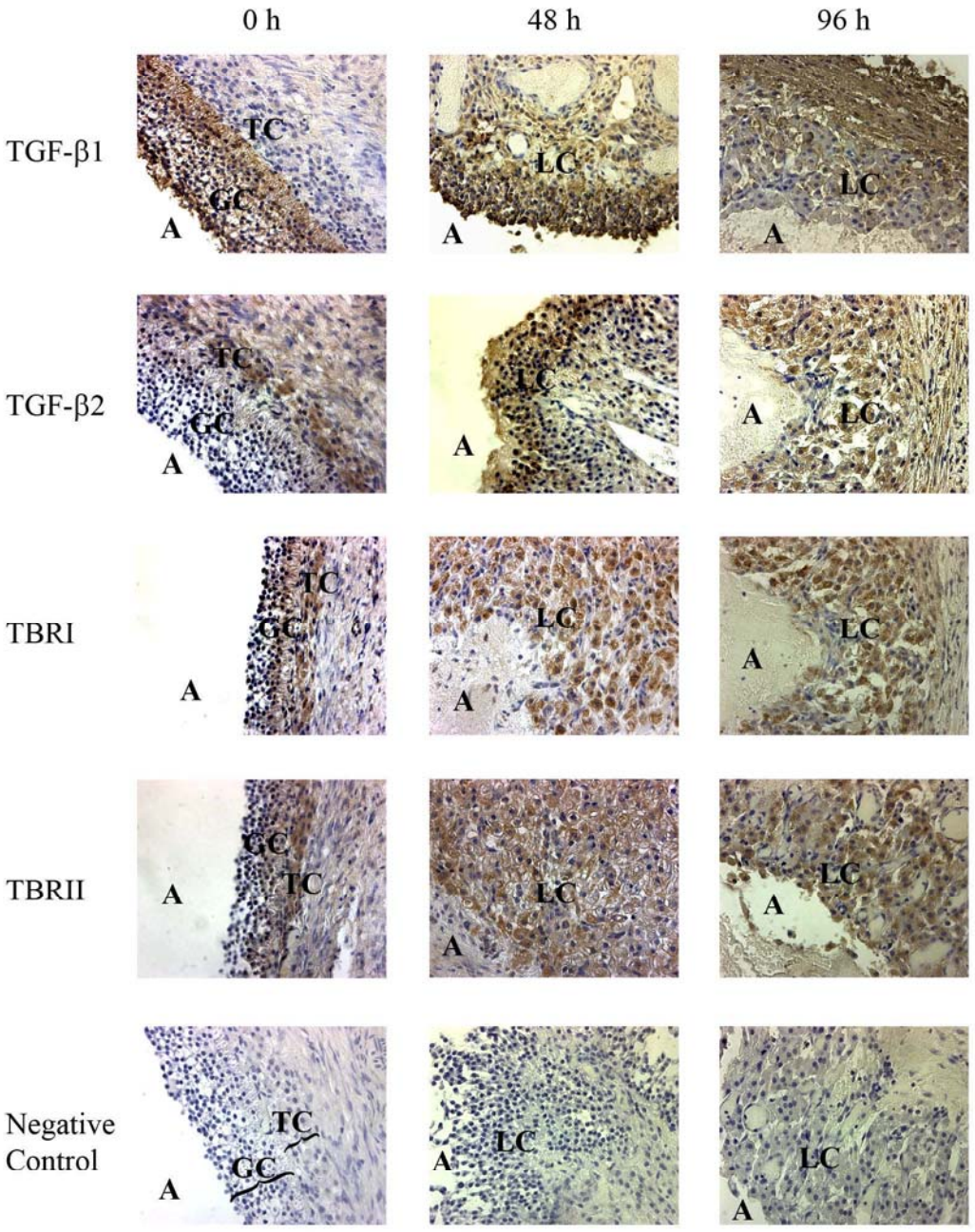

Figure 10. Immunohistochemical localization of TGF-β ligands and receptors in hCG-treated follicles

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Expression of TGF-β and components of its signaling pathway in cultured porcine granulosa cells: a possible role of TGF-β in luteinization?

Rajagopal Sriperumbudur and John E. Gadsby

Summary

The purpose of this study was to investigate the role of TGF-β in luteinization by examining the expression of TGF-β ligands (i.e. TGF-β1, 2 and 3) and components of its signaling pathway [the TGF-β receptors, TβRI and II, the Smad proteins, Smad 2, 3, 4, 6 and 7, and the Smad Anchor for Receptor Activation, (SARA)] in porcine granulosa cells induced to luteinize in vitro with either LH/IGF-1, various doses of TGF-β1 or a combination of the two treatments. Granulosa cells were obtained from porcine follicles collected at a local abattoir, and were placed into culture in M199 containing 10% fetal bovine serum on collagen I-coated culture dishes. Granulosa cells were treated with either LH (250 ng/ml) plus IGF-I (10 ng/ml) (LH/IGF-1), TGFβ-1 (10 and 100 ng/ml), LH/IGF-1 + TGF-β1 10 ng/ml or LH/IGF-1 + TGF-β1 100ng/ml) for 48 hours. To monitor the progress of luteinization, spent medium from the cultures was assayed for progesterone and the expression of genes known to be upregulated by LH [P450aromatase, P450scc, StAR, CyclinD2, 3β-HSD and connective tissue growth factor (CTGF)] was measured by semi-quantitative RT-PCR. Expression of mRNA for the components of the TGF-β signaling pathway was also assessed in granulosa cell after 48 hours of culture. The combination treatment of LH/IGF-1 plus TGF-β1 (10 ng/ml) increased progesterone production in the granulosa cells, compared to the individual treatments themselves, suggesting a possible synergistic effect of LH and TGF-β1 in

luteinization of the cells. Furthermore, LH/IGF-1 treatment appeared to up-regulate most components of the TGF-β signaling pathway, indicating the possibility that TGF-β may at least in part mediate the luteinizing action of LH/IGF-1. Although definite conclusions cannot be drawn because the number of viable cultures is limited, the numerical trends are very interesting nonetheless. Because TGF-β1 synergizes with LH/IGF-1 in increasing progesterone production and LH/IGF-1 may up-regulate components of the TGF-β signaling pathway in luteinizing granulosa cells, we suggest that TGF-β signaling may play a role in mediating the LH-surge induced luteinization in vivo.

Introduction

Luteinization is the process by which the postovulatory follicle differentiates to become the CL (Murphy, 2000). Through this process, the theca, granulosa, and the non-steroidogenic cells of the follicle all differentiate and integrate to form the CL, transforming the cyst-like, fluid-filled follicle into the solid mass of luteal tissue whose primary function is secretion of progesterone. Although it is known that the preovulatory surge of LH initiates luteinization, the exact mechanisms that control this process are still unclear.

Members of the TGF-β superfamily are involved in every stage of follicular development, ovulation and luteinization (Knight P.G. et al., 2003, 2006). They are involved in processes such as granulosa and theca cell proliferation and atresia, steroidogenesis, gonadotropin receptor expression, oocyte maturation, ovulation and luteinization (reviewed in [1]).

Although there is evidence of expression of TGF-β ligands and their signaling pathway components during follicular development, there is very little information on the exact role of these proteins on ovulation and luteinization. Prolactin, a peptide that enhances progesterone production, and TGF-β have been shown to have similar effects on cultured rat luteal cells; they both suppress 20α-HSD, whose activity decreases progesterone levels [2]. In another study, human granulosa-lutein cells treated with TGF-β1 showed decreased apoptosis [3].Taken together, it appears that TGF-β, in concert with prolactin, supports CL function by increasing progesterone levels and decreasing apoptosis. Previous studies from our laboratory (described in Chapter 2) have shown that mRNA for TGF-β3 and TβRII were upregulated in peri-ovulatory porcine follicles in response to hCG treatment, demonstrating that components of the TGF-β pathway are upregulated in the peri-ovulatory follicle in

response to LH-like gonadotropins. We also demonstrated that there was a distinct compartmentalization of ligand expression: TGF-β1 was immunolocalized to granulosa cells and TGF-β2 to theca cells in the peri-ovulatory follicle, but as luteinization progressed, both ligands were immunolocalized to luteal cells.

Other studies also point to the involvement of TGF-β in luteinization. In the marmoset, when follicles were cultured and induced to luteinize, TβRII expression was localized to luteinizing cells only [4, 5]. In addition, TNF-α, an inhibitor of luteinization, downregulated TβRII [6]. In the chicken, TGF-β1 and activin both increase expression of FSH- receptor and LH- receptor in undifferentiated granulosa cells, thus increasing the ability of the granulosa cells to produce more progesterone by increasing P450scc and StAR [7]. In the CL of gonadotropin-treated rhesus monkey, the mRNA and protein expression of TGF-β1, TβRI, TβRII and StAR increased when the CL was functional and decreased when the CL was regressing [8]. Treatment with TNF-α when the CL was functional decreased expression of all of these parameters.

To better understand the role of TGF-β in luteinization, in vitro studies using porcine granulosa cells were performed. Granulosa cells were stimulated to luteinize in the presence of LH/IGF-1, TGF-β1 or a combination of the two. Granulosa cells were used because there is evidence that the receptors for TGF-β are expressed in granulosa cells [9] and from our own studies (Chapter 2), and hence, are the sites of TGF-β signaling.

Methods and materials

Porcine LH was obtained from the National Hormone and Pituitary Program, NIDDK (Baltimore, MD). IGF-1 and TGF-β1 were purchased from R & D systems (Minneapolis,

MN). Collagen-I coated plates were purchased from BD Biosciences (Bedford, MA). Fetal calf serum and other cell culture reagents were purchased from Cambrex Bioscience (Baltimore, MD). Percoll solution was purchased from Sigma Chemical Co. (St. Louis, MO).

Granulosa Cell Isolation and Culture

Ovaries from sows exhibiting estrous cycles were collected from a local abattoir (City Packing, Burlington, NC) and transported in Medium-199 at room temperature. The approximate time from obtaining ovaries to collecting granulosa cells was 2 hours. Just before processing, the ovaries were briefly dipped into a series of 70% EtOH to remove bacterial contamination and washed sequentially with 0.9% saline and M-199. Granulosa cells were harvested using a procedure modified from the original protocol by Picton et al [10]. Briefly, medium size (5-7 mm) follicles that appeared vascular and healthy were trimmed from the surrounding ovarian stroma, placed in a Petri dish with culture medium and hemisected. An inoculation loop was used to gently scrape the granulosa cells into the medium. Staining of the scraped linings with hematoxylin and eosin revealed that the basement membrane and the theca layer below it were intact, confirming that there was no thecal cell contamination (data not shown). The resultant cell suspension was washed at room temperature in M-199. Red blood cells were removed using Percoll separation and the granulosa cells were washed in M-199 again to remove the Percoll. Cell number and viability were determined using Trypan blue dye exclusion. Cells were resuspended in culture medium M-199 (HBSS with L-Glutamine, 25 mM HEPES and 1.4 g/L NaHCO3) supplemented with 0.1% BSA, 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 0.5 μg/ml fungizone. The cells were seeded at a density of 3.75 to 4 x 10^5 viable cells in 500 μl aliquots per well of a 48-well Collagen-I coated plate and incubated

in a humidified chamber with 5% CO_2 at 37ºC. The day of plating was designated as day 0 (D0). The cells were allowed to attach for 48h, and on day 2 (D2), spent media was removed and treatments were added: control (culture medium), 250 ng/ml dose of LH with 10 ng/ml IGF-1 (LH/IGF-1), TGF-β1 at 10 and 100 ng/ml, and a combination of LH /IGF-1/TGF-β1 (10 ng/ml) and LH/IGF-1/TGF-β1 (100 ng/ml). Each treatment consisted of six wells. Cultures were terminated on D4. Spent medium was removed on D4 for P_4 assay and cells were lysed in Tri-reagent for RNA extraction upon termination of cultures. Within each treatment group, RNA was pooled to yield sufficient amounts for reverse transcription. To ensure comparable cell numbers between treatments, spent media was also used in the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI) as per the manufacturer's protocol (data not shown).

A total of 26 cultures were attempted. Of these, only 14 cultures were carried out to completion on D4. The others were abandoned due to reasons such as poor cell viability and bacterial or fungal contamination. Of the 14 completed cultures, data from 10 cultures are not shown here because of the variability in them due to attempts to standardize the culture methods; all of these cultures were different due to the differences in the type of culture wells used, the percentage of FBS in the plating medium and the dosage and combinations of the treatments.

Progesterone RIA

Progesterone (P_4) was measured in the medium to monitor the progress of luteinization of the GC cultures. Samples were diluted (10-500 fold) in PBS-gel buffer and were assayed without extraction. Progesterone standards used in each assay were also diluted in PBS-gel. Diluted media samples were assayed using the Coat-A-Count P_4 assay (DPC,

Los Angeles, CA) according to the manufacturer's instructions as described previously [11]. The inter-assay coefficient of variation was 2.5% and intra-assay variation ranged from 0.8% to 4%.

For each assay, progesterone values for each treatment group were averaged. The fold-increase of a treatment over control was calculated as the ratio of the average progesterone value for that treatment group divided by the average progesterone value for the control group. Finally, fold increases over control were averaged across assays and the least squares means ± SEM values were graphed.

Semi-quantitative RT-PCR:

Total RNA was extracted from granulosa cells from four separate cultures. Only cultures that showed a progesterone increase of 4 to 10 fold for the LH/IGF-1 treatment were used. Of the four cultures used for progesterone analysis, PCRs for only two cultures yielded amplicons. Cells exposed to the combination treatments of LH/IGF-1 plus TGF-β1 did not yield any amplicons. This data is summarized in Table 2.

The RNA was pooled between wells within each treatment to obtain sufficient quantities for reverse transcription. RNA extraction was performed with TRI-Reagent® (MRC, Inc., Cincinnati, OH) and the RNAqueous kit (Ambion, Inc., Austin, TX). The total RNA was subsequently DNAse-treated with Turbo-DNA free enzyme (Ambion) to minimize genomic DNA contamination. Total RNA was reverse transcribed to generate cDNA using Omniscript RT kit (Qiagen, Valencia, CA). To ensure that there was no genomic contamination, -RT controls for all samples were also included in the reverse transcription reaction. To determine the linear phase of amplification, a pooled RNA sample across all samples was reverse transcribed and PCR was performed with each primer pair.

Amplification by PCR was performed on the pool cDNA using porcine-specific primers (Sigma-Genosys, Woodlands, TX) for TGF-β ligands, receptors and Smad proteins (See Table 1 for primer sequences and amplicon sizes) for a total of 40 cycles, and 2 tubes per sample were removed every 5 cycles beginning with cycle 20. Amplicons were visualized on ethidium bromide-stained 2 % agarose gels and band densities were determined using the Alpha Imager gel documentation system (Alpha Innotech, San Leandro, CA). The densities were then graphed to determine the number of cycles which corresponded to the linear phase of amplification for each primer pair. Nucleotide sequencing of all PCR products was performed at the Gene Technologies Labarotories, Texas A & M University (College Station, TX) and compared with existing porcine sequences from the GenBank database. All gene products were confirmed as authentic based on published nucleotide sequences. Thus, for individual samples, PCR was performed under the following conditions: all samples were placed in AB biosystem's 9600 thermocycler for 3 min at 94ºC (initial denaturation), denaturation at 94ºC for 30 seconds. The standard PCR program included annealing at 50ºC for 30 seconds, extension at 72ºC for 60 seconds for appropriate numbers of cycles (specified in Table 2) for each primer, and a final extension at 72ºC for 5 minutes. Semi-quantitative analysis was performed on PCR products obtained during the linear phase of PCR amplification, after normalizing levels of gene expression to expression levels of Histone (H)2A, as a housekeeping gene. Absence of amplicons bands for the –RT samples confirmed the absence of genomic DNA contamination (data not shown). To confirm that the granulosa cells underwent luteinization in culture, we also examined several other genes (P450scc, StAR, 3-β HSD, Aromatase, Cyclin D2 and CTGF), which are known to be responsive to LH and to be altered during luteinization in vitro [12-14] Semi-quantitative analysis was

performed on PCR products obtained during the linear phase of PCR amplification, after normalizing levels of gene expression to expression levels of Histone (H)2A, as a housekeeping gene. The primer sequences (5'-3'), extension cycle numbers and expected product sizes for the various genes examined are given in Table 2. PCR for each mRNA sample was performed in duplicate, and the intra-assay CV ranged from 5.9% to 9.7%.

Results

The raw mean progesterone values from four cultures used in this study are given in Table 3. Fold increases in progesterone values over control are given in Fig. 1. The progesterone data are expressed as least squares means ± SEM. The progesterone data were analyzed using one-way analysis of variance, followed by Duncan's multiple range test to determine differences between treatments. The normalized band densities relative to H2A for both cultures and the raw progesterone values (ng/ml) are given in Table 4. The fold increases in mRNA expression of the three pro-luteinization enzymes StAR, P450scc and 3β-HSD and TGF-β system components, as well as the other luteinization-associated genes for both cultures are given in Table 5. It was evident from table 4 that the fold changes in mRNA expression of most of the genes examined occurred in opposite directions in the two cultures. Therefore, it was necessary to determine which of the two cultures was most likely to produce the progesterone secretion pattern seen in Figure 1. To do this, a correlation analysis was performed after combining data across the four treatments between the raw progesterone values and the raw normalized band densities for each of the three pro-luteinization enzymes StAR, P450scc and 3β-HSD. The Pearson correlation coefficient and the corresponding p-values for these three enzymes for both cultures are given in Table 6. It is evident that

between the two cultures, data for mRNA expression from culture 2 correlated best with the progesterone data, as evidenced by the high Pearson correlation coefficients for StAR (0.989, p=0.011) and P450scc (0.990, p=0.010). Therefore, mRNA expression data from only culture 2 are presented in figures 2 and 3. The mRNA data are represented as densities normalized to H2A. Fig. 2 shows mRNA expression patterns for luteinization-associated genes. Fig. 3 shows mRNA expression patterns for TGF-β pathway components.

Progesterone:

Figure 1 shows the granulosa cell responsiveness in terms of progesterone secretion following treatment with LH/IGF-1, TGF-β and their combination, from four different cultures. LH/IGF-1 increased progesterone secretion relative to untreated control cultures by approximately 6-fold. TGF-β1 at the 10 ng/ml and 100 ng/ml doses was only slightly stimulatory (less than 1.5 fold increase). In combination with LH/IGF-1, TGF-β1 at the 10 ng/ml and 100 ng/ml doses increased progesterone production over the LH/IGF-1 treatment, with a significant increase at the 10 ng/ml dose.

mRNA expression:

Luteinization-associated genes:

Figure 2 shows that LH/IGF-1 appeared to alter the expression of luteinization-associated genes like P450scc, StAR, 3β-HSD, aromatase, Cyclin D2 and CTGF. The expression of StAR, 3β-HSD, P450scc and aromatase numerically increased over 2-fold and the expression of CTGF and Cyclin D2 numerically increased by less than 2-fold. TGF-β1 did not appreciably alter the expression of all of these genes except 3β-HSD, whose expression numerically increased over 2-fold at the 100 ng/ml dose.

TGF-β ligands:

As shown in Figure 3, top panel, LH/IGF-1 treatment numerically increased the expression of the ligand TGF-β3 over 2-fold above control values. TGF-β1 itself numerically increased the expression of the ligand TGF-β1 over 2-fold at the 100 ng/ml dose.

TGF-β receptors:

As shown in Figure 3, middle panel, LH/IGF-1 treatment numerically increased the expression of both receptors. TGF-β1 did not appear to alter the expression of both receptor sub-types.

Smads and SARA:

As shown in Figure 3, bottom panel, LH/IGF-1 treatment numerically increased the expression of Smad 3 over 2-fold. TGF-β1 did not appear to alter the expression of all Smads and SARA.

Correlation between TGF-β components and luteinization

This study is based on mRNA expression data from a single culture. Therefore, it is difficult to draw definite conclusions about the role of TGF-β in luteinization in cultured granulosa cells from it. To glean more information from this dataset, such as which, if any, of the TGF-β system components might be involved in granulosa cell luteinization in vitro, a multiple correlation was performed after combining data across the four treatments between the TGF-β components and each of the following: the raw progesterone values and the raw normalized band densities for the three pro-luteinization enzymes StAR, P450scc and 3β-HSD. The Pearson correlation coefficients and the corresponding p-values for all of the TGF-β components which correlated significantly ($p \leq 0.08$) are presented in Table 7.

Discussion

In this study we have shown that using porcine granulosa cell culture system, we have shown that LH/IGF-1 can be used to successfully induce luteinization in these cells. LH/IGF-1 produced approximately a 7-fold increase in progesterone levels compared to the control. In addition, mRNA expression for genes associated with luteinization, namely, StAR, 3β-HSD, P450scc and aromatase numerically increased in granulosa cells induced to luteinize with LH/IGF-1 treatment. Similar observations were made in Chapter 2, which were to be expected given the widely reported luteinizing actions of LH or hCG in multiple species including the pig. In the pig, progesterone levels have been shown to increase in parallel with increases in mRNA for P450scc, and mRNA and protein for StAR [13]. In the current study we also showed no changes in CyclinD2 expression, which was also similar to our findings hCG treated porcine follicles. In addition, CTGF expression, which has previously shown to increase in the granulosa cells of the luteinizing follicle in the pig [17], did not show any changes in response to LH/IGF-1.

LH/IGF-1 treatment also numerically increased mRNA expression for the TGF-β components TGF-β3, TβRI, TβRII and Smad3. Similar increases in TGF-β3 and TβRII mRNA expression were observed when follicles were exposed to hCG in vivo (Chapter 2). The increases in TGF-β3 in this study and in the in vivo study in Chapter 2 suggest that this isoform may be involved in the luteinization process more than the other two isoforms. The increase in TβRII is also in agreement with the in vivo study in Chapter 2 as well as other studies in the marmoset and the pig [4, 5, 6].

TGF-β1 on its own did not produce appreciable increase in progesterone production by granulosa cells. However, in combination with LH/IGF-1, TGF-β1 caused a significant

increase in progesterone production, compared to either treatment on its own. This suggests that there may be a synergistic stimulatory effect on progesterone secretion, especially at the 10 ng/ml dose of TGF-β1. This might be explained by the observation of increased TGF-β receptors in response to LH/IGF-1, or by stimulation of 3β-HSD by TGF-β, which when combined with elevated P450scc and StAR caused by LH/hCG, further elevated progesterone compared to either TGF-β1 or LH/IGF alone. There is evidence that TGF-β upregulates LH receptor expression in granulosa cells from prehierarchal follicles in the chicken [7], thus indirectly enhancing the luteinizing actions of LH. In the rat, TGF-β has been shown to increase 3β-HSD and P450scc mRNA expression in theca-interstitial cells [16], and may have similar effects in granulosa cells. Alternatively, since the effects of TGF-β1 on steroidogenesis are not striking, it is possible that TGF-β may not play a role in luteinization. But taking into consideration all the literature evidence that is available on the roles of TGF-β in various tissues, and our preliminary observations from the in vitro studies described here, we believe that TGF-β may promote luteinization indirectly by enhancing the actions of LH and by contributing to aspects of luteinization other than steroidogenesis. The numerical increases in the luteinization-promoting enzymes 3β-HSD by TGF-β may also explain the increase in progesterone production by the LH/IGF-1 and TGF-β1 combination treatment. TGF-β is known to have widespread biological actions on the extracellular matrix, angiogenesis, inhibiting apoptosis, blocking inflammation and inhibiting immune function, any of which may subserve a role in the development of the corpus luteum.

The correlation analysis between components of the TGF-β pathway and progesterone values and the three pro-luteinization enzymes presented in Table 7 provide a glimpse of which of the TGF-β components are most likely to play a role in promoting

luteinization. The ligand TGF-β3, as well as both receptors TβRI and TβRII correlated strongly with the progesterone values as well as the expression of the enzymes StAR and P450scc. These findings are in agreement with the results from Chapter 2 where we showed that mRNA expression of TGF-β3 and TβRII increased in follicles treated with hCG, and from other studies in the marmoset and the pig [4, 5, 6]. There were no strong correlations between these components and 3β-HSD, even though LH/IGF-1 and both doses of TGF-β numerically increased 3β-HSD. This could be due to combining the mRNA or progesterone values across treatments for the correlation analysis. None of the other components of the TGF-β pathway showed strong correlations with progesterone values or the three pro-luteinization enzymes. Based on the correlation analysis, we recommend that the mRNA expression of the three TGF-β components, namely TGF-β3, TβRI and TβRII, which correlated best with the progesterone values and the enzymes StAR and P450scc, should be examined more closely and followed up by examining the corresponding protein expression patterns, to determine their role in luteinization. The other components may not contribute to luteinization, based on the correlation analysis, or contribute in ways other than increasing steroidogenic enzymes and progesterone.

The differences in gene expression patterns between the two cultures considered in this study, that led to mRNA data from culture 1 to be discarded, can be due to a variety of reasons. Most sows are culled for reproductive abnormalities, and cultured granulosa cells from such an animal may not respond to treatments as expected. Moreover, the exact stage of the estrous cycle when the follicles are harvested is unknown in ovaries obtained from the slaughterhouse. This can make a difference in the response of granulosa cells to luteinizing treatments in vitro. From our observations (unpublished data), granulosa cells that may have

been stimulated by endogenous LH before harvest do not respond very well, in terms of progesterone production, to LH/IGF-1 treatment in vitro. Finally, in our experience, maintaining a primary granulosa cell culture has been extremely difficult as evident from the number of cultures attempted versus the number of cultures from which data were obtained. Due to the fact that data from only one culture was used to examine mRNA expression patterns, the findings described here can only be considered preliminary at best, and need to be verified using further studies. For future studies, it may be better to use ovaries harvested from normally cycling animals in which the exact stage of estrous cycle is known.

FIGURE LEGENDS

Figure 1. Effect of treatment on progesterone levels in granulosa cell cultures. Progesterone values (ng/ml) were averaged from 4 different cultures and are expressed as least squares means ± SEM.

Figure 2. mRNA expression for LH-responsive genes in luteinizing granulosa cells in vitro. The values represent fold increases above the control treatment. Quantification of mRNA was performed using semi-quantitative PCR, with band densities expressed as the ratio relative to H2A (n = 1 culture per treatment).

Figure 3. mRNA expression of TGF-β ligands (top panel), receptors (middle panel), Smads and SARA (bottom panel) in luteinizing granulosa cells in vitro. The values represent fold increases above the control treatment. Quantification of mRNA was performed using semi-quantitative PCR, with band densities expressed as the ratio relative to H2A (n = 1 culture per treatment).

Table 1. Summary of the number of cell cultures attempted, number of cultures for which progesterone values were obtained and the number of cultures used for analysis of mRNA expression

Total number of cell cultures attempted	**Number of cell cultures for which progesterone data obtained**	**Number of cultures for which mRNA expression data obtained**
26	14	2

Table 2. Primers used for semi-q RT-PCR

Gene	Primer sequence (5'-3')	Extension cycles	Product size (bp)
TGF-β1	F:CTGTGTCTGTCCACCATTCATTTG R:CAACTTTGCTATGTCTGTCTCCCC	33	496
TGF-β2	F:CATCTCCTGCTAATGTTGTTGCC R:CGGTTCTAAATCCTGGGACACG	30	324
TGF-β3	F:CAGACTGGAAGATGCTTGGAGC R:CTTAGAGGGGTCAGGAGAGGAAAC	33	477
TβRI	F:GCAGTATGTCTTCTAGCCTGCCT R:TTCGCAGGCAGCTAACTGTATCC	33	524
TβRII	F:CTGAGGCAGAACACATCTGAGC R:TCGCTGTGCAGGTGTGCAATGC	30	305
Smad2	F:GAAGAGAAGTGGTGTGAGAAAGCAG R:AATACTGGAGGCAAAACTGGTGTC	30	428
Smad3	F:TGGAGGAGGTGGAGAAATCAGAAC R:CACACTCGCTTGCTCACTGTAATC	33	541
Smad4	F:CCTGAGTATTGGTGTTCCATTGC R:TGATGCTCTGCCTTGGGTAATC	33	598
Smad6	F:CGTCAGCATCTTCTACGACCTACC R:TATCTGGGGAGGGTTCTTCCG	33	500
Smad7	F:TACTGGGAGGAGAAGACGAGAGTG R:TGGCTGACTTGATGAAGATGGG	33	241
SARA	F:TCCACATTTCTTCTCTCCCACTTC R:CAGTTTCTGAGTCACAGCCACAAC	33	230
H2A	F:AGGACGACTAGCCATGGACGTGTG R:CCACCACCAGCAATTGTAGCCTTG	28	210
Cyclin-D2	F:CCCCTACCCCCTTCAAAAACTC R:TGTTGTTGACACTTCTGCTCCTCAC	33	351
Aromatase	F:CATCACCAAGCACCTGGACAAG R:TAGTTCCTCTTCAACCTGGGGG	33	472
CTGF	F:CCACCTTCCCACACCTAATAC	33	573

Table 2 cont

	R:ACACCGTTCCTCCAAAGACG		
StAR	F: GAGTGGTTCCATTCTTCCAGAGC R: CAACTATCCCTTCCATCCGTCTC	25	564
P450scc	F:TGATGCTGTCTACCAGATGTTCCAC R:TTGCTTGTGTCTCCTTGGGC	33	481
3β-HSD	F:ATCCACACCAGCAGCATAGAGG R:TGTCAGGACGCCATTGTTCTCC	33	348

Table 3. Progesterone values (ng/ml) expressed as means ± SEM from 4 granulosa cell cultures

Treatment	Culture 1	Culture 2	Culture 3	Culture 4
Control	41.6 ± 1.5	125.4 ± 6	49.7 ± 56.9	259.1 ± 38.5
LH/IGF-1	174.4 ± 14.4	832.3 ± 74.7	5281.8 ± 1816.9	1816 ± 246.8
TGF-β (10 ng/ml)	48.8 ± 1.5	109.1 ± 3.9	932.7 ± 270.8	332.3 ± 13
TGF-β (100 ng/ml)	48 ± 2.6	107.5 ± 2.6	497.1 ± 56.9	391.7 ± 52.5
LH/IGF-1 plus TGF-β (10 ng/ml)	694.7 ± 29.3	1429.6 ± 62	1019.4 ± 149.5	2723.1 ± 273.3
LH/IGF-1 plus TGF-β (100 ng/ml)	461.8 ± 39.1	752.4 ± 36	8318.3 ± 4686.5	1226.2 ± 144.2

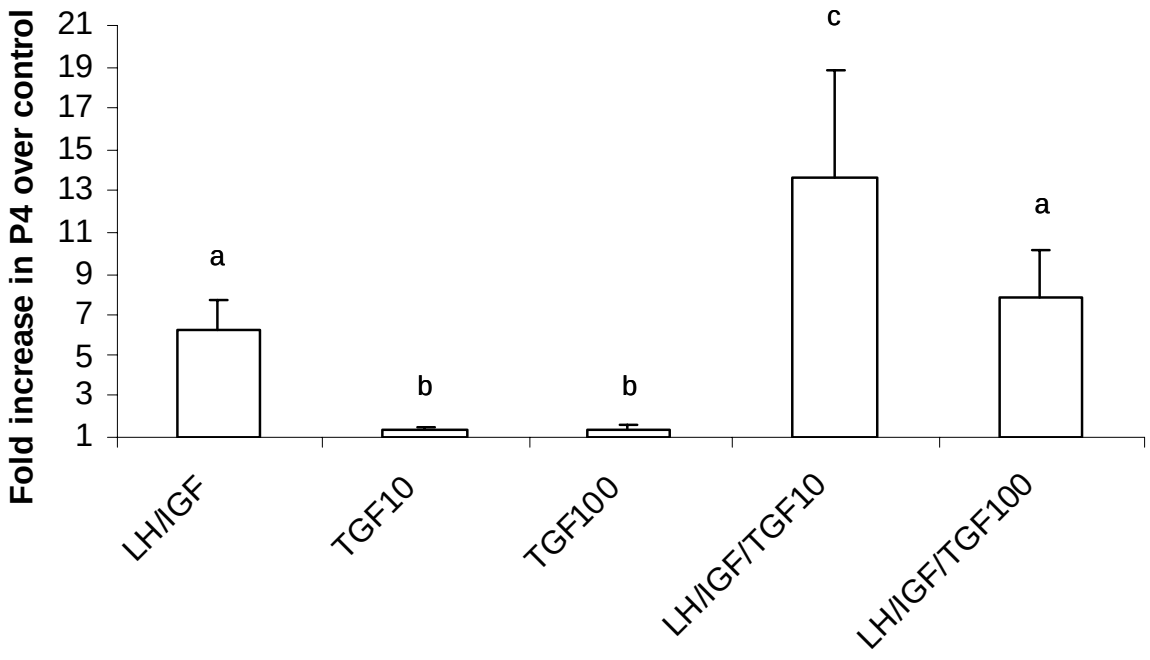

Figure 1. Effect of treatment on progesterone levels in granulosa cell cultures

Table 4. Raw normalized* densities of amplicon bands used to calculate mean densities for graphs in figures 2 and 3, and raw progesterone values (last row)

Gene	Culture 1				Culture 2			
	Control	LH/IGF-1	TGF 10	TGF 100	Control	LH/IGF-1	TGF 10	TGF 100
TGF-β1	7.1	6.6	11.3	74.3	18.6	34	31.3	51.5
TGF-β2	5.1	6.6	44.3	100	14.0	15.0	25.3	15.8
TGF-β3	19.4	5.5	49.5	125.7	11.6	64	21.2	3
TβRI	2	3.3	10.3	5.7	10.5	30	3	4
TβRII	19.4	18.7	55.7	254.3	18.6	58	6.1	6.9
Smad2	28.6	34.1	74.2	254.3	59.3	84	25.3	55.4
Smad3	38.8	63.7	49.5	240	38.4	78	21.2	48.5
Smad4	8.2	9.9	69.1	74.3	39.5	76	62.6	5
Smad7	16.8	121.9	115.7	344.2	108.3	113.6	91.3	101.7
SARA	100.6	132.4	56.5	352	132.1	17.8	48.4	94.9
Cyclin-D2	102	120.4	114.3	344.2	98.7	119.1	31.8	103
Aromatase	8.4	7.5	8.5	46.9	11.1	30.1	12.4	13.6
CTGF	14	60.2	33.9	78.2	41.4	71.2	41.5	31.2
StAR	7	24.1	7.1	19.6	11.1	56.1	6.9	5.4
P450scc	57.1	67	19.6	200	24.4	91	36.4	25.7
3β-HSD	25.1	150.4	127	379.4	60.5	143.8	71.9	135.5
Raw progesterones (ng/ml)	125	832	109	107	42	174	49	48

* indicates that densities were normalized to H2A densities

Table 5. Fold increases in normalized* mRNA expression over control in granulosa cell cultures

Gene	Culture 1			Culture 2		
	LH/IGF-1	**TGF 10**	**TGF 100**	**LH/IGF-1**	**TGF 10**	**TGF 100**
StAR	**3.4**	1	**2.8**	**5.1**	*0.6*	*0.5*
P450scc	1.2	*0.3*	**3.5**	**3.7**	1.5	1.1
3β-HSD	**6**	**5.1**	**15.1**	**2.4**	1.2	**2.2**
TGF-β1	*0.9*	1.6	**10.5**	1.8	1.7	**2.8**
TGF-β2	1.3	**8.7**	**19.6**	1.1	1.8	1.1
TGF-β3	*0.3*	**2.6**	**6.5**	**5.5**	1.8	*0.3*
TβRI	1.7	**5.2**	**2.9**	**2.9**	*0.3*	*0.4*
TβRII	1	**2.9**	**13.1**	**3.1**	*0.3*	*0.4*
Smad2	1.2	**2.6**	**8.9**	1.4	*0.4*	*0.9*
Smad3	1.6	1.3	**6.2**	**2**	*0.6*	1.3
Smad4	1.2	**8.4**	**9.1**	1.9	1.6	*0.9*
Smad7	**7.3**	**6.9**	**20.5**	1	*0.8*	*0.9*
SARA	1.3	*0.6*	**3.5**	*0.1*	*0.4*	*0.7*
Cyclin-D2	1.2	1.1	**3.4**	1.2	*0.3*	1
Aromatase	*0.9*	1	**5.6**	**2.7**	1.1	1.2
CTGF	**4.3**	**2.4**	**5.6**	1.7	1	*0.8*

* indicates that densities were normalized to H2A densities

Table 6. Correlation between progesterone values and expression of enzymes involved in progesterone synthesis

	Gene	Pearson correlation coefficient	p-value
Culture1	P450scc	-0.169	0.831
	StAR	0.727	0.273
	3β-HSD	-0.107	0.893
Culture2	P450scc	0.990	0.010
	StAR	0.989	0.011
	3β-HSD	0.655	0.34

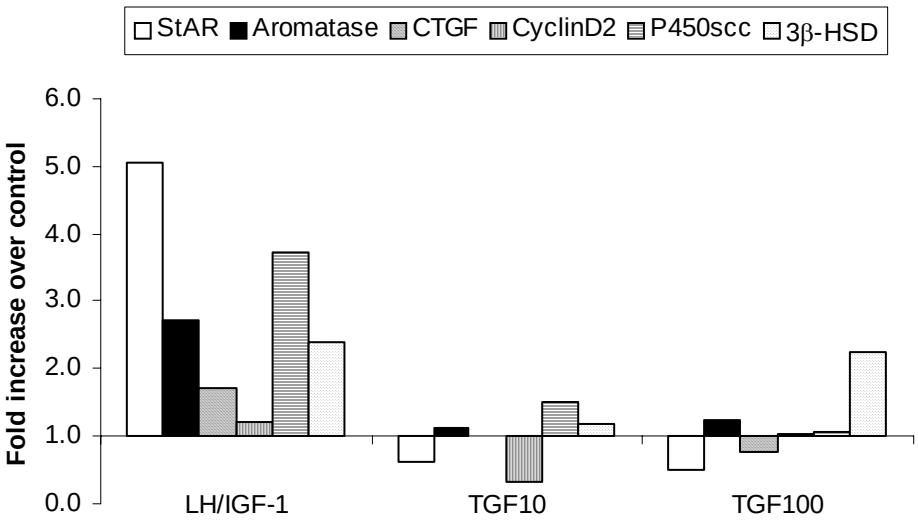

Figure 2. mRNA expression patterns of LH-responsive genes in luteinizing granulosa cells in vitro

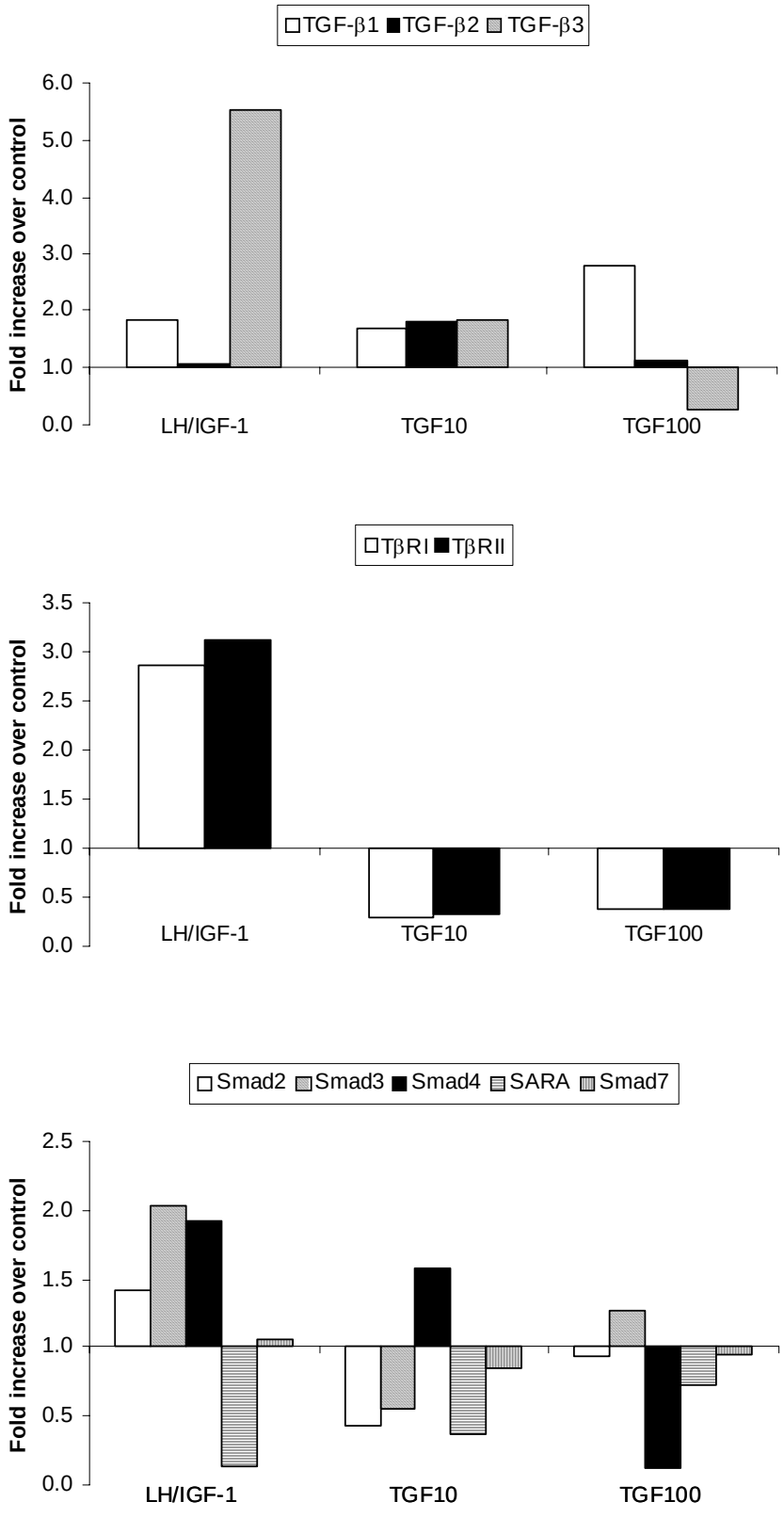

Figure 3. mRNA expression patterns of some TGF-β pathway components and LH-responsive genes in luteinizing granulosa cells in vitro

Table 7. Multiple correlation* between TGF-β components and progesterone values and mRNA expression of pro-luteinization enzymes

TGF-β component	Correlation with progesterone values	Correlation with StAR mRNA expression	Correlation with P450scc mRNA expression
TGF-β3	0.963	0.963	0.986
	0.037	0.037	0.014
TβRI	0.95	0.984	0.919
	0.05	0.016	0.081
TβRII	0.96	0.99	0.933
	0.04	0.01	0.067

*each cell contains the Pearson correlation coefficient on top and the p-value below it

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GENERAL CONCLUSIONS

The corpus luteum (CL) is essential for a successful pregnancy. In the pig, the lifespan of the CL is 13-16 days [1]. Understanding the role of genes and their products that regulate the normal formation, maintenance and regression of this transient endocrine gland is paramount in studying the control of luteinization. Abnormalities in this process can result in conditions such as luteal insufficiency and infertility. Of commercial importance to pig producers is the ability to short cycle a sow to synchronize breeding or to minimize the number of open days that a sow is not pregnant. The porcine CL is refractory to the luteolytic actions of $PGF_{2\alpha}$ until after day 12 of the estrous cycle [2-4], therefore limiting options available for estrus synchronization procedures. A better understanding of the process of luteinization can help address these problems. The research presented in this dissertation examines the role of TGF-β in luteinization.

The purpose of the first study was to examine message and protein expression of components of the TGF-β signaling pathway in porcine follicles induced to luteinize with an in vivo administration of hCG. Follicles were examined at 0, 1, 12, 24, 48 and 96h post-hCG. The results of this study revealed that (1) luteinization was induced successfully with hCG treatment in the treated animals as evidenced by ovulation between 24 and 48h post-hCG and luteinization thereafter, and by the increase in key enzymes and proteins involved in luteinization such as StAR and aromatase and (2) hCG upregulated some components of the TGF-β pathway: the ligand TGF-β3 was upregulated at 12h post-hCG and its receptor TβRII was upregulated at 96h post-hCG. However, this increase did not translate to increased receptor at the protein level. Due to the unavailability of a porcine-specific antibody, protein expression patterns of the ligand

were not examined to see if they matched the mRNA expression patterns. However, one of the intracellular components of the TGF-β pathway, phospho-Smad3, showed a tendency to increase at the 12h time point after hCG, which was the same time point that the ligand mRNA increased. These findings indicated that hCG upregulated some components of the TGF-β pathway at the message level, but this did not translate into increase in protein levels. One possible reason for this discrepancy can be that we only looked at follicles at certain time points. These components could be induced rapidly but are expressed only transiently, with peak levels of message and protein occurring at an intermediate time point post-hCG that was not examined. Another possibility is that Western blotting is not a sensitive enough methodology for assessment of small changes in protein expression levels.

Immunolocalization of the ligands demonstrated a distinctive pattern in follicles up to the 24h time point post-hCG: TGF-β1 was localized exclusively to granulosa cells while TGF-β2 was localized to only theca cells. The receptors TβRI and TβRII were immunolocalized to both cell types in these follicles. After ovulation, the distinction between granulosa and theca cells could not be made because of morphological changes in these cell types associated with luteinization. Labeled as luteal cells, they showed immunolocalization of both ligands and receptor types examined.

The second study examined the expression of mRNA for TGF-β pathway components in granulosa cells induced to luteinize in vitro with LH/IGF-1. The results of this study revealed that (1) LH/IGF-1 successfully induced luteinization of granulosa cells, as evidenced by the increase in progesterone levels and a possible upregulation of key enzymes and proteins involved in luteinization such as StAR, P450scc, 3β-HSD and

aromatase; and, (2) TGF-β did not increase progesterone production on its own, but when combined with LH/IGF-1, it exhibited a synergistic increase in progesterone levels. Preliminary studies to examine patterns of mRNA expression for components of TGF-β pathway indicated it is possible that LH/IGF-1 may upregulate these genes. We had difficulty maintaining granulosa cells in culture and were limited to using mRNA expression data from only one of two viable cultures for this study. Definitive patterns of expression may become apparent if more viable cultures are added to this dataset in the future.

In conclusion, TGF-β may or may not be involved in luteinization in the pig. Data from our studies suggests it is possible that TGF-β plays a role in this process. A cell model proposed to incorporate possible ways in which TGF-β may be involved in luteinization is given in Figure 1. TGF-β has been shown to upregulate LH receptor expression in undifferentiated granulosa cells in the chicken, thus increasing their ability to produce more progesterone by increasing P450scc and StAR [5]. In rodents, TGF-β has been shown to increase progesterone production in cultured luteal cells by suppressing 20α-HSD, an enzyme that decreases progesterone [6], but in the sheep [7], TGF-β decreases progesterone in cultured granulosa cells. From our second study, it is evident that TGF-β on its own does not increase progesterone secretion in luteinizing porcine granulosa cells. Therefore, TGF-β may indirectly increase progesterone secretion in the pig by increasing LH receptor levels, thus increasing the response of granulosa cells to LH. In the rat, TGF-β has been shown to increase 3β-HSD and P450scc mRNA expression in theca-interstitial cells [8], and may have similar effects in granulosa cells. In our second study, TGF-β numerically increased 3β-HSD mRNA expression by over 2-

fold. We also observed a strong correlation, if we ignored treatment effect, between the expressions of TGF-β3, TβRI and TβRII and progesterone levels as well as expressions of StAR and P450scc, the major enzymes involved in progesterone synthesis. From both our studies, as well as other studies in the marmoset [9] and the pig [10], TβRII expression increases in the luteinizing follicle in response to LH. We have also shown that the mRNA expression of TGF-β3 increases in the preovulatory follicle in response to hCG. Taken together, it appears that TGF-β increases the response of granulosa cells to LH by increasing LH receptor expression, while LH in turn upregulates some components of the TGF-β pathway such as TGF-β3 and TβRII.

TGF-β has also been shown to increase the expression of CTGF [11], a promoter of extracellular matrix production, in luteinizing porcine granulosa cells, although we did not observe it in our studies. VEGF, an angiogenic factor, has also been shown to be upregulated by TGF-β [12] in mouse macrophages. In a similar manner, TGF-β may upregulate VEGF in the luteinizing follicle. Taken together, TGF-β may promote luteinization in ways other than increasing steroidogenesis, such as extracellular matrix formation and the formation of new blood vessels by facilitating endothelial cell migration into the developing corpus luteum.

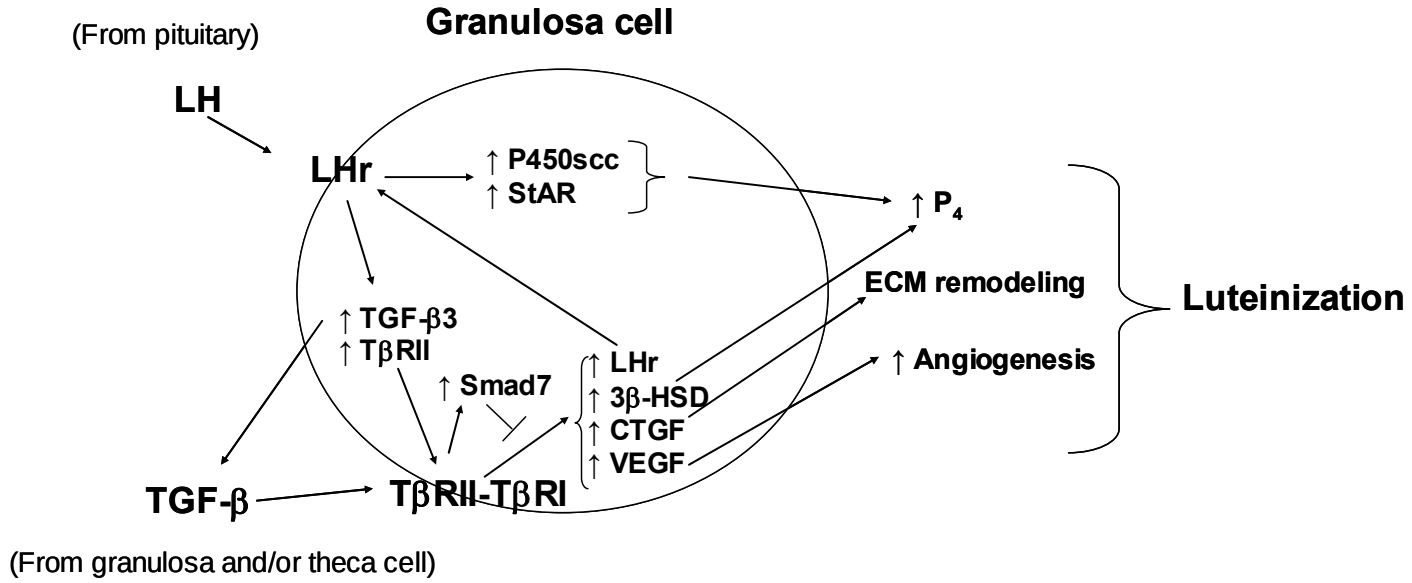

Figure 1. Possible cell model for TGF-β augmenting LH in promoting luteinization.

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APPENDIX

Table A1. List of genes upregulated by at least 2 fold compared to the 0h control after hCG treatment from cDNA expression analysis

GENE	CATEGORY	1h	24h	48h
Proteosome Comp. C5	Proteosomal (cytoplasmic) proteins	4.4	3.1	3.2
Homeobox protein 2.1 HOX-2A	Transcription activators and repressors	3.5	2.3	3.4
cAMP-dep. Protein kinase I alpha reg. Sub.	Intracellular kinase network members (non-receptor protein kinases)	3.4	2.4	2.2
27-kDa heat shock protein (HSP27)	Heat shock proteins	3.3	2.3	2.8
Macrophage-specific colony-stimulating factor (CSF-1)	Growth factors, cytokines, and chemokines	3.3	11	3.8
Leucine-rich repeat protein SHOC-2	Other intracellular transducers/effectors/modulators	3.1	3.8	3.6
Activated RNA polymerase II transcriptional coactivator p15	RNA polymerase	3.1	2.4	3.9
Zinc finger X-chromosomal protein (ZFX)	Other receptors; nuclear proteins	2.9	2.5	3.2
Neural-cadherin precursor (N-cadherin; NCAD)	Cell-cell adhesion receptors	2.8	5.7	3.4
Nuclease-sensitive element DNA-binding protein (NSEP)	Basic transcription factors	2.7	2.7	2.5
cAMP-dependent protein kinase type II beta regulatory	Intracellular kinase network members (non-receptor protein kinases)	2.6	0.4	0

subunit (PRKAR2B)				
Fibronectin receptor beta subunit	Cell-cell adhesion receptors	2.5	7.3	5.9
Linker for activation of T-cells (LAT)	Kinase activators and inhibitors/cell cycle	2.4	2.4	2.7
Helix-loop-helix protein	Transcription activators and repressors	2.4	0.2	0.4
Natural killer enhancing factor (NKEFB)	Xenobiotic transporters	2.3	2.9	3.3
Prothymosin alpha (ProT-alpha)	Other cell cycle proteins	2.3	2.1	2.8
TRAF-interacting protein (I-TRAF)	Transcription activators and repressors	2.3	2.2	2.2
Defender against cell death 1 (DAD1)	Other apoptosis-associated proteins	2.2	2.4	2.1
Transforming growth factor beta2 precursor (TGF-beta2)	**Growth factors, cytokines, and chemokines**	**2.2**	**2.7**	**3**
Rho GDP dissociation inhibitor 1 (RHO-GDI 1)	GTP/GDP exchangers and G-protein GTPase activity modulators	2.1	2.5	4.4
Ras-related protein RAB2	G-proteins	2.1	2.1	2.6
Growth arrest & DNA-damage-inducible protein (GADD45)	Apoptosis associated proteins	2	6.3	3.9