THE ROLES OF OOCYTE- AND THECA CELL-DERIVED BONE MORPHORGENETIC PROTEINS IN HUMAN GRANULOSA CELLS

by

Hsun-Ming Chang

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Abstract

Exerting a broad range of biological effects in various tissues, bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor β superfamily. Accumulating evidence indicates that ovarian BMPs are critical regulators of ovarian function and play important roles in the female reproductive system. Mutual communication between oocytes and the surrounding somatic cells is mandatory for normal follicle development, as these locally expressed growth factors function mainly as paracrine/autocrine effectors in granulosa cells.

Previous studies have shown that oocytes may secrete an anti-luteinization factor and theca cells may have luteinization-inhibiting activities. We therefore hypothesized that during the late follicular stage, oocyte-derived and theca cell-derived BMPs may prevent premature luteinization by down-regulating severalovulation-related genes.This study aims to investigate the anti-luteinization effects of oocyte- and theca cell-derived BMPs in human granulosa cells.An established immortalized human granulosa cell line (SVOG), granulosa cell tumor cell line (KGN) and primary granulosa-lutein cells were used as study models.Several parameters of luteinization were investigated following exposure to recombinant human BMP4, BMP7 or BMP15. Dual pharmacological and siRNA-based approaches were used to examine the underlying mechanisms and verify the specificity of the effects.

Our studies demonstrate that oocyte-derived BMP15 functions as a paracrine factor to decrease progesterone production and that theca cell-derived BMP4 and BMP7 down-regulate PTX3 expressionin human granulosa cells. In addition, all three growth factors decrease intercellular communication by down-regulating Cx43-coupled gap junction formation. Furthermore, both

BMP4 and BMP7 increase the production of a luteinization inhibitor activin A by up-regulating the expression of inhibin βA subunit and furin. Interestingly, different BMPs act through differential subsets of type I receptor-driven SMAD-dependent pathways. These results suggest that oocytes and theca cells may play important roles in the prevention of premature luteinization, a process that is essential for normal ovarian function and fertility.Our findings provide important insight into ovarian biology, and may lead to the development of novel therapeutic tools for fertility regulation.

Preface

This study was approval by the University of British Columbia Research Ethics Board.

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List of abbreviations

3βHSD	3*β*-Hydroxysteroid dehydrogenase
aa	Amino acid
ACVR	Activin receptor
ALK	Activin receptor-like kinase
AMH	Anti-müllerian hormone
AMHR2	AMH type II receptor
ANOVA	Analysis of variance
BMP	Bone morphogenetic protein
BMPR	BMP receptor
BMPR2ECD	BMP type II receptor extracellular domain
bp	Base pair
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid homolog
CL	Corpus luteum
Co-SMAD	Common SMAD
COC	Cumulus-oophorus complex
Cx	Connexin
COX2	Cyclo-oxygenase 2, prostaglandin-endoperoxide synthase 2
CRE	cAMP-response elements
CREB	CRE-binding protein

dNTP	Deoxynucleotide triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxynucleic acid
DNase	Deoxyribonuclease
E2	17β-Estradiol
ECD	Extracellular domain
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-lined immunosorbant assay
ER	Endoplasmic reticulum
Erk	Extracellular signaling-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FLRG	FST-related gene
FSD	FST domain
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
FSTL	FST-like proteins
FST	Follistatin
g	Acceleration of gravity
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCT	Granulosa cell tumor
GDF	Growth differentiation factor

GDNF	Glial cell-derived neurotrophic factor
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GS-domain	Glycine-serine rich domain
GVBD	Geminal vesicle breakdown
h	Hour
HAS2	Hyaluronan synthase 2
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
hGL	Human granulosa-lutein
IαI	Inter-α tripsin inhibitor
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
I-SMAD	Inhibitory SMAD
IVF	*In vitro* fertilization
JNK	c-Jun N-terminal kinase
Kb	Kilobase
KDa	Kilodaltons
KGF	Keratinocyte-growth factor
KL	Kit ligand
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor

μ	Micro
MAPK	Mitogen-activated protein
MADH	Mad-homologues
ml	Milliliter
min	Minutes
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
n	Nano
NFκB	Nuclear factor kappa B
OHSS	Ovarian hyperstimulation syndrome
p	Pico
P450scc	P450 cholesterol side-chain cleavage enzyme
P45017α	P450 17α-dehydroxylas/17,20-lyase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PKA	Protein kinase A
PKC	Protein kinase C
PI3K	Phosphatidylinositol-3-kinase
RNA	Ribonucleic acid

R-SMAD	Receptor-regulated SMAD
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SBE	SMAD binding element
SD	Standard deviation
SEM	Standard error of the mean
SDS	Sodium dodecyl sulphate
Sec	Second
Ser	Serine
SF-1	Steroidogenic factor 1
siRNA	Small interfering RNA
SMAD	Sma- and Mad-related protein
StAR	Steroidogenic acute regulatory protein
Taq	*Thermus acuaticus*, source of a DNA polymerase
TEMED	N,N,N',N'-tetramethylethlenediamine
TGF	Tranforming growth factor
Thr	Threonine
Tris	Tris (hydroxyl methyl) aminomethane
TSG-6	Tumor necrosis factor-stimulated gene 6 protein

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Chapter 1: Introduction

1.1 The ovary

In mammals, the ovary is a part of the female reproductive system which is essential to the perpetuation of the species. The physiological function of the ovary is the periodic production and release of meiotically competent oocytes and the synthesis and secretion of the steroid hormones, estradiol, androgen and progesterone. During the reproductive period, these activities are repeatedly integrated in a continual process of follicle development, ovulation and corpus luteum formation and regression.

Ovarian follicles are the basic functional units of the female reproductive system, and each follicle may correspond to a different developmental stage in the ovary. To achieve full ovulatory and steroidogenic capability, these follicles must progress through a highly coordinated regulatory process that involves a number of neural, neuroendocrine, paracrine and endocrine control systems (1). The primary roles of gonadotropin-releasing hormone (GnRH), the pituitary gonadotropins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) and the gonadal hormones (estrogen and progesterone) in the regulation of ovarian function have been well established. However, increasing evidence has shown that follicular development and normal ovarian function also rely on various locally produced cytokines and growth factors that exert their effects in an autocrine/paracrine manner (2). Among these locally produced factors are members of the transforming growth factor-β (TGF-β) superfamily, which include TGF-βs, growth differentiation factors (GDFs), activins and inhibins and bone morphogenetic proteins (BMPs). Studies on a variety of species have demonstrated that these growth factors are

expressed in the ovary and play critical roles in the regulation of folliculogenesis, oogenesis and ovarian functions; however, their functions in humans are either poorly defined or unknown.

1.1.1 Folliculogenesis

In humans, the reproductive life span of women is mainly determined by the number of primordial follicles, which are composed of oocytes in the diplotene stage surrounded by a layer of flattened pregranulosa cells (3). These primordial follicles remain in a quiescent phase until they are recruited into a pool of primary follicles, at which stage the flattened pregranulosa cells become cuboidal granulosa cells. This process is accompanied by an increase in the size of oocytes surrounded by the zona pellucida (4). The recruitment of dormant primordial follicles into a growing follicle pool begins from the fetal stage and continues until the time of menopause when the ovarian reserve is depleted (5). Several members of the TGF-β superfamily, such as theca cell-derived BMP4 and BMP7 and oocyte-expressed GDF9, play an important role in the process of transition from primordial follicles into the primary follicles (6-8). Thereafter, the mitotic expansion of single-layered granulosa cells results in their transformation into multi-layered granulosa cells, accompanied by an increase in the oocyte diameter and the development of the theca cell layer and basal lamina, a typical characteristic of secondary follicles (also known as pre-antral follicles) (3). In humans, the process of development into a secondary follicle from a primary follicle takes months and is not mediated by the effects of gonadotropins (FSH-independent stage).

With stimulation from FSH, these secondary follicles grow, survive and develop into antral follicles that are characterized by the further proliferation of follicle cells (granulosa and theca cells), increased vascularization, growth of oocytes, and the formation of a fluid-filled antrum.

During this stage, a cohort of antral follicles is cyclically recruited for further growth. Furthermore, FSH becomes a critical determinant of the selection of a dominant follicle from this cohort, which finally reaches the preovulatory stage, while most other follicles are undergoing atresia. Under the effect of the gonadotropin surge, the dominant follicle ruptures and releases the mature oocyte for fertilization, and the remaining granulosa and theca cells form the corpus luteum (see Figure 8.1).

1.1.2 Inhibition of premature luteinization

During the antral follicle stage, a very important physiologic function is the prevention of premature luteinization to maintain follicle growth and somatic cell proliferation. Animal studies have shown that oocyte-derived GDF9, BMP6 and BMP15 act to inhibit premature luteinization by suppressing gonadotropin-driven progesterone production (9). After the release of the oocyte at ovulation, the inhibitory effects of these oocyte-derived growth factors are lost and followed by the luteinization. The mechanisms of these growth factors may be distinct, as BMP15 exerts its effect by suppressing FSH receptors (10), whereas BMP6 inhibits the activity of adenylyl cyclase (11). In concert with the actions of granulosa cell-derived BMPs (BMP2, BMP5 and BMP6), oocyte-derived GDF9 and BMP15 have also been shown to promote follicular survival by stimulating cell proliferation and inhibiting luteinization (12). Acting as paracrine modulators, theca cell-derived BMP4 and BMP7 can enhance cell proliferation while suppressing progesterone and androgen production (2). Meanwhile, activin A, TGF-β and BMP6 originating in granulosa cells up-regulate FSH receptor expression and enhance FSH-induced aromatase activity in large antral follicles (2). Taken together, these data highlight the integral functions of

the oocyte, granulosa cells and theca layer in the follicular structure, which protects the growing follicles from undergoing atresia resulting from premature luteinization induced by LH.

1.1.3 Cumulus expansion

During the development of the antral follicle, which featuresthe formation ofa fluid-filled antrum, the granulosa cells originally surrounding the oocyte differentiate into two anatomically and functionally distinct sublineages. Mural granulosa cells line the follicle wall and reside close to the basement membrane and theca cells, whereas the cumulus cells, whichare intimately associated with the oocyte, form an elaborate structure called the cumulus oophorus complex (COC) (13). These two types of cells exhibit highly divergent responses to gonadotropins and growth factors during the periovulatory stage (14). The LH surge initiates the process of ovulation by reactivating oocyte meiosis, generating tissue restructuring and inducing the expansion of the COC (15, 16). After the LH surge, COC expansion (cumulus expansion) characterized by hyaluronan-based extracellular matrix production is initiated under the control of several endocrine and oocyte-derived factors (17-19). During the assembly of the extracellular matrix, pentraxin 3 (PTX3) functions as an aggregating reagent by linking to the tumor necrosis factor-stimulated gene 6 protein (TSG-6), which is bound to the distinct hyaluronan strand, to form a stable and sustainable hyaluronan network(20, 21). This process is essential for oocyte maturation and ovulation, for effective transportation of the oocyte through the fallopian tubes and for successful fertilization (20, 21). Knockout mice lacking PTX3 expression are subfertile and display defects in the integrity of the COC, as well as failure during *in vivo* fertilization(17, 19).

1.1.4 Steroidogenesis in the ovary

The human ovary is an important steroid-producing endocrine organ that synthesizes all three classes of sex hormones: estrogens, progestins and androgens. The androgens produced by the ovary are critical not only as obligate precursors to estrogens, but also as clinically important secreted substances. Because cholesterol is the building block of all types of steroid hormones, steroidogenesis is the synthetic process used in transforming the cholesterol into alternative types of steroids. Each step of steroidogenesis is mediated by many enzymes and differs from tissue to tissue. Structurally, these steroidogenic enzymes are either dehydrogenases or members of the cytochrome P450 family of oxidative enzymes. Therefore, the number of carbon atoms in cholesterol or any other steroid derivatives will only be reduced, and never increased, by these enzymes during steroidogenesis.

In the gonads and adrenal glands, before the first reaction in the biosynthesis of steroid hormones, cholesterol must be transferred from the outer to the inner mitochondrial membrane, a process that is carried out by steroidogenic acute regulatory protein (StAR) (22). This regulatory protein is critical because StAR controls the rate-limiting step in steroidogenesis. Gene mutations in *StAR* lead to an autosomal recessive disease, congenital lipoid adrenal hyperplasia. In this disease, the synthesis of adrenal and gonadal steroids is severely impaired (23, 24). In the ovary, the expression of StAR primarily occurs in steroid-producing cells, including the granulosa, theca and luteal cells (22, 25).

Ovarian steroid production starts with the conversion of cholesterol (27 carbons) into pregnenolone (21 carbons), which involves hydroxylation of the carbons positions 20 and 22, and the subsequent cleavage of the side chain by the cytochrome P450 side-chain cleavage enzyme (P450scc) (26). This enzymatic step takes place within the mitochondria and is one of

the principal effects of anterior pituitary-derived tropic hormone stimulation. In particular, FSH and LH bind to their G protein-coupled receptors (FSH and LH receptors, respectively) on the cell membrane and activate adenylate cyclase, which further increases intracellular cyclic AMP (27). The actions of cyclic AMP are mediated by protein kinase A (PKA), which leads to gene transcription encoding steroidogenic enzymes and the accessory proteins (28).

The conversion of pregnenolone to progesterone (21 carbons) is catalyzed by 3β-hydroxysteroid dehydrogenase (3βHSD) (29). In some cells, progesterone can be hydroxylated, and the side chain is further cleaved by cytochrome P450 17α-hydroxylase/17, 20-lyase (encoded by *P450c17*) to become androstenedione (19 carbons). Alternatively, pregnenolone can be directly hydroxylated and converted to dehydroepiandrosterone (DHEA), followed by the cleavage of the side chain. The structure of androstenedione can be reversibly transformed into testosterone (19 carbons) by the enzymatic reaction of 17β-hydroxysteroid dehydrogenase (17βHSD).

Aromatization is an enzymatic reaction mediated by P450 aromatase (aromatase), which is located in the endoplasmic reticulum (30). Aromatase is encoded by the *CYP19A1* gene and contains several promoter sites that respond to multiple factors, including cytokines, cyclic nucleotides, gonadotropins, glucocorticoids and growth factors (31). Both androstenedione and testosterone can be converted to estrone and estradiol (both contain 18 carbons), respectively (30).

1.2 Oocyte-somatic cell interactions

It has been widely accepted that oocytesare not the passive recipients of developmental signals from the cumulus/granulosa cells while they reside inside the growing follicles; rather, they are

active modulators and governors of follicle growth and ovulation. Within this microenvironment, a highly coordinated interplay between oocytes and the somatic supporting cells of the ovarian follicles governs the development and maturation of these cells,as well as acquisition of meiotically competent oocytes (32). To achieve this goal, the oocytes promote follicle growth and direct granulosa differentiation by secreting paracrine growth factors that act on the neighboring granulosa cells, which in turn modulate oocyte development and maturation. In addition to pituitary-secreted gonadotropins, oocyte-secreted GDF9 and BMP15 also participate in the regulation of several specific target genes related to ovulation and luteinization (33, 34). However, the identities of these oocyte-derived growth factors modulating such critical ovarian functions remain largely unknown. A comprehensive understanding of the nature of oocyte-somatic cell interactions at various follicle stages is critical to the development of clinical approaches for the women suffering from ovulation dysfunction and infertility.

1.2.1 Oocyte-secreted factors regulate granulosa cell function

Based on animal studies (sheep and mouse), both genetic deficiencies and experimental ablation of the oocyte can lead to the failure of folliculogenesis, indicating the important role of oocyte paracrine signaling in follicular development. In this regard, oocyte-secreted factors regulate follicular development by modulating various activities of the granulosa cells, including cell growth and cell differentiation (35). *In vitro s*tudies have revealed that factors secreted by oocytes may have a potent mitogenic effect to promote cumulus/mural granulosa cell DNA synthesis and cell proliferation (36, 37). Moreover, this oocyte-derived mitogen also interacts with and augments the growth-promoting activities of several well-known granulosa cell regulators, including IGF-I, FSH and androgens (38-41). On the other hand, oocytes are potent

regulators of cumulus/granulosa cell differentiation (32). Specifically, oocytes can regulate FSH-stimulated estradiol and progesterone synthesis by cumulus and mural granulosa cells (42-44) and suppress FSH-induced LH receptor mRNA expression (45). Collectively, these data support the previously proposed hypothesis that oocytes are capable of inhibiting follicular luteinization (46).

1.2.2 Oocyte-secreted factors maintain the cumulus cell phenotype

In antral follicles, the functional characteristics of cumulus cells and mural granulosa cells are markedly distinct. Compared to mural granulosa cells, cumulus cells have a higher proliferation rate, lower steroidogenic capacity, lower LH receptor expression level and the capacity to secrete hyaluronic acid for cumulus expansion (38, 40, 45). In bovine antral follicles, cumulus cells have a higher capacity to incorporate tritiated thymidine and produce lower levels of progesterone than the mural granulosa cells from the same follicles (40). These findings were further confirmed by an experiment that used a microsurgical technique to remove the oocyte contents from the cumulus-oophorus complex. This oocyte-lacking complex then displays an *in vitro* phenotype that is more similar to that of mural granulosa cells (47). Interestingly, coculturing this oocyte-lacking complex with denuded oocytes eliminated this mural granulosa cell-like phenotype and restored the features of cumulus cells (40). Collectively, these results suggest that oocytes are responsible for the active maintenance of the cumulus cell phenotype by releasing certain paracrine factors. The maintenance of this specialized microenvironment during antral follicle development is critical to facilitating the oocyte meiotic resumption and cytoplasmic maturation in preparation for fertilization.

1.2.3 Oocyte-secreted factors regulate cumulus expansion

Cumulus expansion is induced by the pre-ovulatory signaling cascade initiated by the LH surge. After the LH surge, cumulus expansion characterized by hyaluronan-based extracellular matrix production is initiated under the control of several endocrine and oocyte-secreted factors (17-19). These mucoid extracellular matrix proteins are produced by cumulus cells. Among these proteins, hyaluronan is the principle structural backbone of the matrix, which is further stabilized by a complicated network of hyaluronan-binding proteins, including PTX3, versican, inter-α trypsin inhibitor (IαI) and TSG-6 (48, 49). In *in vitro* culture systems, either oocytes or oocyte-conditioned medium are required for the response of cumulus cells to FSH, cyclic AMP or EGF for hyaluronan synthesis (47, 50, 51). The key enzyme that polymerizes and elongates hyaluronan chains into the intercellular space in cumulus cells is hyaluronansynthase type 2 (HAS2), a transmembrane protein (52). In *in vitro* expansion systems, exogenous GDF9, but not BMP15, has been shown to replace oocytes or oocyte-conditioned medium in the induction of cumulus expansion (53). Results from real-time PCR demonstrated that GDF9 stimulated the mRNA expression of HAS2 (53). It has been shown that an inflammatory-like reaction, featuring the induction of cyclooxygenase-2 (COX-2) expression and prostaglandin synthesis by granulosa cell is required for cumulus expansion and subsequent ovulation. Failed prostaglandin synthesis leads to defective cumulus expansion, impaired ovulation and infertility (54, 55). In mouse preovulatory granulosa cells, oocyte-derived GDF9 stimulates COX2 mRNA within 2 hours and subsequent prostaglandin E2 production within 6 hours, suggesting that COX2 is a direct downstream target of GDF9 (56).

1.2.4 Ineraction between FSH/EGFR and the TGFβ/GDF9 signaling pathway

In mouse preovulatory follicles, LH stimulates the production of several EGF-like factors, amphiregulin, epiregulin and betacellulin, which further induce cumulus expansion and ovulation (57).In addition, the activation of EGFR signaling together with stimulation by the cyclic AMP pathway in mouse cumulus cells may induce cumulus expansion and oocyte maturation (57). Our recent studies have demonstrated that three EGF-like factors triggered by LH induced prostaglandin E2 production by up-regulating COX2 expression in human granulosa cells (58). Interestingly, the activation of the SMAD2/3 signaling pathway (downstream of GDF9) is involved in porcine cumulus expansion (59). Furthermore, treatment of SB431542, a BMP type I receptor (ALK4/5/7) kinase inhibitor, led to the complete blockage of GDF9- and oocyte-activated cumulus expansion in the FSH/EGF-stimulated mouse cumulu-oophorus complex (33, 60). It is likely that the SMAD2/3 phosphorylation induced by the TGFβ/GDF9 produced by the cumulus/oocyte contributes to linking the gonadotropin/EGFR induction of cumulus expansion. Taken together, close cross talk between the FSH/EGFR and TGFβ/GDF9 signaling pathways is a prerequisite for the functional activities of cumulus expansion and final oocyte maturation.

1.2.5 Expression of the cumulus matrix components is associated with oocyte quality

Gene expression in the cumulus and the synthesis of the cumulus-oophorus complex has been linked to oocyte quality and developmental capacity. In addition, the extent of expansion of the cumulus is used as a criterion for oocyte selection in IVF procedures (61, 62). Oocyte quality is closely correlated with the expression of CD44 in the cumulus cells, as well as the concentrations of hyaluronan in the follicular fluid (63). Moreover, the expression levels of COX2, HAS2 and PTX3 are correlated with oocyte quality (64, 65).

1.3 Somatic theca cells

In the ovarian follicle, the oocyte and supporting somatic cells (theca and granulosa cells) constitute three principal and distinct components with specific functions in oogenesis, folliculogenesis and steroidogenesis. In terms of development, theca cells are derived from the mesenchymal tissue, whereas granulosa cells are derived from the epithelial tissue. These two types of cells are highly differentiated and are well coordinated to achieve the goal of generating meiotically competent oocytes prepared for fertilization. During the developmental process, a strict regulatory system is controlled by gonadotropins, gonadal steroids, cytokines and various ovarian growth factors in a stage-specific manner (66).

Theca cells play a critical role in folliculogenesis from many different perspectives. First of all, theca cells are the principle source of ovarian androgen, which are the steroid precursors for estrogen production in the neighboring granulosa cells. In addition, animal studies in both *in vivo* and *in vitro* systems have shown that theca cell-derived BMP4 and BMP7 promote follicular growth beyond the primary follicle stage (7, 67). Moreover, theca cell- and stroma-derived hepatocyte growth factor (HGF) and keratinocyte-growth factor (KGF) (also known as fibroblast growth factor-7, FGF-7) have been shown to promote the induction of kit-ligand in granulosa cells. Through bi-directional communication, kit-ligand, in turn, stimulates the expression of HGF and KGF in theca cells. KGF originating in the theca cells has been implicated as a positive regulator of the transition from a primordial to primary follicle (68, 69). Interestingly, in mice, the depletion of the *GDF9* gene leads to the impairment of theca cell recruitment to surround the basement membrane of the ovarian follicles, indicating a close interaction between oocyte and somatic theca cells (70).

During the rapid growth period of pre-antral and antral follicles, theca cells also participate in the modulation of steroidogenesis. In this regard, theca cells act as luteinization inhibitors, because both BMP4 and BMP7 promote FSH-induced estradiol production, while attenuating FSH-induced progesterone synthesis (71). BMP4 and BMP7 may also enhance basal and insulin-like growth factor (IGF)-stimulated estradiol synthesis in cows and suppress progesterone and androgen synthesis in mice (2). Other paracrine effects include the enhancement of granulosa cell proliferation and induction of inhibin A, activin A and follistatin secretionby BMP4 and BMP7 (2).

Apart from the preantral folliculogenesis and steroidogenesis, two-way signaling between oocytes and theca cells with their derived growth factors has been shown to play a critical role in follicular atresia. Higher concentrations of LH can induce apoptosis in the granulosa cells of antral follicles, whereas theca-derived BMP7 together with oocyte-derived GDF9 are thought to prevent the apoptotic cell death associated with atresia (46). On the other hand, FSH stimulates the meiosis-arresting factor originating from the granulosa cells and theca cells directly, augmenting FSH-stimulated meiosis-inhibiting activity. In bovine follicles, geminal vesicle breakdown (GVBD) was delayed when the follicles were cultured with bovine theca cells; this meiosis-arresting effect was intensified by the addition of FSH (72).

Taken together, these data suggest that ovarian theca cells act as inhibitors of luteinization of the granulosa cells, protecting them from premature luteinization. Indeed, somatic theca cells have a more significant role in the regulation of ovarian function than was previously believed.

1.4 Intraovarian cell-cell communication

Gap junctions mediate cell-cell communication by allowing the passage of ions, metabolites and small signaling molecules (up to approximately 1 kDa) between adjacent cells (73, 74). A single gap junction is composed of two connexons (or hemichannels) that join the apposed cell membranes and span the intercellular space (75). Each connexon consists of six symmetric subunits of various isotypes (there are 20 members in mice and 21 members in humans) of the transmembrane protein connexin (76-78). In many tissues, these connexin-coupled channels form clusters at membrane junctions called gap junction plaques (73). In mammals, ovarian follicles are the functional units of female reproductive biology. Coordination withinthis physiological compartment between the oocyte, the cumulus/granulosa cells and the theca cells, relies on functional gap junctions (79). In the developing follicles, gap junctions between cumulus/granulosa cells are composed primarily of connexin43 (Cx43), whereas connexin37 (Cx37) mainly contributes to the gap junctions that connect the oocyte to the circumjacent cumulus cells (80, 81). The importance of connexins in ovarian development and folliculogenesis has been demonstrated by studies examiningconnexin knockout mice. Mice lacking Cx37 exhibit an abolishment of intercellular coupling between oocytes and cumulus cells, disrupted follicle development at the antrum formation stage, ovulatorydysfunction and incompetent oocytes (81). Cx43 is expressed strongly in the granulosa cells throughout all stages of follicular development, and Cx43-coupling gap junctions are required to sustain granulosa cell proliferation (82, 83). The ablation of Cx43 leads to a reduced number of germ cells in the fetal gonads, retarded growth of oocytes and failed fertilization (82).

Given the pivotal role of Cx43-coupled gap junctions in sustaining normal ovarian function, the study of the regulation of gap junction activity and its formative proteins has been a subject

of considerable research. There is accumulating evidence that the cyclic expression of Cx43 in granulosa cells of growing follicles is developmentally and hormonally regulated (84-88). In the rat, the expression patterns of Cx43 in ovarian follicles positively correlate with changes inthe serum levels of gonadotropins, indicating a role for gonadotropins in the regulation of Cx43 expression in the female reproductive system (80, 87). Specifically, FSH up-regulates the expression of Cx43 mRNA and protein, whereas LH or human chorionic gonadotropin down-regulates Cx43 expression, leading to the subsequent loss of intercellular coupling within rat granulosa cell layers (79, 80, 87). In addition to the endocrine effects of gonadotropins, locally released steroid hormones such as estrogen, progesterone and androgen have been reported to regulate Cx43 and gap junction intercellular (GJIC) activity in several species, including humans (89-91).

1.5 TGFβ superfamily

1.5.1 Lignads

The transforming growth factor β (TGFβ) superfamily of growth factors, which contains over 40 members, including TGFβs (TGFβ1, TGFβ2 and TGFβ3), activins (at least 7 members), inhibins (inhibin A and inhibin B), GDFs (at least 9 members), BMPs (more than 20 members), glial cell-derived neurotrophic factor (GDNF), nodal, lefty and anti-Müllerian hormone (AMH), are pivotal for the development and homeostasis of multicellular animals (92). During their evolution, these ligands and their downstream signaling pathways have been well conserved, and they are involved in the regulation of diverse cellular functions, including proliferation, adhesion, motility, migration, apoptosis, and differentiation (93). In addition, the actions of these ligands are modulated in a time- and space-dependent manner during embryonic development and

organogenesis. Moreover, members of this superfamily share a common feature, as the mature bioactive forms of their ligands are homodimers or heterodimers covalently linked by a disulfide bond between conserved Cys residues. Initially, TGFβ superfamily members start with a pre-proprotein, which is composed of a C-terminal mature region, a pro-region (with 200-300 amino acids) and a short N-terminal signal peptide (94). The signal peptide directs the pre-protein to the endoplasmic reticulum and the subsequent secretory pathway, whereas variations in the pro-regions are responsible for the dimerization and folding of the mature proteins that determine the unique structures of the ligands (95).

1.5.2 Signaling receptors

The members of the TGFβ superfamily exert their effects on target cells by acting as ligand dimers to bind and activate heteromeric complexes of type I and type II transmembrane serine/threonine (Ser/Thr) kinase receptors (93, 96). To date, five type II receptors (TβR2, BMPR2, ACVR2A, ACVR2B, and AMHR2) and seven type I receptors (also known as activin receptor-like kinase 1-7) (ALK 1-7) have been identified in mammals (97, 98). These receptors share a similar structure, including an N-terminal extracellular domain, a transmembrane domain and a C-terminal intracellular Ser/Thr kinase domain. Each ligand can trigger the signaling complexes by binding to the N-terminal extracellular domains of different combinations of type II and type I receptors.

1.5.3 Signal transduction pathway

Receptor activation results from the phosphorylation of the intracellular kinase domains, leading to the activation and phosphorylation of the downstream signaling molecules, receptor-

regulated SMAD (R-SMAD; SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8): SMAD2/3 in response to activins, GDFs and TGFβs, whereas SMAD1/5/8 in response to BMPs, and AMH (See Figure 1.1 and Figure 1.2). Upon activation, R-SMAD associates with a common SMAD (Co-SMAD or SMAD4) and translocates into the nucleus to regulate target gene expression by interacting with various transcription factors, co-activators and co-repressors (99, 100). SMAD activation can be further modulated by many factors, including the inhibitory SMAD (I-SMAD), SMAD6 and SMAD7, which are the negative regulators of SMAD activity (100, 101).

1.5.4 The roles of TGFβ superfamily members in the regulation of ovarian function

Members of the TGFβsuperfamily play critical roles in many cellular and physiological functions (100). To date, accumulating evidence has shown that these growth factors are also involved in the intraovarian regulation of follicular development (review in (102)). Studies from animals have revealed that oocytes, granulosa cells and theca cells express various TGFβsuperfamily members in a developmental-stage-related manner. These growth factors play pivotal roles in the different stages of folliculogenesis, including the initiation of primordial follicles, proliferation and apoptosis of somatic cells, synthesis of steroid hormones, expression of gonadotropin receptors, oocyte maturation, ovulation, luteinization and formation of the corpus luteum (2, 103). However, most of these studies were performed using animal models or naturally occurring genetic mutations in sheep and humans. Therefore, a detailed investigation of the functional roles of the members of TGFβ superfamily in ovarian biology remains a subject of considerable research.

1.5.5 Bone morphogenetic proteins (BMPs)

In the TGFβ superfamily, BMPs comprise one of largest subgroups of polypeptide homodimers with pivotal physiological functions in morphogenesis and organogenesis in both vertebrates and invertebrates (104, 105). BMPs were initially identified due to their ability to induce ectopic bone formation; however, BMPs are also essential mediators of many developmental and physiological processes (106).

To date, over 20 BMP members have been identified in human tissues, some of which are expressed in the ovary. The development and physiological functions of the basic units of the female reproductive system, the ovarian follicles, are modulated by the spatial and tissue-specific expression of BMP family members (12). Specifically, granulosa cells express BMP2, BMP5 and BMP6; oocytes express BMP6 and BMP15; and theca cells express BMP4 and BMP7 (2). Increasing evidence suggests that BMP in theca cells is a putative luteinization inhibitor acting to inhibit progesterone production (71) and to promote the survival and development of growing follicles (7). In particular, treatment with BMP4 and BMP7 attenuates FSH-stimulated progesterone production in rat granulosa cells (71). In bovine granulosa cells, BMP4, BMP6 and BMP7 enhance basal and IGF-stimulated estradiol, inhibin A, activin A and FST secretion, but suppress progesterone secretion (107). In contrast, in rat granulosa cells BMP6 attenuates FSH-induced progesterone production but hasno effect on FSH-induced estradiol production (108). Similarly, BMP5 suppresses basal and FSH-induced progesterone production in rat granulosa cells (109). On the other hand, BMP2 enhances FSH-induced estradiol secretion in sheep granulosa cells (110). These discrepancies may be attributed to inter-species differences or potential mechanisms by which the BMP-driven activation and inhibition of gene expression is tissue- or cell type-dependent (111). Because most of the knowledge and understanding of the

putative roles of BMPs in ovarian function is based on studies in mice, rats, cows and sheep, a comprehensive examination of the functional roles of BMPs in human granulosa cell is urgently required.

1.5.6 BMP15 and GDF9

BMP15 (also known as GDF9B) is a close relative of GDF9 and displays a similar oocyte-specific ovarian expression pattern (112). These oocyte-derived growth factors, in coordination with the other intrafollicular signals, regulate follicular development and oocyte maturation, predominantly acting on the surrounding granulosa cells via paracrine signaling (113).

BMP15 is specifically expressed in oocytes from the early follicular stage through ovulation in several species, including humans (114, 115). Studies in sheep have identified a number of *BMP15* mutations associated with an increased ovulation rate in heterozygotes and infertility in homozygotes (116, 117). Likewise, in humans, an important role for BMP15 in female fertility is suggested by studies identifying *BMP15* mutations in women with premature ovarian failure, polycystic ovary syndrome and dizygotic twins (118-121). However, findings from gene-knockout mice indicate that BMP15 may not regulate folliculogenesis and the ovulation rate in the same manner across all species. Indeed, female homozygous *BMP15* null mice have normal follicular development and display only mild subfertility due to minor defects in their ovulation and fertilization rates(122). However, *in vitro* studies with rat granulosa cells have demonstrated that BMP15 promotes cell proliferation, suppresses FSH receptor expression and stimulates Kit ligand expression (10, 123, 124). Given the persistent expression of BMP15 by oocytes throughout follicular development (including ovulated cumulus-oocyte complexes) (115), and positive correlations between follicular fluid BMP15 levels and the numbers of high-quality

oocytes and embryos (125), BMP15 likely plays a broader role in the regulation of follicular development and function.

In addition to their regulatory roles in folliculogenesis, oocyte-derived growth factors are important regulators of ovarian steroidogenesis. Although animal studies have identified many of the known biological functions of GDF9 and BMP15 in granulosa cells; it is uncertain to what extent these functions accurately approximate their real activities in human granulosa cells (122, 126, 127). Indeed, GDF9 has no effects on basal or FSH-induced StAR levels or progesterone production in human granulosa-lutein cells (128, 129), even though StAR and/or progesterone production are up-regulated by GDF9 in mouse and rat granulosa cells (53, 130). In contrast, BMP15 inhibits the FSH-induced StAR levels and progesterone production but has little effect on the basal levels in rat and hen granulosa cells (10, 123, 131). As of yet, the role of BMP15 in the regulation of steroidogenesis in human granulosa cells has not been defined.

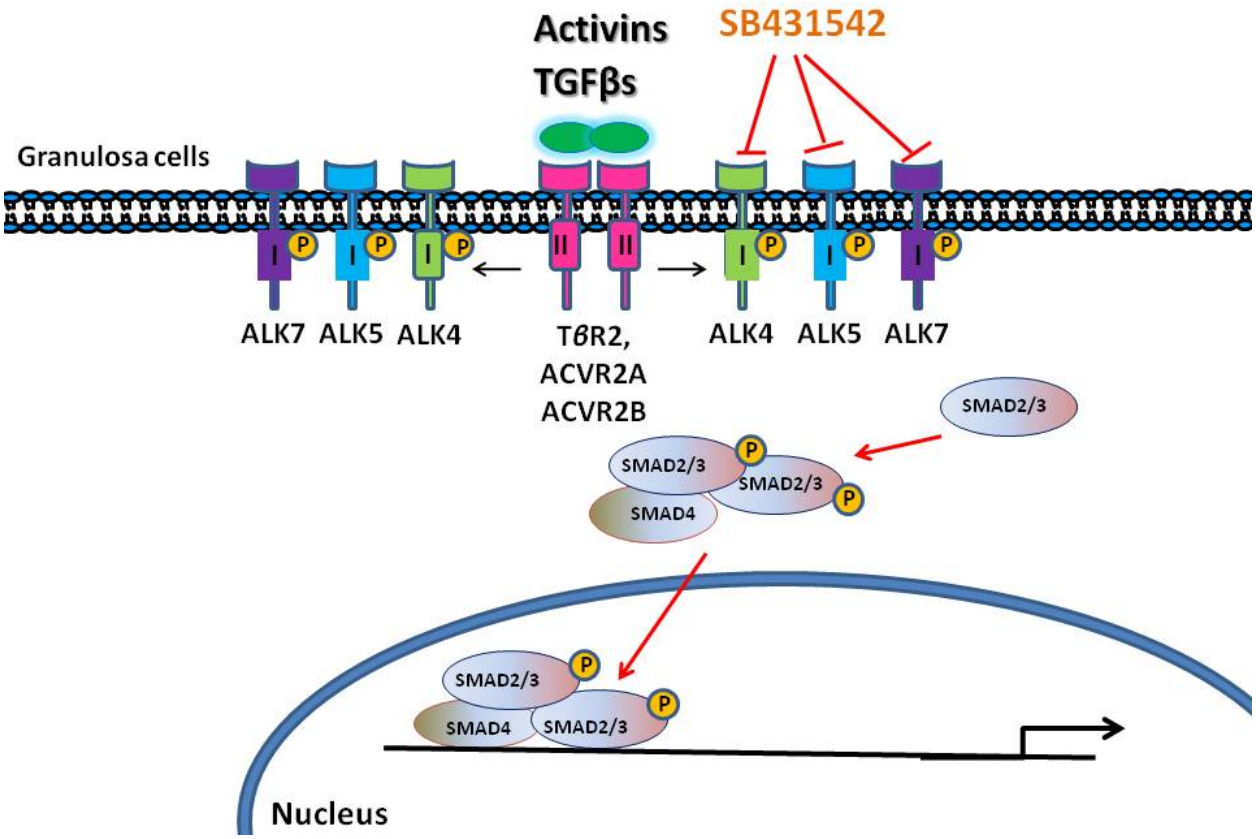


Figure 1.1 Inhibition of activin/TGFβ and SMAD2/3 signaling by the inhibitor SB431542

Inhibition of activin/TGFβ and SMAD2/3 signaling by the inhibitor SB431542. Activins and TGFβs signal to granulosa cells by binding initially to the three activin type II receptors, ActRIIA, ActRIIB and/or TGFβIIB (also known as ACVR2A, ACVR2B and TβR2, respectively), and by recruiting type I receptors, ALK4, ALK5 and/or ALK7. These activated type I receptors phosphorylate the intracellular mediators of signaling, SMAD2 and/or SMAD3. Upon activation, phosphorylated SMAD2/SMAD3 associate with SMAD4 and translocate into the nucleus to regulate the target genes. SB431542 is a potent and selective inhibitor of ALK4, ALK5 and ALK7 type I receptors.

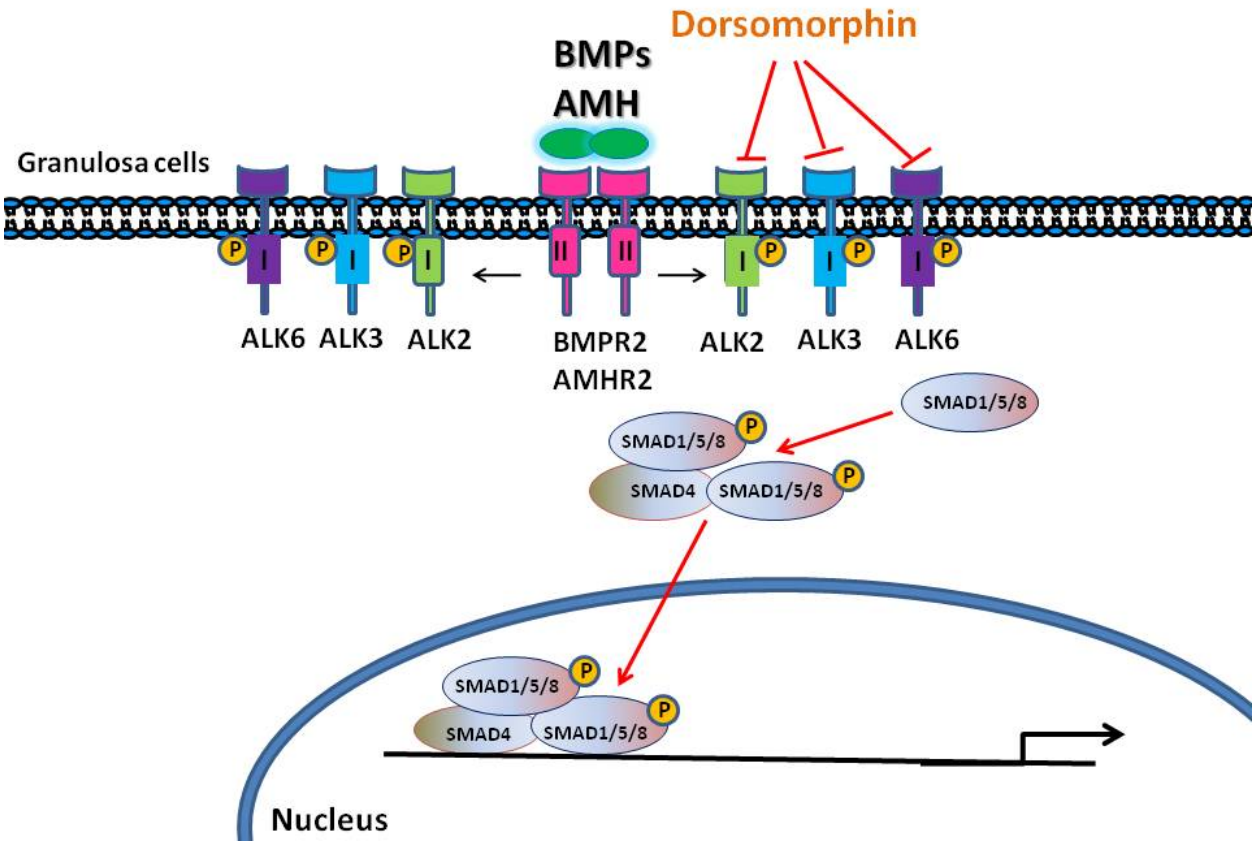


Figure 1.2 Inhibition of BMP/AMH and SMAD1/5/8 signaling by the inhibitor dorsomorphin

BMPs and AMH signal to granulosa cells by binding initially to the two type II receptors, BMPR2 and AMHR2, and then by recruiting type I receptors, ALK2, ALK3 and/or ALK6. Activated type I receptors phosphorylate the intracellular mediators of signaling, SMAD1, SMAD5 and/or SMAD8. Upon activation, phosphorylated SMAD1/5/8 associate with SMAD4 and translocate into the nucleus to regulate the target genes. Dorsomorphin is a potent and selective inhibitor of ALK2, ALK3 and ALK6 type I receptors.

Chapter 2: Rationale and objectives

2.1 Rationale

In mural granulosa cells, luteinization is the final differentiation stage that is provoked by an ovulatory stimulus. Premature luteinization,which is associated with high serum progesterone levels, may adversely affect oocyte quality and the implantation environment (132). A pioneering study showed that the removal of the oocyte-cumulus complex led to the precocious luteinization of rabbit follicles *in vivo* (46), indicating that oocytes may secrete an anti-luteinization factor. Subsequent studies have supported the long-standing hypothesis that oocytes can regulate the production of progesterone, a hallmark of luteinized cells, by granulosa cells from the time of follicular development throughout ovulation (10, 53). Aside from progesterone, the underlying mechanisms that modulate the luteinization process include intracellular signaling pathways, cell adhesion factors, intracellular cholesterol and oxysterols (133). The trafficking and exchange of these molecules among cumulus granulosa cells relies on functional Cx43-channeled gap junctions. Therefore the expression pattern of Cx43 can be regarded as another parameter of luteinization. Previous clinical data have also demonstrated that a reduction in Cx43 expression within cumulus cells at the time of oocyte collection yields good embryo competence (134).

Increasing evidence suggests that BMP in theca cells is a putative luteinization inhibitor and acts to inhibit progesterone production (71), promoting the survival and development of growing follicles (7) in granulosa cells. In particular, treatment with BMP4 and BMP7 attenuates FSH-stimulated progesterone production in rat granulosa cells (71). In bovine granulosa cells, BMP4 and BMP7 suppress progesterone secretion (107). A higher concentration of LH can induce

apoptosis in the granulosa cells of antral follicles, whereas thecal BMP7 together with oocyte GDF9 have been thought to prevent the apoptotic cell death associated with atresia (46). On the other hand, FSH stimulates the meiosis-arresting factor originating from granulosa cells and theca cells that directly augments FSH-stimulated meiosis-inhibiting activity. In bovine follicles, geminal vesicle breakdown (GVBD) was delayed during culture with bovine theca cells; this meiosis-arresting effect was intensified by adding FSH (72). Collectively, these data highlight the integral functions of the thecal layer in the follicle structure that protects the growing follicles from undergoing atresia resulting from premature luteinization incurred by LH (135).

Taken together, these results lead us to propose that the oocytes and their somatic theca cells may secrete anti-luteinization factors to modulate the functions of the neighboring granulosa cells in a paracrine manner.

2.2 Overall hypothesis

Our central hypothesis is that during the late follicular stage, oocyte-derived and theca cell-derived BMPs may prevent premature luteinization by down-regulating several ovulation-related genes.

2.3 Aim of the study

The general aim of this study was to investigate the anti-luteinization effects of oocyte- and theca cell-derived BMPs in human granulosa cells.

2.4 Study models

To clearly understand the functions and underlying mechanisms of the ovarian physiology, materials that are suitable for *in vitro* studies are required. During the terminal stage of folliculogenesis, granulosa cells proliferate and undergo differentiation and luteinization. Therefore, human granulosa-lutein cells obtained from infertile women undergoing their in vitro fertilization procedure have often been used to study the follicular functions during the periovulatory stage. Since these cells are obtained from individual patients and never combined, we are only able to obtain a relatively small number of cells for each experiment. Because of this obstacle, a substitute human granulosa cell line has become an attractive option. We previously produced a non-tumorigenic immortalized human granulosa-lutein cell line (SVOG) by transfecting human granulosa-lutein cells with the SV40 large T antigen (136). SVOG cells are steroidogenic and provide a feasible model system to study the biological functions of granulosa cells in humans(136). In addition, to obtain more information on human granulosa cellfunction, we also include a human granulosa cell tumor-derived cell line (KGN) to examine the cell responsiveness to the growth factors.

2.5 The specific objectives of this study

Objective 1: To investigate the effects of oocyte-derived GDF9 and BMP15 on the regulation of steroidogenesis in human granulosa cells. (Presented in Chapter 3)

1) To investigate the effect of GDF9 and BMP15 on the expression of steroidogenic enzymes

2) To determine the effects of GDF9 and BMP15 on SMAD signaling

3) To investigate the SMAD1/5/8 signaling-mediated down-regulation of StAR

4) To determine the specific BMP type I receptor (ALK 2/3/6) involved in the BMP15-induced SMAD1/5/8 phosphorylation and down-regulation of StAR

5) To examine the effect of BMP15 on progesterone production

Objective 2: To investigate the effects of oocyte-derived GDF9 and BMP15 on the regulation of cell-cell communication in human granulosa cells. (Presented in Chapter 4)

1) To investigate the effect of GDF9 and BMP15 on the expression of Cx43

2) To examine the localization and distribution of Cx43 in SVOG cells

3) To determine the effects of GDF9 and BMP15 on SMAD signaling

4) To investigate the SMAD signaling-mediated down-regulation of Cx43

5) To examine the effects of BMP15 on gap junction intercellular communication

6) To confirm the effect of BMP15 on Cx43 expression in human granulosa-lutein (hGL) cells

Objective 3: To investigate the effects of theca cell-derived BMP4 and BMP7 on the regulation of cell-cell communication in immortalized human granulosa cells. (Presented in Chapter 5)

1) To investigate the effect of BMP4 and BMP7 on the expression of Cx43 in SVOG cells

2) To determine the effects of BMP4 and BMP7 on SMAD signaling

3) To investigate the SMAD signaling-mediated down-regulation of Cx43

4) To examine the effects of BMP4 and BMP7 on gap junction intercellular communication

Objective 4: To investigate the effects of theca cell-derived BMP4 and BMP7 on the regulation of pentraxin 3 expression. (Presented in Chapter 6)

1) To investigate the effect of BMP4 and BMP7 on the expression of PTX3 in SVOG cells

2) To confirm the effects of BMP4 and BMP7 on the expression of PTX3 in hGL and KGN cells

3) To determine the effects of BMP4 and BMP7 on SMAD signaling

4) To investigate the SMAD signaling-mediated down-regulation of PTX3

5) To determine the specific BMP type I receptor(s) (ALK 2/3/6) involved in the BMP4- and BMP7-induced SMAD1/5/8 phosphorylation and down-regulation of PTX3

Objective 5: To investigate the effects of theca cell-derived BMP4 and BMP7 on the regulation of furin expression and activin A production in immortalized human granulosa cells (Presented in Chapter 7)

1) To investigate the effect of BMP4 and BMP7 on the expression of inhibin subunits and activin A production in SVOG cells

2) To investigate the effects of BMP4 and BMP7 on the expression of furin in SVOG cells

3) To examine the localization and distribution of furin in SVOG cells

4) To determine the effects of BMP4 and BMP7 on SMAD signaling

5) To investigate the SMAD signaling-mediated up-regulation of furin

6) To determine the involvement of furin in BMP4- and BMP7-induced increases in activin A production

Chapter 3: BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells

3.1 Introduction

Transforming growth factor-β (TGF-β) superfamily members, including TGF-β, activins/inhibins,growth differentiation factors (GDFs), anti-Müllerian hormone and bone morphogenetic proteins (BMPs), play crucial roles in the regulation of ovarian functions (102). Among them, the oocyte-derived growth factors GDF9 and BMP15 (also known as GDF9B), in coordination with the other intrafollicular signals, regulate follicular development and oocyte maturation, predominantly acting on surrounding granulosa cells via paracrine signaling (113).

BMP15 is specifically expressed in oocytes from the early follicular stage through ovulation in several species, including humans (114, 115). Studies in sheep have identified a number of *BMP15* mutations associated with increased ovulation rate in heterozygotes and infertility in homozygotes (116, 117). Likewise in humans, an important role for BMP15 in female fertility is suggested by studies identifying *BMP15* mutations in women with premature ovarian failure, polycystic ovary syndrome and dizygotic twins (118-121). However, findings from gene-knockout mice indicate that BMP15 may not regulate folliculogenesis and ovulation rate in the same manner in all species. Indeed, female homozygous *BMP15* null mice have normal follicle development and display only mild subfertility due to minor defects in ovulation and fertilization rate (122). However, *in vitro* studies with rat granulosa cells have demonstrated that BMP15 promotes cell proliferation, suppresses FSH receptor expression and stimulates Kit ligand expression (10, 123, 124). Given the persistent expression of BMP15 by oocytes throughout follicle development (including ovulated cumulus-oocyte complexes) (115), and positive

correlations between follicular fluid BMP15 levels and the numbers of high-quality oocytes and embryos (125), BMP15 likely plays a broader role in the regulation of follicle development and follicular function.

In addition to their regulatory roles in folliculogenesis, oocyte-derived growth factors are important regulators of ovarian steroidogenesis. While animal studies have identified many of the known biological functions of GDF9 and BMP15 in granulosa cells; it is uncertain to what extent these accurately model their functions in human granulosa cells (122, 126, 127). Indeed, GDF9 has no effects on basal or FSH-induced steroidogenic acute regulatory protein (StAR) levels or progesterone production in human granulosa-lutein cells (128, 129), whereas StAR and/or progesterone production are up-regulated by GDF9 in mouse and rat granulosa cells (53, 130). In contrast, BMP15 inhibits FSH-induced StAR levels and progesterone production, but has little effect on basal levels, in rat and hen granulosa cells (10, 123, 131). As yet, the role of BMP15 in the regulation of steroidogenesis in human granulosa cells has not been defined. In the present study, we investigated the effects of BMP15 on steroidogenic enzyme expression and progesterone production as well as the potential underlying mechanisms of these effects in human granulosa cells.

3.2 Materials and methods

Cells and cell culture

A non-tumorigenic immortalized human granulosa-lutein cell line (SVOG), previously produced by transfecting human granulosa-lutein cells with the SV40 large T antigen (136), and a human granulosa cell tumor-derived cell line, KGN, were used in this study. SVOG or KGN cells were counted with a hemocytometer, and cell viability was assessed by trypan blue (0.04%)

exclusion. The cells were seeded (4-8 × 10^5 cells per well in 6-well plates) and cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin (Invitrogen, Life Technologies, NY), 100 μg/ml streptomycin sulfate (Invitrogen, Life Technologies) and 1X GlutaMAX (Invitrogen, Life Technologies). The culture medium was changed every other day in all experiments and cells were maintained in serum-free medium for 24 h prior to growth factors treatments.

Antibodies and reagents

Polyclonal rabbit anti-StAR (sc-25806), polyclonal rabbit anti-SMAD1/5/8 (N-18; sc-6031-R) and monoclonal mouse anti-α-tubulin (B-5-1-2; sc-23948) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-phospho-SMAD1 ($Ser^{463/465}$)/SMAD5 ($Ser^{463/465}$)/SMAD8 ($Ser^{426/428}$) antibody was obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit and donkey anti-goat IgGs were obtained from Bio-Rad Laboratories (Hercules, CA) and Santa Cruz Biotechnology, respectively. Recombinant human BMP15, dorsomorphin dihydrochloride (dorsomorphin) and DMH-1 were obtained from R&D Systems (Minneapolis, MN). Chinese Hamster Ovary (CHO) cell-derived recombinant human BMP15 was >90% pure (SDS-PAGE) and supplied lyophilized from a 0.2 μm filtered solution of 4mM HCl with 0.1% BSA as a carrier protein. Recombinant human GDF9 from BioVision Incorporated (Milpitas, CA) was *E. coli*-derived, >98% pure (SDS-PAGE and HPLC) and supplied lyophilized with no additives.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Madison, WI). Each 20 μl qPCR reaction contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were StAR, 5'- AAA CTT ACG TGG CTA CTC AGC ATC -3' (sense) and 5'- GAC CTG GTT GAT GCT CTT G -3' (antisense); P450 side chain cleavage enzyme (P450scc) (CYP11A1), 5'- CAG GAG GGG TGG ACA CGA C -3' (sense) and 5'- AGG TTG CGT GCC ATC TCA TAC -3' (antisense); 3β-hydroxysteroid dehydrogenase (3β-HSD), 5'- GCC TTC CAG ACC AGA ATT GAG AGA -3' (sense) and 5'- TCC TTC AAG TAC AGT CAG CTT GGT -3' (antisense); FSH receptor (FSHR), 5'- AAC ACC CAT CCA AGG AAT GG -3' (sense) and 5'- GGG CTA AAT GAC TTA GAG GGA CAA -3' (antisense); LH receptor (LHR), 5'- ACA CTT TAT TCT TCC ATG CTT GCT GAG -3' (sense) and 5'- ATT AAA AGC ATC TGG TTC AGG AGC ACA -3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'- ATG GAA ATC CCA TCA CCA TCT T -3' (sense) and 5'- CGC CCC ACT TGA TTT TGG -3' (antisense). qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by evaluating amplification efficiencies by means of calibration curves and ensuring that the plot of log input amount *vs.* ΔCq (also known as ΔCT) has a slope < |0.1|. Alternatively, TaqMan gene expression assays for ALK2, ALK3 and ALK6 (Hs00153836_m1, Hs01034913_g1 and Hs00176144_m1, respectively; Applied Biosystems) were performed in triplicate on

corresponding cDNA samples. For each 20 µl TaqMan reaction, 4 µl cDNA was mixed with 5 µl RNase-free water, 10 µl 2X TaqMan gene expression master mix (Applied Biosystems) and 1 µl 20X TaqMan gene expression assay. The PCR parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq ($2^{-\Delta\Delta Cq}$) method with GAPDH as the reference gene.

Western blot analysis

After treatment, cells were washed with cold PBS and lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µM aprotinin, 1 µM leupeptin and 1 mM PMSF) (Cell Signaling) containing protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 x *g* for 15 min at 4°C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 1 h in TBS containing 0.05% Tween 20 and 5% nonfat dried milk and incubated overnight at 4°C with the relevant primary antibodies. After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence reagents or a SuperSignal West Femto Chemiluminescence Substrate (Pierce, Rockford, IL), followed by exposure to CL-XPosure film (Thermo Fisher, Waltham, MA). Membranes were stripped with

stripping buffer (50 mM Tris-HCl pH 7.6, 10 mmol/l β-mercaptoethanol and 1% SDS) at 50°C for 30 min and reprobed with mouse anti-α-tubulin antibody as a loading control.

Measurement of progesterone

Following the specified treatments, culture medium was assayed immediately or stored at –80°C until assayed. Progesterone accumulation in conditioned medium was measured as per the manufacturer's instructions using a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Inter- and intra-assay coefficients of variation for this assay were less than 10% and the detection limit was 10 pg/ml. Each sample was measured in triplicate, and secreted progesterone levels were normalized to total cellular protein content.

Small interfering RNA (siRNA) transfection

We performed transient knockdown assays with ON-TARGET*plus* Non-targeting Control Pool or separate ON-TARGET*plus* SMARTpools targeting ALK2, ALK3 or ALK6 (Thermo Fisher Scientific, Lafayette, CO). Cells were pre-cultured to 50% confluence in antibiotic-free DMEM/F12 medium containing 10% charcoal/dextran-treated fetal bovine serum and then transfected with 25 nM siRNA using Lipofectamine RNAiMAX (Life Technologies) for 48 h. Knockdown efficiency for each target was confirmed by RT-qPCR.

Statistical analysis

Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests using PRISM software (GraphPad Software, Inc., San Diego, CA). The results are presented as

the mean ± SEM of at least three independent experiments. Data were considered significantly different from each other if $P < 0.05$.

3.3 Results

BMP15, but not GDF9, decreases StAR expression in SVOG cells

To investigate the effect of oocyte-derived BMP15 and GDF9 on steroidogenesis, SVOG human immortalized granulosa cells were treated for 24 h with increasing concentration of human recombinant BMP15 or GDF9 (1, 10, 100 or 500 ng/ml), and the expression of key steroidogenic enzymes was examined. As shown in Figure 3.1, neither BMP15 nor GDF9 affected the mRNA levels of P450scc or 3β-HSD. In addition, treatment with 100 ng/ml GDF9 has no effect on P450scc and 3β-HSD mRNA levels at earlier time points (1, 3 or 6 h; data not shown). Interestingly, treatment with BMP15 significantly decreased StAR mRNA levels, whereas treatment with GDF9 did not (Figure 3.2A). The concentration-dependent suppression of StAR protein levels by BMP15, but not GDF9, was confirmed by Western blot analysis (Figure 3.2B). Moreover, treatment with BMP15 (100 ng/ml) reduced StAR mRNA and protein levels in a time-dependent manner, whereas GDF9 had no effect at any time point examined (Figures 3.2C and 3.2D). Finally, the effect of GDF9 on gonadotropin receptor expression was examined toconfirm the bioactivity of recombinant GDF9 in SVOG cells. As shown in Figure 3.2D and 3.2E, treatment with GDF9 significantly increased FSH and LH receptor mRNA levels in a concentration dependent manner.

BMP15 activates SMAD1/5/8 signaling in SVOG and KGN cells

BMP family members exert their effects by binding to transmembrane serine/threonine kinase receptors. Ligand binding and formation of a ternary complex involving type I (also known as activin receptor-like kinases, ALKs) and type II receptors results in receptor transphosphorylation and subsequent phosphorylation of the downstream signaling molecules SMAD1/5/8 (137). BMP15-induced phosphorylation of SMAD1 has been demonstrated in rat primary granulosa cells and a human granulosa tumor cell line (138). To determine whether BMP15 activates SMAD1/5/8 in SVOG cells, SMAD phosphorylation was measured following treatment with BMP15 (100 ng/ml) for 30 min or 60 min. As shown in Figure 3.3A, treatment with BMP15 increased the phosphorylation levels of SMAD1/5/8 at both time-points. Previous studies have demonstrated that BMP15 and GDF9 signal through SMAD1/5/8 and SMAD2/3, respectively (138, 139). Treatment with GDF9 did not alter SMAD1/5/8 phosphorylation in SVOG cells (Figure 3.3B), however we have previously demonstrated GDF9-induced SMAD2/3 phosphorylation in primary human granulosa-lutein cells (140). Likewise, the ability of BMP15, but not GDF9, to increase the phosphorylation levels of SMAD1/5/8 at both 30 and 60 min was confirmed in KGN cells, a human granulosa tumor cell line (Figure 3.3C).

ALK3 mediates BMP15-induced SMAD1/5/8 signaling in SVOG and KGN cells

Although BMP ligands must bind both type I and type II receptors prior to activating downstream signaling, pathway specificity is mainly determined by the type I receptor (141). To date, three type I receptors (ALK2, ALK3 and ALK6) have been implicated in BMP-induced SMAD1/5/8 phosphorylation (141). To determine whether ALK2, ALK3 or ALK6 are required for BMP15-induced SMAD1/5/8 activation, SVOG cells were treated with BMP15 in the

presence or absence of 1 μM dorsomorphin or 0.5 μM DMH-1. Dorsomorphin is a specific inhibitor of ALK2/3/6, while DMH-1 is a selective inhibitor of ALK2/3 (142, 143).As shown in Figure 3.4A, treatment of SVOG cells with either dorsomorphinor DMH-1 abolished BMP15-induced SMAD1/5/8 phosphorylation. These results suggest that ALK2 or ALK3, but not ALK6, is required for the activation of SMAD1/5/8 by BMP15 in SVOG cells.

To further confirm which ALK(s) mediates BMP15-induced SMAD1/5/8 activation, specific siRNAs were used to knockdown endogenous ALK2, ALK3 or ALK6 in SVOG and KGN cells. As shown in Figure 3.4B and 3.4C, transfection of SVOG or KGN cells with ALK2, ALK3 or ALK6 siRNA significantly down-regulated the mRNA levels of only the targeted ALKs. Interestingly, only knockdown of ALK3 abolished BMP15-induced SMAD1/5/8 phosphorylation, whereas down-regulation of ALK2 or ALK6 was without effect (Figure 3.4D and 3.4E). These results demonstrate that ALK3 type I BMP receptor is required for downstream SMAD1/5/8 signaling in response to BMP15 in both SVOG and KGN cells.

ALK3 is required for BMP15-induced down-regulation of StAR in SVOG cells

Dual pharmacological and siRNA-based approaches were next used to examine the requirement for ALK3 in BMP15-induced down-regulation of StAR. Consistent with the results for BMP15-induced SMAD1/5/8 activation, both dorsomorphin and DMH-1 abolished the inhibitory effects of BMP15 on StAR mRNA and protein levels in SVOG cells (Figures 3.5A and 3.5B). Similarly, in SVOG cells siRNA-mediated knockdown of ALK3, but not ALK6, abolished BMP15-induced down-regulation of StAR mRNA (Figure 3.5C). Unexpectedly, ALK2 knockdown significantly down-regulated basal StAR mRNA levels, although further reductions were observed with BMP15 treatment (Figure 3.5C). Western blot analysis

producedsimilar results concerning the effects of ALK knockdown on BMP15-induced down-regulation of StAR protein levels (Figure 3.5D).

BMP15 suppresses progesterone accumulation in SVOG cells in a dorsomorphin- and DMH-1-sensitive manner

Since StAR is a key regulatory protein in steroidogenesis, we next examined the influence of BMP15 on progesterone accumulation in SVOG cells. As shown in Figure 3.6A, treatment with different concentrations (1, 10, 100 or 500 ng/ml) of BMP15 decreased progesterone accumulation in conditioned medium in a concentration dependent manner. Moreover, the inhibitory effects of BMP15 (100 ng/ml) on progesterone accumulation were abolished by co-treatment with either dorsomorphin (1 μM) or DMH-1 (0.5 μM) (Figure 3.6B). These results indicate the similar BMP type I receptor requirements (likely ALK3) for BMP15-mediated reductions in StAR expression and progesterone accumulation.

3.4 Discussion

There is compelling evidence that oocytes play key roles in folliculogenesis and that the establishment of bidirectional communication between the oocyte and its neighboring supporting cells is essential for the development of a meiotically competent oocyte (144). Previous pioneer experiments have demonstrated that auto-transplantation of intact rat preovulatory follicles results in non-luteinization, whereas oocyte-free explants of either the follicle wall or granulosa cells do undergo morphological luteinization (145). Subsequent studies also confirmed that removal of oocyte from the dominant follicles from rabbits causes spontaneous luteinization of corresponding granulosa and theca cells and elevated progesterone secretion (46). Thus, the

oocyte is thought to be instrumental in preventing the premature luteinization of follicular cells, possibly via the secretion of inhibitors of luteinization.

The present study describes, for the first time, the effects of recombinant human BMP15 on the expression of key steroidogenic enzymes and progesterone production in human granulosa cells. We show that exogenous BMP15 inhibits progesterone production in human granulosa cells by down-regulating the mRNA and protein levels of the rate-limiting regulatory protein StAR. Indeed, the magnitude of BMP15-induced decreases in StAR mRNA and protein levels were consistent with those of progesterone accumulation in conditioned medium. *In vitro* studies with chicken granulosa cells have demonstrated inhibitory effects of BMP15 on gonadotropin-induced, but not basal, StAR protein levels and progesterone production (131). In addition, BMP15 does not affect basal but reduces FSH-induced StAR mRNA levels by suppressing FSH receptor expression in rat granulosa cells (123). In contrast, our results suggest that BMP15 has pronounced effects on basal StAR expression and progesterone production in human granulosa cells, notwithstanding any as of yet unknown effects on gonadotropin-induced responses.

GDF9 is a close relative of BMP15 and displays a similar oocyte-specific expression pattern (112). GDF9 inhibits 8-Br-cAMP-induced StAR expression and progesterone production in human granulosa and theca cells (129). Consistent with our previous findings, GDF9 has no effects on basal StAR protein levels or progesterone production (128). Accordingly, our results therefore suggest divergent roles for BMP15 and GDF9 in the regulation of human ovarian steroid hormone production, likely as inhibitors of luteinization. Secretion of BMP15 from oocytes reduces the expression of StAR and, in turn, suppresses the synthesis of progesterone. In addition, oocyte-derived GDF9 interacts with gonadotropin to inhibit progesterone production.

However, in post-ovulatory luteinized granulosa cells, the absence of BMP15 and GDF9 leads to elevated StAR expression and increased production of progesterone (113).

In rat preantral follicles, exogenous GDF9 has no effect on basal FSH receptor levels, while addition of GDF9 antisense suppresses FSH receptor levels, indicating that endogenous GDF9 can increase basal FSH receptor levels (146). Consistent with these data, our results also show that GDF9 increases FSH receptor mRNA levels in human granulosa-lutein cells. In preovulatory mouse granulosa cells, incubation of the cells with GDF9 suppresses basal and FSH-induced LH receptor mRNA levels (53). These results are inconsistent with our findings that GDF9 increases LH receptor mRNA levels in human granulosa-lutein cells. While this discrepancy could result from species differences, it likely also reflects differences in follicular stage and differentiation (i.e. human granulosa cell models are luteinized and rodent models are not). We and others have demonstrated endogenous GDF9 expression in human granulosa-lutein cells (147, 148), yet its roles in luteal phase granulosa cell function are unknown. In this context, the up-regulation of LH receptor could indicate a role for autocrine/paracrine GDF9 in promoting corpus luteum formation and function following ovulation.

Significant reductions in StAR expression and progesterone accumulation were observed with 10 ng/ml BMP15, and maximal reductions with 500 ng/ml. To date, studies examining BMP15 levels in human follicular fluid have involved only relative quantification by Western blot (125, 149, 150). In the absence of quantified follicular fluid concentrations, it remains uncertain whether the effective concentrations of BMP15 identified in our study are biologically relevant. However, Wu *et al.*(149) performed Western blot with 0.4-1 µl of unconcentrated follicular fluid followed by detection with enhanced chemiluminescent substrate. Considering the volumes used and estimated limits of detection of enhanced chemiluminescent substrates (mid-high fg to low-

mid pg), one might conservatively estimate levels at the limit of detection to be anywhere in the range of 0.5-50 ng/ml. Moreover, it is evident that those studies were performed well above the limit of detection, suggesting that our effective BMP15 concentrations of 10 or 100ng/ml are very likely biologically relevant. Lastly, it is worth noting that follicular fluid concentrations of GDF9 have been reported to be in the range of 55 to 459 ng/ml (151). Nevertheless, future studies will be required to quantitatively determine BMP15 levels in human follicular fluid.

A clear understanding of the molecular determinants of the cellular responses to BMP15 is critical to the development of pharmacological strategies for clinical use. However, in spite of the apparentimportance of BMP15 in female fertility, the receptors responsible for mediating the biological effects of BMP15 remain poorly defined. It is generally understood that BMP-induced SMAD1/5/8 activation is mediated by either ALK2, ALK3 or ALK6 (also known as ActRIA, BMPRIA and BMPRIB, respectively) (152). Currently, ALK6 is widely regarded as the ALK for BMP15; however this is largely based on indirect evidence, with no study definitively addressing which ALK(s) are required for the biological effects of BMP15 in human granulosa cells. Non-cell co-immunoprecipitation studies with BMP15 and recombinant ALK extracellular domain (ECD)/IgG Fc chimeras demonstrated greater co-immunoprecipitation with ALK6-ECD than with ALK2-ECD or ALK3-ECD (138). Likewise, incubation of COV434 human granulosa tumor cells with [^{125}I]BMP15^{S356C} followed by immunoprecipitation of cross-linked receptor-ligand complexes demonstrated greater binding to ALK6 than to ALK2 or ALK3 (153).

In addition to the aforementioned immunoprecipitation studies, Moore *et al.* examined the inhibitory effects of ALK-ECDs on progesterone production and thymidine incorporation in primary rat granulosa cells (138). The inhibitory effects of BMP15 on FSH-induced progesterone production were modestly inhibited by ALK6-ECD whereas ALK3-ECD and ALK2-ECD were

without effect. In contrast, all three ALK-ECDs were equally effective partial inhibitors of BMP15-stimulated thymidine incorporation. More compelling evidence supporting the involvement of ALK6 stems from the inability of human BMP15 to induce SMAD1/5/8 phosphorylation in granulosa cells from ALK6$^{-/-}$ mice (154). However, the relevance of these findings to humans is uncertain since in mouse granulosa cells human GDF9 is largely inactive and human BMP15 uncharacteristically activates both SMAD1/5/8 and SMAD2/3 signaling, the latter also being abolished in ALK6$^{-/-}$ knockout mice. Nevertheless, Peng *et al.* also demonstrated that co-treatment with the ALK2/3/6 inhibitor LDN-193189 abolished BMP15-induced SMAD1/5/8 phosphorylation in COV434 human granulosa tumor cells (154).

The present study used two BMP type I receptor inhibitors (dorsomorphin and DMH-1) to examine which receptors are required for the biological effects of BMP15 in human granulosa cells. Co-treatment with the ALK2/3/6 inhibitor dorsomorphin (143) abolished the effects of BMP15 on SMAD1/5/8 phosphorylation, StAR expression and progesterone production. Surprisingly, the effects of BMP15 were also inhibited by DMH-1, a potent inhibitor of ALK2/3 but not ALK6 (142, 143). Considering the limitations of these inhibitors, we therefore used specific siRNAs to precisely define which ALK(s) mediate the actions of BMP15. Knockdown of ALK3, but not ALK6 or ALK2, abolished the effects of BMP15 on SMAD1/5/8 phosphorylation and StAR expression. Our results strongly suggest that ALK6 and ALK2 are not the major physiological receptors for BMP15 in SVOG and KGN cells. Rather, we provide convincing evidence that the biological effects of BMP15 are mediated by ALK3, which has important implications for the development of specific translational approaches.

Interestingly, we found that knockdown of ALK2 dramatically decreased basal StAR mRNA and protein levels, but did not abolish the effects of BMP15 on StAR expression. Our results

suggest a positive role for ALK2 in maintaining basal StAR expression, which is consistent with a previous study demonstrating significant reductions in FSH-induced progesterone production following treatment of rat granulosa cells with ALK2-ECD (138). Precisely which TGF-β superfamily member acts via ALK2 to enhance StAR expression in human granulosa cells is unknown. The BMP5/6/7/8 subgroup of BMPs are thought to utilize ALK2, ALK3 or ALK6 (152); however BMP5, BMP6 and BMP7 have been shown to exert suppressive effects on basal or FSH-induced progesterone production in rat and/or bovine granulosa cells (71, 107-109). Likewise, anti-müllerian hormone is thought to signal via ALK2, ALK3 or ALK6 (155), but also exerts suppressive effects on basal progesterone production in human granulosa cells (156). Future studies aimed at addressing how ALK2 contributes to the regulation of progesterone production by granulosa cells will be of great interest.

In summary, we have shown that oocyte-derived BMP15, but not GDF9, reduces basal progesterone production by suppressing the expression of the rate-limiting regulatory protein StAR in human granulosa cells. Moreover, our results indicate that the effects of BMP15 are mediated by ALK3 and most likely involve SMAD1/5/8 signaling (See Figure 3.7). These findings provide evidence that oocyte may play a crucial role in preventing premature luteinization by modulating progesterone production especially during the late stage of follicle development.

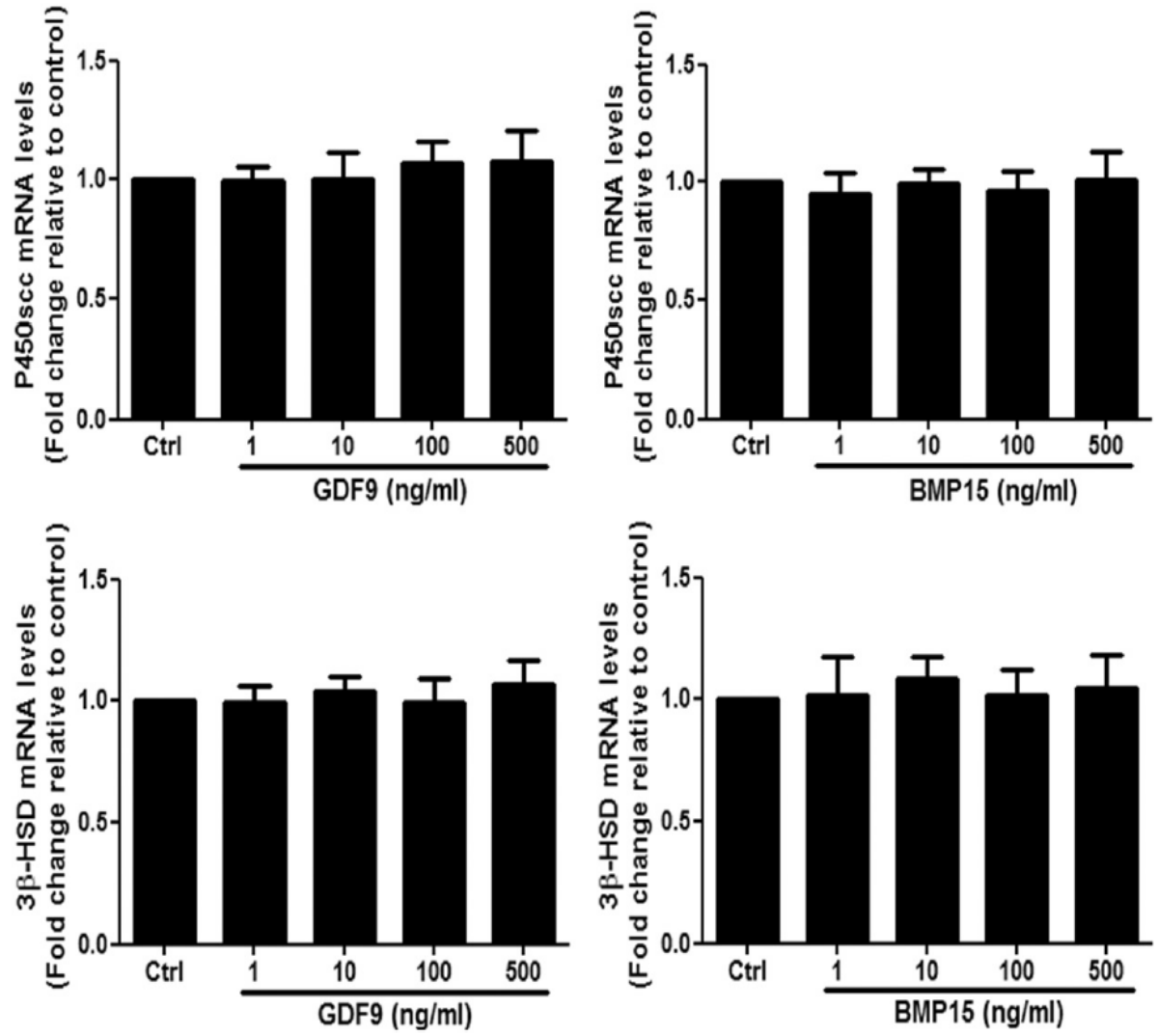
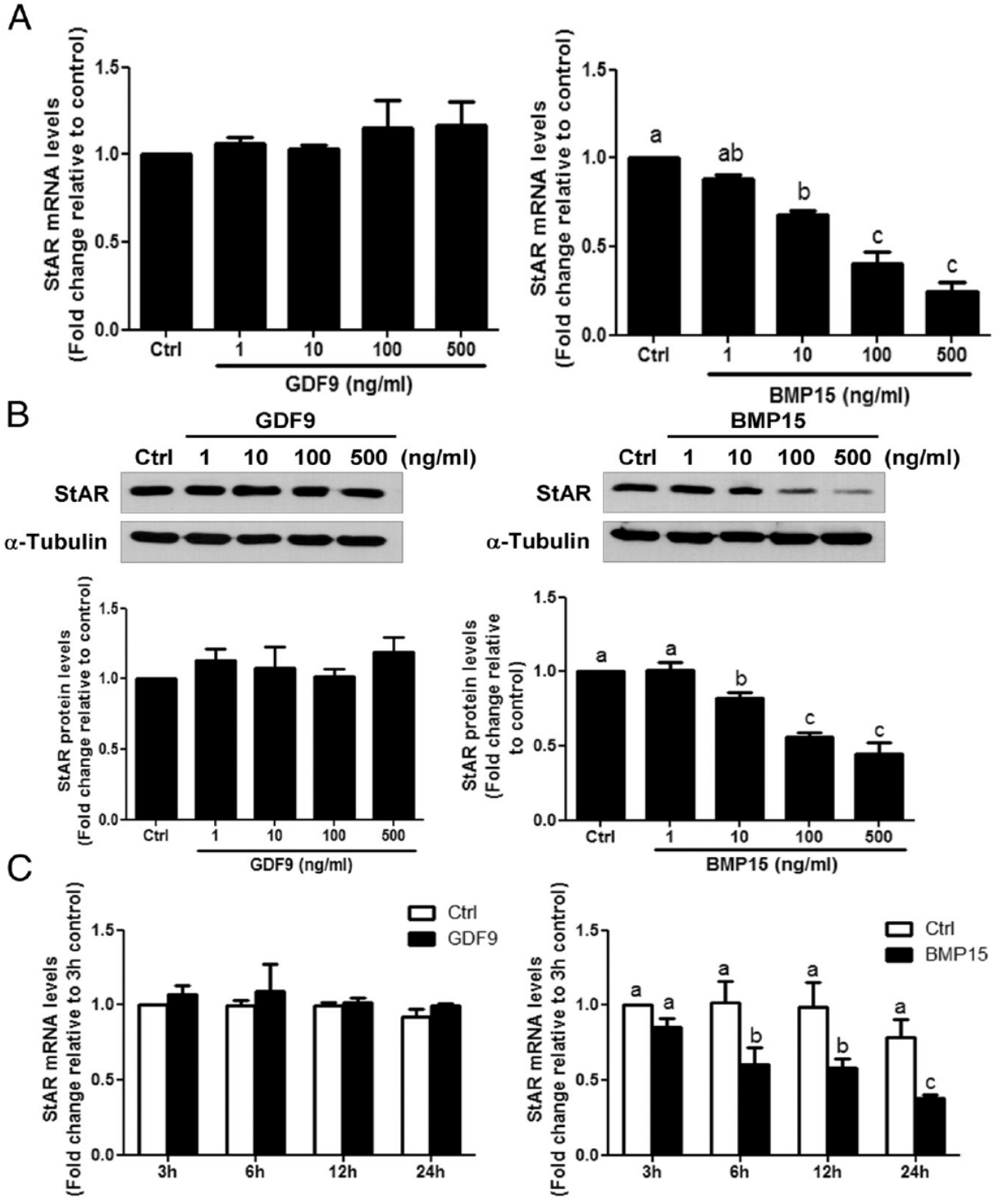


Figure 3.1 Neither GDF9 nor BMP15 affects the mRNA levels of P450scc or 3β-HSD in SVOG cells

Cells were treated for 24 h with vehicle control (Ctrl) or different concentrations (1, 10, 100 or 500 ng/ml) of GDF9 or BMP15 and the mRNA levels of P450scc and 3β-HSD were examined by RT-qPCR. Results are expressed as the mean ± SEM of at least 3 independent experiments.


A
StAR mRNA levels
(Fold change relative to control)
1.5
1.0
0.5
0.0
Ctrl
1
10
100
500
GDF9 (ng/ml)
a
ab
b
c
c
BMP15 (ng/ml)
B
GDF9
BMP15
Ctrl 1 10 100 500 (ng/ml)
StAR
α-Tubulin
StAR protein levels
(Fold change relative to control)
StAR protein levels
(Fold change relative
to control)
C
Ctrl
GDF9
BMP15
StAR mRNA levels
(Fold change relative to 3h control)
3h
6h
12h
24h

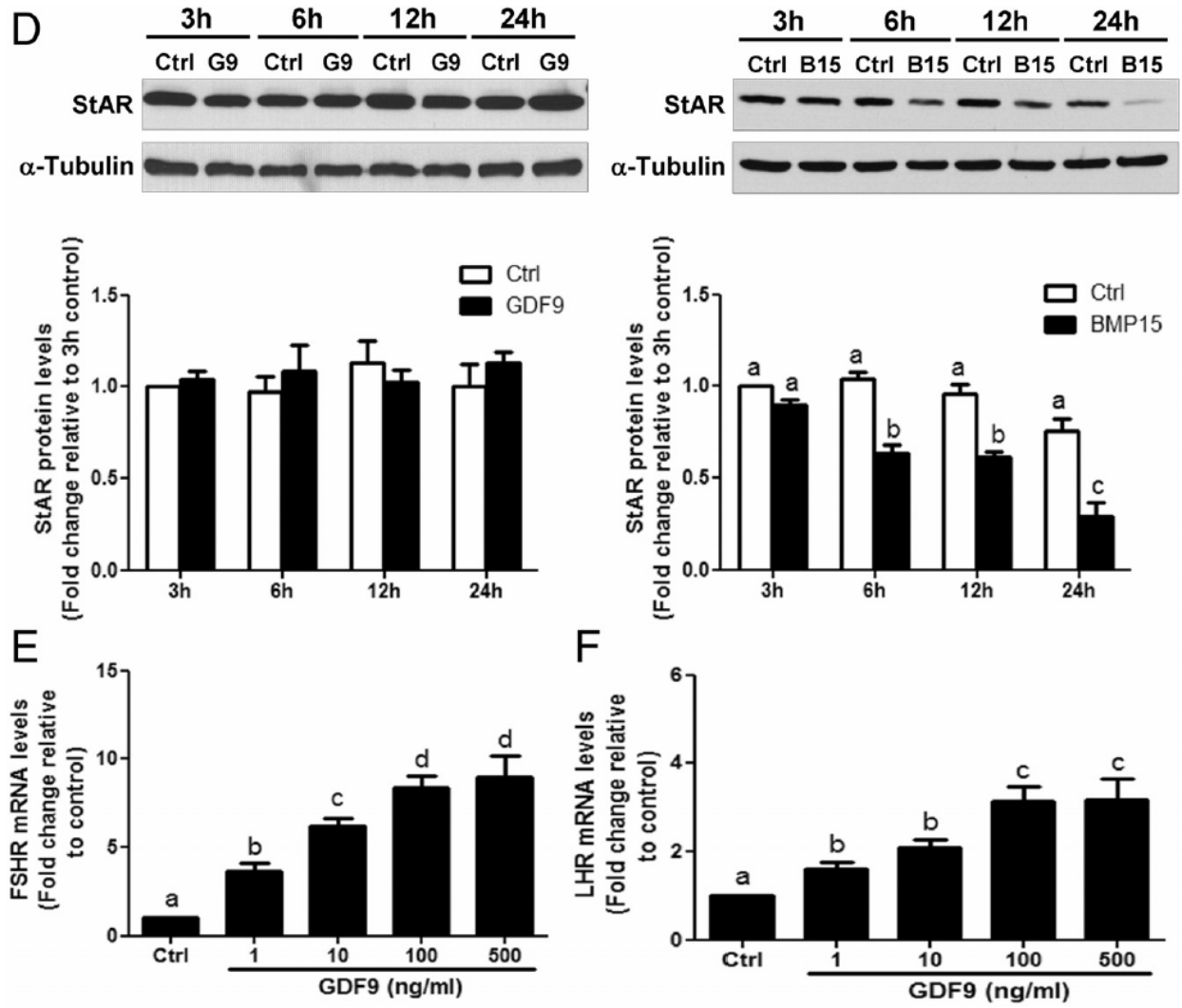


Figure 3.2 BMP15, but not GDF9, down-regulates StAR expression in SVOG cells

(A and B) Cells were treated for 24 h with vehicle control (Ctrl) or different concentrations (1, 10, 100 or 500 ng/ml) of GDF9 or BMP15 and StAR mRNA (A) and protein (B) levels were examined by RT-qPCR and Western blot, respectively. (C and D) Alternatively, SVOG cells were treated with 100 ng/ml GDF9 (G9) or BMP15 (B15) for 3, 6, 12 or 24 h, afterwhich StAR mRNA (C) and protein (D) levels were examined by RT-qPCR and Western blot, respectively. (E and F) Cells were treated for 24 h with vehicle control (Ctrl) or different concentrations (1,

10, 100 or 500 ng/ml) of GDF9, and FSHR (E) and LHR (F) mRNA levels were examined by RT-qPCR. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different, e.x. a V.S. ab indicates not significantly different ($P<0.05$).

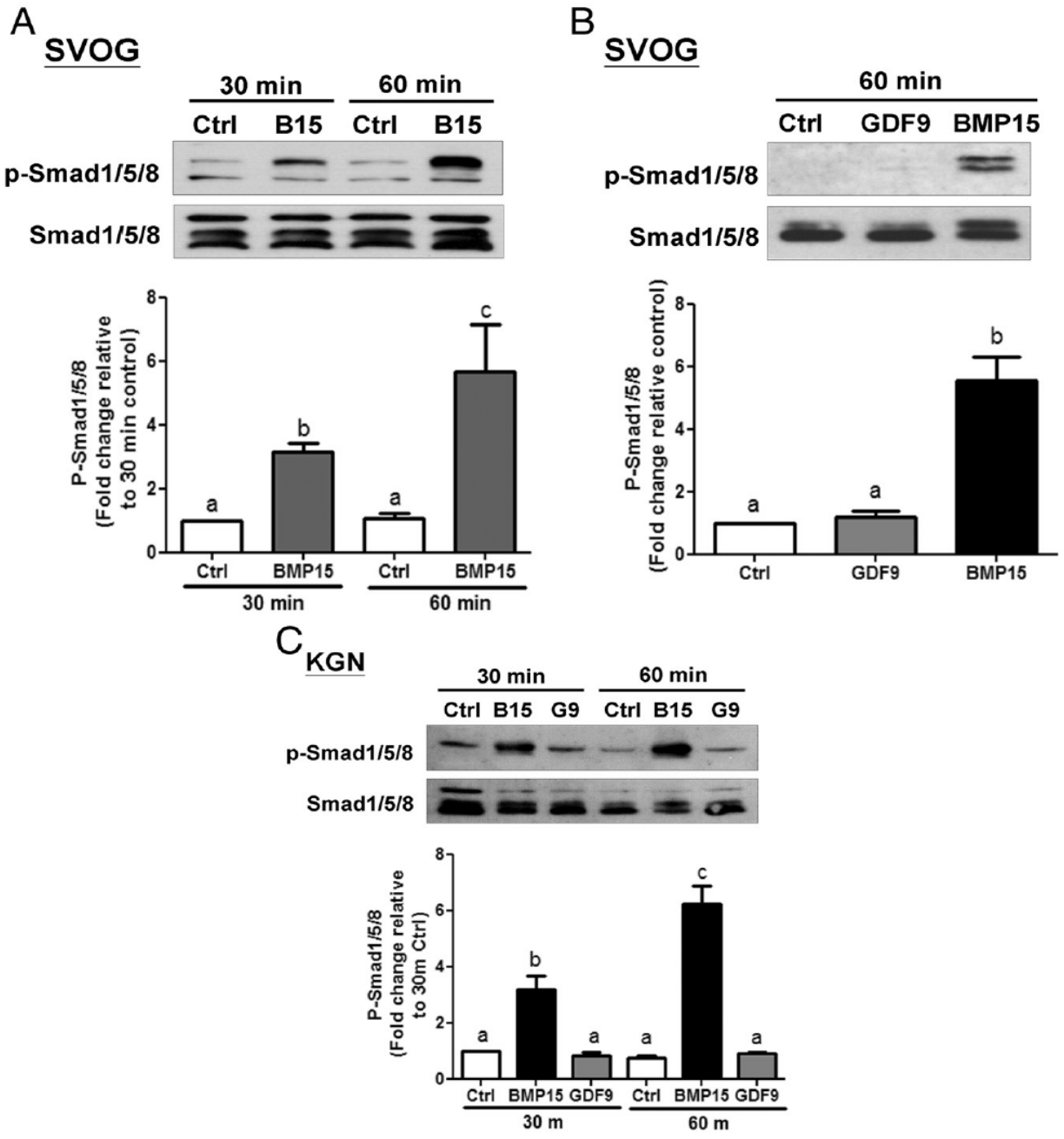
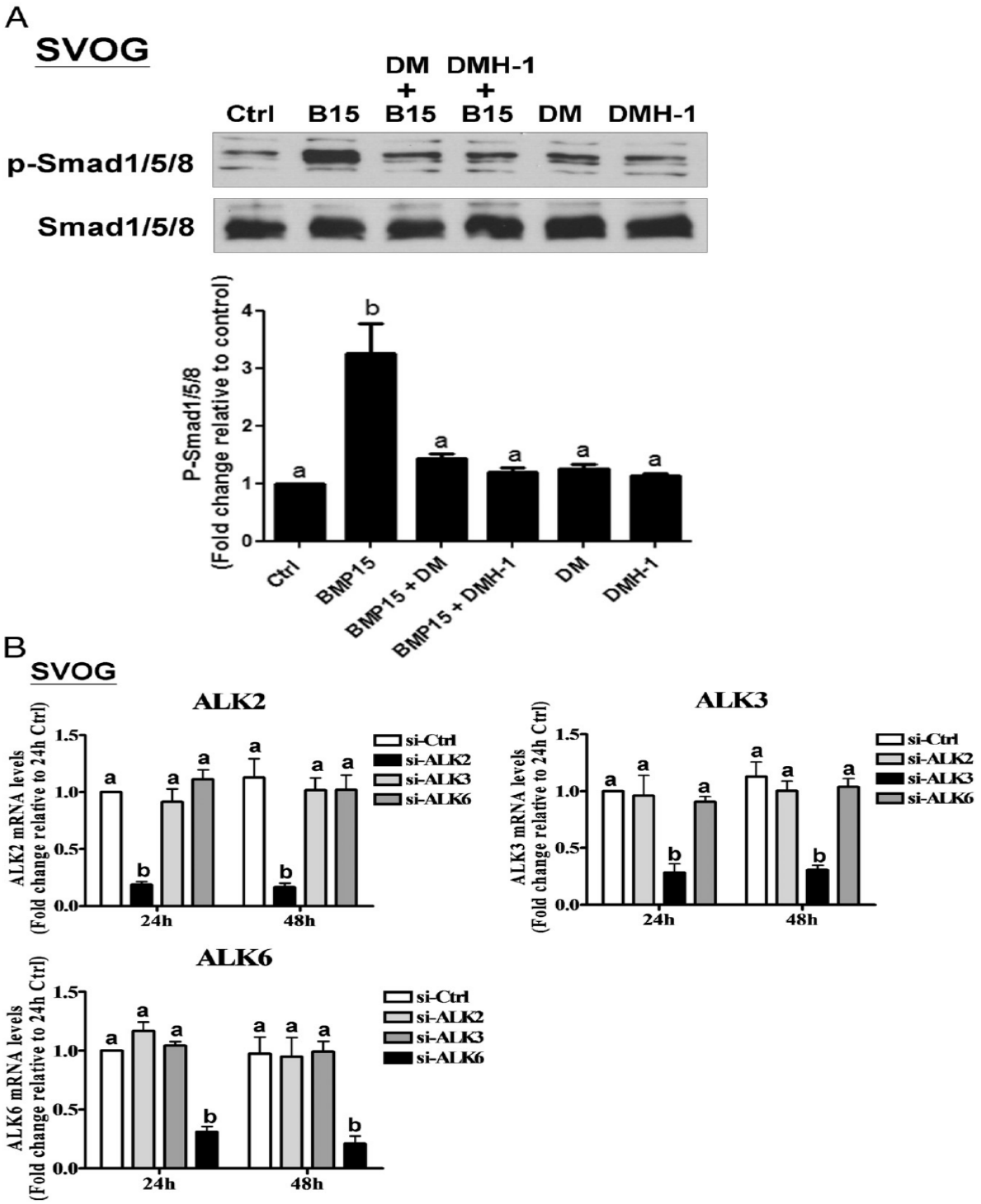


Figure 3.3 BMP15, but not GDF9, induces SMAD1/5/8 phosphorylation in SVOG and KGN cells

(A) SMAD1/5/8 phosphorylation was examined by Western blot following treatment with vehicle control (Ctrl) or 100 ng/ml BMP15 (B15) for 30 or 60 min in SVOG cells. (B) SVOG

cells were treated for 60 min with 100 ng/ml GDF9 or BMP15 and phosphorylated SMAD1/5/8 levels were examined by Western blot. (C) KGN cells were treated for 30 or 60 min with 100 ng/ml BMP15 or GDF9 and SMAD1/5/8 phosphorylation was examined by Western blot. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different ($P<0.05$).


A
SVOG
Ctrl
B15
DM + B15
DMH-1 + B15
DM
DMH-1
p-Smad1/5/8
Smad1/5/8
P-Smad1/5/8 (Fold change relative to control)
Ctrl
BMP15
BMP15 + DM
BMP15 + DMH-1
DM
DMH-1
B
SVOG
ALK2
ALK2 mRNA levels (Fold change relative to 24h Ctrl)
si-Ctrl
si-ALK2
si-ALK3
si-ALK6
24h
48h
ALK3
ALK3 mRNA levels (Fold change relative to 24h Ctrl)
ALK6
ALK6 mRNA levels (Fold change relative to 24h Ctrl)

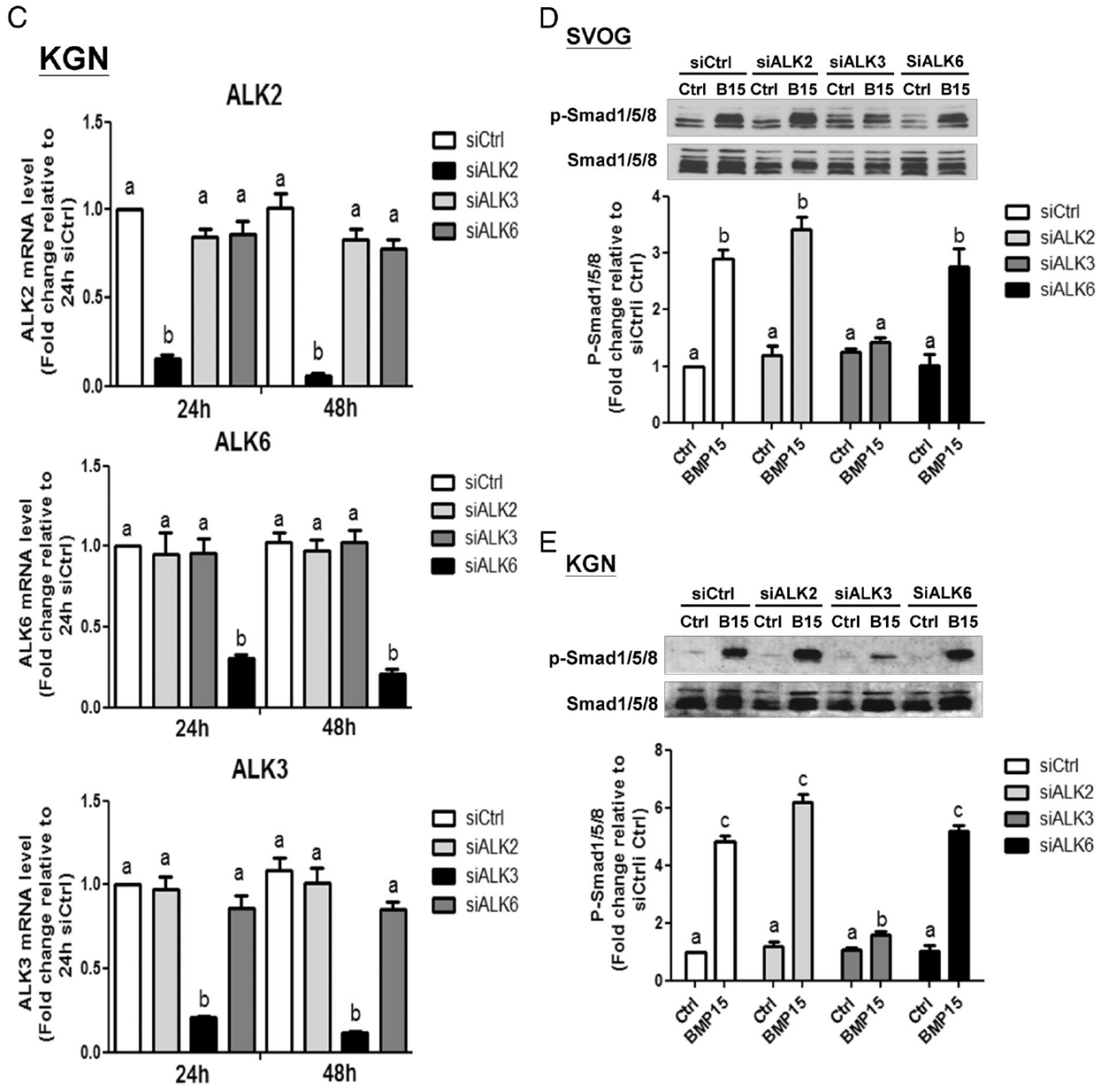


Figure 3.4 ALK3 mediates BMP15-induced SMAD1/5/8 phosphorylation in SVOG and KGN cells

(A) SMAD1/5/8 phosphorylation was examined in SVOG cells following treatment for 60 min with vehicle control or 100 ng/ml BMP15 in the absence or presence of BMP type I receptor inhibitors dorsomorphin (DM, 1 μM) or DMH-1 (0.5 μM). (B-E) SVOG or KGN cells were

transfected for 24 or 48 h with 25 nM control siRNA (siCtrl), ALK2 siRNA (siALK2), ALK3 siRNA (siALK3) or ALK6 siRNA (siALK6). The specificity and efficiency of knockdown for each siRNA was evaluated by RT-qPCR in (B) SVOG and (C) KGN cells. Thereafter,(D) SVOG or (E) KGN cells were transfected with siRNA for 48 h, treated for 60 min with vehicle control (Ctrl) or 100 ng/ml BMP15 (B15) and phosphorylated SMAD1/5/8 levels were examined by Western blot. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different ($P<0.05$).

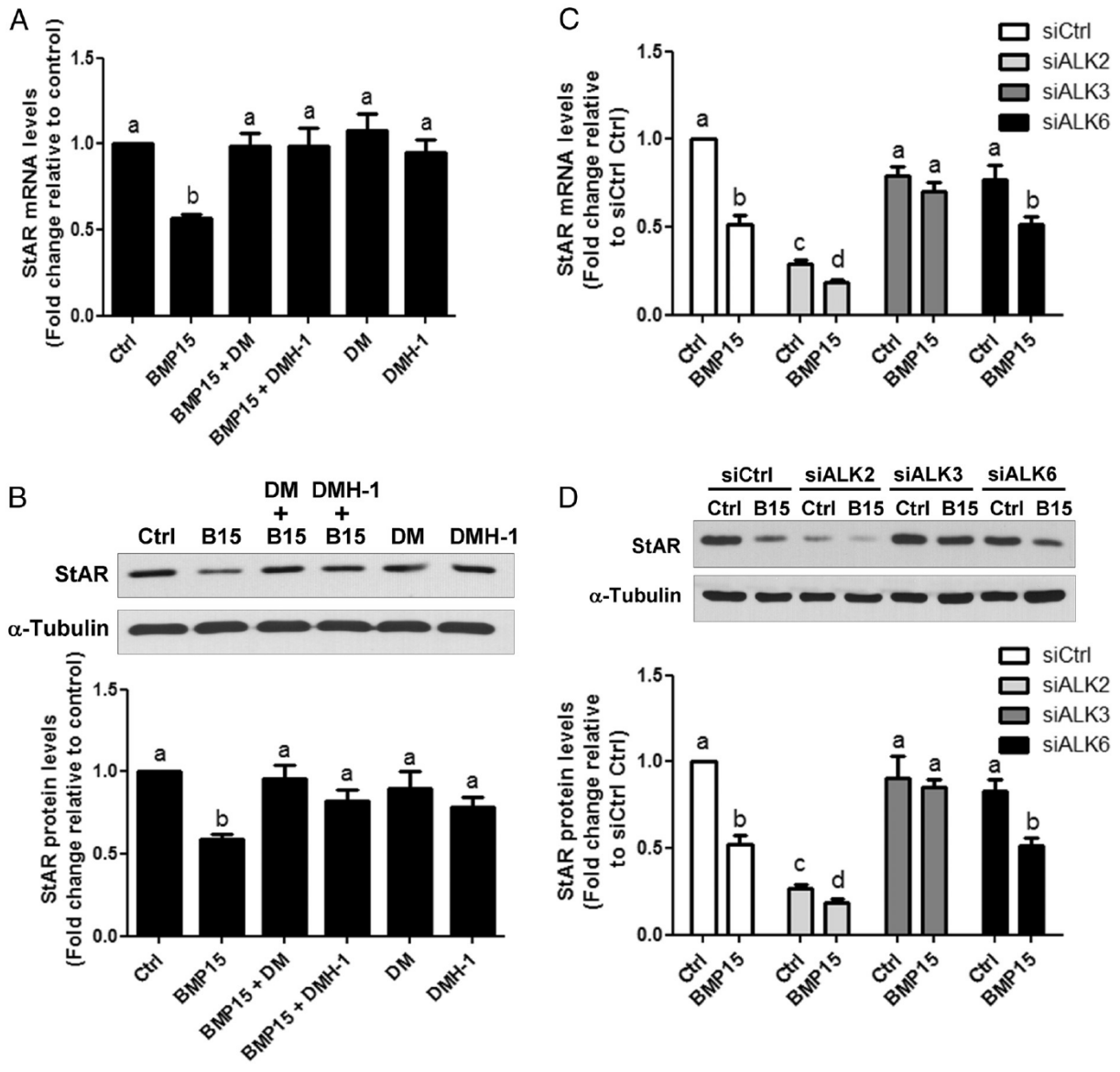


Figure 3.5 ALK3 mediates the suppressive effects of BMP15 on StAR expression in SVOG cells

(A and B) Cells were treated for 24 h with vehicle control (Ctrl) or 100 ng/ml BMP15 (B15) in the presence or absence of the BMP type I receptor inhibitors dorsomorphin (DM, 1 μM) or DMH-1 (0.5 μM), and StAR mRNA (A) and protein (B) levels were examined by RT-qPCR and Western blot, respectively. (C and D) Alternatively, the effects of BMP15 (100 ng/ml, 24 h) on

StAR mRNA (C) and protein (D) levels were examined in cells previously transfected for 48 hours with 25 nM control siRNA (siCtrl), ALK2 siRNA (siALK2), ALK3 siRNA (siALK3) or ALK6 siRNA (siALK6). Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different ($P<0.05$).

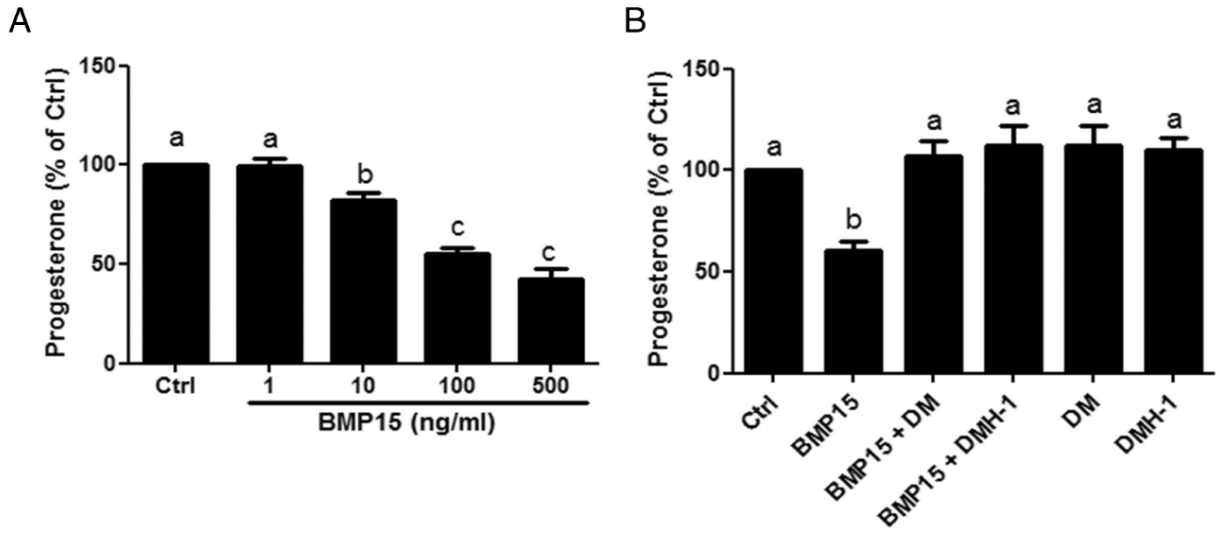


Figure 3.6 BMP15 suppresses progesterone accumulation in SVOG cells in a dorsomorphin- and DMH-1-sensitive manner

(A) Cells were treated for 24 h with vehicle control (Ctrl) or different concentrations (1, 10, 100 or 500 ng/ml) of BMP15 and progesterone accumulation in conditioned medium was measured by enzyme immunoassay. (B) Alternatively, cells were treated for 24 h with vehicle control (Ctrl) or 100 ng/ml BMP15 in the presence or absence of the BMP type I receptor inhibitors dorsomorphin (DM, 1 μM) or DMH-1 (0.5 μM), and progesterone accumulation in conditioned medium was measured by enzyme immunoassay. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different (P<0.05).

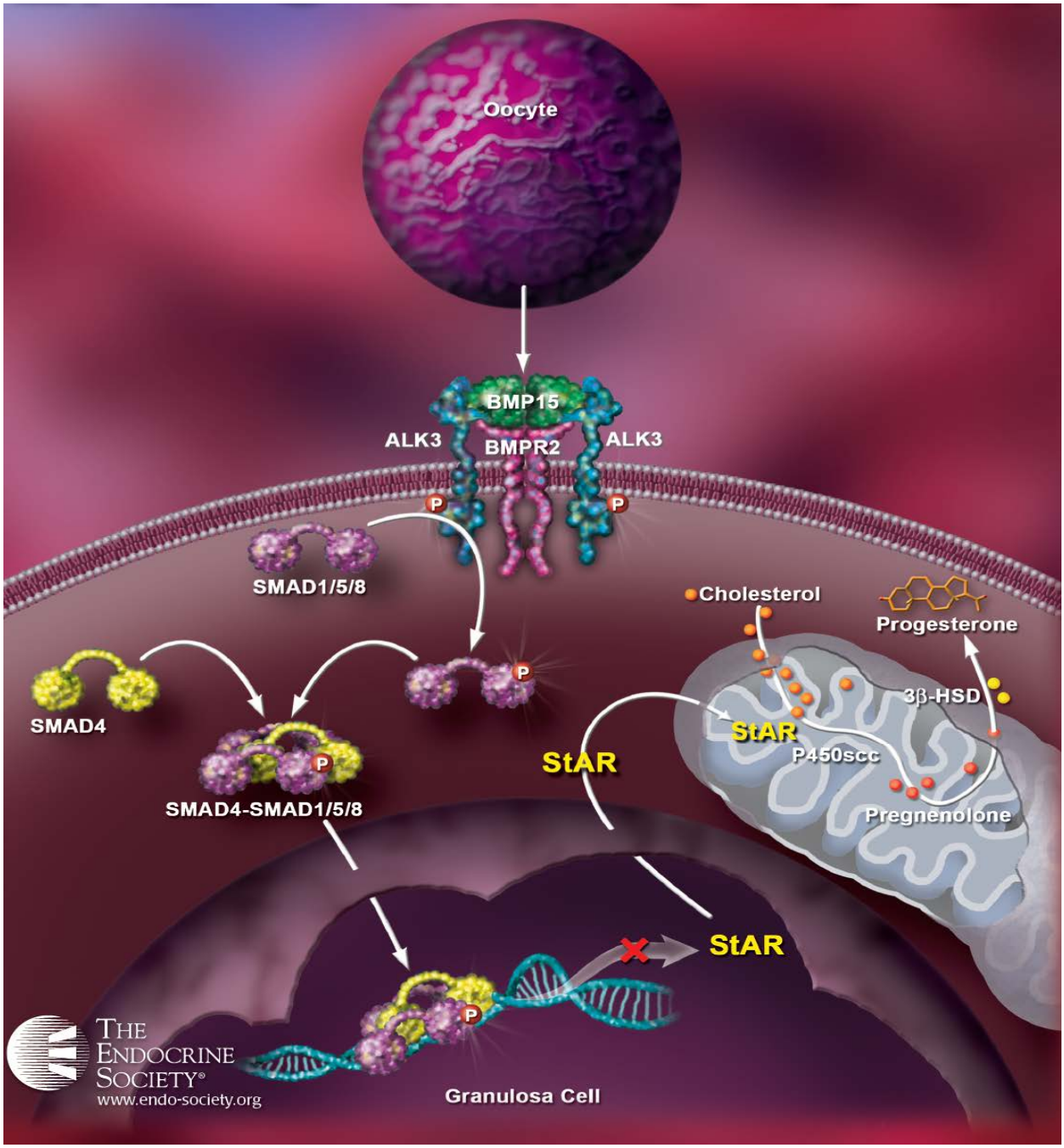


Figure 3.7 Proposed model for paracrine actions of BMP15 on StAR expression and progesterone production in human granulosa cells

Oocyte-derived BMP15 homodimers bind to a heterotetrameric receptor complex comprised ofBMP type I (ALK3) and type II (BMPR2) receptors. In the ligand-induced receptor complex, BMPR2 phosphorylates ALK3 leading to its activation and subsequent phosphorylation of receptor-regulated SMAD1/5/8. Phosphorylated SMAD1/5/8 binds with common SMAD4 to form aheterotrimeric complex thattranslocates into the nucleus where it binds DNA and suppressesStARtranscription. Reduced protein levels of the rate-limiting regulatory protein StAR result in decreased transport of cholesterol from the outerto the inner mitochondrial membrane, thereby diminishing granulosa cell progesterone production. BMP15, bone morphogenetic protein 15; StAR, steroidogenic acute regulatory protein; BMPR2, BMP receptor type II; ALK3, activin receptor-like kinase 3; SMAD, Sma- and Mad-related protein; P450scc, P450 side-chain cleavage enzyme; 3β-HSD, 3 β-hydroxysteroid dehydrogenase. *Molecular Endocrinology* December 2013 Cover Figure. Copyright (2013), with permission from The Endocrine Society.

Chapter 4: Oocyte-derived BMP15 but not GDF9 down-regulates connexin43 expression and decreases gap junction intercellular communication activity in immortalized human granulosa cells

4.1 Introduction

It has been widely accepted that oocytesare not passive recipients of developmental signals from cumulus/granulosa cells while residing inside the growing follicles but are active modulators and governors of follicle growth and ovulation. Within this microenvironment, a highly coordinated interplay between oocytes and the somatic supporting cells of the ovarian follicles governs the development and maturation of these cells and the acquisition of meiotically competent oocytes (32). In addition to pituitary-secreted gonadotropins, the oocyte-derived growth factors growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) also participate in the regulation of several specific target genes related to ovulation and luteinization (33, 34). As members of the transforming growth factor β (TGF-β) superfamily, both GDF9 and BMP15 bind to their type I and type II membrane receptors and trigger serine/threonine protein kinase activity. This ligand-receptor binding-induced activation further phosphorylates specific SMAD transcription factors: SMAD2/3 in response to GDF9 and SMAD1/5/8 in response to BMP15 (137). Subsequently, phosphorylated and activated SMAD2/3 or SMAD1/5/8 heterodimerize with a common SMAD, SMAD4, and then translocateinto the nucleus where they modulatethe transcription of several target genes (157).

Gap junctions mediate cell-cell communication by allowing the passage of ions, metabolites and small signaling molecules (up to approximately 1 kDa) between adjacent cells (73, 74). A single gap junction is composed of two connexons (or hemichannels) that conjoin the apposed

cell membrane and span the intercellular space (75). Each connexon consists of six symmetric subunits of various isotypes (there are 20 members in the mouse and 21 members in the human genome) of the transmembrane protein connexin (76-78). In many tissues, these connexin-coupled channels form clusters at membrane junctions called gap junction plaques (73). In mammals, ovarian follicles are the functional units of female reproductive biology. The coordination between this physiological compartment, the oocyte, the cumulus/granulosa cells and the theca cells, relies on functional gap junctions (79). In the developing follicles, gap junctions between cumulus/granulosa cells are composed primarily of connexin43 (Cx43), whereas connexin37 (Cx37) mainly contributes to the gap junctions that connect the oocyte to the circumjacent cumulus cells (80, 81). The importance of connexin in ovarian development and folliculogenesis has been demonstrated by studies of connexin knockout mice. Mice lacking Cx37 exhibit an abolishment of intercellular coupling between oocytes and cumulus cells, disrupted follicle development at the antrum formation stage, ovulation dysfunction and incompetent oocytes (81). Cx43 is expressed strongly in granulosa cells throughout all stages of follicle development, and Cx43-coupling gap junctions are required to sustain granulosa cell proliferation (82, 83). The ablation of Cx43 leads to a reduced number of germ cells in fetal gonads, retarded growth of oocytes and failed fertilization (82).

Given the pivotal role of Cx43-coupled gap junctions in sustaining normal ovarian function, the study of the regulation of gap junction activity and its forming proteins has been a subject of considerable research. Accumulating evidence has shown that the cyclic expression of Cx43 in granulosa cells of growing follicles is developmentally and hormonally regulated (84-88). In the rat, the expression pattern of Cx43 in ovarian follicles positively correlates with changes in serum levels of gonadotropins, indicating a role for gonadotropins in the regulation of Cx43

expression in the female reproductive system (80, 87). Specifically, FSH up-regulates the expression of Cx43 mRNA and protein, whereas LH or human chorionic gonadotropin down-regulates Cx43 expression and leads to the subsequent loss of intercellular coupling within rat granulosa cell layers (79, 80, 87). In addition to the endocrine effects of gonadotropins, locally released steroid hormones such as estrogen, progesterone and androgen have been reported to regulate Cx43 and gap junction intercellular (GJIC) activity in several species, including humans (89-91).

Furthermore, our recent study identified exciting new roles for the theca cell-derived TGF-β superfamily members BMP4 and BMP7 in the regulation of Cx43 expression in human granulosa cells (158). Moreover, we have clarified an important mechanism by which BMP4 and BMP7 modulate GJIC activity (158). These data indicate that paracrine/juxtacrine signaling may be involved in the regulation of cell-cell communication,yet the roles of oocyte-derived growth factors in the regulation of granulosa cell-related connexins remain to be determined. In the present study, GDF9 was compared to BMP15 with respect to their effects on the expression of Cx43 in a human granulosa cell line and primary human granulosa-lutein cells. We further investigated these effects relative to GJIC activity and the potential underlying cellular mechanisms of action.

4.2 Materials and methods

Preparation of primary human granulosa-lutein (hGL) cells

Primary hGL cells were obtained with informed patient consent following approval from the University of British Columbia ethics review board. The controlled ovarian stimulation protocol for *in vitro* fertilization patients consisted of either luteal-phase naferelin acetate (Synarel, Pfizer,

Kirkland, Quebec, Canada) or follicular phase GnRH antagonist (Ganirelix; Merck Canada) down regulation. Gonadotropin stimulation began menstrual cycle day 2 with human menopausal gonadotropin(hMG; Menopur, Ferring, Canada)and recombinant FSH (Puregon, Merck, Canada), and was followed by human chorionic gonadotropin administration 34 h before oocyte retrieval, based on follicle size. Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval as previously described(159).

Simian virus 40 large T antigen–immortalized human granulosa cell (SVOG) culture

In this study, we used SVOG, a non-tumorigenic immortalized human granulosa cell line, which was previously produced by transfecting human granulosa-luteal cells (from a patient undergoing *in vitro* fertilization) with the SV40 large T antigen (136). Cells were counted with a hemocytometer, and cell viability was assessed by trypan blue (0.04%) exclusion. The cells were seeded (2-4 × 10^5 cells per ml in 6-well plates) and cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Cells were cultured in Dulbecco's Modified Eagle's Medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin (Invitrogen, Life Technologies, NY), 100 μg/ml streptomycin sulfate (Invitrogen, Life Technologies) and 1X GlutaMAX (Invitrogen, Life Technologies). The culture medium was changed every other day in all experiments. Finally, cells were maintained in serum-free medium for 24 h before receiving the growth factor treatment.

Antibodies and reagents

Polyclonal rabbit anti-connexin43 (#3512) (1:1000) and polyclonal rabbit anti-phospho-SMAD1 ($Ser^{463/465}$)/SMAD5 ($Ser^{463/465}$)/SMAD8 ($Ser^{426/428}$) (#9511) (1:1000) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal rabbit anti-SMAD1/5/8 (N-18) (sc-6031-R) (1:1000) and monoclonal mouse anti-α-tubulin (B-5-1-2) (sc-23948) (1:3000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Bio-Rad Laboratories (Hercules, CA). Recombinant human BMP15 and dorsomorphin dihydrochloride (dorsomorphin) were obtained from R&D Systems (Minneapolis, MN). Recombinant human GDF9 was obtained from BioVision Incorporated (Milpitas, CA). SB-431542 was obtained from Sigma-Aldrich Corp..

Reverse transcription and real-time quantitative PCR (RT-qPCR)

Cells were washed with cold phosphate buffered saline (PBS), and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Madison, WI). Each 20-μl qPCR reaction contained 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 20 ng cDNA and 250 nM of each specific primer. The primers used were GJA1 (Cx43), 5'- TAC CAA ACA GCA GCG GAG TT -3' (sense) and 5'- TGG GCA CCA CTC TTT TGC TT -3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'- ATG GAA ATC CCA TCA CCA TCT T -3' (sense) and 5'- CGC CCC ACT TGA TTT TGG -3' (antisense). qPCR was performed on an Applied

Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate (Applied Biosystems). The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of the PCR products. Assay performance was validated by evaluating the amplification efficiencies by means of calibration curves and by ensuring that the plot of the log input amount *vs.* ΔCq (also known as ΔCt) had a slope < |0.1|. The PCR parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq ($2^{-\Delta\Delta Cq}$) method with *GAPDH* as the reference gene.

Western blot analysis

After treatment, cells were washed with cold PBS and lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μM aprotinin, 1 μM leupeptin and 1 mM PMSF) (Cell Signaling) containing a protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 x *g* for 15 min at 4°C to remove cellular debris, and protein concentrations were quantified using a DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 1 h in Tris-buffered solution containing 0.05% Tween 20 and 5% nonfat dried milk and incubated overnight at 4°C with the relevant primary antibodies. After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence reagents or a SuperSignal West Femto Chemiluminescence Substrate (Pierce, Rockford, IL), followed by

exposure to CL-XPosure film (Thermo Fisher, Waltham, MA). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mmol/l β-mercaptoethanol and 1% SDS) at 50°C for 30 min and re-probed with a mouse anti-α-tubulin antibody used as a loading control.

Small interfering RNA (siRNA) transfection

Transient knockdown assays were performed using 25 nM GJA1 (Cx43)-targeting siRNA (ON-TARGET*plus*SMARTpool), 25 nM SMAD4-targeting siRNA (ON-TARGET*plus* SMARTpool) or 25 nM control siRNA (ON-TARGET*plus* Non-targeting Pool) purchased from Thermo Fisher Scientific (Lafayette, CO). Cells were pre-cultured to 50% confluence in antibiotic-free DMEM/F12 medium containing 10% charcoal/dextran-treated fetal bovine serum, after which they were transfected with siRNA for 48 h using Lipofectamine RNAiMAX (Dharmacon, Life Technologies). The efficiency of knockdown for each target was confirmed by Western blot.

Immunofluorescence staining

Cells were plated on glass cover slips, fixed with 4% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. After they were washed with PBS, the cover slips were mounted on microscope slides and blocked with Dako Protein Block (Dako, Mississauga, Ontario, Canada) for 1 h followed by incubation with a connexin43 antibody (1:50 diluted in Dako Protein Block) overnight. An Alexa Fluor 555 donkey anti-rabbit IgG (Life Technologies) was used as a secondary antibody. Finally, the cells were counterstained with chromosomal dye Hoechst 33258 (Sigma-Aldrich), rinsed with PBS, mounted in Gelvatol and

imaged under a Zeiss Axiophot fluorescence microscope equipped with a digital camera (Q Imaging, Burnaby, BC, Canada).

Scrape loading and dye transfer assay

Several methods have been used to examine the intercellular communication (GJIC) between cells including fluorescence return after photobleaching (FRAP), thymidine-uridine nucleotide transfer and metabolic coupling of 6-thioguanine metabolites. However, the scrape loading and dye transfer assay is the most sensitive assay to measure intercellular communication (160).

To determine the effects of BMP15 on GJIC between human granulosa cells, we performed a scrape loading and dye transfer assay, which is based on monitoring the transfer of the fluorescent dye Lucifer yellow (MW 457.2) from one cell into the adjacent cell via functional gap junctions. The scrape loading technique has been used to introduce macromolecular substances into cultured cells effectively by producing a transient tear in the cell membrane. Lucifer yellow cannot diffuse through intact cell membranes; however, its low molecular weight allows it to transfer to adjacent cells through intact gap junctions. After treatment, fully confluent cells were washed with PBS and scraped using a surgical blade prior to the addition of fluorescent dye (0.5% Lucifer yellow CH, potassium salt, Life Technology). After a 5-min incubation, the cells were washed thoroughly to remove background fluorescence, fixed with 4% paraformaldehyde and imaged with a Zeiss Axiophot fluorescence microscope equipped with a digital camera. GJIC was evaluated and measured as the distance from the scrape line to the furthest extent of the dye-coupled cells.

Statistical analysis

The results were analyzed by one-way ANOVA followed by Tukey's multiple comparison test in PRISM software (GraphPad Software, Inc., San Diego, CA). The results are presented as the mean ± SEM of at least three independent experiments. Data were considered significantly different if $P < 0.05$.

4.3 Results

BMP15 but not GDF9 down-regulates Cx43 expression in SVOG cells

The concentrations of GDF9 in human follicular fluid range from 54.38 to 458.91 ng/ml (151), whereas there is no reported data on the concentration of BMP15 in serum or in follicular fluid. Because Cx43 is the dominant connexin in human granulosa cells and this connexin isoform is associated with a good prognosis for the corresponding oocyte (161), we first investigated the effects of GDF9 and BMP15 on Cx43 expression in SVOG cells. Cells were treated with increasing concentrations of recombinant human GDF9 or BMP15 (1, 10 or 100 ng/ml) for 24 h, and the levels of Cx43 mRNA were examined. The results show that treatment with BMP15 significantly decreased Cx43 mRNA levels in a concentration-dependent manner, while treatment with GDF9 did not affect basal Cx43 mRNA levels (Figure 4.1A). Moreover, treatment with 50 ng/ml BMP15, but not GDF9, significantly down-regulated Cx43 mRNA levels at any time point (3, 6, 12 or 24 h) examined (Figure 4.1B). The concentration-dependent suppression of Cx43 protein levels by BMP15, but not GDF9, was confirmed by Western blot analysis (Figure 4.1C). Moreover, treatment with BMP15 (50 ng/ml) reduced Cx43 protein levels at different time points (3, 6, 12 or 24 h) with the maximal effect observed 12 h after treatment, whereas GDF9 had no effect at any time point examined (Figure 4.1D). However,

neither BMP15 nor GDF9 affected basal Cx37 mRNA levels at any concentration or time point examined (data not shown).

Localization and distribution of Cx43 in SVOG cells

To study the localization and distribution of Cx43 in human granulosa cells, we performed immunofluorescence staining in SVOG cells following a transfection of control siRNA or GJA1 (Cx43)-targeting siRNA for 48 h. A Western blot analysis showed that Cx43-targeting siRNA decreased Cx43 protein levels by 85-90% relative to control siRNA (Figure 4.2A). Our immunostaining results showed that Cx43 was expressed clustered as red fluorescent plaques at the sites of cell-cell contacts, which is the typical appearance of aggregated Cx43 channels in gap junction plaques (Figure 4.2B). Consistent with the Western blot results, transfection of the cells with Cx43-targeting siRNA for 48 h dramatically decreased the amount of Cx43 immunostaining in SVOG cells (Figure 4.2B).

Dorsomorphin abolishes the BMP15-induced down-regulation of Cx43 in SVOG cells

To date, three TGF-β type I receptors, ALK2, ALK3 and ALK6, have been demonstrated to be involved in BMP-mediated cellular functions (162). It has been shown that dorsomorphin, a selective inhibitor of BMP type I receptor, specifically inhibits the function of ALK2, ALK3 and ALK6 (143, 163). To investigate whether ALK2, ALK3 and ALK6 are involved in the BMP15-induced down-regulation of the Cx43 expression, cells were treated with 50 ng/ml BMP15 in the presence or absence of 5 μM dorsomorphin. As shown in Figure 4.2C, BMP15-induced down-regulation of Cx43 protein was abolished by co-treatment with dorsomorphin. Consistent with the Western blot results, an immunostaining assay showed that treatment with BMP15 decreased

the expression of Cx43 and that this decrease was abolished by co-treatment with dorsomorphin (Figure 4.2D).

To further confirm that other BMP type I receptors are not involved in the BMP15-induced down-regulation of Cx43, cells were treated with 50 ng/ml BMP15 in the presence or absence of 5 μM SB-431542, an inhibitor of ALK4, ALK5 or ALK7 (164). As shown in Figure 4.2E, co-treatment of SB-431542 did not affect BMP15-induced down-regulation of the Cx43 protein. Consistent with the Western blot results, an immunostaining assay showed that treatment with BMP15 decreased the expression of Cx43 and that this decrease was not altered by co-treatment with SB-431542 (Figure 4.2F).

BMP15 activates Smad1/5/8 signaling in SVOG cells

To examine whether treatment with BMP15 activates SMAD1/5/8, SVOG cells were treated with BMP15 or GDF9 (50 ng/ml) for 30, 45 or 60 min. The results showed that treatment with BMP15 increased the phosphorylation levels of SMAD1/5/8 after 30-, 45- and 60-min stimulations, whereas treatment with GDF9 did not alter the phosphorylation level of SMAD1/5/8 at any time point (Figure 4.3A). To further investigate whether ALK2, ALK3 and ALK6 are involved in BMP15-induced SMAD1/5/8 activation, cells were treated with BMP15 in the presence or absence of 5 μM dorsomorphin. As shown in Figure 4.3B, treatment of the cells with dorsomorphinabolished the BMP15-induced SMAD1/5/8 phosphorylation, whereas treatment of the cells with SB-431542 did not alter BMP15-induced SMAD1/5/8 phosphorylation (Figure 4.3C).

The SMAD signaling pathway is required in SVOG cells for the BMP15-induced down-regulation of Cx43

Before regulating the transcription of target genes, the common mediator Smad4 forms heteromeric complexes with R-SMAD, SMAD2/3 or SMAD1/5/8 (100). To investigate whether SMAD signaling is required for the BMP15-induced down-regulation of Cx43, SMAD4 siRNA was used to knockdown the endogenous expression of the common SMAD, SMAD4. Western blot analysis was used to examine the knockdown efficiency. As shown in Figure 4.4A, after transfection for 48 h, SMAD4 siRNA down-regulated SMAD4 protein levels by 80-90% relative to control siRNA. In addition, transfection with SMAD4 siRNA for 48 h abolished the suppressive effect of BMP15 on Cx43 protein (Figure 4.4B).

BMP15 decreases gap junction intercellular communication (GJIC) in SVOG cells

To investigate whether the down-regulation of Cx43 contributes to decreased GJIC activity, we evaluated cellular GJIC using a scrape-loading dye transfer technique. As shown in Figure 4.5A, treatment with 50 ng/ml BMP15 for 24 h reduced the number of Lucifer yellow dye-coupled cell layers on either side of the scrape. In addition, a 30-min pre-treatment with 5 μM dorsomorphin abolished the BMP15-mediated decrease in GJIC activity (Figure 4.5A). Similarly, the transfection of 25 nM SMAD4 siRNA for 48 h reversed the suppressive effects of BMP15 (50 ng/ml) on GJIC activity (Figure 4.5B). These results indicate that BMP15 decreases GJIC activity in SVOG cells, most likely via a BMP type I receptor-driven SMAD-dependent signaling pathway.

BMP15 but not GDF9 down-regulate Cx43 in primary human granulosa-lutein (hGL) cells

Non-immortalized primary hGL cells were used to further confirm the function of GDF9 and BMP15 in human granulosa cells. Primary hGL cells obtained from infertile patients undergoing an *in vitro* fertilization (IVF) procedure were cultured in vehicle control with or without GDF9 and BMP15 (1, 10 and 100 ng/ml), and the results showed that BMP15 decreased Cx43 mRNA and protein levels in a concentration-dependent manner (Figure 4.6A and 4.6B). Consistent with the results in SVOG cells, treatment of hGL cells with GDF9 did not affect basal Cx43 mRNA and protein levels (Figure 4.6A and 4.6B).

4.4 Discussion

The bi-directional communication between oocytes and their surrounding follicle cells is essential for coordinating and fulfilling the differentiation and maturation of these follicle compartments from one developmental stage to the next. In mural granulosa cells, luteinization is the final differentiation stage that is provoked by the ovulatory stimulus. Premature luteinization that is associated with high serum progesterone levels may adversely affect oocyte quality and the implantation environment (132). A pioneer study has shown that the removal of the oocyte-cumulus complex led to the precocious luteinization of rabbit follicles *in vivo* (46), indicating that oocytes may secrete an anti-luteinization factor. Subsequent studies have supported the long-standing hypothesis that oocytes can regulate the production of progesterone, a hallmark of luteinized cells, by granulosa cells from the time of follicle development throughout ovulation (10, 53). Aside from progesterone, the underlying mechanisms that modulate the luteinization process include intracellular signaling pathways, cell adhesion factors, intracellular cholesterol

and oxysterols (133). The trafficking and exchange of these molecules between cumulus granulosa cells relies on functional Cx43-channeled gap junctions. Therefore the expression pattern of Cx43 can be regarded as another parameter of luteinization. Previous clinical data have also demonstrated that a reduction in Cx43 expression within cumulus cells at the time of oocyte collection yields good embryo competence (134). Our results showing that oocyte-derived BMP15 decreases GJIC activity by down-regulating Cx43 expression suggest that oocytes may play an important role in the prevention of premature luteinization.

A comprehensive understanding of the molecular mechanism of the cellular response to BMP15 is crucial for developing new pharmacological strategies for clinical treatment. In the present study, the knockdown of SMAD4, the central component of the TGF-β superfamily signaling pathway, reversed the inhibitory effects of BMP15 on Cx43 expression and GJIC activity in human granulosa cells. Consistent with our previous results, SMAD4 associates with SMAD1/5/8, which constitutes a well-characterized signaling pathway downstream of BMPs, and may mediate the suppressive effect of BMP15 on Cx43 expression. Moreover, the same effects did not occur when the cells responded to GDF9, which exerts its action through SMAD2/3 signaling. It has been shown that unlike mouse GDF9, human GDF9 is initially produced in an inactive form because the human GDF9 prodomain is designed to limit mature growth factor (165). A study using combined Northern blot and *in situ* hybridization analysis showed that BMP15 is coincidently expressed with GDF9 in the oocyte beginning at the primary follicle stage and continuing through ovulation in mice(112, 166). Therefore, our results indicate that although GDF9 and BMP15 are close relatives and display a similar oocyte-specific expression pattern (112), their effects vary with respect to the origin of these growth factors. Previous *in vitro* studies have shown that in many species including human, GDF9 and BMP15

may exhibit distinct effects on reproductive functions (167). In particular, GDF9 suppresses FSH-induced progesterone and estradiol production (130); whereas BMP15 suppresses only FSH-induced progesterone production (108) in rodent granulosa cells. In addition, BMP15 has been shown to suppress bovine cumulus granulosa cell apoptosis; however GDF9 has no effect on apoptosis in bovine cumulus granulosa cells (168). Furthermore, our recent studies also showed that treating SVOG cells with GDF9 or BMP15 elicits differential effects on the mRNA and protein levels of StAR, a critical regulatory protein that regulates the rate-limiting step in steroid biosynthesis (169).

In a conditional knockout study, ovarian-specific Smad4 knockout mice are subfertile with multiple defects in folliculogenesis (170). In these mice, the depletion of SMAD4 resulted in the disrupted regulation of steroidogenesis, leading to the premature luteinization of granulosa cells and subsequently to premature ovarian failure (170). These results highlight the critical role of a SMAD4-driven signaling pathway in the timing of granulosa cell differentiation. Although it is increasingly apparent that BMP superfamily members activate both SMAD and other signaling pathways (171), our results suggest that BMP15 modulates Cx43-based gap junction activity most likely through a SMAD-dependent signaling pathway. However, the present data are unable to provide information as to whether SMAD1/5/8 transcription factors interact with Cx43 (GJA1) promoter sequences in the nucleus following exposure to BMP15. Future studies aimed at addressing this question by generating Cx43 (GJA1) promoter constructs and transfecting them into granulosa cells would be of great interest.

In conclusion, the current study demonstrates that BMP15 down-regulates Cx43 in primary human granulosa-lutein cells and a transformed human granulosa cell line. In addition, the reduction of Cx43 contributes to a decrease in the activity of gap junction intercellular

communication. Moreover, the present results indicate that the SMAD1/5/8 signaling pathway is required for the BMP15-induced down-regulation of Cx43. Taken together, these *in vitro* studies suggest that the oocyte plays a critical role in the local regulation of cell-cell communication.

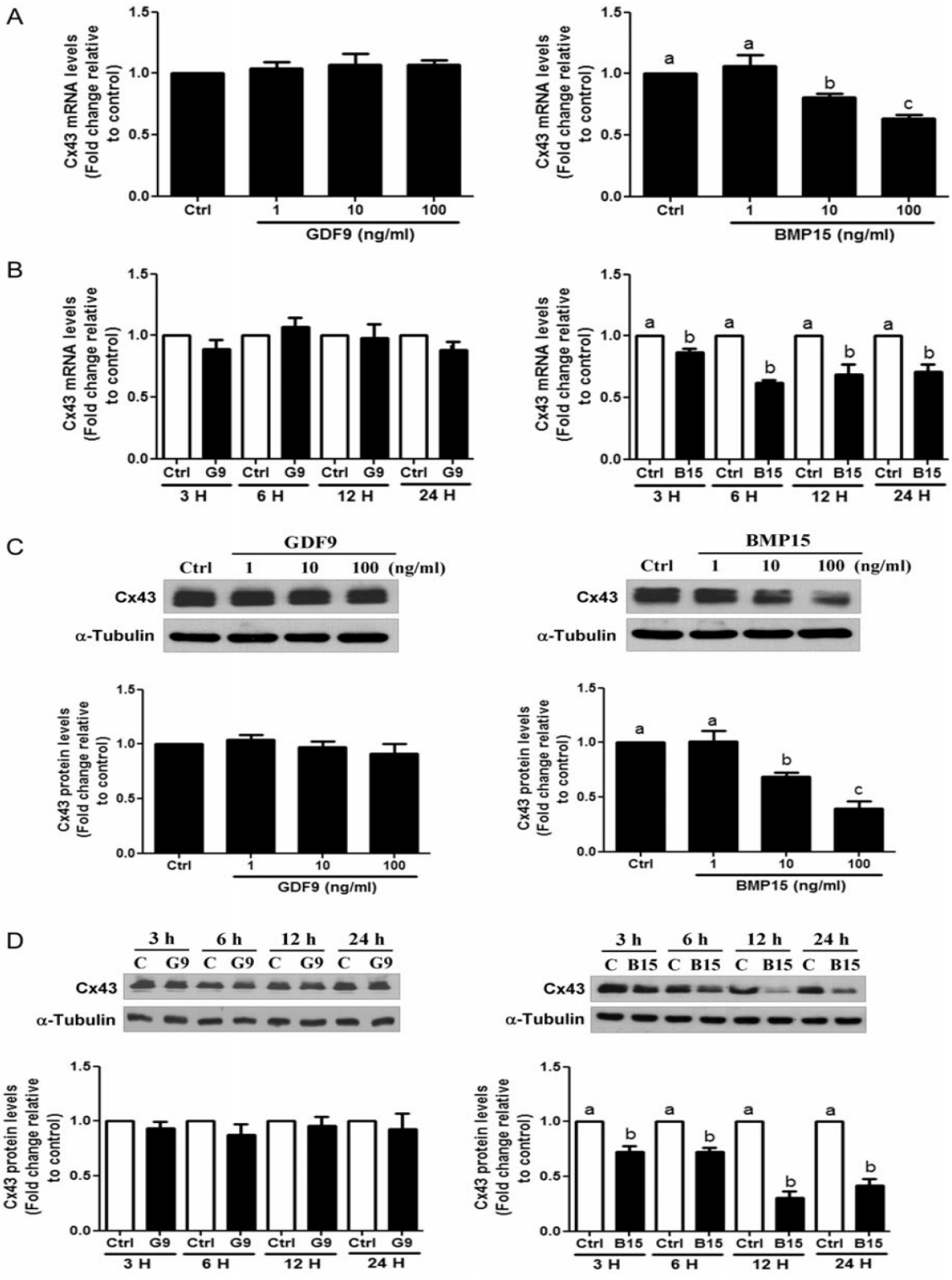

A
Cx43 mRNA levels (Fold change relative to control)
Ctrl
1
10
100
GDF9 (ng/ml)
BMP15 (ng/ml)
B
G9
B15
3 H
6 H
12 H
24 H
C
GDF9
BMP15
(ng/ml)
Cx43
α-Tubulin
Cx43 protein levels (Fold change relative to control)
D
3 h
6 h
12 h
24 h
C
G9
B15

Figure 4.1 BMP15 but not GDF9 down-regulates Cx43 mRNA expression in SVOG cells

A and C, Cells were treated with different concentrations (1, 10 or 100 ng/ml) of GDF9 or BMP15 for 24 h; Cx43 mRNA (A) and protein (C) levels were examined by RT-qPCR and Western blot analysis. B and D, Cells were treated with 50 ng/ml GDF9 or BMP15 for 3, 6, 12 or 24 h; Cx43 mRNA (B) and protein(D) levels were examined by RT-qPCR. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P <0.05). Ctrl, control. B15, BMP15. G9, GDF9.

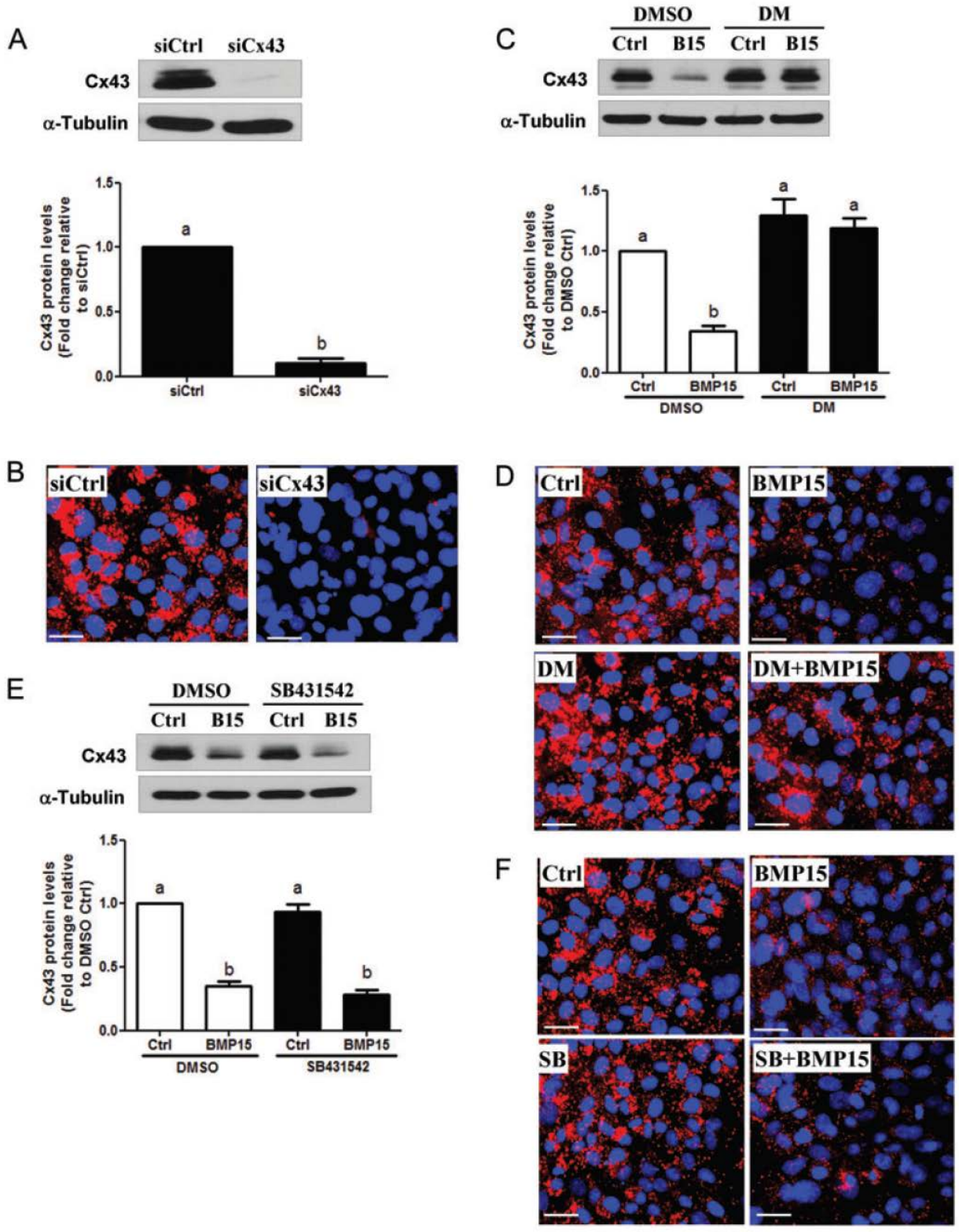

A
siCtrl
siCx43
Cx43
α-Tubulin
Cx43 protein levels (Fold change relative to siCtrl)
1.5
1.0
0.5
0.0
a
b
siCtrl
siCx43
C
DMSO
DM
Ctrl
B15
Ctrl
B15
Cx43
α-Tubulin
Cx43 protein levels (Fold change relative to DMSO Ctrl)
a
b
a
a
Ctrl
BMP15
Ctrl
BMP15
DMSO
DM
B
siCtrl
siCx43
D
Ctrl
BMP15
DM
DM+BMP15
E
DMSO
SB431542
Ctrl
B15
Ctrl
B15
Cx43
α-Tubulin
Cx43 protein levels (Fold change relative to DMSO Ctrl)
a
b
a
b
Ctrl
BMP15
Ctrl
BMP15
DMSO
SB431542
F
Ctrl
BMP15
SB
SB+BMP15

Figure 4.2 The BMP15-induced down-regulation of Cx43 in SVOG cells is abolished by treatment with the BMP type I receptor inhibitor dorsomorphin

A and B, To localize the distribution of Cx43 in SVOG cells, cells were transfected with 25 nM control siRNA (siCtrl) or (GJA1) Cx43 siRNA (siCx43) for 48 h. The knockdown efficiency of each siRNA was examined by Western blot analysis (A). Following the transfection of the cells with 25 nM siCtrl or siCx43 for 48 h, the cells were fixed in 4% paraformaldehyde in PBS, and examined for Cx43 immunofluorescence (red) (B). C and D, Cells were treated with 50 ng/ml BMP15 for 24 h in the presence of vehicle control (DMSO) or 5 μM dorsomorphin. The levels of Cx43 protein were examined by Western blot analysis (C) or immunofluorescence microscopy (D). (E and F) Cells were treated with 50 ng/ml BMP15 for 24 h in the presence of vehicle control (DMSO) or 5 μM SB431542. The levels of Cx43 protein were examined by Western blot analysis (E) or immunofluorescence microscopy (F). Cell nuclei were stained with Hoechst 33258. Scale bar represents 50 μm. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < 0.05$). Ctrl, control. B15, BMP15. DM, dorsomorphin.

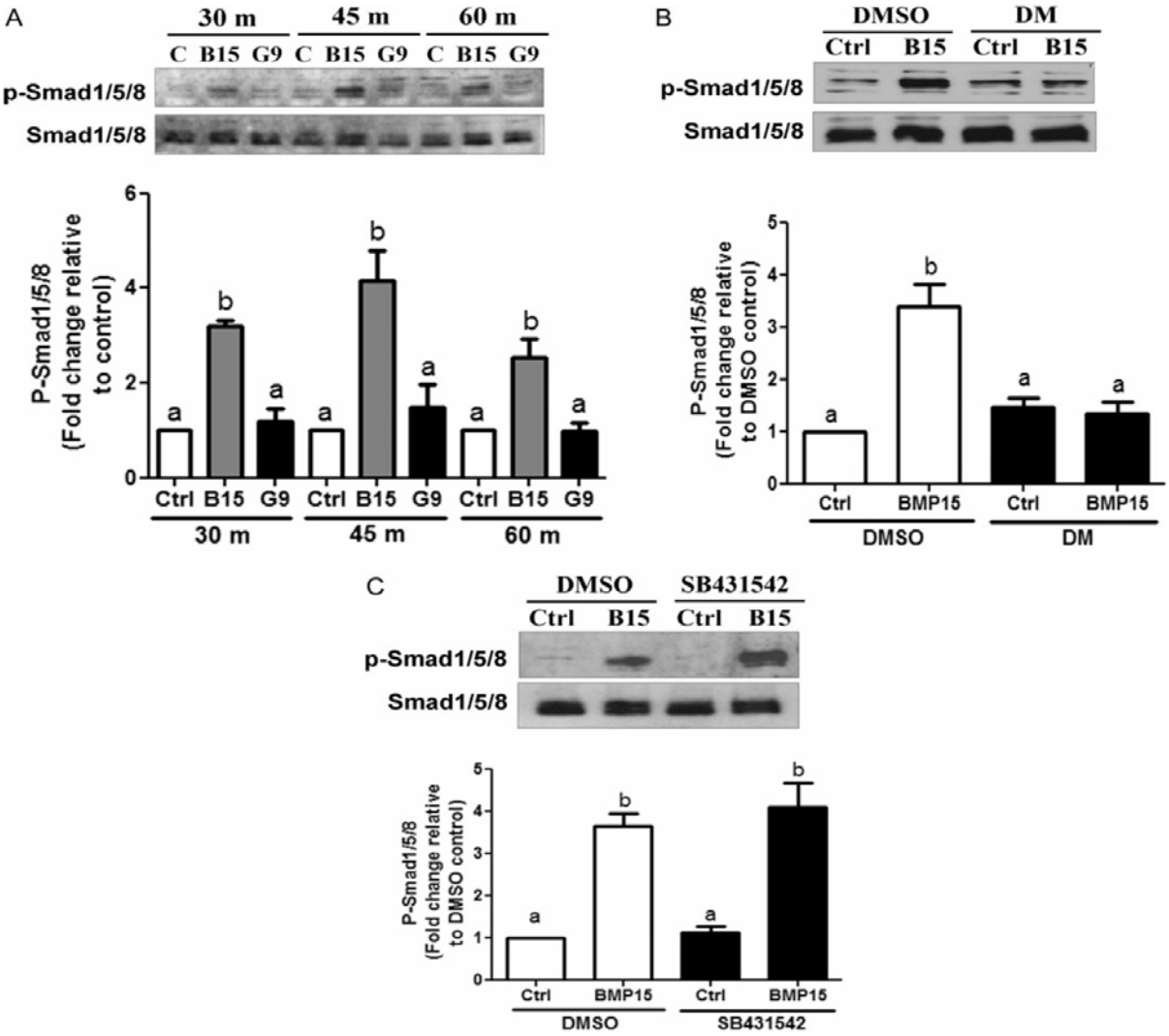


Figure 4.3 BMP15 but not GDF9 activates the SMAD1/5/8 signaling pathway in SVOG cells

A, Cells were treated with 50 ng/ml BMP15 or GDF9 for 30, 45 or 60 min; the phosphorylation levels of SMAD1/5/8 were examined by Western blot analysis. B, Cells were treated with 50 ng/ml BMP15 for 60 min in the presence of vehicle control (DMSO) or 5 μM dorsomorphin; the phosphorylation levels of SMAD1/5/8 were examined by Western blot analysis. C, Cells were treated with 50 ng/ml BMP15 for 60 min in the presence of vehicle control (DMSO) or 5 μM SB431542; the phosphorylation levels of SMAD1/5/8 were examined by Western blot

analysis.The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P<0.05). Ctrl, control. DM, dorsomorphin. B15, BMP15. G9, GDF9.

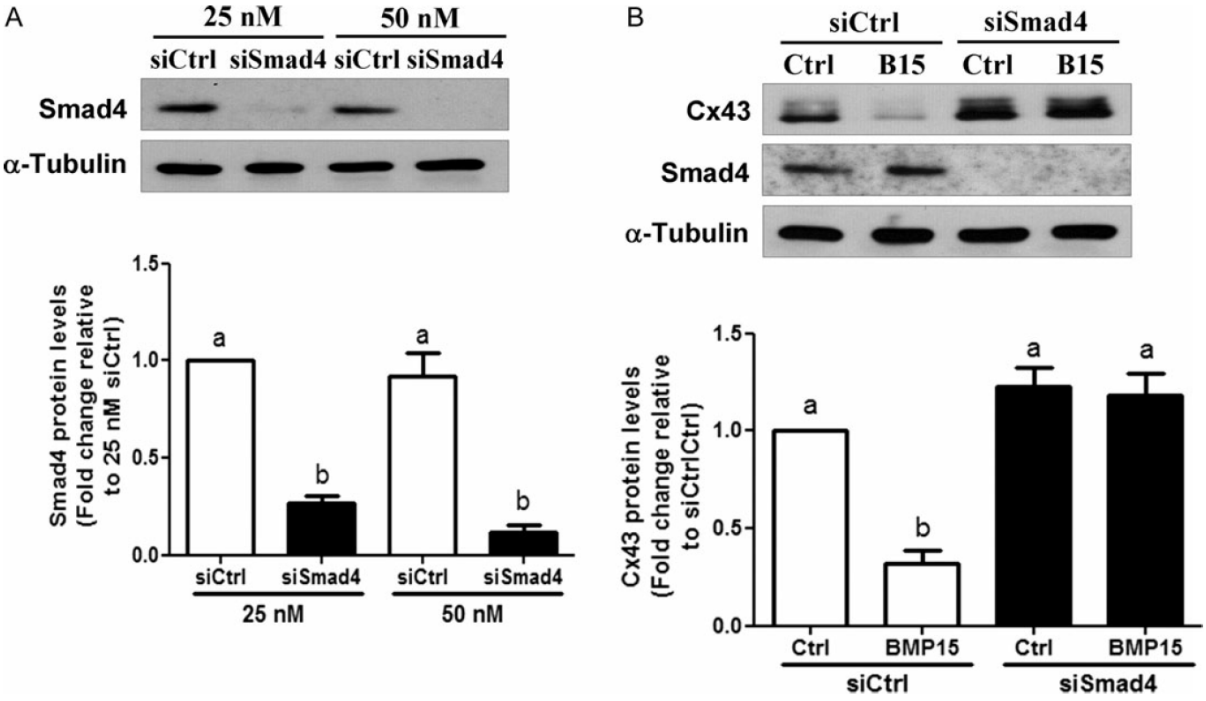
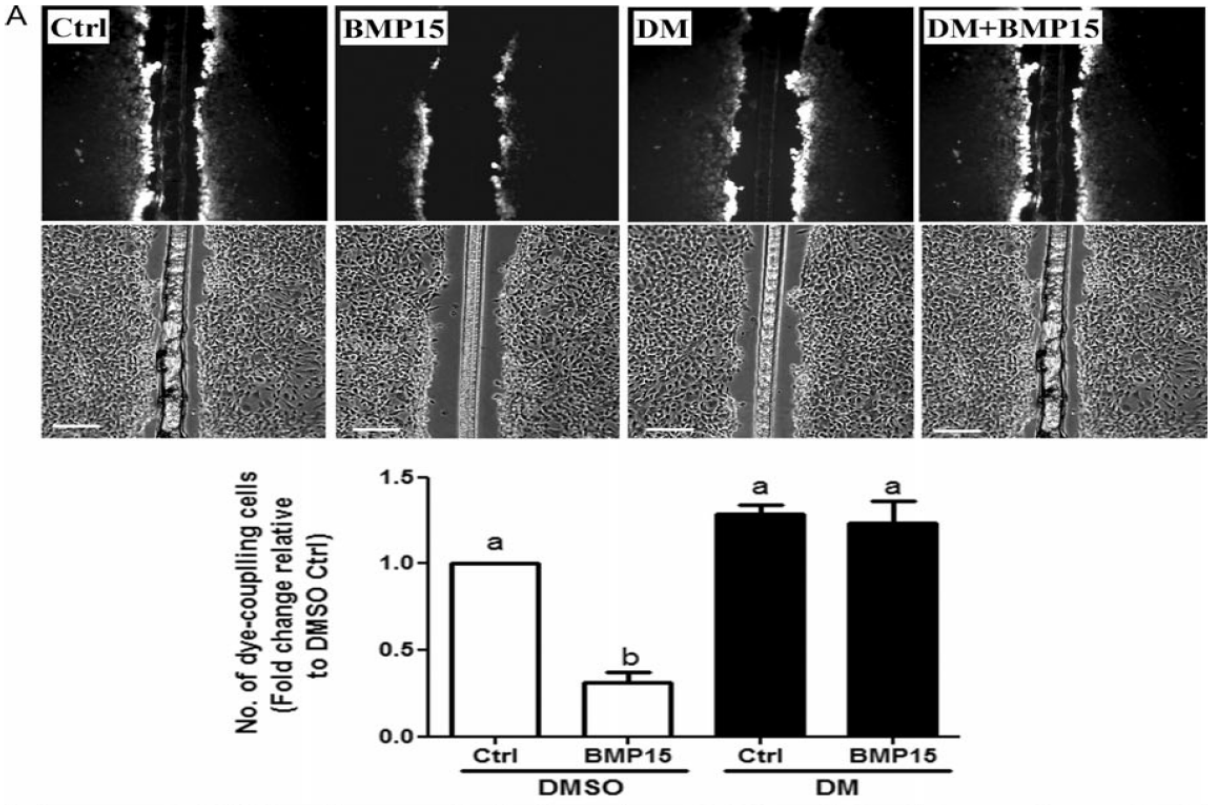


Figure 4.4 Knockdown of SMAD4 abolishes the BMP15-induced down-regulation of Cx43 in SVOG cells

A, Cells were transfected for 48 h with transfection reagent (Lipofectamine RNAiMAX), 25 nM or 50 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4). The knockdown efficiency of SMAD4 siRNA was examined by Western blot analysis. B, Cells were transfected for 48 h with 25 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4) and then treated for 24 h with 50 ng/ml BMP15 or vehicle control. The levels of Cx43 protein were examined by Western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments.

Values marked by different letters are significantly different ($P<0.05$). Ctrl, control. B15, BMP15.


A
Ctrl
BMP15
DM
DM+BMP15
No. of dye-coupllling cells
(Fold change relative
to DMSO Ctrl)
1.5
1.0
0.5
0.0
a
b
a
a
Ctrl
BMP15
Ctrl
BMP15
DMSO
DM

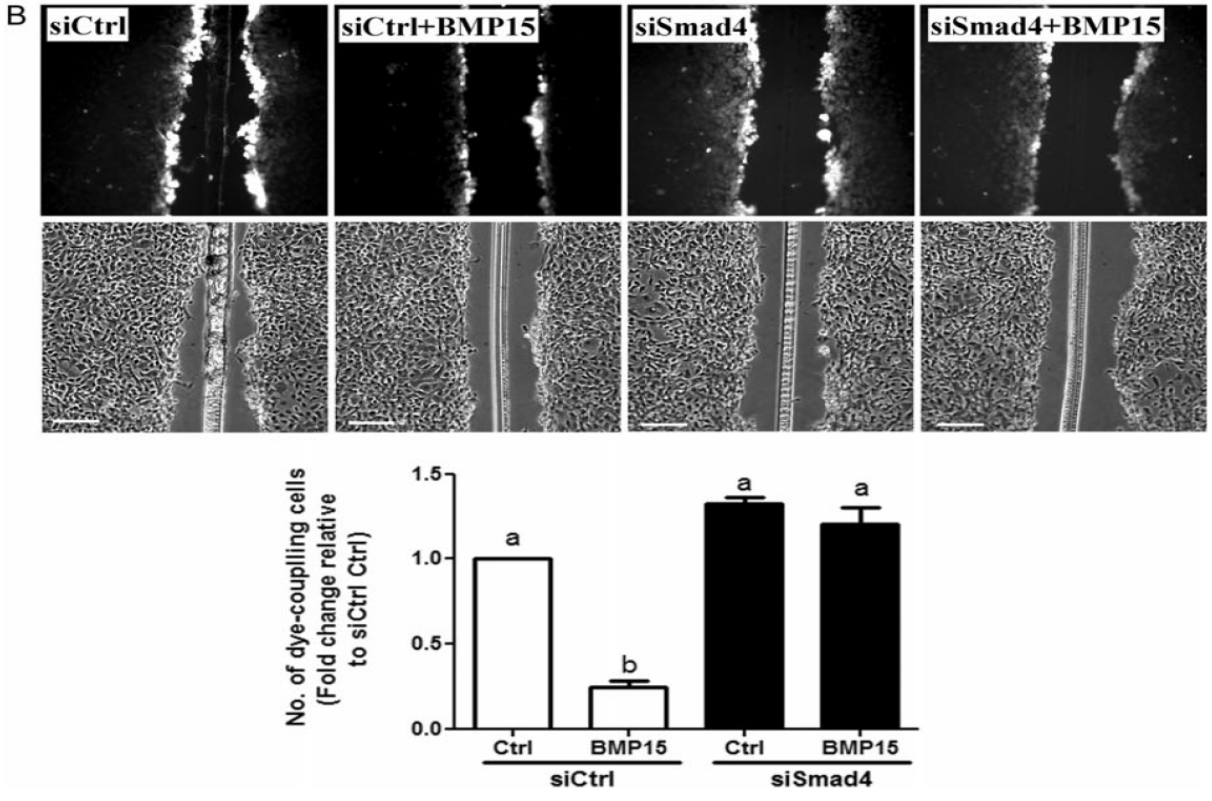

B
siCtrl
siCtrl+BMP15
siSmad4
siSmad4+BMP15
No. of dye-coupllling cells
(Fold change relative
to siCtrl Ctrl)
1.5
1.0
0.5
0.0
a
b
a
a
Ctrl
BMP15
Ctrl
BMP15
siCtrl
siSmad4

Figure 4.5 BMP15 decreases GJIC activity in SVOG cells

A. Fully confluent cells were treated with 50 ng/ml BMP15 for 24 h in the presence or absence of 5 μM dorsomorphin (DM). B. Cells were transfected for 48 h with 25 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4) and then treated for 24 h with 50 ng/ml BMP15. The GJIC activity was measured by monitoring the transfer of Lucifer yellow fluorescent dye between cells, and the images were captured utilizing a fluorescence microscope (top panel). The corresponding phase contrast micrographs are shown in the bottom panel. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P<0.05$). Ctrl, control. DM, dorsomorphin.

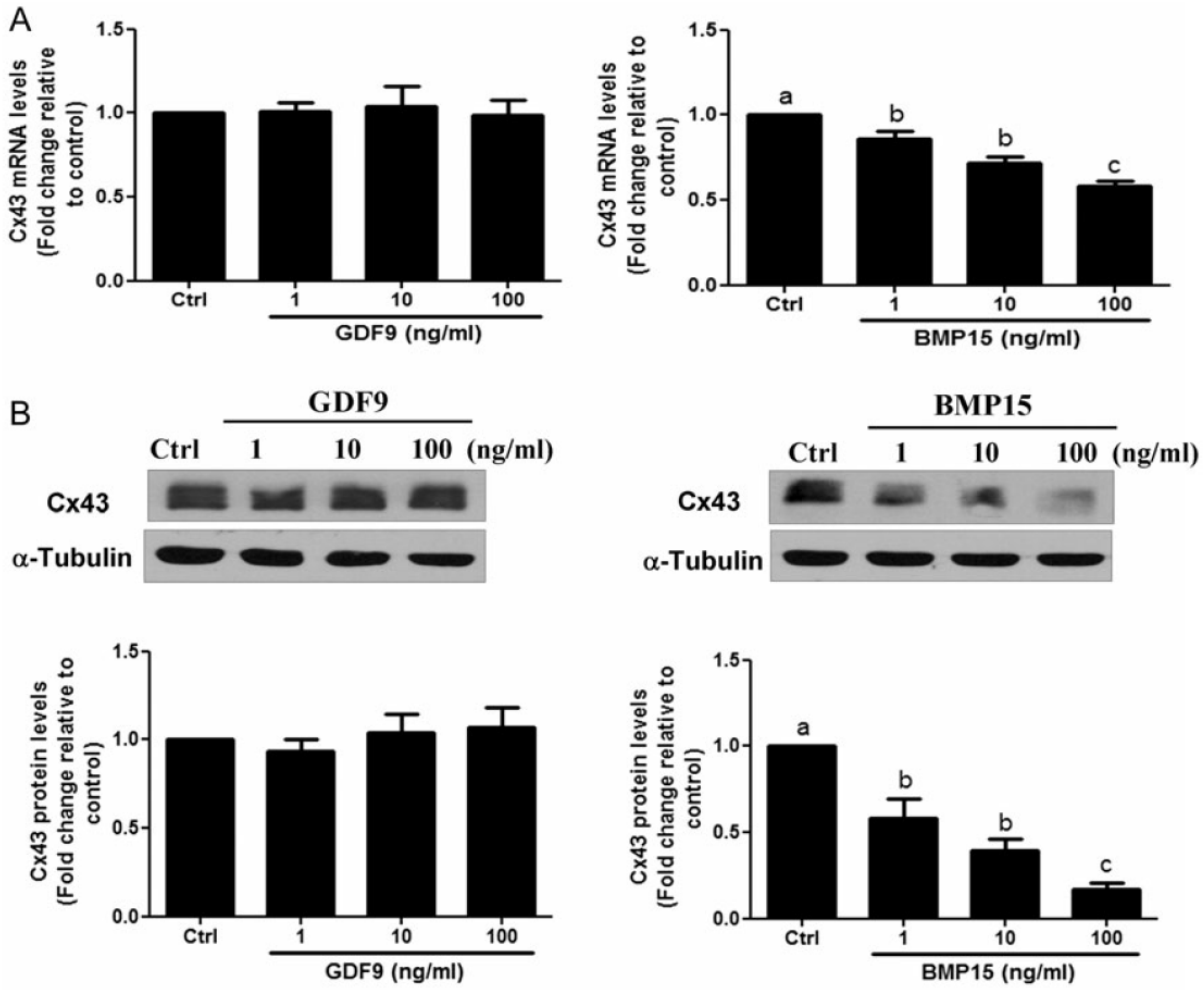


Figure 4.6 BMP15, but not GDF9, down-regulates Cx43 protein expression in hGL cells

hGL cells were treated with different concentrations (1, 10 or 100 ng/ml) of GDF9 or BMP15 for 24 h; Cx43 mRNA (A) and protein (B) levels were examined by RT-qPCR and Western blot analysis. The results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P <0.05). hGL, human granulosa-lutein. Ctrl, control. G9, GDF9. B15, BMP15.

Chapter 5: Theca-derived BMP4 and BMP7 down-rgulate connexin43 expression and decrease gap junction intercellular communication activity in immortalized human granulosa cells

5.1 Introduction

Gap junctions are specialized channels that directly connect the cytoplasm of cells and facilitate the transfer of various small molecules, metabolites and ions between neighboring cells (172, 173). One gap junction is composed of two docking hemichannels (or connexons), which span the cell membrane and bridge the intercellular space (75). The hemichannels on each side of the bordering cells consist of six symmetric rings of monomers, called connexins (76, 77). Recent studies utilizing tandem mass spectrometry, phosphorylation site-specific antibodies and site-directed mutagenesis have shown that a number of kinases and connexin phosphorylation are involved in the regulation of gap junction intercellular communication (GJIC) (173-176). Moreover, increasing evidence has shown that connexin-based channels between granulosa cells and the oocyte not only provide nutrition and metabolite transport but also play a crucial role in the maintenance of oocyte meiotic arrest (161, 177).

The connexins constitute a multigene family of membrane-spanning proteins, with at least twenty members identified in the sequenced rodent genome and twenty-one, in the human genome (178, 179). In the most widely used nomenclature system for members of this family, the word “connexin” (Cx) is followed by a number indicating the molecular weight (in kilodaltons) of the connexin isoform (78, 179). Gene knockout studies have demonstrated that connexin-coupled gap junction communication plays an important role in germ cell development and ovarian folliculogenesis (81, 82, 180). In developing follicles, Cx37 contributes to the gap

junctions that connect the oocyte to the surrounding granulosa cells, whereas gap junctions between granulosa cells contain predominantly Cx43 (81, 82, 181). Mice deficient in Cx37 exhibit a lack of coupling between oocytes and granulosa cells, disrupted folliculogenesis at the stage of antrum formation, failed ovulation and infertility (81). Cx43 is expressed abundantly in granulosa cells and is present at all stages of follicle development, and its ablation completely abolishes coupling among these cells, leading to a reduced number of germ cells in the fetal gonads, retarded oocyte growth and follicular arrest at the primary or early secondary follicle stage (82). It has also been suggested that Cx43-coupling channels are required to sustain granulosa cell proliferation (82, 83).

In the ovary, the cyclic expression of Cx43 has been detected during follicular growth, indicating that Cx43 is developmentally and hormonally regulated. In the developing and mature follicles, the expanding granulosa cells continue to express abundant Cx43 until the time of ovulation, after which the level of Cx43 dramatically decreases. Very low amounts of Cx43 mRNA or protein are detected in follicles undergoing atresia (80, 88, 182). Bone morphogenetic protein 4 (BMP4) and BMP7 are theca cell-derived growth factors that directly affect granulosa cell function, mainly as paracrine factors (6, 183). In the rat ovary, BMP4 and BMP7 are expressed by ovarian stromal and/or theca cells and have been shown to promote early folliculogenesis, stimulate granulosa cell proliferation, maintain follicle survival and prevent premature luteinization and/or atresia (2, 6). As a member of the transforming growth factor β (TGF-β) superfamily, BMP exerts its effects by binding to transmembrane serine/threonine kinase receptors. Upon ligand binding to the type II receptor (BMPRII), type I receptors (also known as active-like kinase, ALK) are recruited and transphosphorylated, and the formation of this ternary complex leads to the subsequent phosphorylation of the downstream signaling

molecules, SMAD1/5/8 (137). Phosphorylated and activated SMAD1/5/8 then associate with a common SMAD (Co-SMAD or SMAD4) and translocate to the nucleus to regulate target gene expression (157).

Given that Cx43 is the dominant connexin in the granulosa cells of human ovaries and that there is evidence of a correlation between the Cx43 mRNA levels in granulosa cells and the competence of the associated oocytes and embryos, it has been suggested that one important function of Cx43-coupled gap junctions is to complete the final processes of folliculogenesis and oogenesis (134, 161, 184). Interestingly, the dramatic spatiotemporal changes of BMP4 and BMP7 in theca cells are accompanied by the expression of Cx43 in granulosa cells (11). These data lead us to propose that BMP4 and BMP7 have a crucial role in modulating Cx43 expression to ensure the proper timing of the events required for the generation of the menstrual cycle.

The role of gonadotropins and steroid hormones in regulating Cx43 expression in the female reproductive system has been investigated in several studies, yet the roles of BMP4 and BMP7 in modulating Cx43 expression remain unknown. In the present study, we investigated the effects of theca cell-derived BMP4 and BMP7 on Cx43 expression in human granulosa cells. The observed inhibitory effects were then examined in relation to GJIC activity and the potential underlying mechanisms.

5.2 Materials and methods

Simian virus 40 large T antigen immortalized human granulosa cell (SVOG) culture

SVOG, a non-tumorigenic immortalized human granulosa cell line previously produced by transfecting human granulosa-luteal cells with the SV40 large T antigen (136), was used in this study. Cells were counted with a hemocytometer, and cell viability was assessed by trypan blue

(0.04%) exclusion. The cells were seeded (2-4 × 10^5 cells per ml in 6-well plates) and cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37 C. Cells were cultured in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/ml penicillin (Invitrogen, Life Technologies, NY), 100 μg/ml streptomycin sulfate (Invitrogen, Life Technologies), and 1X GlutaMAX (Invitrogen, Life Technologies). The culture medium was changed every other day in all experiments.

Antibodies and reagents

Polyclonal anti-Connexin 43 and anti-phospho-SMAD1($Ser^{463/465}$)/SMAD5($Ser^{463/465}$)/SMAD8($Ser^{426/428}$) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal anti-SMAD1/5/8 (N-18) (sc-6031-R) and polyclonal anti-actin (C-11) (sc-1615) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad Laboratories (Hercules, CA), and horseradish peroxidase-conjugated donkey anti-goat IgG was obtained from Santa Cruz Biotechnology. Recombinant human BMP4, recombinant human BMP7, and dorsomorphin dihydrochloride were obtained from R&D Systems (Minneapolis, MN). Lucifer yellow was obtained from Molecular Probes (Life Technologies).

Reverse transcription and quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS, and total RNA was extracted with TRIzol Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. RNA (2μg) was reverse transcribed into first-strand cDNA with random primers and MMLV reverse

transcriptase (Promega, Medison, WI). Each 20 µl RT-qPCR reaction contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were as follows: GJA1 (Cx43), 5'-TAC CAA ACA GCA GCG GAG TT-3' (sense) and 5'-TGG GCA CCA CTC TTT TGC TT-3' (antisense) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'- ATG GAA ATC CCA TCA CCA TCT T -3' (sense) and 5'- CGC CCC ACT TGA TTT TGG -3' (antisense). RT-qPCR was performed on the Applied Biosystems 7300 Real-Time PCR System equipped with a 96-well optical reaction plate (Applied Biosystems). Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Cq}$.

Western blot analysis

After treatment, cells were washed with cold PBS and lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µM aprotinin, 1 µM leupeptin and 1 mM PMSF) (Cell Signaling) containing a protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 *xg* for 15 min at 4 C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 1 h in TBS containing 0.05% Tween 20 and 5% nonfat dried milk and incubated overnight at 4 C with the relevant primary antibodies. After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) reagents or a

SuperSignal West Femto Chemiluminescence Substrate (Pierce, Rockford, IL), followed by exposure to CL-X Posure film (Thermo Fisher, Waltham, MA). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mmol/l β-mercaptoethanol and 1% SDS) at 50 C for 30 min and reprobed with goat anti-actin antibody as a loading control.

Immunofluorescence staining

To study the localization and distribution of Cx43 in granulosa cells, immunofluorescence staining was performed in SVOG cells cultured in vehicle and BMP4 or BMP7 with or without dorsomorphin. Briefly, cells were plated on glass cover slips, fixed with 4% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. After washing with PBS, the cover slips were mounted on microscope slides and blocked with Dako Protein Block (Dako, Mississauga, Ontario, Canada) for 1 h, followed by incubation with Connexin43 antibody (diluted in Dako Protein Block) overnight. Alexa Fluor 555 donkey anti-rabbit IgG (Life Technologies) were used as a secondary antibody. Finally the cells were counterstained with chromosomal dye Hoechst 33258 (Sigma-Aldrich), rinsed with PBS, mounted in Gelvatol and imaged under a Zeiss Axiophot fluorescence microscope equipped with a digital camera (Q Imaging, Burnaby, BC, Canada).

Scrape loading and dye transfer assay

To determine the effects of BMP4 and BMP7 on gap junction intercellular communication between human granulosa cells, a scrape loading and dye transfer assay, which is based on monitoring the transfer of the fluorescent dye Lucifer yellow (MW 457.2) from one cell into the adjacent cell via functional gap junctions, was performed (185). The scrape loading technique

has been used to introduce macromolecular substances into cultured cells effectively by producing a transient tear on the cell membrane. Lucifer yellow cannot diffuse through intact cell membranes; however, its low molecular weight allows this fluorescent dye to transfer among adjacent cells by passing through intact gap junctions (185). Fully confluent cells were washed with PBS and scraped using a surgical blade before fluorescent dye (0.5% Lucifer yellow CH, potassium salt, Life Technology) was added. After incubation for 5 min, the cells were washed thoroughly to remove background fluorescence, fixed with 4% paraformaldehyde and imaged with a Zeiss Axiophot fluorescence microscope equipped with a digital camera. The GJIC was evaluated and measured as the distance from the scrape line to the furthest extent of the dye-coupled cells.

Small interfering RNA (siRNA) transfection

We performed transient knockdown assays using 25 nM SMAD4-targeting siRNA (ON-TARGET*plus* SMARTpool) or 25 nM control siRNA (ON-TARGET*plus* Non-targeting Pool) purchased from Thermo Fisher Scientific (Lafayette, CO). Cells were pre-cultured to 50% confluence in antibiotics-free DMEM/F12 medium containing 10% FBS, after which they were transfected with siRNA using Lipofectamine RNAiMAX (Dharmacon, Life Technologies) for 48 h. The efficiency of the SMAD4 knockdown was confirmed by Western blot analysis.

Statistical analysis

Results were analyzed by One-way ANOVA followed by Tukey's multiple comparison tests in PRISM software (GraphPad Software, Inc., San Diego, CA). The results are presented as the

mean ± SEM of at least three independent experiments. Data were considered significantly different from each other if $P < 0.05$.

5.3 Results

BMP4 and BMP7 down-regulate Cx43 in SVOG cells

The concentrations of BMP7 in human follicular fluid are known to range from 50 to 130 ng/ml (186), and the concentrations of BMP4 in human serum samples range from 0.4 to 1.3 ng/ml (187). To investigate whether theca cell-derived BMP4 and BMP7 induce the down-regulation of Cx43, SVOG cells were treated with increasing concentrations (1, 10 and 100 ng/ml) of human recombinant BMP4 or BMP7. As shown in Figure 5.1A, treatment with BMP4 for 12 h down-regulated Cx43 mRNA levels in a concentration-dependent manner, and treatment with BMP4 for 24 h down-regulated Cx43 protein levels in a concentration-dependent manner (Figure 5.1B). Treatment with BMP7 for 24 h produced similar effects on Cx43 mRNA and protein levels (Figures 5.1C and 5.1D). In addition, treatment with 30 ng/ml of BMP4 or BMP7 resulted in a time-dependent inhibitory effect on Cx43 expression (Figure 5.1E). Interestingly, BMP4 showed more potent effects than BMP7 on the down-regulation of Cx43 (Figure 5.1F).

Dorsomorphin abolishes the BMP4- and BMP7-induced down-regulation of Cx43 in SVOG cells

Three type I receptors, ALK2, ALK3 and ALK6, have been shown to be involved in BMP-mediated cellular functions (162). Dorsomorphin, a selective inhibitor of BMP type I receptors, has been shown to specifically inhibit the function of ALK2, ALK3 and ALK6 (143, 163). To

examine whether ALK2, ALK3 and ALK6 are required for the BMP4 and BMP7-induced down-regulation of Cx43, cells were treated with BMP4 or BMP7 in the presence or absence of 1 μM dorsomorphin. As shown in Figure 5.2A, treatment with dorsomorphinabolished the BMP4 and BMP7-induced down-regulation of Cx43. In addition, immunostaining results showed that Cx43 was expressed as red fluorescent plaques at the sites of contact between adjacent cells (Figure 5.2B). Consistent with the Western blot results, treatment with BMP4 or BMP7 decreased the expression of Cx43, and these decreases were abolished by co-treatment with dorsomorphin (Figure 5.2B). These results indicated that ALK2, ALK3 and ALK6 are required for the inhibitory effects of BMP4 and BMP7 on Cx43 expression in SVOG cells.

BMP4 and BMP7 activate SMAD1/5/8 signaling pathway in SVOG cells

To examine whether treatment with BMP4 and BMP7 activates SMAD1/5/8, cells were treated with BMP4 or BMP7 (30 ng/ml) at different time points (30 min, 45 min and 60 min). As shown in Figure 5.3A, treatment with BMP4 or BMP7 increased the phosphorylation levels of SMAD1/5/8, with a maximum effect at 30 min, followed by a decrease after 60 min of treatment. To further examine whether ALK2, ALK3 and ALK6 are required for BMP4 and BMP7-induced SMAD1/5/8 activation, cells were treated with BMP4 or BMP7 in the presence or absence of 1μM dorsomorphin. As shown in Figure 5.3B, treatment with dorsomorphinabolished the BMP4 and BMP7-mediated increase in SMAD1/5/8 phosphorylation. These results indicated that ALK2, ALK3 and ALK6 are required for the BMP4 and BMP7-induced activation of SMAD1/5/8 in SVOG cells.

The SMAD1/5/8 signaling pathway is required for the BMP4- and BMP7-induced down-regulation of Cx43 in SVOG cells

To examine whether SMAD1/5/8 is required for the BMP4 and BMP7-induced down-regulation of Cx43, SMAD4 siRNA was used to knock down the endogenous expression of the common SMAD, SMAD4 because SMAD4 is necessary for SMAD1/5/8 function. Western blot analysis showed that after transfection for 48 h, SMAD4 siRNA down-regulated SMAD4 protein levels by 70-80% relative to the transfection reagent control (iMAX) and control siRNA (Figure 5.4A). Treatment with SMAD4 siRNA for 48 h abolished the inhibitory effects of BMP4 and BMP7 on Cx43 expression (Figure 5.4B). These data indicated that SMAD1/5/8 signaling is involved in the BMP4- and BMP7-induced down-regulation of Cx43.

The effects of BMP4 and BMP7 on gap junction intercellular communication (GJIC) in SVOG cells

To investigate the effects of down-regulating Cx43 expression on GJIC activity, cellular GJIC was analyzed using the scrape-loading dye transfer technique. As shown in Figure 5.5A, BMP4 or BMP7 treatment (24 h) reduced the number of Lucifer yellow dye-coupled cell layers on either side of the scrape. In addition, pre-treatment dorsomorphin for 30 min abolished the BMP4- and BMP7-mediated decrease in GJIC. Similarly, treatment with SMAD4 siRNA for 48 h reversed the inhibitory effects of BMP4 and BMP7 on GJIC activity (Figure 5.5B). These results indicated that BMP4 and BMP7 decreased GJIC activity in SVOG cells.

5.4 Discussion

Gap junctions mediate various biological functions in granulosa cells. Given the evidence that changes in Cx43 levels throughout the estrous cycle are highly correlated with the profiles of serum FSH and LH, it has been suggested that the expression of Cx43 in granulosa cells is mainly regulated by pituitary gonadotropins (80, 87, 88, 188). In the rat, the exogenous administration of FSH up-regulated the expression of Cx43 mRNA and the synthesis of Cx43 protein in the ovarian follicles and granulosa cell lines, whereas follicles incubated with LH or injected with human chorionic gonadotropin exhibited down-regulated Cx43 expression and the subsequent loss of intercellular coupling within the granulosa cell layers (79, 88, 189, 190). In addition to gonadotropins, steroid hormones have also been reported to regulate Cx43 in a large number of mammalian species, including humans (89-91, 191). It has been shown that high androgen levels decrease Cx43 levels and GJIC activity in a human granulosa cell line (91). However, to date, the effects of BMPs on granulosa cells have not been investigated. In the present study, we show for the first time that exogenous BMP4 and BMP7 inhibit gap junction function by down-regulating its major component, Cx43.

In the growing follicles, Cx43-coupled gap junctions facilitate the synchronization of follicular development, and the levels of Cx43 mRNA and protein increase until ovulation occurs (80, 88, 182). Furthermore, mutant follicles from Cx43 knockout mice failed to undergo meiotic maturation and could not be fertilized, indicating that Cx43-containing gap junction channels are required for oocyte maturation and subsequent luteinization (82). Our results provided evidence that BMP4 and BMP7 may exert their biological functions in the human ovary by inhibiting GJIC through the down-regulation of Cx43 expression. In rat *in vivo* studies, injections of recombinant BMP7 into the ovarian bursa caused pronounced inhibitory effects on the ovulation

rate and serum progesterone levels (6). Taken together, the results of previous studies and our findings suggest that at the periovulatory stage, both BMP4 and BMP7 are involved in inhibiting ovulation and preventing luteinization. However, future studies investigating the physiological roles of BMP4 and BMP7 at the periovulatory stage of granulosa cells will be required. Furthermore, it has been proposed that the high androgen levels in granulosa cells decreased Cx43 expression and GJIC activity in polycystic ovary syndrome (PCOS) patients, as shown by impaired folliculogenesis and anovulation (91). Whether serum or follicular levels of BMP4 and BMP7 are increased in PCOS patients and whether these increased levels contribute to the etiology of these multi-pathogenetic processes warrant further investigation.

Outside of the reproductive system, members of the TGF-β superfamily have been demonstrated to modulate Cx43 expression. A recent study showed that TGF-β1 induced an epithelial-to-mesenchymal transition (EMT) by reducing Cx43 and GJIC activity in human renal cells (192). BMPs and growth and differentiation factors (GDFs) have been reported to play an important role in rat skeletogenesis by modulating the expression of gap junction gene Gja1 (Cx43α1) (193, 194). In mouse osteosarcoma cells, BMP2, BMP4 and GDF5, but not BMP6 and BMP7, positively regulate Cx43α1 promoter activities (194). However, the regulatory effects of the TGF-βsuperfamily members, particularly BMPs, on Cx43 expression in the reproductive system remain largely unknown. In the present study, instead of using Cx43 promoter constructs, we directly demonstrated that treatment with BMP4 or BMP7 down-regulated Cx43 mRNA and protein levels in SVOG cells. Interestingly, BMP7 was previously found not to affect the Cx43 promoter activity in mouse osteosarcoma cells, in contrast to our results (194). These discrepancies may be attributed to inter-species differences or potential mechanisms in which the BMP-driven activation and inhibition of gene expression is tissue- or cell type-dependent (111).

To date, over 20 BMPs have been identified in humans, and studies have shown that some of these growth factors are expressed in the ovary. Specifically, oocytes express BMP6 and BMP15; granulosa cells express BMP2, BMP5 and BMP6; and theca cells express BMP4 and BMP7 (2). Further investigation will be required to determine whether oocyte and granulosa cell-derived BMPs exert Cx43-regulatory functions similar to those of the theca cell-derived BMPs in human granulosa cells.

The PKA and MAPK signaling pathways mediate gonadotropin-regulated Cx43 (79, 189). Although the regulatory effects of BMPs on Cx43 have been reported in mouse osteosarcoma cells, the underlying molecular mechanisms that mediate the regulatory functions of BMP on Cx43 remain uncharacterized. The results presented here demonstrated that SMAD1/5/8, which constitutes a well-known signaling pathway downstream of BMPs, mediated the inhibitory effects of BMP4 and BMP7 on Cx43. The analysis of human and mouse Cx43 promoters showed significant sequence conservation between these two species. Importantly, two putative SMAD binding sites have been identified (194). Although we did not provide direct evidence that SMAD1/5/8 could bind to the Cx43 promoter, the results of previous and present studies suggest that SMAD1/5/8 may be involved in the BMP4- and BMP7-induced down-regulation of Cx43 in human granulosa cells.

Unlike animal studies, in which granulosa cells from different stages of differentiation can be used to assess hormonal actions, nearly all studies of the human ovary are constrained to the use of highly differentiated granulosa cells obtained from women undergoing *in vitro* fertilization. In the present study, we used the SV40 large T antigen immortalized human granulosa cell line (SVOG) as our experimental model. SVOG cells are steroidogenic and provide a feasible model system to study the biological functions of granulosa cells in humans

(136). However, we are aware that immortalization may affect the behavior of cells, because the SV40 large T antigen has been shown to target multiple cellular pathways (195). Therefore, in the future, it will be important to investigate the effect of BMP4 and BMP7 on Cx43 expression in primary cultures of human granulosa cells.

In summary, the present study demonstrates that BMP4 and BMP7 down-regulate Cx43 in human granulosa cells. Our results suggest that the SMAD1/5/8 signaling pathway is required for the BMP4- and BMP7-induced down-regulation of Cx43. Moreover, the down-regulation of Cx43 contributes to the inhibition of gap junction intercellular communication, indicating that theca cell-derived BMP4 and BMP7 may be involved in the inhibition of ovulation and luteinization.

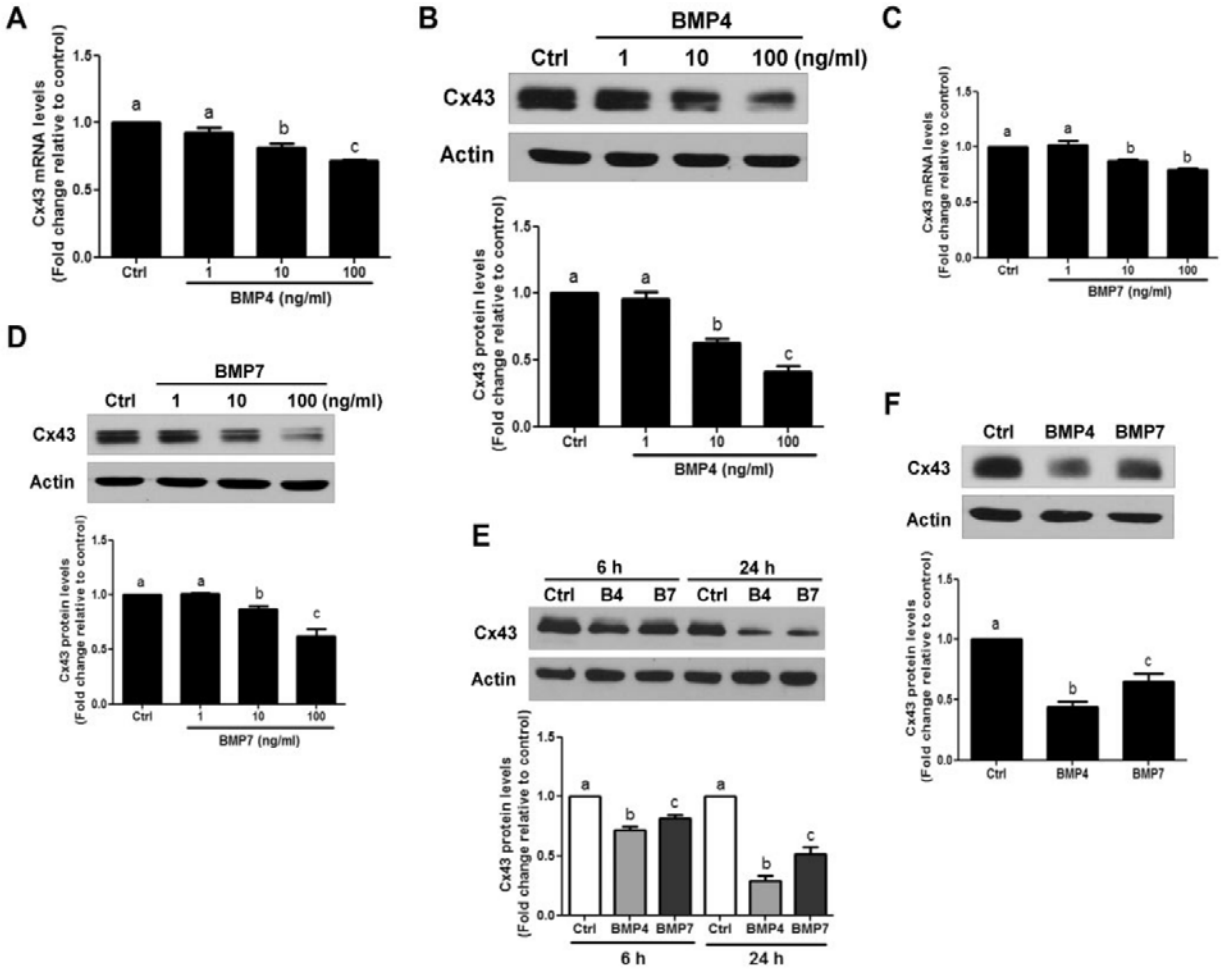


Figure 5.1 BMP4 and BMP7 down-regulate Cx43 mRNA and protein levels in SVOG cells

A and C, Cells were treated with different concentrations (1, 10 and 100 ng/ml) of BMP4 (A) or BMP7 (C) for 12 h; the levels of Cx43 mRNA were examined by RT-qPCR. B and D, Cells were treated with different concentrations (1, 10 and 100 ng/ml) of BMP4 (B) or BMP7 (D) for 24 h; the levels of Cx43 protein were examined by Western blot. E, Cells were treated with 30 ng/ml of BMP4 or BMP7 for 6 h and 24 h; the levels of Cx43 protein were examined by Western blot. F, Cells were treated with 30 ng/ml of BMP4 or BMP7 for 24 h; the levels of Cx43 protein were examined by Western blot. The results are expressed as the means ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P<0.05).

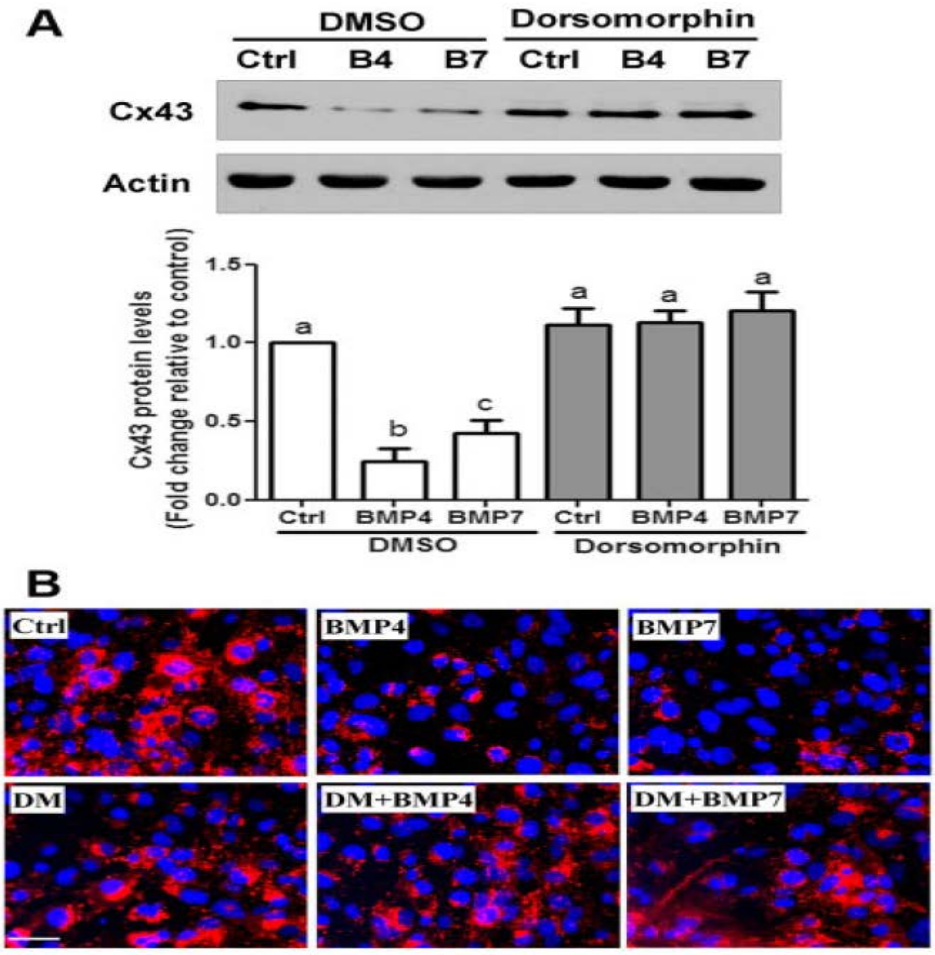


Figure 5.2 The BMP4- and BMP7-induced down-regulation of Cx43 in SVOG cells is abolished by treatment with the BMP type I receptor inhibitor dorsomorphin

A, Cells were treated with 30 ng/ml of BMP4 or BMP7 for 24 h in the presence of vehicle control (DMSO) or 1 μM dorsomorphin. The levels of Cx43 protein were examined by Western blot. The results are expressed as the means ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P<0.05). B, Cells were treated for 24 h with vehicle control (Ctrl) or 30 ng/ml BMP4 or BMP7 and fixed in cold methanol, and Cx43 levels (red) were examined by immunofluorescence microscopy. Cell nuclei were stained with Hoechst 33258. Scale bars represent 50 μm.

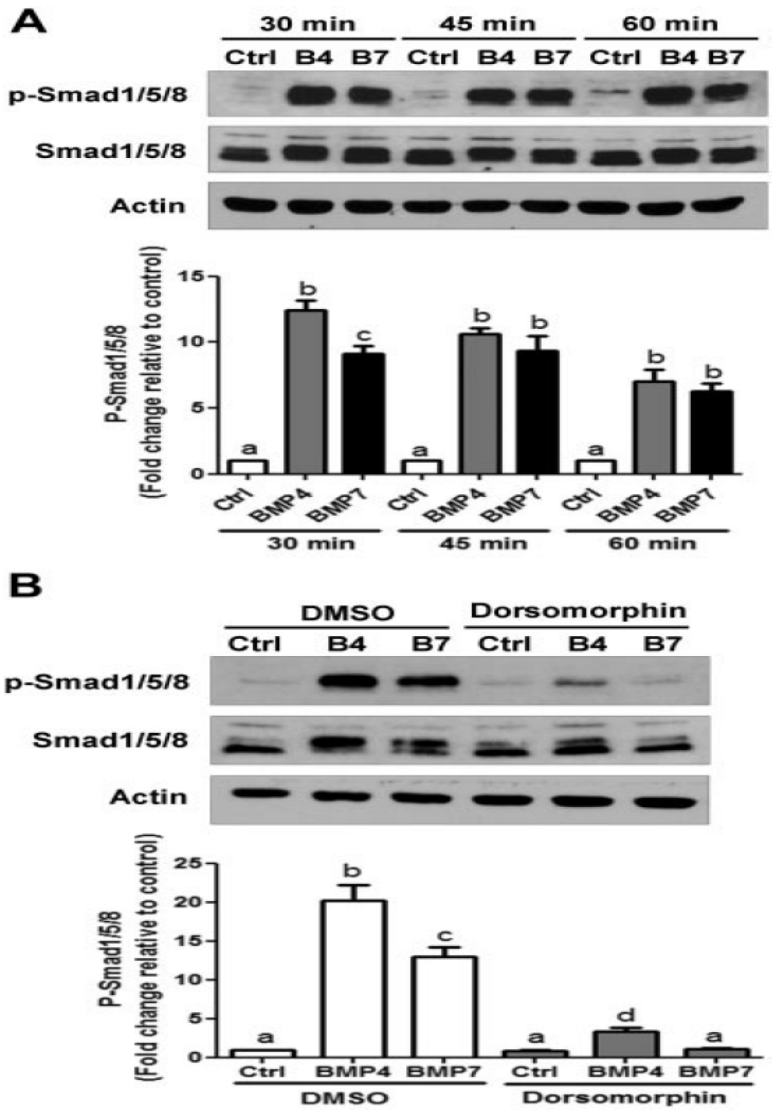


Figure 5.3 BMP4 and BMP7 activate the SMAD1/5/8 signaling pathway in SVOG cells

A, Cells were treated with 30 ng/ml of BMP4 or BMP7 for 30, 45 and 60 min. The phosphorylation levels of SMAD1/5/8 were examined by Western blot. B, Cells were treated with 30 ng/ml of BMP4 or BMP7 for 30 min in the presence of vehicle control (DMSO) or 1 μM dorsomorphin. The levels of SMAD1/5/8 phosphorylation were examined by Western blot. The results are expressed as the means ± SEM of at least three independent experiments. Values marked by different lettersletter are significantly different (P<0.05).

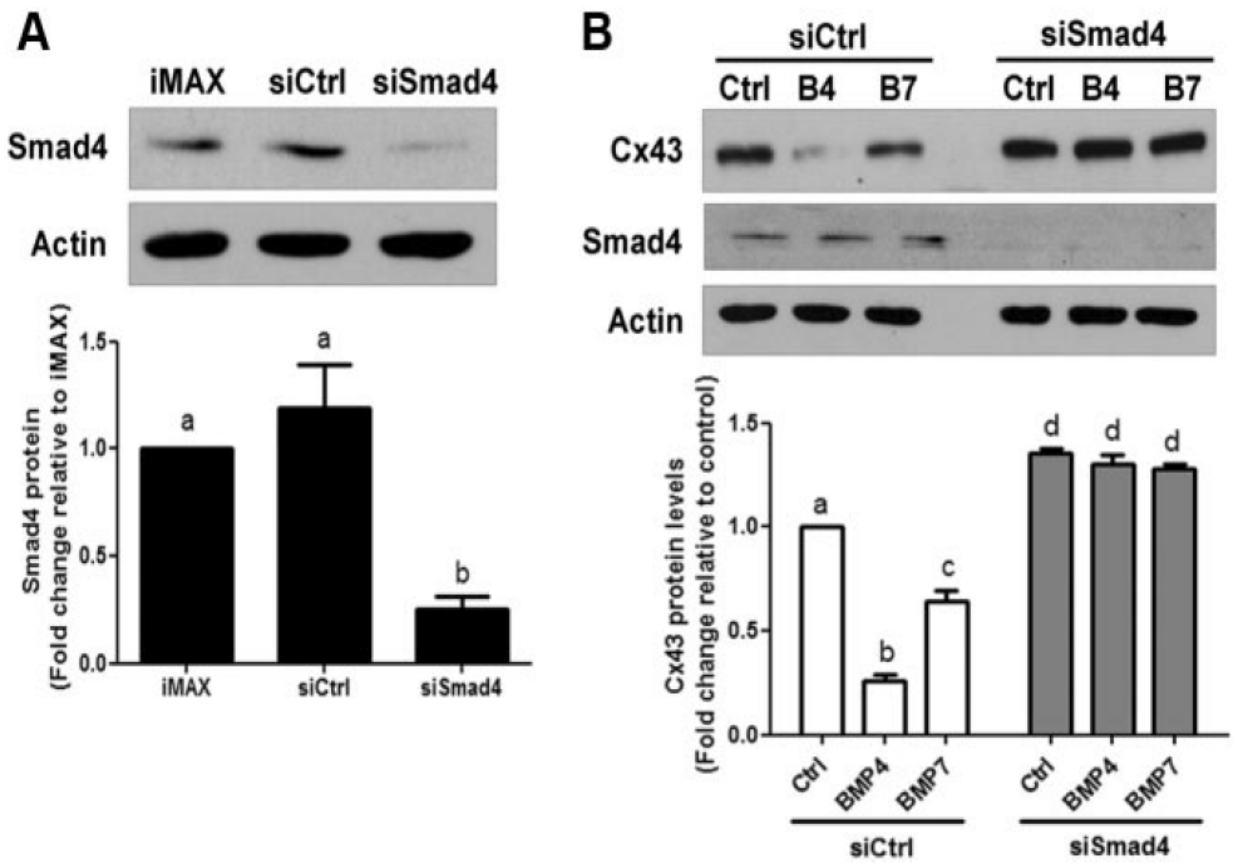


Figure 5.4 SMAD4 knockdown abolishes the BMP4- and BMP7-induced down-regulation of Cx43 in SVOG cells

A, Cells were transfected for 48 h with a transfection reagent (iMAX), 25 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4). The knockdown efficiency of SMAD4 siRNA was examined by Western blot. B, Cells were transfected for 48 h with 25 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4) and then treated with 30 ng/ml of BMP4 or BMP7 for 24 h. The levels of Cx43 were examined by Western blot. The results are expressed as the means ± SEM of at least three independent experiments. Values marked by different letters are significantly different (P <0.05).

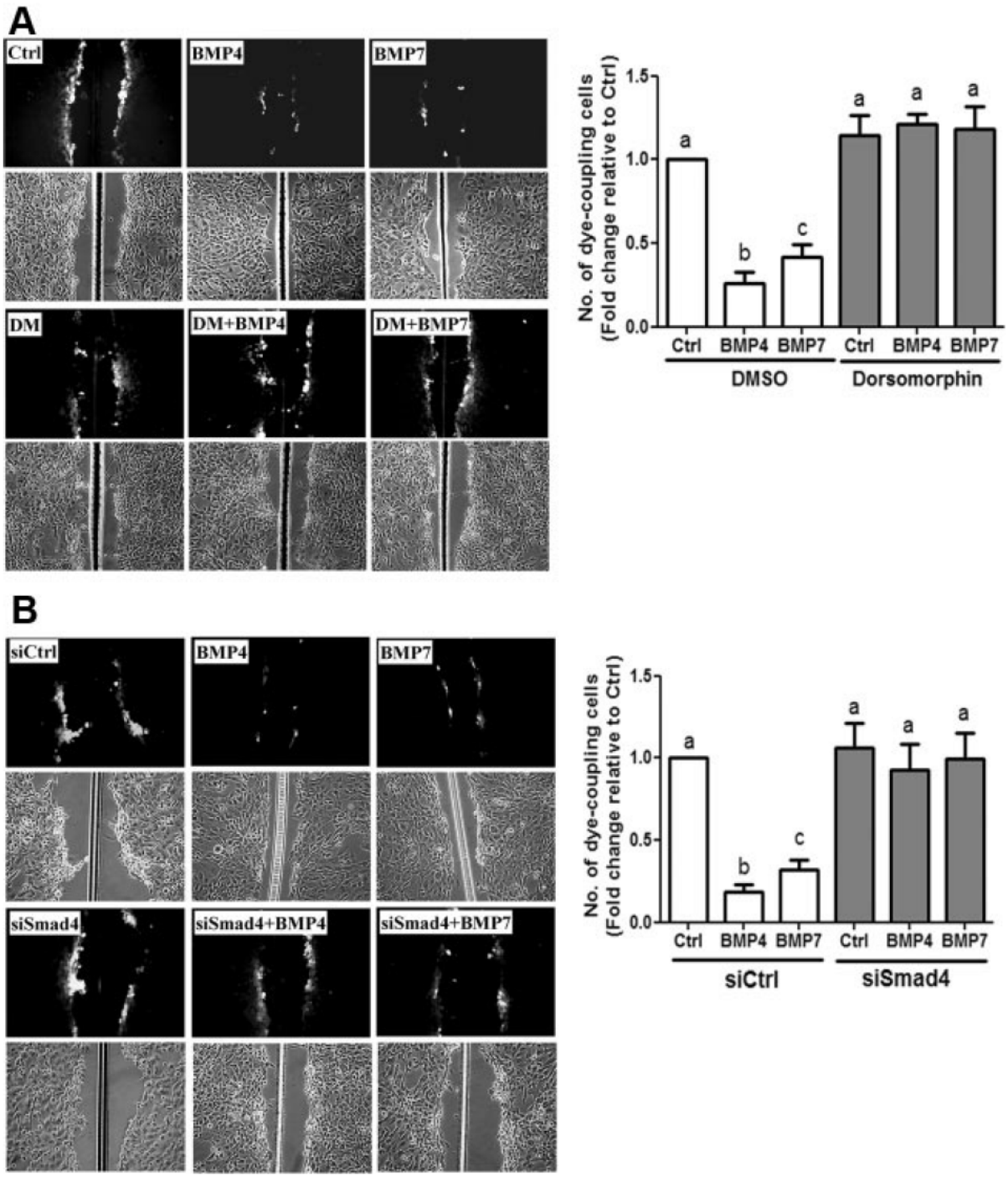


Figure 5.5 BMP4 and BMP7 inhibit gap junction intercellular communication (GJIC) in SVOG cells

A, Fully confluent cells were treated with 30 ng/ml of BMP4 or BMP7 for 24 h in the presence or absence of 1μM dorsomorphin. B, Cells were transfected for 48 h with 25 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4), and then treated with 30 ng/ml of BMP4 or BMP7 for 24 h. The GJIC was measured by monitoring the transfer of fluorescent dye between cells, and images were captured using a fluorescence microscope (top panel). The corresponding phase contrast micrographs are shown in the bottom panel. The results are expressed as the means ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P<0.05$).

Chapter 6: Theca-derived BMP4 and BMP7 down-regulate pentraxin 3 in human granulosa cells

6.1 Introduction

The bone morphogenetic protein (BMP) family is the largest among the TGF-β superfamily of growth factors. Studies of animals with naturally occurring mutations and targeted deletions of certain BMP family genes have shown that BMPs play crucial roles in mammalian fertility (12). The development and physiological functions of the basic units of the female reproductive system, ovarian follicles, are modulated by the spatial and tissue-specific expression of BMP family members (12). Specifically, granulosa cells express BMP2, BMP5 and BMP6; oocytes express BMP6 and BMP15; and theca cells express BMP4 and BMP7 (2). Increasing evidence suggests that BMP in theca cells is a putative luteinization inhibitor acting to inhibit progesterone production (71), to promote the survival and development of growing follicles (7) and to decrease cell-cell communication (158) in granulosa cells. In particular, treatment with BMP4 and BMP7 attenuates FSH-stimulated progesterone production in rat granulosa cells (71). In bovine granulosa cells, BMP4 and BMP7 suppress progesterone secretion (107). Our previous results also demonstrated that both BMP4 and BMP7 decrease cell-cell communication by down-regulating connexin 43-coupled gap junction formation in human granulosa cells (158). Taken together, all these data highlight the integral functions of the theca layer in the follicle structure that protect the growing follicles from undergoing atresia that results from premature luteinization incurred by LH (135).

Signal transduction studies have revealed that BMPs act through two types of serine/threonine kinase receptors, type I and II subtypes (100). Three type I receptors, activin receptor-like kinase

(ALK)2 (or ActRIA), ALK3 (or BMPRIA) and ALK6 (or BMPRIB), have been shown to bind BMP ligands and induce the phosphorylation of downstream receptor-regulated SMADs (R-SMADs), SMAD1/5/8 (137). Upon phosphorylation, the activated R-SMADs associate with a common SMAD, SMAD4, and translocate into the nucleus and participate in the BMP-induced gene transcription with other transcription factors (196). To date, despite the obvious importance of BMPs in female fertility, the corresponding BMP type I receptors (ALKs) that mediate the biological effects of BMPs remain poorly defined.

Pentraxin 3 (PTX3) belongs to the pentraxin superfamily and is expressed in a variety of human tissues (197, 198). Current research suggests that PTX3 is a multifunctional protein that plays an important role in innate immune responses, inflammation, matrix deposition and female fertility (17, 199, 200). In the female reproductive system, PTX3 is expressed and produced in both cumulus and mural granulosa cells during the preovulatory periods, indicating the close temporal correlation to extracellular matrix formation by the cumulus-oophorus complex (COC) (17, 20). Furthermore, PTX3 protein has been detected by Western blotting in the human cumulus matrix and by ELISA in the follicular fluid (3.2-27.9 ng/ml) (20). After the LH surge, COC expansion (cumulus expansion) characterized by hyaluronan-based extracellular matrix production is initiated under the control of several endocrine and oocyte-derived factors (17-19). This process is essential for oocyte maturation and ovulation, for effective transportation of the oocyte through the fallopian tubes and for successful fertilization (20, 21). During the assembly of the extracellular matrix, PTX3 functions as an aggregating reagent by linking the TSG-6 protein, which is bound to the distinct hyaluronan strand, and forming a stable and sustainable hyaluronan network (20, 49). Knockout mice lacking the expression of PTX3 are subfertile and display defects in the integrity of COC and failure of *in vivo* fertilization (17, 20).

Given that optimal cumulus expansion is essential for fertility, the mechanism and regulation of cumulus expansion components has been an important topic in reproductive biology. In COC, the expression of PTX3 is mainly modulated by FSH and human chorionic gonadotropin (17). Previous studies have shown that either solitary effect or synergistic action from FSH, cyclic AMP, EGF or PGE2 is able to induce PTX3 expression in cultured cumulus cells (47, 50, 201). In addition, an oocyte-derived growth factor, growth differentiation factor 9 (GDF9), another member of the TGFβ superfamily, is required for the temporal expression of PTX3, assembly of hyaluronan matrix and formation of cumulus expansion during the periovulatory stage (17). Interestingly, conditioned medium from goat cultured theca cells decreased expansion rates of mouse COC (202). These data led us to propose that theca cells may secrete cumulus expansion-inhibiting factors that are capable of maintaining cumulus oophorus in meiotic arrest before the time of ovulation (203).

The roles of gonadotropins, cytokines and oocyte-specific growth factors in modulating PTX3 expression have been investigated in several studies; however, the roles of theca cell-derived BMP4 and BMP7 in regulating PTX3 expression have not been defined. We aim to investigate the effects of BMP4 and BMP7 on PTX3 expression and production as well as the potential underlying mechanisms of these effects in human granulosa cells.

6.2 Materials and methods

Preparation of primary human granulosa-lutein (hGL) cells

Primary hGL cells were obtained with informed patient consent following approval from the University of British Columbia Research Ethics Board. The controlled ovarian stimulation protocol for *in vitro* fertilization patients consisted of either luteal-phase naferelin acetate

(Synarel, Pfizer, Kirkland, Quebec, Canada) or follicular phase GnRH antagonist (Ganirelix; Merck Canada) down-regulation. Gonadotropin stimulation began on menstrual cycle day 2 with human menopausal gonadotropin (hMG; Menopur, Ferring, Canada) and recombinant FSH (Puregon, Merck, Canada), and was followed by human chorionic gonadotropin administration 34-36 h before oocyte retrieval, based on follicle size. Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval as previously described (159).

Simian virus 40 large T antigen–immortalized human granulosa cell (SVOG) and KGN cell culture

A non-tumorigenic immortalized human granulosa-lutein cell line (SVOG), previously produced by transfecting human granulosa-lutein cells with the SV40 large T antigen (136), and a human granulosa cell tumor-derived cell line, KGN, were used in this study. SVOG or KGN cells were counted with a hemocytometer, and cell viability was assessed by trypan blue (0.04%) exclusion. The cells were seeded (4-8 × 10^5 cells per well in 6-well plates) and cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON) supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin (Invitrogen, Life Technologies, NY), 100 μg/ml streptomycin sulfate (Invitrogen) and 1X GlutaMAX (Invitrogen). The culture medium was changed every other day in all of the experiments, and cells were maintained in serum-free medium for 24 h prior to growth factor treatment.

Antibodies and reagents

Polyclonal rabbit anti-SMAD1/5/8 (N-18; sc-6031-R) and polyclonal goat anti-actin (C-11; sc-1615) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-phospho-SMAD1 ($Ser^{463/465}$)/SMAD5 ($Ser^{463/465}$)/SMAD8 ($Ser^{426/428}$) antibody was obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit, goat anti-mouse and donkey anti-goat IgGs were obtained from Bio-Rad and Santa Cruz Biotechnology, respectively. Recombinant human BMP4, recombinant human BMP7, dorsomorphin dihydrochloride (dorsomorphin) and DMH-1 were obtained from R&D Systems (Minneapolis, MN). SB431542 was obtained from Sigma-Aldrich Corp.. Recombinant human BMP4 and BMP7 are derived from Chinese Hamster Ovarian (CHO) cell line. These proteins are purified using polyacrylamide gel electrophoresis (SDS-PAGE) with purity more than 98% and are lyophilized from a 0.2 μm filtered solution in HCl with bovine serum albumin as a carrier protein.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Madison, WI). TaqMan gene expression assays for PTX3, ACVR1 (ALK2), BMPR1A (ALK3), BMPR1B (ALK6), SMAD4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs00173615_m1, Hs00153836_m1, Hs01034913_g1, Hs00176144_m1, Hs00929647_m1 and Hs02758991_g1, respectively; Applied Biosystems) were performed in triplicate on corresponding cDNA samples. For each 20 μl TaqMan reaction, 4 μl cDNA was mixed with 5 μl

RNase-free water, 10 µl 2X TaqMan gene expression master mix (Applied Biosystems) and 1 µl 20X TaqMan gene expression assay. qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. The PCR parameters for the reaction were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Three separate experiments were performed on different cultures, and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq ($2^{-\Delta\Delta Cq}$) method with GAPDH as the reference gene.

Western blot analysis

After treatment, cells were washed with cold PBS and lysed in lysis buffer (Cell Signaling) containing protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 x *g* for 15 min at 4°C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked for 1 h in TBS containing 0.05% Tween 20 and 5% nonfat dried milk and incubated overnight at 4°C with the relevant primary antibodies. After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence reagents or a SuperSignal West Femto Chemiluminescence Substrate (Pierce, Rockford, IL), followed by exposure to CL-XPosure film (Thermo Fisher, Waltham, MA). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mmol/l β-mercaptoethanol and 1% SDS) at 50°C for 30 min and reprobed with goat anti-actin antibody as a loading control. Quantified phosphorylated SMAD1/5/8 levels are normalized to T-SMAD1/5/8.

Measurement of PTX3

Following the specified treatments, culture medium was assayed immediately or stored at –80°C until it was assayed. PTX3 accumulation in conditioned medium was measured according to the manufacturer's instructions using a solid phase sandwich enzyme immunoassay Quantikine kit (R&D Systems, Minneapolis, MN). The inter- and intra-assay coefficients of variation for this assay were less than 6% and the detection limit ranged from 0.007-0.116 ng/ml. Each sample was measured in triplicate, and secreted PTX3 levels were normalized to total cellular protein content.

Small interfering RNA (siRNA) transfection

We performed transient knockdown assays with ON-TARGET*plus* Non-targeting Control Pool or separate ON-TARGET*plus* SMARTpools targeting ALK2, ALK3, ALK6 or Smad4 (Thermo Fisher Scientific, Lafayette, CO). Cells were pre-cultured to 50% confluence in antibiotic-free DMEM/F12 medium containing 10% charcoal/dextran-treated fetal bovine serum and then transfected with 25 nM siRNA using Lipofectamine RNAiMAX (Life Technologies) for 48 h. Knockdown efficiency for each target was confirmed by RT-qPCR or Western blot analysis.

Statistical analysis

PRISM software (GraphPad Software, Inc., San Diego, CA) was used to perform one-way ANOVA followed by Tukey's multiple comparison tests. Results are presented as the mean ± SEM of at least three separate experiments performed on different cultures and were considered significantly different from each other if $P < 0.05$.

6.3 Results

BMP4 and BMP7 down-regulate PTX3 expression in SVOG cells

To investigate whether theca cell-derived BMP4 and BMP7 induce the down-regulation of PTX3, SVOG cells were treated with a vehicle control or increasing concentrations (1, 10 or 100 ng/ml) of recombinant human BMP4 or BMP7. As shown in Figure 6.1A, treatment with BMP4 or BMP7 for 6 h down-regulated PTX3 mRNA levels in a concentration-dependent manner. In addition, a time course change following BMP4 or BMP7 treatment demonstrated that a decrease in the PTX3 mRNA level was observed in both treatments as early as 3 h, reaching maximal suppressive effects at 12 h and persisting until 24 h (Figure 6.1B). Moreover, the suppressive effects of BMP4 and BMP7 on PTX3 accumulation were confirmed by an enzyme immunoassay (ELISA). Treatment with increasing concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7 for 24 h decreased PTX3 accumulation in the conditioned medium of cultured SVOG cells (Figure 6.1C).

BMP4 and BMP7 down-regulate PTX3 expression in hGL and KGN cells

Non-immortalized primary hGL cells were used to further confirm the function of BMP4 and BMP7 in human granulosa cells. Primary hGL cells obtained from infertile patients undergoing an *in vitro* fertilization (IVF) procedure were cultured in a vehicle control with or without BMP4 and BMP7 (1, 10 or 100 ng/ml), and the results showed that both BMP4 and BMP7 decreased PTX3 mRNA levels in a concentration-dependent manner (Figure 6.2A). To obtain more information on the function of human granulosa cells, we examined cell responsiveness to BMP4 and BMP7 in KGN cells. Consistent with the results from SVOG and hGL cells, treatment with

BMP4 or BMP7 decreased the PTX3 mRNA levels in a concentration-dependent manner in KGN cells (Figure 6.2B).

BMP4 and BMP7 activate SMAD1/5/8 signaling in SVOG cells

To determine whether BMP4 and BMP7 activate SMAD1/5/8 in SVOG cells, SMAD phosphorylation was measured following treatment with BMP4 or BMP7 (30 ng/ml) for 30 min or 60 min. As shown in Figure 6.3A, treatment with BMP4 or BMP7 increased the phosphorylation levels of SMAD1/5/8 at both time points.

Although signaling in the BMP pathway begins with the binding to both type I and II receptors, pathway specificity is mainly determined by the type I receptor (141). Of the seven distinct type I receptors in humans, ALK2, ALK3 and ALK6 have been implicated in BMP-induced SMAD1/5/8 phosphorylation (141). To determine whether ALK2, ALK3 or ALK6 are required for BMP4- and BMP7-induced SMAD1/5/8 activation, cells were treated with 30 ng/ml BMP4 or BMP7 in the presence or absence of 10 μM dorsomorphin or 1 μM DMH-1. Dorsomorphin is a specific inhibitor of ALK2/3/6, and DMH-1 is a selective inhibitor of ALK2/3 (142, 143). As shown in Figure 6.3B and 6.3C, treatment with either dorsomorphin or DMH-1 abolished BMP7-induced SMAD1/5/8 phosphorylation, indicating that ALK2 or ALK3, but not ALK6, is required for the activation of SMAD1/5/8 by BMP7 in SVOG cells. Interestingly, treatment with dorsormorphin, but not DMH-1, abolished BMP4-induced SMAD1/5/8 phosphorylation, indicating that ALK6 is required for the BMP-4 driven activation of SMAD1/5/8 (Figure 6.3B and 6.3C).

To further confirm that other BMP type I receptors are not involved in the BMP4- or BMP7-induced activation of SMAD1/5/8 phosphorylation, cells were treated with 30 ng/ml BMP4 or

BMP7 in the presence or absence of 10 μM SB431542, an inhibitor of ALK4, ALK5 or ALK7 (164). As shown in Figure 6.3D, co-treatment with SB431542 did not affect BMP4- or BMP7-induced SMAD1/5/8 phosphorylation.

ALK3/ALK6 mediate BMP4-induced SMAD1/5/8 phosphorylation, whereas ALK2/ALK3 mediate BMP7-induced SMAD1/5/8 phosphorylation in SVOG cells.

To further confirm which ALK(s) mediate(s) BMP4- and BMP7-induced SMAD1/5/8 activation, specific siRNAs were used to knockdown endogenous ALK2, ALK3 or ALK6 in SVOG cells. As shown in Figure 6.4A, transfection of SVOG cells with ALK2, ALK3 or ALK6 siRNA significantly down-regulated the mRNA levels of only the targeted ALK. Interestingly, dual knockdown of both ALK3 and ALK6 completely abolished BMP4-induced SMAD1/5/8 phosphorylation (Figure 6.4B and 6.4C), whereas the down-regulation of both ALK2 and ALK3 completely reversed the BMP7-induced SMAD1/5/8 phosphorylation (Figure 6.4D and 6.4E). These results demonstrate that ALK3/ALK6 and ALK2/ALK3 type I BMP receptors are required for downstream SMAD1/5/8 signaling in response to BMP4 and BMP7, respectively, in SVOG cells.

ALK3/ALK6 mediate BMP4-induced down-regulation of PTX3, whereas ALK2/ALK3 mediate BMP7-induced down-regulation of PTX3 in SVOG cells

Dual pharmacological and siRNA-based approaches were next used to examine the requirement for ALK3/ALK6 and ALK2/ALK3 in BMP4- and BMP7-induced down-regulation of PTX3. Consistent with the results for SMAD1/5/8 activation, dorsomorphin, but not SB431542 abolished the inhibitory effects of BMP4 and BMP7 on PTX3 mRNA in SVOG cells

(Figures 6.5A and 6.5B). Similarly, in SVOG cells siRNA-mediated knockdown of both ALK3 and ALK6 abolished BMP4-induced down-regulation of PTX3 mRNA (Figure 6.5C). Likewise, dual knockdown of ALK2 and ALK3 reversed the BMP7-induced down-regulation of PTX3 mRNA (Figure 6.5D).

The SMAD signaling pathway is required in SVOG cells for the BMP4- and BMP7-induced down-regulation of PTX3

Before translocating to the nucleus and regulating the transcription of target genes, the common mediator SMAD4 forms heteromeric complexes in tandem with R-SMAD, SMAD2/3 or SMAD1/5/8 (100). To investigate whether the observed suppressive effects of BMP4 and BMP7 on PTX3 were SMAD-dependent, SMAD4 siRNA was used to knockdown the endogenous expression of the common SMAD, SMAD4. The knockdown efficiency was examined by Western blot analysis. As shown in Figure 6.6A, after transfection for 24 h and 48 h, SMAD4 siRNA down-regulated SMAD4 protein levels by 80-90% relative to control siRNA. In addition, transfection with SMAD4 siRNA for 48 h abolished the suppressive effect of BMP4 or BMP7 on PTX3 mRNA (Figure 6.6B and 6.6C).

6.4 Discussion

During the development of the antral follicle, which is featured by fluid-filled antrum formation, granulosa cells originally surrounding the oocyte differentiate into two anatomically and functionally distinct sublinages. Mural granulosa cells (MGCs) line the follicle wall and reside close to the basement membrane and theca cells, whereas the cumulus cells (CCs), in an intimate association with the oocyte, form an elaborate structure called COC (13). These two

types of cells exhibit highly divergent responses to gonadotropins and growth factors during the periovulatory stage (14). The LH surge initiates the process of ovulation by reactivating oocyte meiosis, creating tissue restructuring and inducing the expansion of the COC (15, 16). Given that PTX3 is the key component of cumulus expansion and is expressed and produced in both MGCs and CCs (20), the study of the regulation of PTX3 has been a subject of considerable research. In CCs, oocyte-secreted GDF9 has been shown to be the up-stream inducer of PTX3 expression and the subsequent cumulus expansion (17). In the present study, we demonstrate that theca cell-derived BMP4 and BMP7 down-regulate PTX3 expression and production in human granulosa cells, indicating that the theca layer may facilitate the separation of COC and MGCs by decreasing the extracellular matrix formation in the neighboring MGCs.

Previous *in vivo* studies have demonstrated that either injection of BMP7 into rat ovarian bursa or treatment of exogenous BMP4 led to an increased number of primary, preantral and antral follicles, whereas a decreased number of primordial follicles indicates that BMP4 and BMP7 may promote the transition of primordial follicles into the pool of growing follicles (6, 7). During the development of rat growing follicles, BMP4 and BMP7 produced by theca cells have been shown to enhance and attenuate the stimulatory effects of FSH on granulosa cells estradiol and progesterone production, respectively (71). However, during the periovulatory stage, the ovary that was treated with BMP7 ovulated less than the contralateral control ovary, indicating that BMP7 may act to inhibit ovulation (6). This finding is supported by our previous studies in which we demonstrated that BMP4 and BMP7 down-regulate connexin43-coupled gap junction formation which is another parameter of ovulation and luteinization (158). Taken together, the previous studies and our findings provide evidence that theca cell-derived growth factors may promote follicular development and ovarian steroidogenesis while simultaneously inhibiting

ovulation and luteinization. Unfortunately these important reproductive functions cannot be further confirmed using conventional knockout mouse models because both BMP4 and BMP7 deficient mice are developmentally lethal (204, 205). It would be of great interest to overcome and bypass this limitation through the generation of conditional mutation animal models to determine the related ovarian function.

A comprehensive understanding of the molecular mechanisms of the cellular response to BMP ligands is essential to the design of more rational targeted therapies for clinical application. Signaling specificity by BMP is mainly determined by the type I receptor (ALK) recruited in the ligand-receptor complex (206). Therefore, the differential determination of which ALK is recruited by BMP is of great importance. Three type I receptors, ALK2, ALK3 and ALK6, have been identified as BMP type I receptors that mediate BMP-specific cellular signaling (141). However, the BMP-ALK interactions are complex because of the existence of various cross-reactivity among different BMP ligands and the ALK type I receptors (12). Furthermore, the same BMP ligand has been shown to bind different ALKs in different cells (12). For instance, BMP6 strongly binds to ALK2 and ALK3 in MCsTs-E1 and C2C12 cells, whereas BMP6 binds most efficiently to ALK6 in ROB-C26 cells (206). To date, despite the apparent importance of BMPs in female fertility, the ligand-receptor relationships between various ovarian BMP ligands and their cognate receptors in human granulosa cells remain poorly defined. In monkey kidney COS-1 cells transfected with cDNAs for ALK2/3/6, BMP4 bound to ALK3 and ALK6 but not ALK2, whereas ALK2 or ALK6 are widely regarded as the ALKs for BMP7 (207, 208). Our previous studies have shown that oocyte-derived BMP15 may bind to ALK3 and activate the down-stream signaling pathway in human granulosa cells (169). Using the same experimental approaches that combined pharmacologic and siRNA-base intervention, we demonstrated that

BMP4 and BMP7 activate the SMAD1/5/8-dependent pathway using differential subsets of BMP type I receptors. Specifically, ALK3 and/or ALK6 are required for the BMP4 ligand, whereas ALK2 and/or ALK3 are required for the BMP7 ligand in human granulosa cells. Furthermore, dual knockdown of either ALK3/ALK6 or ALK2/ALK3 reversed the suppressive effects of BMP4 or BMP7 on PTX3 expression, respectively. The finding that a particular TGFβ ligand can bind to several TGFβ receptors of either types is called ligand-receptor promiscuity, a phenomenon that is commonly present in the BMP/GDF subfamily (152). These concerted interactions of different BMP ligands and receptors may generate compensatory cellular signals, which are beneficial and crucial for follicle development and synchronization.

In summary, we have shown that theca cell-derived BMP4 and BMP7 down-regulate PTX3 expression and production in human granulosa cells. Moreover, our results indicate that the suppressive effects of BMP4 and BMP7 are mediated by differential subsets of ALKs and most likely involve the SMAD-dependent signaling pathway. These findings shed light on the functional roles of theca cells in preventing ovulation and premature luteinization.

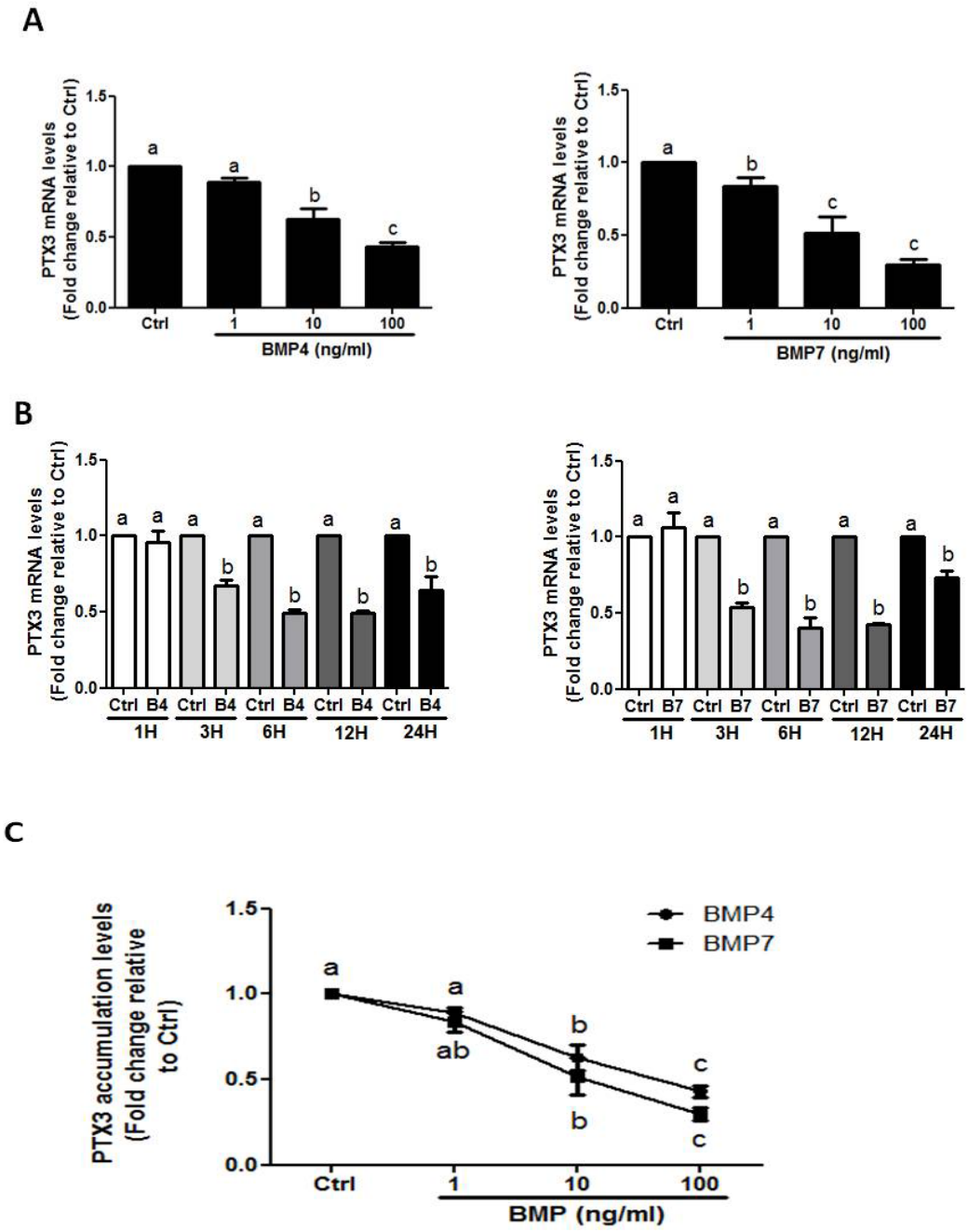


Figure 6.1 BMP4 and BMP7 down-regulate PTX3 expression in SVOG cells

A, Cells were treated for 6 h with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7 and PTX3 mRNA levels were examined by RT-qPCR. B, Cells were treated

with 30 ng/ml BMP4 or BMP7 for 1, 3, 6, 12 or 24 h, after which PTX3 mRNA levels were examined by RT-qPCR. C, Alternatively, cells were treated for 24 h with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7. PTX3 accumulation in the conditioned medium was measured by enzyme immunoassay. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different ($P<0.05$). Ctrl, control. B4, BMP4. B7, BMP7.

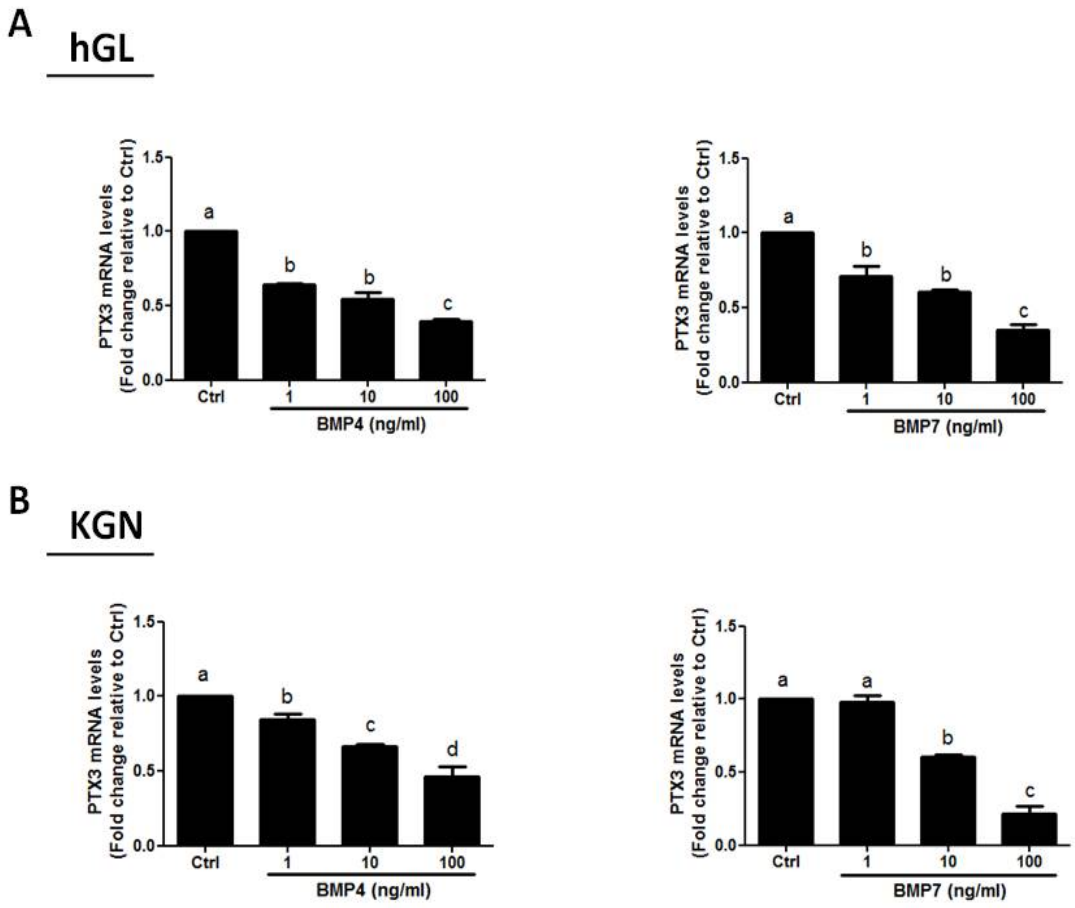
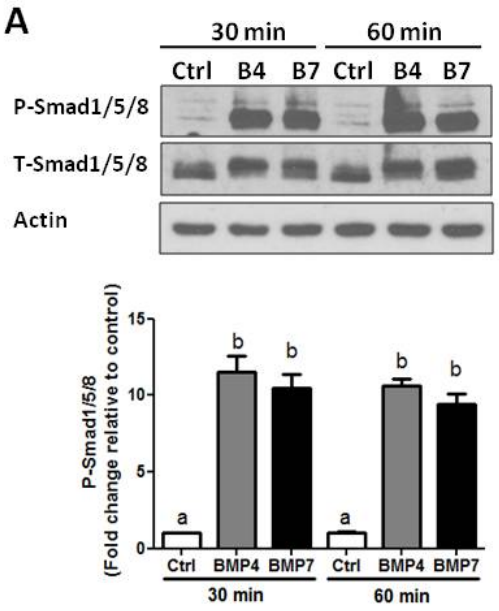
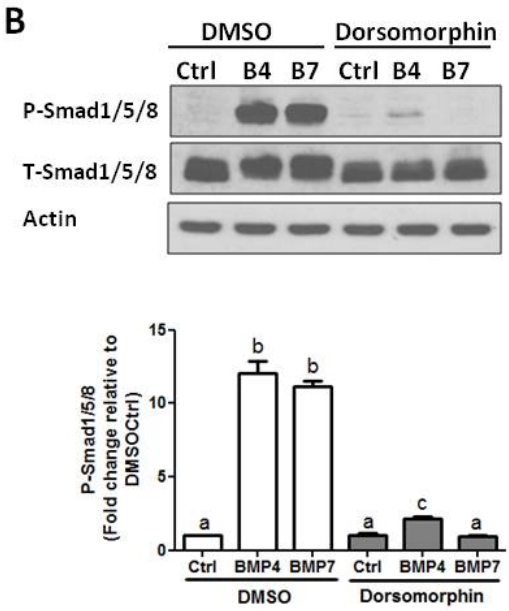
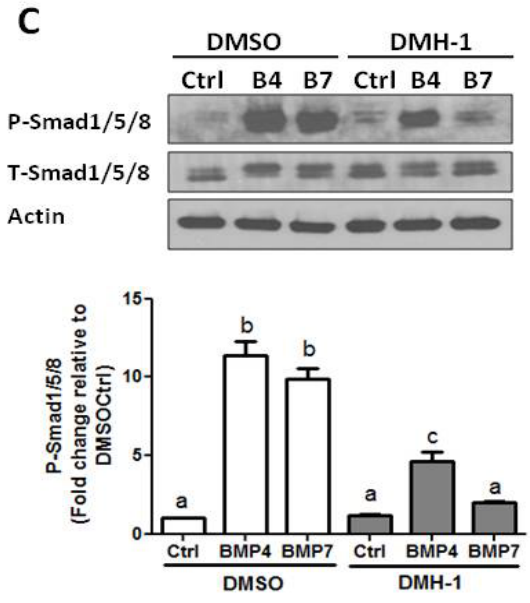


Figure 6.2 BMP4 and BMP7 down-regulate PTX3 expression in hGL and KGN cells

hGL cells (A) or KGN cells (B) were treated with vehicle control or different concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7 for 6 h and PTX3 mRNA levels were examined by RT-qPCR. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different (P<0.05). Ctrl, control. hGL, human granulosa-lutein.


A
30 min
60 min
Ctrl B4 B7 Ctrl B4 B7
P-Smad1/5/8
T-Smad1/5/8
Actin
P-Smad1/5/8 (Fold change relative to control)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
30 min
60 min


B
DMSO
Dorsomorphin
Ctrl B4 B7 Ctrl B4 B7
P-Smad1/5/8
T-Smad1/5/8
Actin
P-Smad1/5/8 (Fold change relative to DMSOCtrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
DMSO
Dorsomorphin


C
DMSO
DMH-1
Ctrl B4 B7 Ctrl B4 B7
P-Smad1/5/8
T-Smad1/5/8
Actin
P-Smad1/5/8 (Fold change relative to DMSOCtrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
DMSO
DMH-1

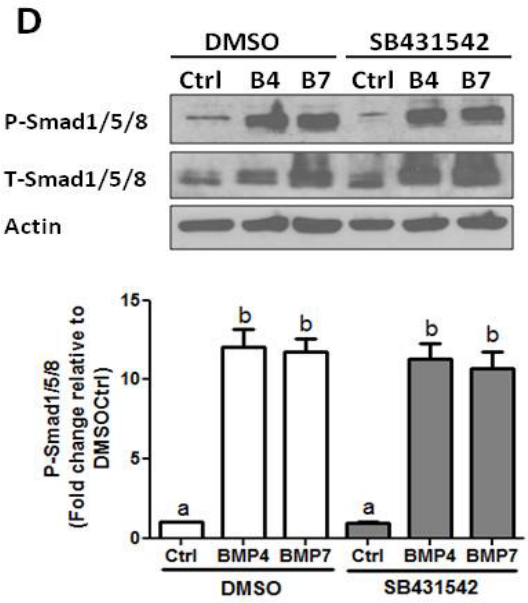
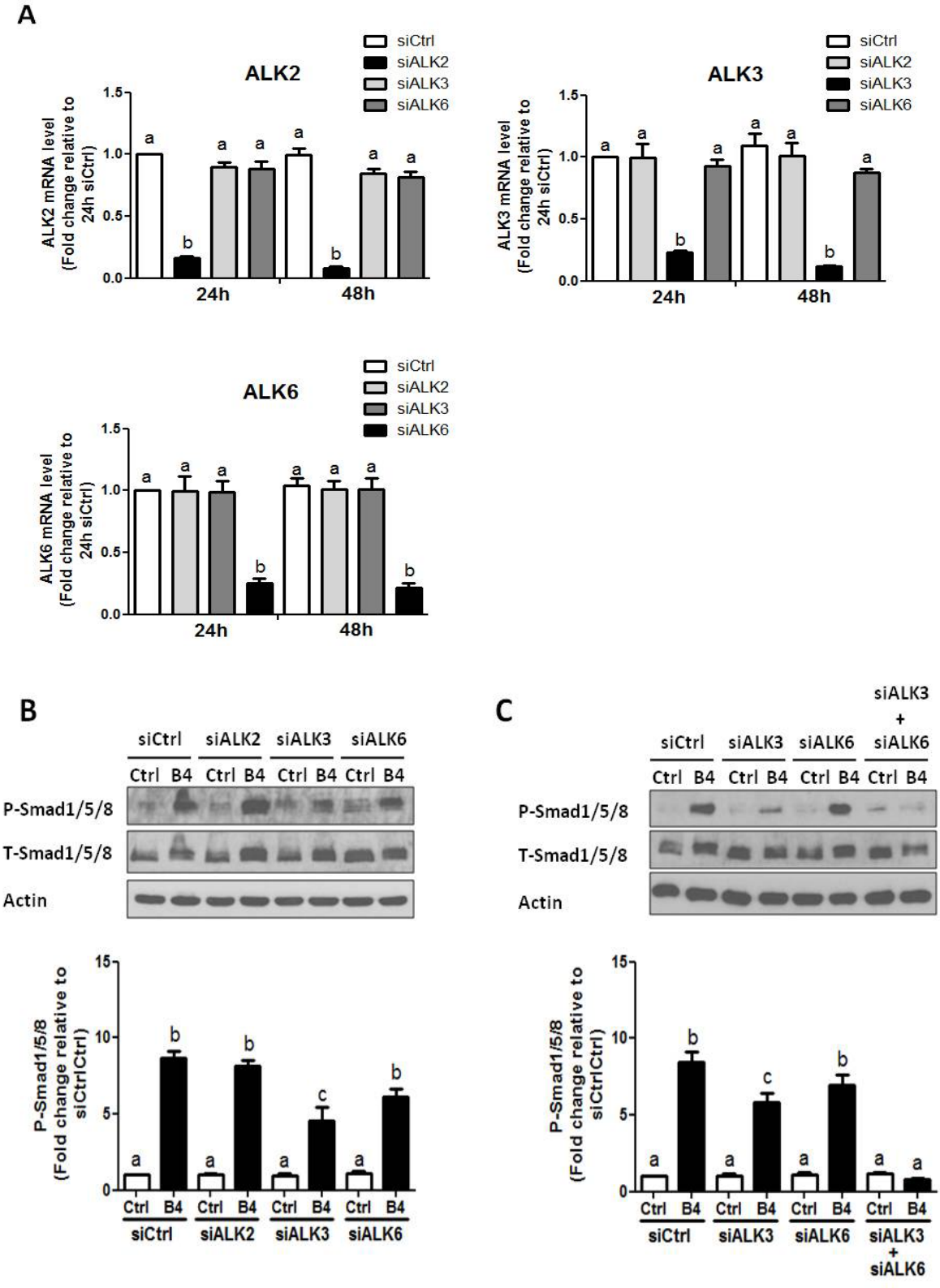

D
DMSO
SB431542
Ctrl B4 B7 Ctrl B4 B7
P-Smad1/5/8
T-Smad1/5/8
Actin
P-Smad1/5/8 (Fold change relative to DMSOCtrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
DMSO
SB431542

Figure 6.3 BMP4 and BMP7 activate the SMAD1/5/8 signaling pathway in SVOG cells

A, Cells were treated with 30 ng/ml BMP4 or BMP7 for 30 or 60 min; the phosphorylation levels of SMAD1/5/8 were examined by Western blot analysis. B-D, Cells were treated with 30 ng/ml BMP4 or BMP7 for 60 min in the presence of vehicle control (DMSO) or 10 μM dorsomorphin (B), 1 μM DMH-1 (C) or 10 μM SB431542 (D); the phosphorylation levels of SMAD1/5/8 were examined by Western blot analysis (quantified data are normalized to T-SMAD1/5/8). Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different ($P<0.05$). Ctrl, control. B4, BMP4. B7, BMP7.


A
ALK2
siCtrl
siALK2
siALK3
siALK6
ALK2 mRNA level (Fold change relative to 24h siCtrl)
24h
48h
ALK3
ALK3 mRNA level (Fold change relative to 24h siCtrl)
ALK6
ALK6 mRNA level (Fold change relative to 24h siCtrl)
B
siCtrl
siALK2
siALK3
siALK6
Ctrl
B4
P-Smad1/5/8
T-Smad1/5/8
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C
siALK3 + siALK6

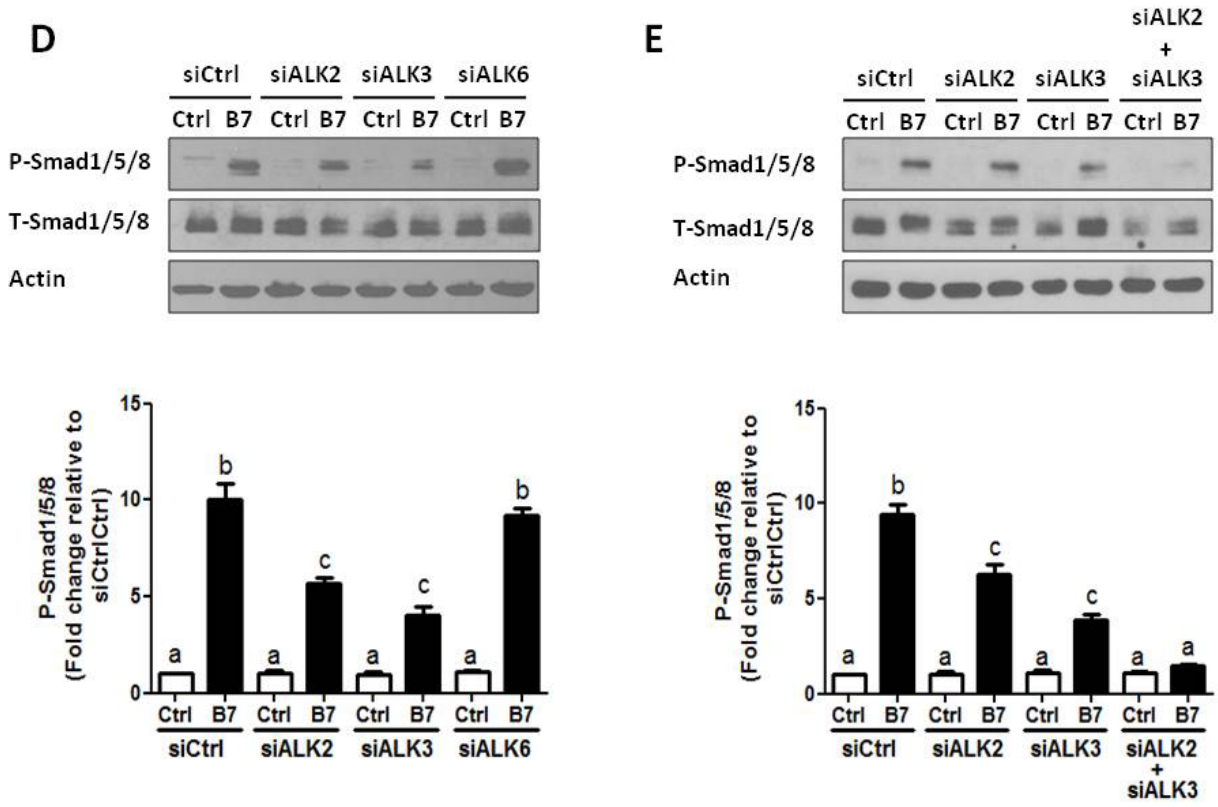


Figure 6.4 ALK3/ALK6 mediate BMP4-induced Smad1/5/8 phosphorylation, whereas ALK2/ALK3 mediate BMP7-induced SMAD1/5/8 phosphorylation in SVOG cells

A, Cells were transfected for 24 or 48 h with 25 nM control siRNA (siCtrl), ALK2 siRNA (siALK2), ALK3 siRNA (siALK3) or ALK6 siRNA (siALK6). The specificity and efficiency of knockdown for each siRNA was evaluated by RT-qPCR. B and C, Thereafter, cells were transfected with siRNAs (siCtrl, siALK2, siALK3, siALK6 or combined siALK3 and ALK6) for 48 h, then treated for 60 min with vehicle control or 30 ng/ml BMP4 and phosphorylated SMAD1/5/8 levels were examined by Western blot. D and E, Alternatively, cells were transfected with siRNAs (siCtrl, siALK2, siALK3, siALK6 or combined siALK2 and ALK3) for 48 h, then treated for 60 min with vehicle control or 30 ng/ml BMP7 and phosphorylated SMAD1/5/8 levels were examined by Western blot (quantified data are normalized to T-

SMAD1/5/8). Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different ($P<0.05$). Ctrl, control. B4, BMP4. B7, BMP7.

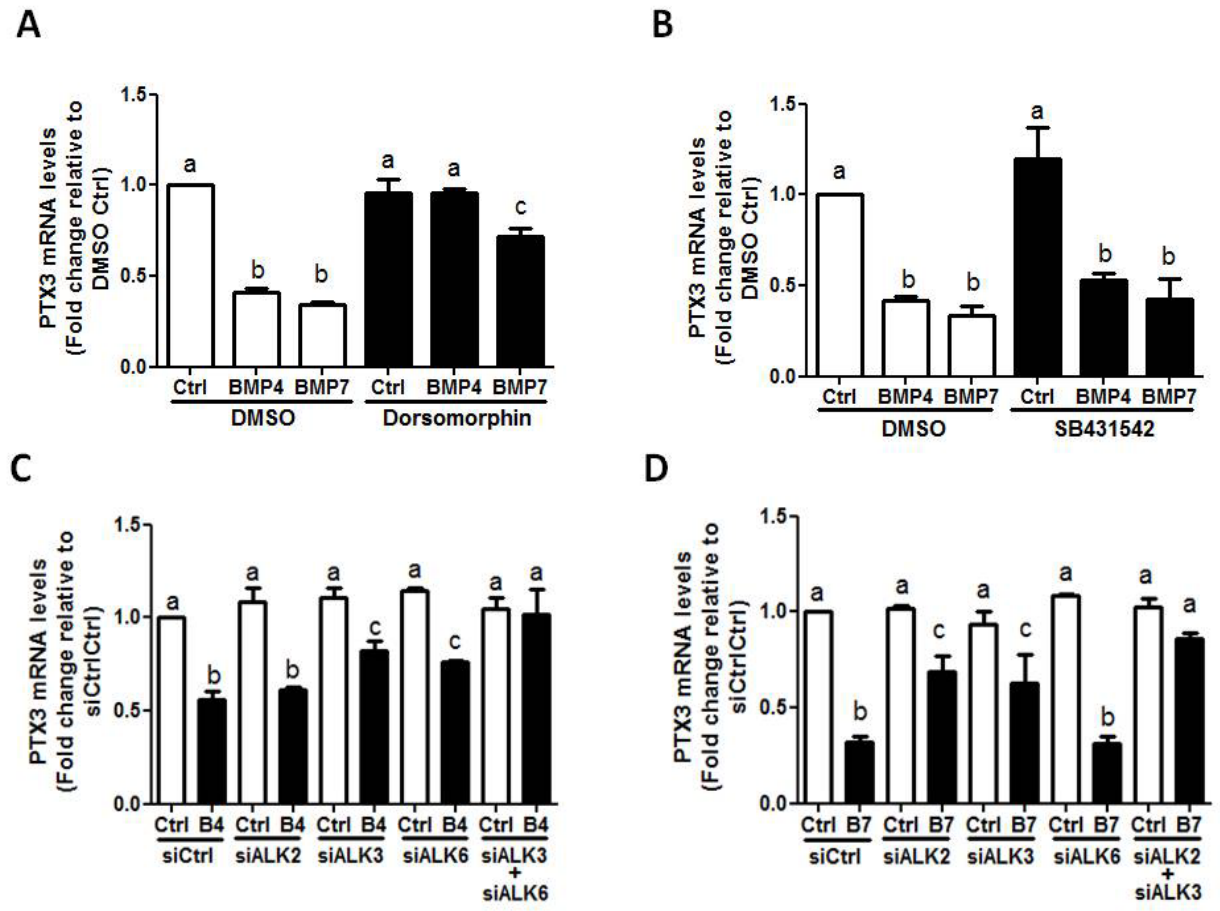


Figure 6.5 ALK3/ALK6 mediate the suppressive effects of BMP4, whereas ALK2/ALK3 mediate the suppressive effects of BMP7 on PTX3 expression in SVOG cells

A and B, Cells were treated with 30 ng/ml BMP4 or BMP7 for 60 min in the presence of vehicle control (DMSO) or 10 μM dorsomorphin (A) or 10 μM SB431542 (B); PTX3 mRNA levels were examined by RT-qPCR. C, Cells were transfected with siRNAs (siCtrl, siALK2, siALK3, siALK6 or combined siALK3 and ALK6) for 48 h, then treated for 60 min with vehicle control or 30 ng/ml BMP4 and PTX3 mRNA levels were examined by RT-qPCR. D, Alternatively, cells were transfected with siRNAs (siCtrl, siALK2, siALK3, siALK6 or combined siALK2 and

ALK3) for 48 h, then treated for 60 min with vehicle control or 30 ng/ml BMP7 and PTX3 mRNA levels were examined by RT-qPCR. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different (P<0.05). Ctrl, control. B4, BMP4. B7, BMP7.

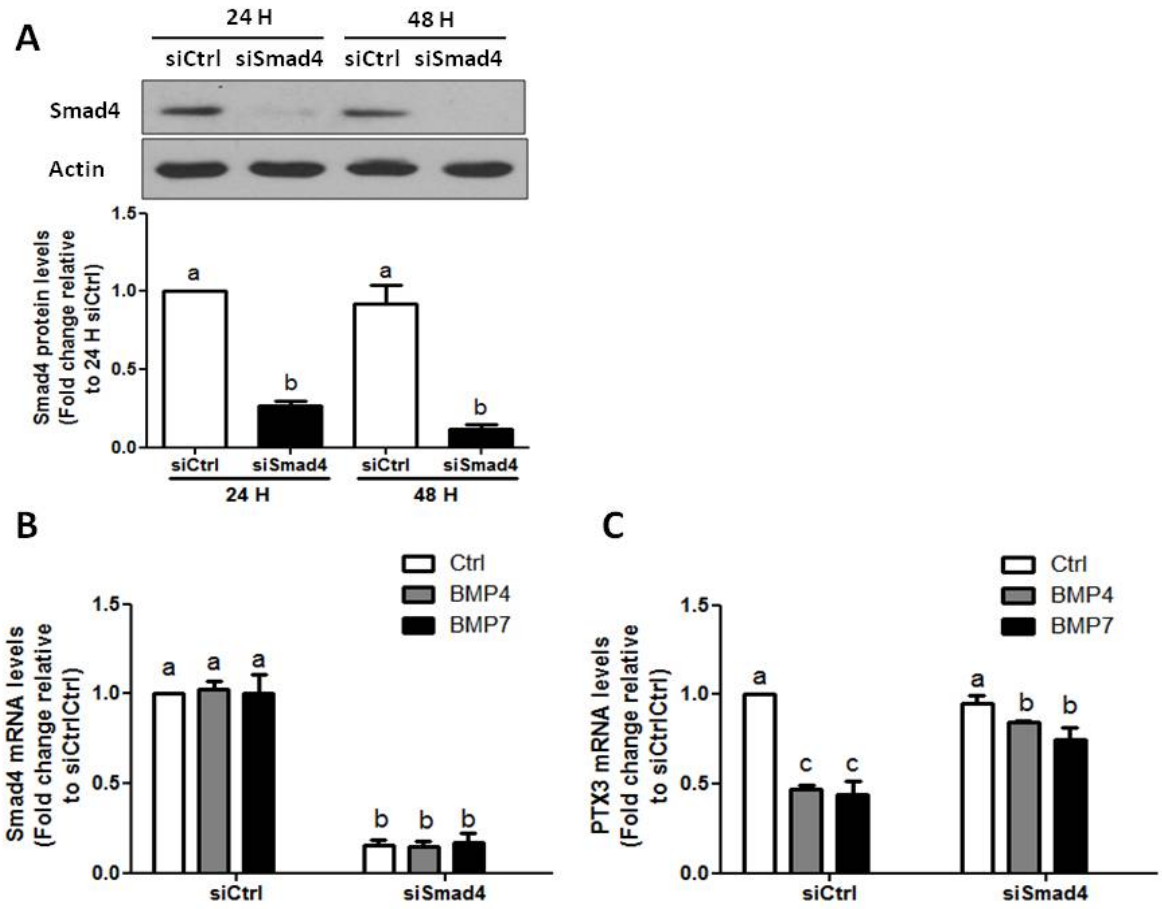


Figure 6.6 SMAD4 knockdown abolishes the BMP4- and BMP7-induced down-regulation of PTX3 in SVOG cells

A, Cells were transfected for 24 h or 48 h with 25 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4). The knockdown efficiency of SMAD4 siRNA was examined by Western blot. B and C, Cells were transfected for 48 h with 25 nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4) and then treated with 30 ng/ml of BMP4 or BMP7. The mRNA levels of Smad4 (B) and PTX3 (C) were examined by RT-qPCR. Results are expressed as the mean ± SEM of at least 3 independent experiments and values without a common letter are significantly different (P<0.05). Ctrl, control. B4, BMP4. B7, BMP7.

Chapter 7: Theca-derived BMP4 and BMP7 increase activin A production by up-regulating inhibin βA subunit and furin expression in human granulosa cells

7.1 Introduction

In the ovary, bone morphogenetic protein (BMP) 4 and BMP7 are locally expressed by ovarian stromal and/or theca cells and exert autocrine/paracrine actions to promote early folliculogenesis, modulate steroidogenesis, stimulate granulosa cell proliferation, maintain follicle survival and prevent premature luteinization (6, 183). Recently, we showed that theca-drived BMP4 and BMP7 can regulate gap junction intercellular communication in human granulosa cells by altering the production of connexin 43, suggesting paracrine regulation of granulosa cell function by theca-derived BMPs (158). BMP ligands initially bind to their cognate type II receptors and then recruit and activate type I receptors, ultimately resulting in the phosphorylation and activation of receptor-regulated SMADs, SMAD1/5/8 (137). As downstream signaling mediators, activated SMAD1/5/8 form oligomers with the common SMAD, SMAD4, and subsequently translocate into the nucleus to regulate target gene expression (157).

Produced locally in the ovary, as well as in many other tissues, activins and inhibins are functionally antagonistic members of the transforming growth factor-β (TGF-β) superfamily that are important mediators of diverse autocrine/paracrine functions (209). Studies on transgenic knockout mice as well as *in vitro* studies on isolated follicular cells and oocytes have demonstrated that granulosa cell-derived activins and inhibins are important regulators of folliculogenesis, steroidogenesis, gonadotropin responsiveness, oocyte maturation and corpus luteum function (210-212). Although the functions of activins have been extensively studied in

human granulosa cells, much less is known about their intracellular regulation, assembly and secretion.

Activins are disulfide-linked homodimers or heterodimers of inhibin/activin β (inhibin β) subunits (inhibin βA or inhibin βB), whereas inhibins are disulfide-linked heterodimers of a common inhibin α subunit and an inhibin β subunit (209, 213). Initially synthesized as large inactive precursors, inhibin subunits are thought to dimerize into mature forms through an enzymatic cleavage process that occurs via proprotein convertases, a family of serine proteases consisting of seven members (214, 215). Furin (PCSK3) is the mammalian prototype enzyme of this family and is responsible for the maturation of several members of the TGF-β superfamily, including TGF-β1, BMP4, nodal, lefty1, lefty2 and anti-Müllerian hormone (216-220). Inhibin subunit precursors contain a recognition motif (RXXR) for cleavage by serine proteases (215, 216), and they have been shown to be substrates for furin cleavage in murine LβT2 gonadotrope cells (221).

In the monkey ovary, the expression of inhibin subunits is differentially regulated during follicular development. Granulosa cells of small antral follicles predominantly express inhibin βB, whereas those of the dominant follicle mainly express inhibin α and inhibin βA (222). During the human menstrual cycle, serum activin A concentrations increase from the mid-follicular phase (approximately 125 pg/ml) to reach their highest concentrations at the time of ovulation (approximately 220 pg/ml) (223). In human antral follicles, the concentration of activinA in follicular fluid is significantly higher in the group of follicles with greater diameter (10 mm *vs.* 5-6 mm) (224).

In the rat ovary, a specific pattern of BMP4 and BMP7 mRNA expression was identified in theca cells during folliculogenesis (11). Theca cells of developing follicles initially express

BMP4 and BMP7 during the transition from primary to secondary follicles. Subsequently, their expression levels increase with continued follicle growth and reach their highest levels in dominant follicles (11). Interestingly, these spatiotemporal alterations in theca cell BMP4 and BMP7 are accompanied by changes in the expression of inhibin βA in granulosa cells, as well as the levels of activin A in peripheral serum and follicular fluid (11, 222-224). Clinically, oocyte culture medium concentrations of activin A are positively correlated with morphological assessments of oocyte quality (225). These findings led to the hypothesis that theca-derived BMP4 and BMP7 could modulate granulosa cell activin A production in a paracrine manner. Indeed, BMP4 and BMP7 have been shown to increase the secretion of inhibin A and activin A in cultured bovine granulosa cells (107). However, whether theca cell-derived BMPs exert similar effects in human granulosa cells remain unknown. Moreover, the mechanisms mediating these effects have yet to be determined. In the present study, we examined the effects of BMP4 and BMP7 on activin A production and investigated the underlying mechanisms in human granulosa cells.

7.2 Materials and methods

Culture of primary and immortalized human granulosa-lutein (hGL) cells

Primary hGL cells were obtained with informed patient consent following approval from the University of British Columbia Research Ethics Board. The controlled ovarian stimulation protocol for *in vitro* fertilization patients was as previously described (226). Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval as previously described (159). The non-tumorigenic immortalized human granulosa cell line (SVOG) was previously produced by transfecting human granulosa-luteal

cells with the SV40 large T antigen (136). Cells were counted with a hemocytometer and cell viability was assessed by trypan blue (0.04%) exclusion. Cells were seeded (2-4 × 10^5 cells per ml in 6-well plates) and cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°Cin Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON). The medium was supplemented with 10% charcoal/dextran-treated fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin plus 100 μg/ml streptomycin sulfate (Invitrogen, Life Technologies) and 1X GlutaMAX (Invitrogen, Life Technologies). The culture medium was changed every other day in all experiments and cells were maintained in serum-free medium for 24 h before the specified treatment.

Antibodies and reagents

Polyclonal rabbit anti-furinconvertase antibody (PA1-062) was obtained from Thermo Scientific (Rockford, IL). Polyclonal rabbit anti-SMAD1/5/8 (N-18; sc-6031-R) and polyclonal goat anti-actin antibodies (C-11; sc-1615) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-phospho-SMAD1 ($Ser^{463/465}$)/SMAD5 ($Ser^{463/465}$)/SMAD8 ($Ser^{426/428}$) antibody was obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit and donkey anti-goat IgG were obtained from Bio-Rad Laboratories (Hercules, CA) and Santa Cruz Biotechnology, respectively. Recombinant human BMP4, recombinant human BMP7, recombinant human activin Aand dorsomorphin dihydrochloride (dorsomorphin) were obtained from R&D Systems (Minneapolis, MN). Furin inhibitor I, Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-CMK) was purchased from EMD Millipore (Billerica, MA).

Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold phosphate buffered saline (PBS) and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA (2μg) was reverse transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Madison, WI). RT-qPCR was performed on the Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates.Each 20 μl RT-qPCR reaction contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were: INHA (inhibin α subunit), 5'-GTCTCCCAAGCCATCCTTTT-3' (sense) and 5'-TGGCAGCTGACTTGTCCTC-3' (antisense); INHBA (Inhibin βA subunit), 5'-CTCGGAGATCATCACGTTTG-3' (sense) and 5'-CCTTGGAAATCTCGAAGTGC-3' (antisense); INHBB (inhibin βB subunit), 5'-ATCAGCTTCGCCGAGACA-3' (sense) and 5'-GCCTTCGTTGGAGATGAAGA-3' (antisense); FURIN, 5'-CCTTCTTCCGTGGGGTTAG-3' (sense) and 5'-GCAGTTGCAGCTGTCATGTT-3' (antisense); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5'-ATGGAAATCCCATCACCATCTT-3' (sense) and 5'-CGCCCCACTTGATTTTGG-3' (antisense). The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by evaluating amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount *vs.* ΔCq has a slope < |0.1|. Three separate experiments were performed on different cultures and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative ΔCq method with GAPDH as the reference gene and using the formula $2^{-\Delta\Delta Cq}$.

Western blot analysis

Following treatment, cells were washed with cold PBS and lysed in lysis buffer (20 mMTris, 150 mMNaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μM aprotinin, 1 μM leupeptin and 1 mM PMSF; Cell Signaling) containing protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000x *g* for 15 min at 4°C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h in TBS containing 0.05% Tween 20 and 5% nonfat dried milk, incubated overnight at 4°C with the relevant primary antibodies, washed, and incubated for 1 h with secondary antibody.Immunoreactive bands were detected using enhanced chemiluminescence (ECL) reagents or SuperSignal West Femto Chemiluminescence Substrate (Pierce, Rockford, IL) followed by exposure to CL-XPosure film (Thermo Fisher, Waltham, MA). Membranes were stripped with stripping buffer (50 mMTris-HCl pH 7.6, 10 mmol/l β-mercaptoethanol and 1% SDS) at 50°C for 30 min and reprobed with total SMAD1/5/8 or actin antibodies as loading controls.

Immunofluorescence staining

SVOG cells were plated on glass coverslips and treatedwith vehicle, BMP4 or BMP7 in the absence or presence of dorsomorphin. Following treatment, cells were, fixed with 4% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Coverslips were washed three times with PBS prior to blocking for 1 h with Protein Block (serum-free; Dako, Mississauga, Ontario, Canada). Furin antibody wasdiluted 1:50

inProtein Block and incubated overnight at 4°C. After three washes with PBS, coverslips were incubated for 1 h with Alexa Fluor 555 donkey anti-rabbit (Life Technologies) diluted 1:500 in Protein Block. Coverslips were washed three more times with PBS andcell nuclei were stained with Hoechst 33258 (Sigma-Aldrich). After washing a final three times with PBS, coverslips were mounted with Gelvatol and examined usinga Zeiss Axiophotfluorescence microscope equipped with a digital camera (Q Imaging, Burnaby, BC, Canada).

Small interfering RNA (siRNA) transfection

We performed transient knockdown assays using 25 nM SMAD4-targeting siRNA (ON-TARGET*plus*SMARTpool), 25 nM furin-targeting siRNA (ON-TARGET*plus*SMARTpool) or 25 nM control siRNA (ON-TARGET*plus*Non-targeting Pool) purchased from Thermo Fisher Scientific (Lafayette, CO). Cells were pre-cultured in antibiotic-free DMEM/F12 medium containing 10% FBS until they were 50-60% confluent and then transfected with siRNA using Lipofectamine RNAiMAX (Life Technologies) for 48 h. Knockdown efficiency for each target was confirmed by Western blot.

Measurement of Activin A

Following the specified treatment, cultured medium was assayed immediately or stored at -80°C until assayed. Activin A accumulation in conditioned medium wasmeasured as per the manufacturer's instructions using a solid phase sandwich enzymeimmunoassay kit (R&D Systems). Inter- and intra-assay coefficients of variation forthis assay were less than 10% and the detection limit was 7.85 pg/ml. Each sample was measured in triplicate andsecreted activin A levels were normalized to totalcellular protein content.

Statistical analysis

Results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests using PRISM software (GraphPad Software, Inc., San Diego, CA). Results are presented as the mean ± SEM of at least three independent experiments. Data were considered significantly different from each other if $P < 0.05$.

7.3 Results

BMP4 and BMP7 up-regulate inhibin βA expression and increase activin A production in SVOG cells

To investigate the effects of theca cell-derived BMP4 and BMP7 on the expression of inhibin subunits in human granulosa cells, SVOG cells were treated for 24 h with increasing concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7. As shown in Figure 7.1, treatment with BMP4 or BMP7 did not affect inhibin α or inhibin βB subunit mRNA levels (Figure 7.1A-D), whereas they increased those of inhibin βA in a concentration-dependent manner (Figure 7.1E and F). Next, we measured the levels of activin A in conditioned medium to determine whether BMP4 and BMP7 enhance the production of activin A by human granulosa cells. SVOG cells were treated with increasing concentrations of BMP4 or BMP7. As shown in Figure 7.1G, treatment for 24 h with BMP4 or BMP7 (1, 10 or 100 ng/ml) increased the accumulation of activin A in a concentration-dependent manner.

BMP4 and BMP7 up-regulate furin in SVOG cells

To investigate whether enhanced proteolytic processing by furin convertase contributes to the up-regulation of activinA production by BMP4 and BMP7, furin mRNA and protein levels were measured in SVOG cells following treatment with BMP4 or BMP7. Interestingly, treatment with BMP4 or BMP7 (1, 10 or 100 ng/ml) up-regulated both furin mRNA (12 h; Figure 7.2A and B) and protein (24 h; Figure 7.2C and D) levels in a concentration-dependent manner. The stimulatory effects of BMP4 and BMP7 (30 ng/ml) on furinm RNA and protein levels were also found to be time-dependent (Figure 7.2E and F).

Dorsomorphin abolishes BMP4- and BMP7-induced production of furin in SVOG cells

To date, three BMP type I receptors (ALK2, ALK3 and ALK6) have been shown to mediate the cellular functions of BMPs. Dorsomorphin is a small-molecule inhibitor of BMP type I receptors that specifically blocks downstream signaling mediated by ALK2, ALK3 and ALK6 (143, 163). To ascertain whether BMP type I receptors are required for the up-regulation of furin, SVOG cells were treated with BMP4 or BMP7 (30 ng/ml) in the presence or absence of 5 μM dorsomorphin. As shown in Figure 7.3A, co-treatment with dorsomorphin abolished the stimulatory effects of BMP4 and BMP7 on furin protein production. Consistent with these findings, treatment with BMP4 or BMP7 increased SVOG cell juxta-nuclearfurin immunostaining, and these increases were abolished by co-treatment with dorsomorphin (Figure 7.3B).

These results indicate that BMP type I receptors are required forthe stimulatory effects of BMP4 and BMP7 on furin expression in SVOG cells. However, BMP-induced activin A production, combined with previous reports of activin A-induced furin expression (221), suggest

that activin signaling may also contribute to increased furin production following treatment with BMP4 or BMP7. Thus, we next investigated whether treatment of SVOG cells with activin A could induce the expression of furin. As shown in Figure 7.3C, treatment for 12 h with activinA (1, 5or 100 ng/ml) up-regulatedfurin mRNA levels in a concentration-dependent manner.To determine whether activin signaling contributes to BMP-induced furin production, SVOG cells were treated for 24 h with BMP4 or BMP7 (30 ng/ml) in the presence or absence of 5 μM SB431542, a selective inhibitor of activin/TGF-β type I receptors (ALK4, ALK5 and ALK7; (164)). Interestingly, co-treatment with SB431542 attenuated, but did not completely abolish, the up-regulation of furin production by BMP4 and BMP7 (Figure 7.3D).

SMAD signaling is required for the up-regulation of furin by BMP4 and BMP7 in SVOG cells

Western blot analysis for phosphorylated and total SMAD1/5/8 was used to investigate the mechanism by whichBMP4 and BMP7 up-regulatefurin expression. Treatment of SVOG cells for 30 or 60 min with BMP4 or BMP7 (30 ng/ml) increased the levels of phosphorylated SMAD1/5/8 (Figure 7.4A). Next, dorsomorphin was used to investigate the role ofBMP type I receptors in BMP4- and BMP7-induced SMAD1/5/8 phosphorylation. Co-treatment of SVOG cells with increasing concentrations (0.1, 1 or 10 μM) of dorsomorphin reduced BMP4-induced (30 ng/ml, 60 min) SMAD1/5/8 phosphorylationin a concentration-dependent manner (Figure 7.4B). Consistent with these findings, both BMP4- and BMP7-induced SMAD1/5/8 phosphorylation were significantly attenuated by co-treatment with 5 μM dorsomorphin (Figure 7.4C).

To examine whether SMAD signaling is required for the up-regulation of furin, SVOG cells were treated with BMPs following siRNA-mediated knockdown of SMAD4, the essential common SMAD necessary for proper functioning of receptor-regulated SMADS (e.g. SMAD1/5/8). Western blot analysis confirmed that treatment for 48 h with 50 nM SMAD4 siRNA down-regulated SMAD4 protein levels by 70-80% relative to treatment with transfection reagent alone or non-targeting control siRNA (Figure 7.4D). Importantly, transfection with SMAD4 siRNA for 48 h prior to treatment with BMP4 or BMP7 (30 ng/ml, additional 24 h) abolished their effects on furin production (Figure 7.4E). These data indicate that SMAD1/5/8-SMAD4 signaling is involved in the up-regulation of furin by BMP4 and BMP7.

Furin contributesto BMP4- and BMP7-induced activinA production in SVOG cells

To investigate the role of furin in BMP4/7-induced activin A production in human granulosa cells, siRNA targeting furin was used to down-regulate furin prior to treatment with BMPs. Western blot analysis demonstrated that treatment for 48 h with 25 nM Furin siRNA decreased furin protein levels by 80-90 % relative to treatment with transfection reagent alone or non-targeting control siRNA (Figure 7.5A). Interestingly, transfection with furin siRNA for 48 h prior to treatment with BMP4 or BMP7 (30 ng/ml, additional 24 h) significantly attenuated, but did not completely abolish, their effects on activinA accumulation in conditioned medium (Figure 7.5B). Importantly, down-regulation of furin did not affect the up-regulation of inhibin βA mRNA by BMP4 or BMP7 (Figure 7.5C). To further confirm a role for furin in BMP4/7-induced activin A production, SVOG cells were treated for 24 h with BMP4 or BMP7 (30 ng/ml) in the presence or absence offurin inhibitor I (5 μM). As shown in Figure 7.5D, co-treatment

with furin inhibitor attenuated, but did not completely abolish, the stimulatory effects of BMP4 and BMP7 on activinA accumulation.

BMP4 and BMP7 up-regulate the expression of inhibinβA and furinin hGL cells

Non-immortalized primary hGL cells were used to further confirm the regulation of inhibin βA subunit and furin by BMPs. Treatment of primary hGL cells for 12 h with BMP4 or BMP7 (1, 10 or 100 ng/ml) increased both inhibin βA subunit and furin mRNA levels in a concentration-dependent manner (Figure 7.6).

7.4 Discussion

During follicular development, bi-directional communication between granulosa and theca cells is important for, among other things, the synthesis of steroid hormones, the supply of factors to regulate oocyte maturation, and the provision of structural support for the growing follicle (227, 228). In the present study, we demonstrate that the theca cell-derived growth factors BMP4 and BMP7 stimulate the biosynthesis, maturation andsecretion of activin A in human granulosa cells. This overall effect arises from the dual functions of BMP4 and BMP7 in the up-regulation of inhibin βA subunit production as well as proteolytic processing by its proprotein convertase, furin. BMP4/7-induced inhibin βA subunit production is independent of furin as knockdown of furin did not attenuate these effects. However, the enhanced production of mature activin A appears to contribute to BMP4/7-induced furin expression as these effects are partially inhibited by a selective inhibitor of activin/TGF-β type I receptors (SB431542). Our findings suggest a novel paracrine mechanism by which theca cells can regulate neighboring granulosa cells to augment the production and secretion of mature activin A needed for follicular

function. Such a mechanism provides an additional layer of regulatory control over locally produced hormonal factors, and may extend to other TGF-β superfamily members processed by furin or to other proprotein convertases and their substrates.

In the ovary, pituitary gonadotropins are the main regulators of activins and inhibins. In rat granulosa cells, treatment with FSH and activin A increases the hybridization of inhibin βA mRNA (229). In cultured hGL cells, the addition of 8-br-cAMP, FSH and human chorionic gonadotropin increases the immunostaining for activin A (230). Outside the reproductive system, members of the TGF-β superfamily have been demonstrated to modulate activin A production. In rat mesenchymal progenitor cells, BMP2 was found to increase inhibin βA mRNA levels and activin A accumulationin in conditioned medium in a time-dependent manner (231). Similarly, treatment of murine LβT2 pituitary gonadotrope cells with activin A increased inhibin α and inhibin βB mRNA levels (221). Previous studies in hGL cells have demonstrated stimulatory effects of BMP6 and BMP7 on inhibin βA subunit mRNA levels, however their effects on activin A production were not evaluated (232, 233). Here, we demonstrate that treatment with BMP4 or BMP7 up-regulates inhibin βA and increases activin A secretion in human granulosa cells. Moreover, we show that increased activinA production occurs, in part, via enhanced proteolytic processing of inhibin βA subunit by its proprotein convertase, furin, which is also up-regulated by BMP4, BMP7 and activin A. These results are consistent with a previous study showing activin A induced expression of inhibin α and βB subunits as well as furin in LβT2 cells (221). Treatment with BMP4, BMP3, TGF-β1, TGF-β2 or TGF-β3 failed to alter furin expression in LβT2 cells (221), however TGF-β1 increased the expression of furin in rat synoviocytes (234). Interestingly, activin A was shown to differentially regulate the expression of proprotein convertases in cultured mouse secondary follicles, enhancing the expression of

PCSK5 (also cleaves inhibin subunits) and decreasing the expression of furin and PCSK6 (235). Thus, the differential regulation of proprotein convertases by TGF-β superfamily members, both within and between cell-types, is likely an important mechanism controlling the production and action of these hormonal factors within ovarian follicles.

To date, mechanisms underlying the differential regulation of furin expression by TGF-β superfamily members remain poorly understood. The *FURIN* gene is composed of at least three distinct promoters (P1, P1A and P1B) of which the P1 promoter is more likely to be a regulated promoter, whereas P1A and P1B have characteristics of housekeeping genes due to their lack of TATA or CCAAT boxes (236). Studies in HepG2 hepatocellular carcinoma cells have demonstrated that the P1 promoter, which contains SMAD2/3-SMAD4 binding elements in the proximal region, is the most sensitive promoter to TGF-β1 stimulation (237). Moreover, knockdown of SMAD2 or SMAD3 abolished activin-induced furin P1-luciferase reporter activity in LβT2 cells (221). Our study is the first to show that the expression of furin can be regulated by BMPs, likely via SMAD1/5/8-SMAD4 signaling. Although we did not provide direct evidence for SMAD1/5/8-SMAD4 binding to the furin promoter, this is suggested by the rapidity of the responses (3 h) and the potent inhibitory effects of the BMP type I receptor inhibitordorsomophin. Unlike LβT2 cells, where only activin A enhances furin expression (221), human granulosa cells display increased furin production in response to BMP4/7 as well as activin A. Future studies will be required to determine whether additional TGF-β superfamily members can regulate furin expression, and to explore whether unique promoter elements mediate responsiveness to SMAD2/3-SMAD4 *vs.* SMAD1/5/8-SMAD4 signaling.

In summary, the present study demonstrates that BMP4 and BMP7 increase the production of activin A byup-regulating inhibin βA subunit and furin expression in human granulosa cells,

likely via SMAD1/5/8-SMAD4 signaling. These results suggest additional roles for theca cells in the regulation of follicular function through their paracrine interactions with granulosa cells.

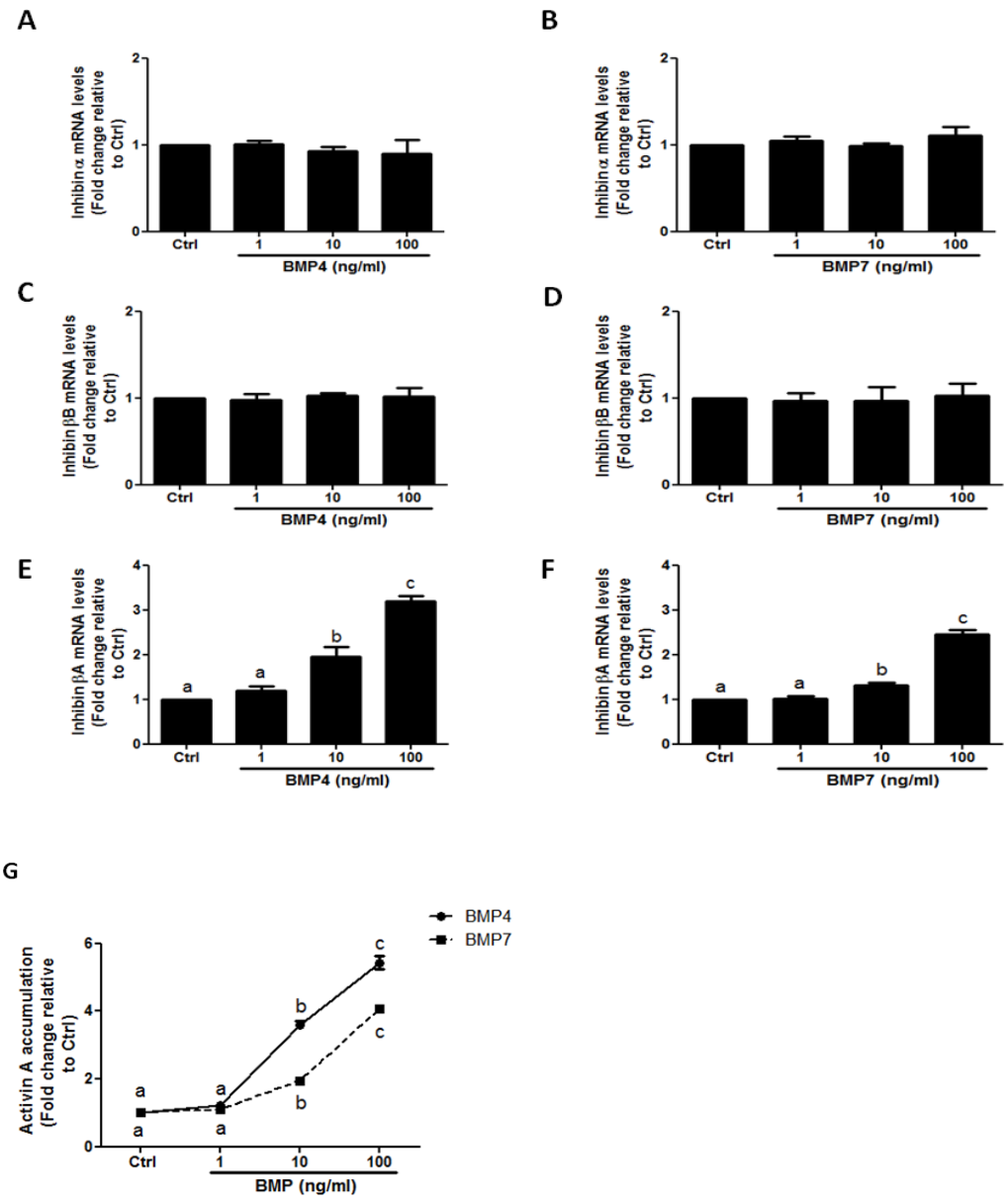
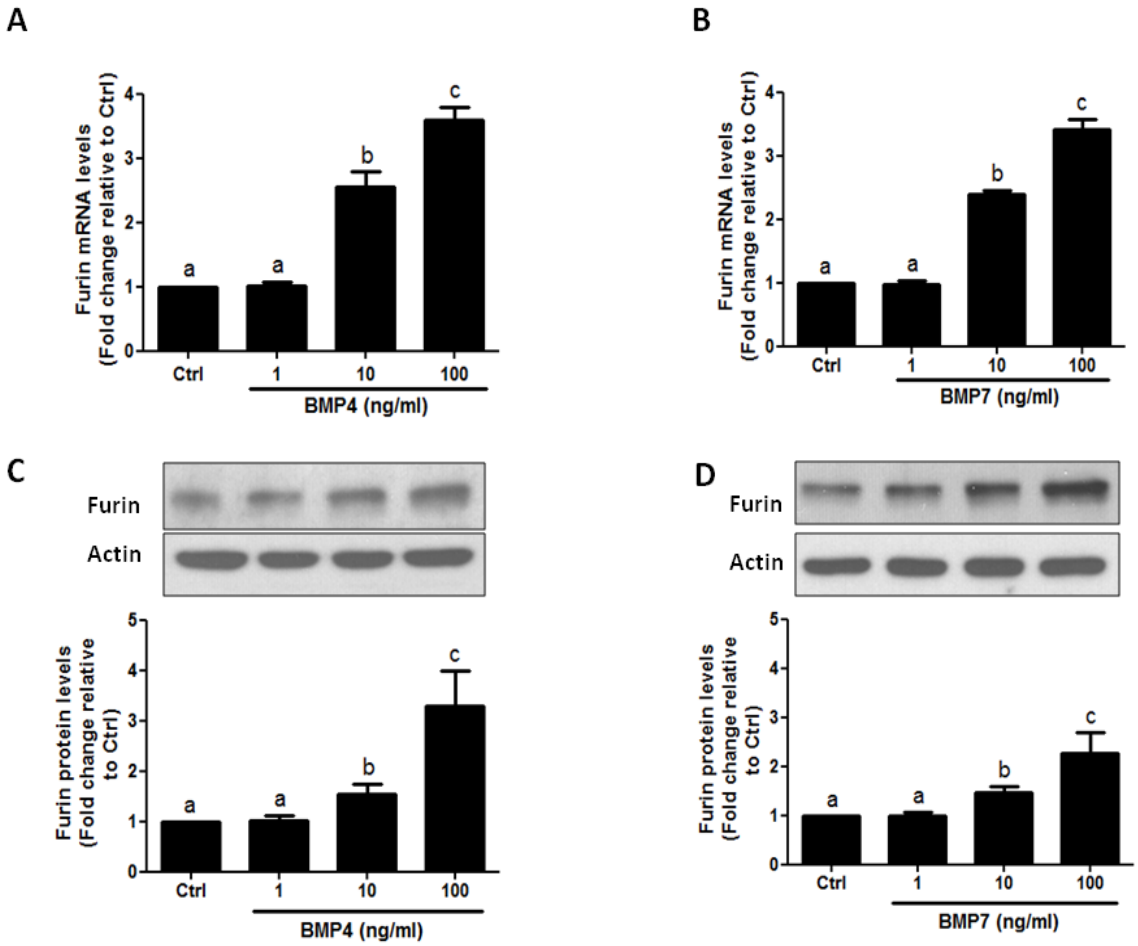


Figure 7.1 BMP4 and BMP7 up-regulate inhibinβA mRNA and increase activinA production in SVOG cells

A-F, Cells were treated for 12 h with different concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7 and inhibin subunit (α, βA or βB) mRNA levels were examined by RT-qPCR. G, Cells were treated for 24 h with different concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7 and

activinA levels in the conditioned medium were measured by enzyme immunoassay (ELISA). Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < 0.05$). Ctrl, control.



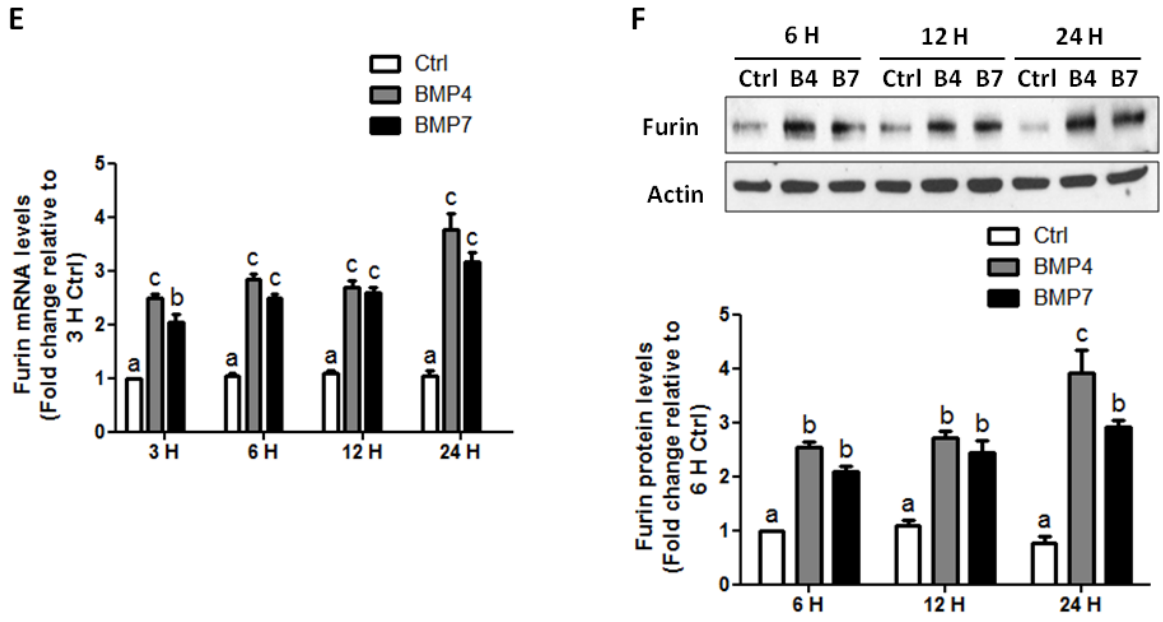
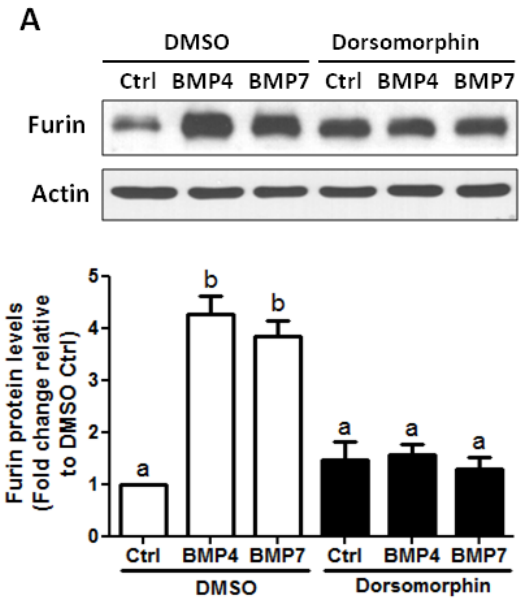
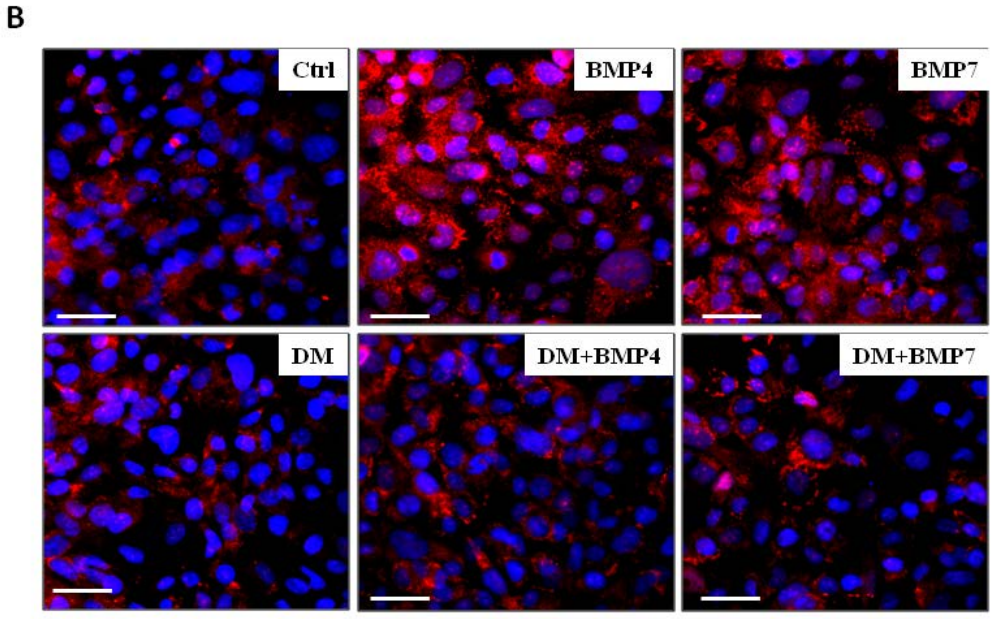


Figure 7.2 BMP4 and BMP7 up-regulate furin mRNA and protein levels in SVOG cells

A andB, Cells were treated for 12 h with different concentrations (1, 10 or 100 ng/ml) of BMP4 (A) or BMP7 (B) and furin mRNA levels were examined by RT-qPCR. Cand D, Cells were treated for 24 h with different concentrations (1, 10 or 100 ng/ml) of BMP4 (C) or BMP7 (D) and furin protein levels were examined by Western blot (quantified data are normalized to actin). E, Cells were treated with 30 ng/ml of BMP4 or BMP7 for 3, 6, 12 or 24 h and furin mRNA levels were examined by RT-qPCR. F, Cells were treated with 30 ng/ml of BMP4 or BMP7 for 6, 12 or 24 h and furin protein levels were examined by Western blot. Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P< 0.05$). Ctrl, control. B4, BMP4. B7, BMP7.


A
DMSO
Dorsomorphin
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
Furin
Actin
Furin protein levels (Fold change relative to DMSO Ctrl)
a b b a a a
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
DMSO
Dorsomorphin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Ctrl
BMP4
BMP7
DM
DM+BMP4
DM+BMP7

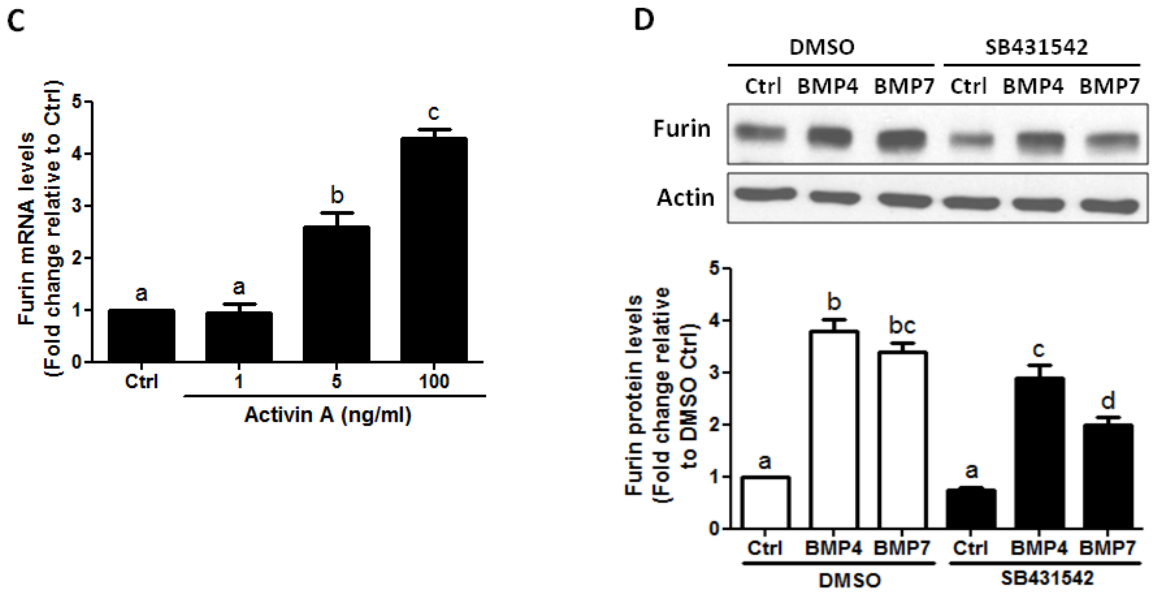
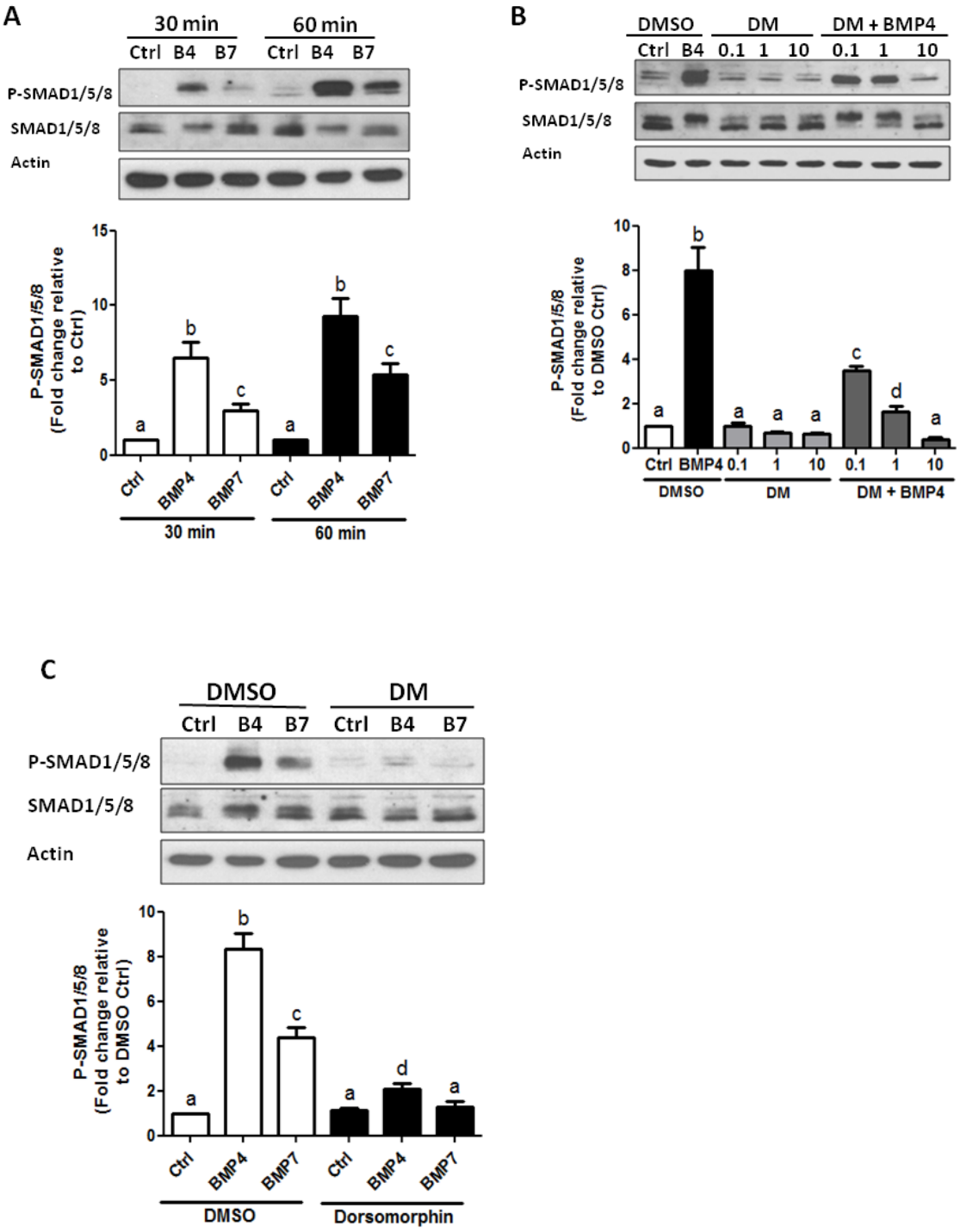


Figure 7.3 Effects of BMP (dorsomorphin) and TGF-β (SB431542) type I receptor inhibitors on BMP4- and BMP7-induced up-regulation of furin in SVOG cells

A, Furin protein levels were examined in cells following treatment for 24 h with 30 ng/ml BMP4 or BMP7 in the absence (DMSO) or presence of 5 μM dorsomorphin (quantified data are normalized to actin). Ctrl, control.B, Furin protein levels (red) were examined by immunofluorescence microscopy following treatment of SVOG cells for 24 h withBMP4 or BMP7 (30 ng/ml) in the absence or presence of 5 μM dorsomorphin (DM). Cell nuclei (blue) were stained with Hoechst 33258. Scale bars: 50 μm. C, Cells were treated for 12 h with different concentrations (1, 5 or 100 ng/ml) of activin A and furin mRNA levels were examined by RT-qPCR. D, Furin protein levels were examined in cells following treatment for 24 h with 30 ng/ml BMP4 or BMP7 in the absence (DMSO) or presence of 5 μM SB431542. Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P< 0.05$). Ctrl, control. DM, dorsomorphin.


A
30 min
60 min
Ctrl B4 B7 Ctrl B4 B7
P-SMAD1/5/8
SMAD1/5/8
Actin
P-SMAD1/5/8 (Fold change relative to Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
30 min
60 min
B
DMSO
DM
DM + BMP4
Ctrl B4 0.1 1 10 0.1 1 10
P-SMAD1/5/8
SMAD1/5/8
Actin
P-SMAD1/5/8 (Fold change relative to DMSO Ctrl)
Ctrl BMP4 0.1 1 10 0.1 1 10
DMSO
DM
DM + BMP4
C
DMSO
DM
Ctrl B4 B7 Ctrl B4 B7
P-SMAD1/5/8
SMAD1/5/8
Actin
P-SMAD1/5/8 (Fold change relative to DMSO Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
DMSO
Dorsomorphin

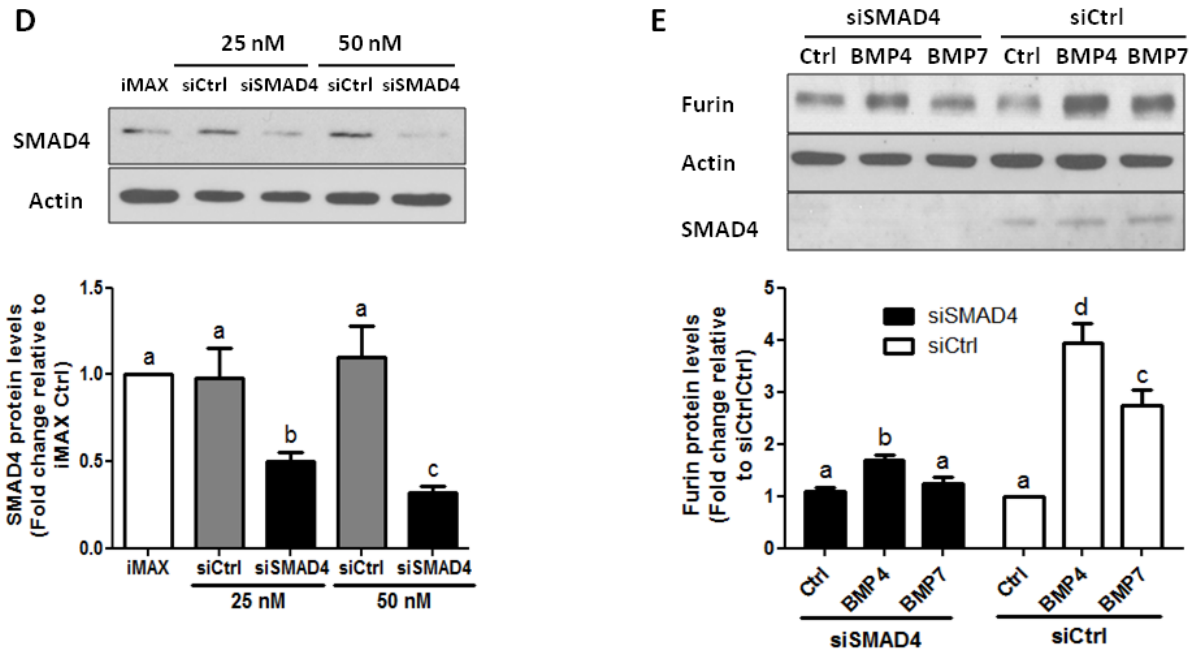
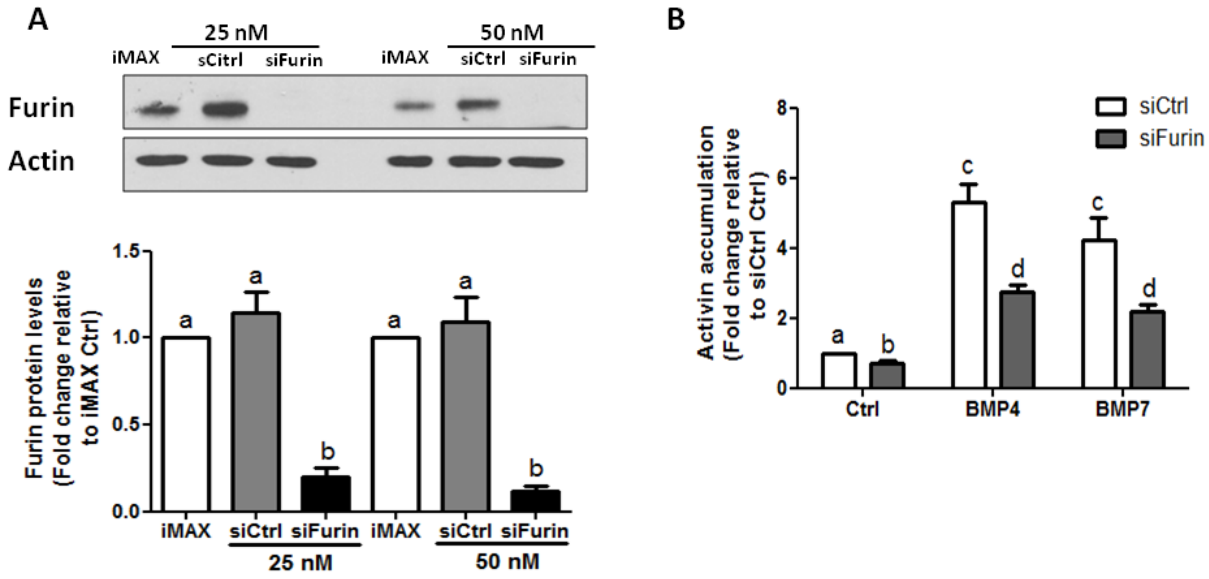


Figure 7.4 SMAD signaling is required for the up-regulation of furin by BMP4 and BMP7 in SVOG cells

A, Cells were treated with BMP4 or BMP7 (30 ng/ml) for 30 or60 minand phosphorylated SMAD1/5/8 levels were examined by Western blot (quantified data are normalized to total SMAD1/5/8 levels). B, Phosphorylated SMAD1/5/8 levels were examined in cells followingtreatmentfor 60 min with BMP4 (30 ng/ml) in absence (DMSO) or presence of different concentrations of dorsomorphin (0.1, 1 or 10 μM). C, Cells were treated for 60 min with BMP4 or BMP7 (30 ng/ml)in the absence or presence of 5 μMdorsomorphin and phosphorylated SMAD1/5/8 levels were examined by Western blot.Ctrl, control; B4, BMP4; B7, BMP7; DM, dorsomorphin.D, Cells were transfected for 48 h with transfection reagent (iMAX), control siRNA (siCtrl; 25 or 50 nM) or SMAD4 siRNA (siSMAD4; 25 or 50 nM),and knockdown efficiency was examined by Western blot(quantified data are normalized to actin). E, Following transfection for 48 h withcontrol or SMAD4 siRNA (50 nM), SVOG cells were

treated for 24 h with BMP4 or BMP7 (30 ng/ml) and furin protein levels were examined by Western blot (quantified data are normalized to actin). Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < 0.05$).



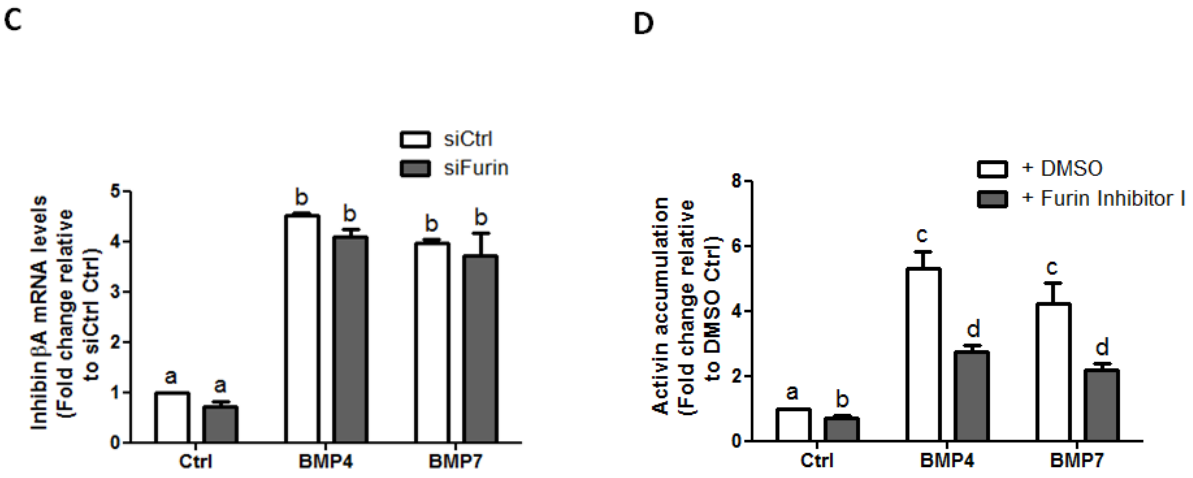


Figure 7.5 BMP4- and BMP7-induced activin A accumulation is attenuated by furin knockdown or co-treatment with furin inhibitor I in SVOG cells

A, Cells were transfected for 48 h with transfection reagent (iMAX), control siRNA (siCtrl; 25 or 50 nM) or furin siRNA (siFurin; 25 or 50 nM), and knockdown efficiency was examined by Western blot (quantified data are normalized to actin). Band C, Cells were transfected for 48 h with control or furin siRNA (25 nM) and then treated for 24 h with BMP4 or BMP7 (30 ng/ml). B, Activin A levels in conditioned medium were examined by enzyme immunoassay. C, Inhibin βA subunit mRNAlevels were examined by RT-qPCR. D, Cells were treated for 24 h with BMP4 or BMP7 (30 ng/ml) in the absence (DMSO) or presence of 5 μMfurin inhibitor I andactivin A levels in conditioned medium were measuredby enzyme immunoassay.Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P< 0.05$). Ctrl, control.

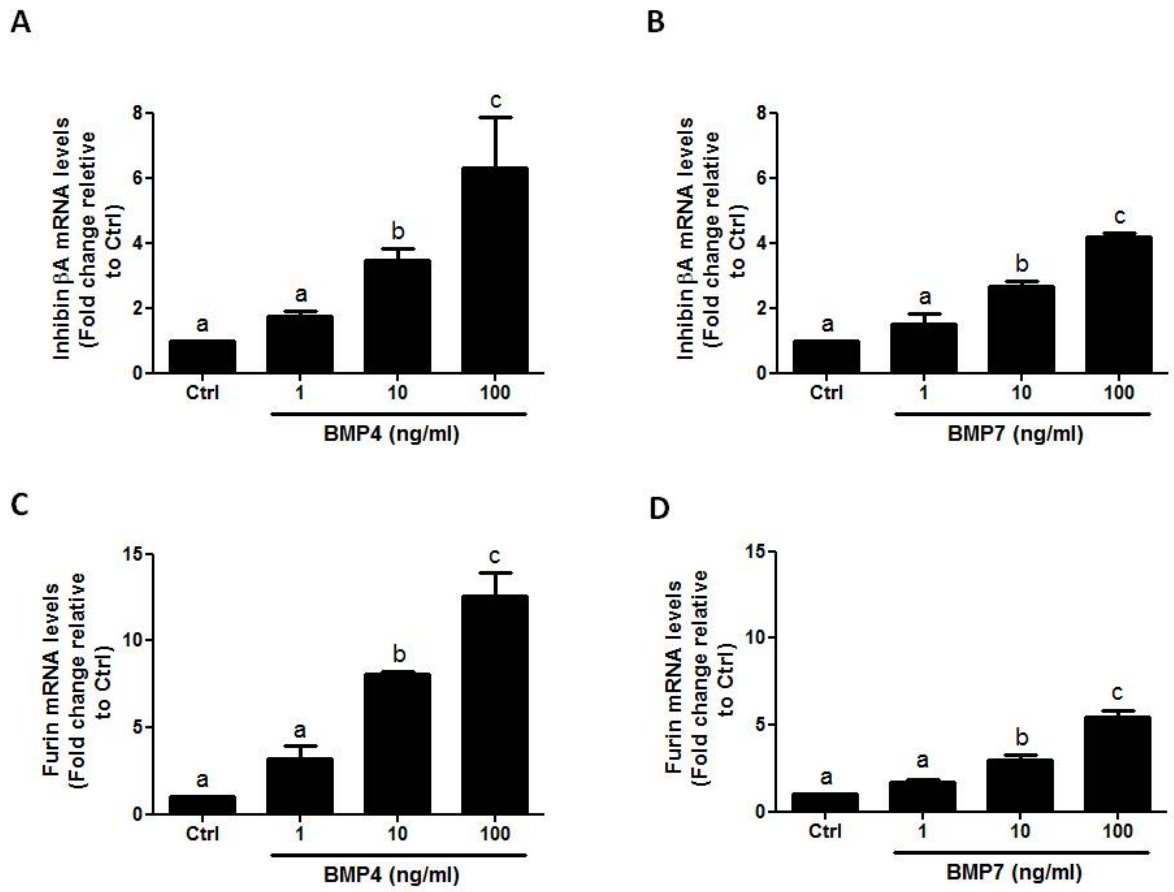


Figure 7.6 BMP4 and BMP7 up-regulate inhibin βA subunit and furin expression in hGL cells

A-D, Primary hGL cells were treated for 12 h with different concentrations (1, 10 or 100 ng/ml) of BMP4 or BMP7 and inhibin βA subunit and furinmRNA levels were examined by RT-qPCR. Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($P < 0.05$). Ctrl, control.

Chapter 8: Conclusion

8.1 Conclusion

The main objective of the current thesis was to investigate the potential anti-luteinization effects of the oocyte- and theca cell-derived BMPs in human granulosa cells and their underlying signaling mechanisms. Several results have provided physiological information about these objectives.

First of all, neither GDF9 nor BMP15 affects the mRNA levels of the P450 side chain cleavage enzyme or of the 3β-hydroxysteroid dehydrogenase in SVOG cells. However, treatment with BMP15, but not GDF9, significantly decreases StAR mRNA and protein levels as well as progesterone production. These suppressive effects, along with the induction of SMAD1/5/8 phosphorylation, are attenuated by co-treatment with two different BMP type I receptor inhibitors: dorsomorphin and DMH-1. Furthermore, the depletion of ALK3 using small interfering RNA reverses the effects of BMP15 on SMAD1/5/8 phosphorylation and StAR expression. Similarly, the knockdown of ALK3 abolishes BMP15-induced SMAD1/5/8 phosphorylation in KGN cells. These results provide evidence that oocyte-derived BMP15 down-regulates StAR expression and decreases progesterone production in human granulosa cells, likely via ALK3-mediated SMAD1/5/8 signaling (see Figure 3.7 and Figure 8.2).Thus, oocytes may play a critical role in the regulation of progesterone to prevent premature luteinization during the late stage of follicular development.

Second, neither GDF9 nor BMP15 affects the mRNA levels of Connexin 37 (Cx37) in SVOG cells. However, treatment with BMP15, but not GDF9, significantly decreasesCx43 mRNA and protein levels andgap junction intercellular communication (GJIC) activity. These suppressive

effects, along with the induction of SMAD1/5/8 phosphorylation, are attenuated by co-treatment with a BMP type I receptor inhibitor, dorsomorphin. Furthermore, knockdown of the central component of the TGF-β superfamilysignaling pathway, SMAD4 reverses the suppressive effects of BMP15 on Cx43 expression and GJIC activity. These findings suggest that oocyte-derived BMP15 decreases GJIC activity between human granulosa cells by down-regulating Cx43 expression, most likely via a SMAD-dependent signaling pathway (see Figure 8.3).

Third, using the same dual inhibition approaches, we also demonstrated that theca cell-derived BMP4 and BMP7 down-regulate Cx43 in human granulosa cells. The results suggest that the SMAD1/5/8 signaling pathway is required for the BMP4- and BMP7-induced down-regulation of Cx43. Moreover, the down-regulation of Cx43 contributes to the inhibition of gap junction intercellular communication, indicating that theca cell-derived BMP4 and BMP7 may be involved in the inhibition of ovulation and luteinization (see Figure 8.3).

Fourth, pentraxin 3 (PTX3) plays a critical role in the assembly of the extracellular matrix of the cumulus oophorus, which is essential for cumulus expansion during ovulation. Theca cell-derived BMP4 and BMP7 are important regulators of folliculogenesis and have been shown to inhibit luteinization. Here, for the first time, we demonstrate that BMP4 and BMP7 significantly decrease PTX3 mRNA and protein production in human granulosa cells (see Figure 8.3). Furthermore, the results indicate that these biological effects are most likely mediated by SMAD-dependent pathways via differential subsets of BMP type I receptors (ALK3 and/or ALK6 for BMP4, ALK2 and/or ALK3 for BMP7) (see Figure 8.4). These findings shed light on the functional roles of theca cells in preventing ovulation and premature luteinization.

Fifth, activins are disulfide-linked homo- or hetero-dimers of inhibin βsubunits (βA or βB) and the formation of mature bioactive ligands depends on the proteolytic processing of precursor

proteins. Activin A has been shown to be a potent inhibitor of premature luteinization. Despite intensive functional studies, the intracellular regulation, assembly and secretion of endogenous activins in human granulosa cells remain to be determined. We demonstrated that both BMP4 and BMP7 increased the mRNA transcription of the inhibin βA subunit without affecting the basal expression levels of the inhibin αand βB subunits. Furthermore, we provide the first charaterization of a paracrine regulatory pathway that controls precursor processing and dimer secretion. Theca cell-derived BMPs (BMP4 and BMP7) induced pro-protein convertase furin expression in concentration- and time-dependent manners. These enhancing effects of BMP4 and BMP7 on activin production and secretion provide a potential mechanism by which theca cells interact with their neighboring cells in the ovary (see Figure 8.2).

In summary, these results describe several important aspects of ovarian physiology. Oocyte- and theca cell-derived BMPs play pivotal roles in the prevention of ovulation and premature luteinization, including decreased progesterone production, decreased intercellular communication, down-regulation of pentraxin 3 and increased activin A formation. Collectively, these data highlight the integral functions of the oocyte, granulosa cells and theca layer in the follicle structure, which protects the growing follicles from undergoing atresia due to premature luteinization induced by LH. Furthermore, different BMP ligands may act through differential subsets of type I receptor-driven downstream signaling pathways (BMP15 interacts with ALK3, BMP4 interacts with ALK3 and/or ALK6 and BMP7 interacts with ALK2 and/or ALK3) (see Figure 8.4). These concerted interactions of different BMP ligands and receptors may generate compensatory cellular signals, which are beneficial and crucial for follicular development and synchronization. Taken together, the information provided in this thesis indicates that oocytes

and theca cells may play broader rolesthan previously acknowledged in the regulation of follicular function through their interactions with granulosa cells.

8.2 Limitations of this study

The results described in this thesis are based on a granulosa cell tumor-derived KGN cell line, an immortalized SVOG cell line or primary hGL cells obtained from women undergoing IVF procedures. We appreciate that these cells are either tumor cells or have been exposed to high pharmacological concentrations of exogenous gonadotropins (FSH and/or LH) during the process of hCG stimulation and luteinization. However, because of the shortage of granulosa cells from unstimulated ovaries, the data generated from our cell models still provideuseful information on ovarian physiology during the peri-ovulatory stages of follicular development. Another limitation of the cell model is that these cells have been cultured in an artificial environment and may not reflect the intraovarian microenvironment *in vivo*. Therefore, the gene expression patterns of receptors and signaling mediators could be different between the *in vitro* and the *in vivo* systems, leading to different responses to the ligand treatment. In the future, a three-dimensional cell culture system or a tissue culture system that includes three types of cells (oocytes, granulosa cells and theca cells) as a mimic of the *in vivo* conditions will provide more practical information regarding the normal ovarian biology. Furthermore, because of these limitations, it is difficult to link the results generated from our *in vitro* model system to the clinical parameters of IVF patients. Whether these conclusions could be applied to explain the normal ovarian physiology or pathology (such as chronic anovulation, premature luteinization and infertility) and any subsequent pharmacological applications remains to be elucidated.

8.3 Future directions

All of these results have increased our fundamental understanding of the functional roles of oocytes and theca cells in human granulosa cells. However, the close and complete relationship between the oocyte and its supporting follicle cells remains far from fully understood. Further comprehensive research regarding the function of human granulosa cells is required to advance our knowledge of ovarian biology. Future directions should include the following:

1) Estradiol production is an important aspect of steroidogenesis in granulosa cells. Whether the interaction of the oocyte, theca cells and granulosa cells play a role in the modulation of estradiol production remains to be investigated.

2) In the study regarding BMP15-induced suppression of progesterone production by down-regulating StAR via ALK3, we found that knockdown of ALK2 dramatically decreased basal StAR mRNA and protein levels, but did not abolish the effects of BMP15 on StAR expression. Our results suggest a positive role for ALK2 inmaintaining basal StAR expression. The preciseTGF-β superfamilymemberthat acts via ALK2 to enhance StAR expression in the humangranulosacellsremains unknown. Future studies aimed at addressing how ALK2 contributes to the regulation of progesterone production by granulosa cells will be of great interest.

3) A previous study has demonstrated that, in mouse granulosa cells, GDF9:BMP15 heterodimers are the most bioactive ligands, althoughthey require ALK6 as a coreceptor in the signalingcomplex (154). In fact, in *in vivo* systems, the question of whether GDF9 and BMP15 actually function as homodimers, heterodimers or both is still unknown. It would be interesting to examine the effects of the synthetic GDF9:BMP15 heterodimers on non-tumorigenic human granulosa cells in the future.

4) We and others have previously demonstrated the presence of endogenous GDF9 expression in human granulosa-lutein cells (147, 148), even though its roles in luteal phase granulosa cell function are unknown.As a result,the up-regulation of the LH receptor induced by exogenous GDF9 could indicate a role for autocrine/paracrine GDF9 in promoting the formation of the corpus luteum and its function following ovulation.

5) In this thesis, only oocyte- and theca cell-derived BMPs were examined todetermine their roles in granulosa cells. It has been shown that BMP2, BMP5 and BMP6 are all expressed in granulosa cells (109, 238). The question of whether these granulosa cell-derived BMPs have similar functions needs to be further investigated.

6) All of the data reported in this study are unable to provide answers as to whether the SMAD1/5/8 transcription factors interact with their target gene (*StAR, Cx43, PTX3 and furin*) promoter sequences in the nucleus following exposure to BMPs. Future studies aimed at addressing this question would be of great interest for the generation of promoterconstructs that can be transfected into granulosa cells.

7) All of these results are based on an *in vitro* culture system; thus, further studies involving animal experiments will advance our knowledge of normal and pathological ovarian biology.

8.4 Significance and translational potential

Our studies represent the first comprehensive research of the functional roles and mechanisms of oocyte-derived BMP15 and theca cell-derived BMP4 and BMP7 in the regulation of human granulosa cells. Deepening our understanding of the normal physiological roles of these potent ovarian regulatory factors will offer important insights into ovarian pathology, such as polycystic ovary syndrome, premature ovarian failure and dysfunctional ovulation. In addition, detailed

information about the mechanisms of action and molecular determinants could be of clinical significance. New knowledge may also lead to thedevelopment of noveltherapeutic methods for fertility regulation, whether the objectiveis to enhance human health, treat infertility, develop alternative forms of contraception or develop new ovulation induction procedures in assisted reproduction.

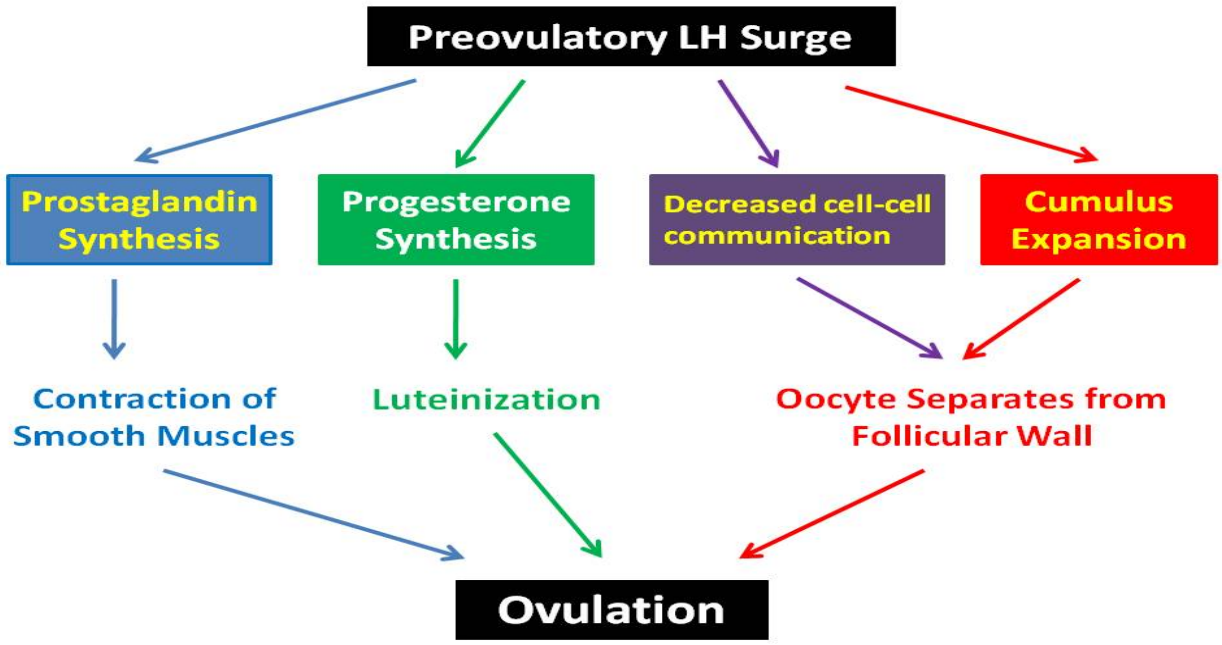


Figure 8.1 Proposed mechanisms involved in the LH surge induced ovulation

The LH surge occurs 34 to 36 hours prior to ovulation. The LH surge stimulates luteinization ofthe granulosacells and stimulates the synthesis of progesterone. Prostaglandins are increased in response to LHand progesterone, leading to the contraction of smooth muscles. Also, the LH surge decreasescell-cell communication by down-regulating connexin-coupled gap junction formation. Furthermore, the LH surge induces the cumulus expansion and the subsequent realease of oocyte cumulus complex from follicular wall.

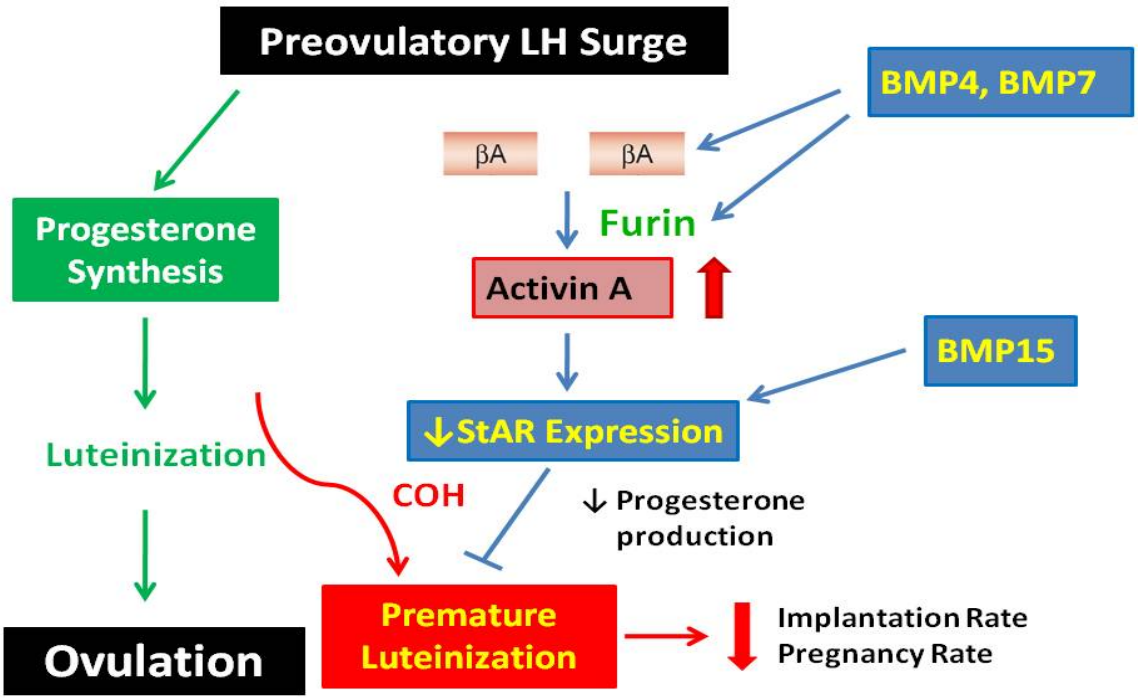


Figure 8.2 Proposed mechanisms involved in the prevention of premature luteinization induced by BMP4, BMP7 and BMP15

Oocyte-derived BMP15 decreases progesterone production bydown-regulating StAR expression. Both theca cell-derived BMP4 and BMP7 increase activin A formation by up-regulating the expression of inhibin βA and proprotein convertase furin. Increased activin A further decreases progesterone production by down-regulating StAR expression. The result of decreased progesterone contributes to the prevention of premature luteinization. βA, inhibin βA. StAR, steroidogenic acute regulatory protein. COH, controlled ovarian hyperstimulation.

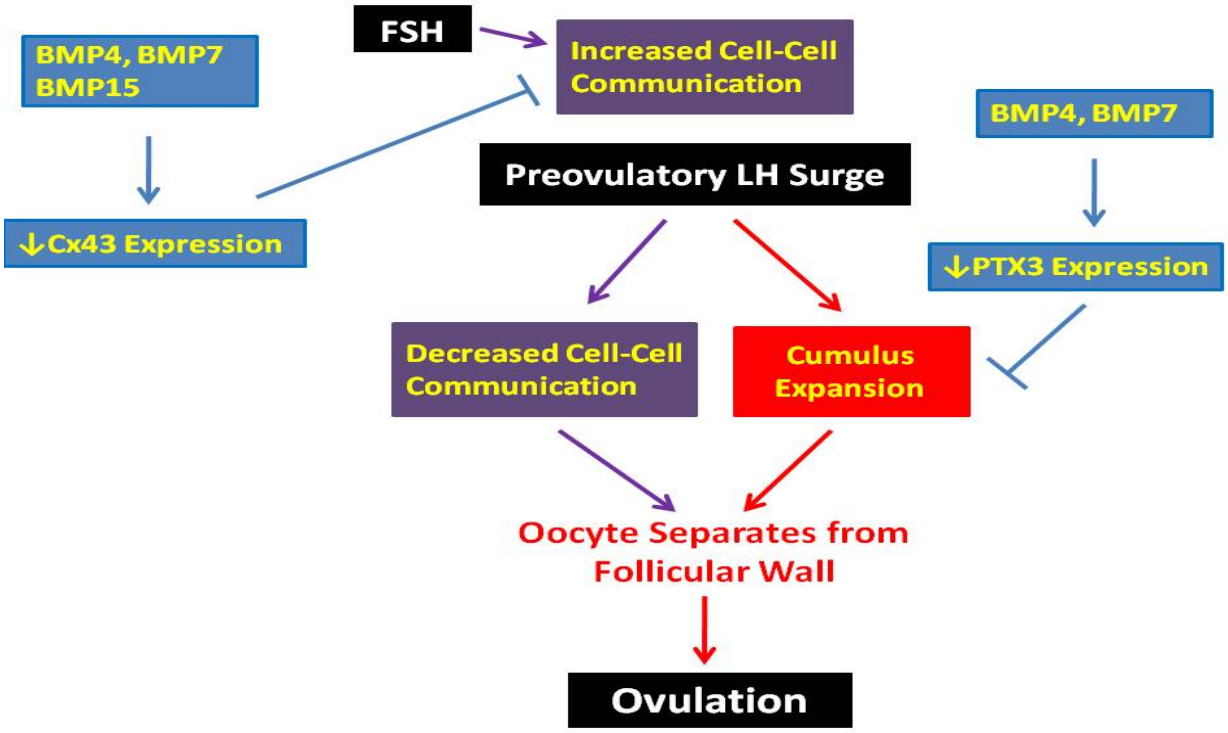


Figure 8.3 Proposed mechanisms involved in the prevention of ovulation and premature luteinization induced by BMP4, BMP7 and BMP15

The underlying mechanisms that modulate ovulation and the subsequent luteinization process include the functional gap junction mediated cell-cell communication and the formation of cumulus expansion. All the BMP4, BMP7 and BMP15 decrease cell-cell communication by down-regulating connexin 43-coupledgap junction formation. Theca cell derived BMP4 and BMP7 disrupt cumulus expansion by down-regulating pentraxin 3 expression. Cx43, connexin 43. PTX3, pentraxin 3.

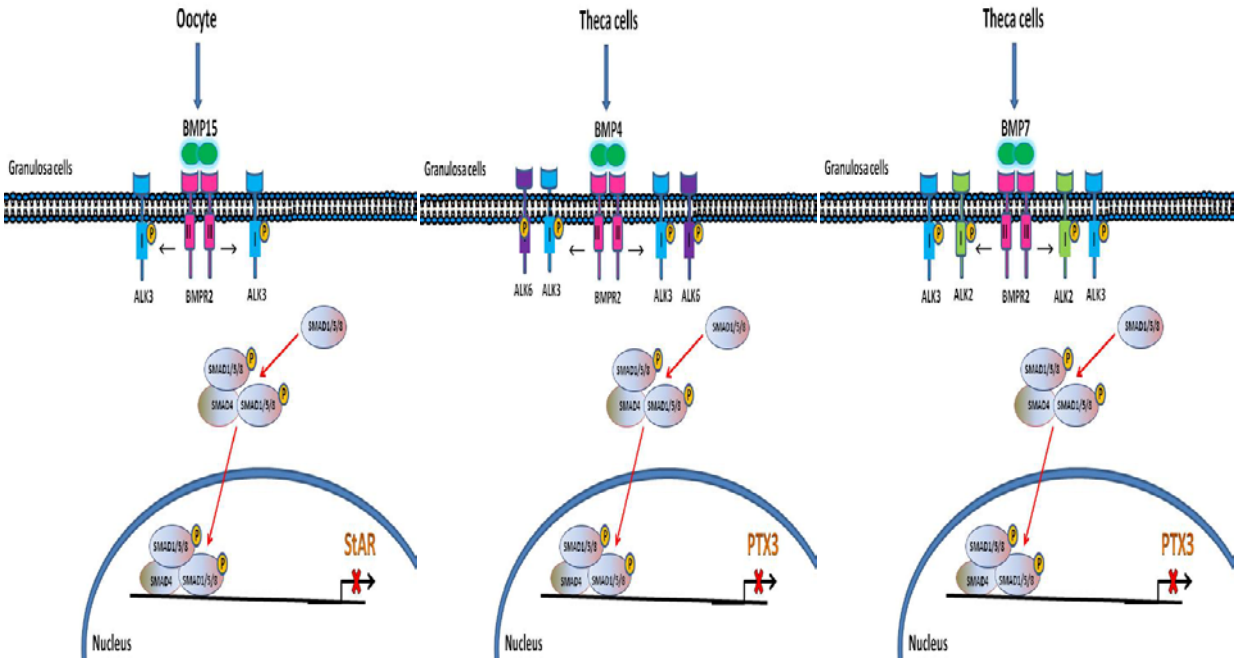


Figure 8.4. Proposed models for ligand-receptor binding of BMPs in human granulosa cells

BMP homodimers bind to heterotetrameric receptor complexes comprised ofBMP type I and type II (BMPR2) receptors. Different BMP ligands act through differential subsets of type I receptor-driven downstream signaling pathways (BMP15 interacts with ALK3, BMP4 interacts with ALK3 and/or ALK6 and BMP7 interacts with ALK2 and/or ALK3, respectively). In the ligand-induced receptor complex, BMPR2 phosphorylates ALKs leading to its activation and subsequent phosphorylation of receptor-regulated SMAD1/5/8. Phosphorylated SMAD1/5/8 binds with common SMAD4 to form a heterotrimeric complex and thentranslocates into the nucleus where it binds DNA and suppressesStAR or PTX3 transcription. StAR, steroidogenic acute regulatory protein; BMPR2, BMP receptor type II; ALK, activin receptor-like kinase; SMAD, Sma- and Mad-related protein; PTX3, Pentraxin 3

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Appendix A: Anti-müllerian hormone inhibits FSH-induced adenylyl cyclase activation, aromatase expression and estradiol production in human granulosa-lutein cells

A.1 Introduction

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors. During male fetal development, AMH is responsible for the regression of the Müllerian ducts, from which the female genital tract develops (239). Postnatally, AMH is primarily produced by Sertoli cells and granulosa cells of the gonads where it plays a crucial role in regulating testicular and ovarian functions (240, 241).

In clinical practice, measurement of serum AMH has been used to assess ovarian reserve, to predict poor response and ovarian hyperstimulation syndrome (OHSS) during ovulation induction, to diagnose polycystic ovarian syndrome (PCOS), to diagnose granulosa cell tumors, and to differentiate ambiguous genitalia (242-245). Potential widespread clinical use of AMH is anticipated because it has the lowest inter- and intra-cycle variability and is the earliest marker to change with age (246).

Human studies to date indicate that AMH is expressed by granulosa cells of developing follicles from the early primary stage to the early antral stage (247, 248). *In vivo* and *in vitro* animal studies examining the function of AMH in the postnatal ovary have shown that AMH plays an important role in two critical selection points of follicular development. First, AMH exerts an inhibitory effect on the initial recruitment of resting primordial follicles. Second, AMH negatively regulates preantral and small antral follicle growth by attenuating their responsiveness to FSH (249). Consistent with a role for AMH in suppressing follicle maturation, higher AMH

concentrations have been shown to correlate with a greater number of immature follicles in rats (250). Apart from its suppressive effects on folliculogenesis, AMH has also been shown to attenuate or inhibit aromatase activities and estradiol production in fetal ovaries from rats, rabbits and cows (251, 252). Based on these studies, it has been postulated that AMH inhibits FSH-induced aromatase activity, thus attenuating granulosa cell production of estradiol from testosterone. Low serum levels of estradiol are correlated with difficulties in selecting a dominant follicle and may lead to anovulation (246). This phenomenon may account, in part, for the hyperandrogenism, oligo/anovulation, and infertility frequently observed in PCOS patients, because it has been found that serum and follicular fluid AMH concentrations are increased in women with this syndrome (253, 254). Yet, only a few studies to date have investigated the inhibitory effects of AMH on FSH-induced aromatase activity in human granulosa cells *in vitro*; and the molecular mechanisms underlying this effect remain unclear (255). Understanding the mechanisms by which AMH regulates steroidogenesis may provide insight into pathogenesis of PCOS and OHSS.

In this study, we investigate the effects of AMH on basal and FSH-induced aromatase expression and estradiol production in human granulosa-lutein (hGL) cells, and elucidate the mechanism by which AMH exerts its effects.

A.2 Materials and methods

Preparation and treatment of hGL cells

Ethical approval for this study was obtained from the Research Ethics Board of the University of British Columbia and informed written consent was obtained from all patients. The controlled ovarian stimulation protocol for *in vitro* fertilization patients consisted of luteal-phase naferelin

acetate (Synarel, Pfizer, Kirkland, Quebec, Canada) and recombinant FSH (Gonal-F, Serono, Ontario, Canada) stimulation followed by human chorionic gonadotropin administration 34 h before oocyte retrieval. Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval as previously described; see supplement Materials and Methods (140). Individual primary cultures were comprised of cells from one individual patient and, in total, samples were obtained from 42 patients. Cells were counted with a hemocytometer and cell viability was assessed by Trypan blue (0.04%) exclusion. Purified hGL cells were seeded (2×10^5 cells per well in 12-well plates) and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 C. Cells were cultured in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich Corp., Oakville, ON) supplemented with 10 % fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/ml penicillin (Life Technologies, Inc/BRL, Grand Island, NY), 100 μg/ml streptomycin sulfate (Life Technologies), and 1X GlutaMAX (Life Technologies). Culture medium was changed every other day in all experiments.

On day 5 of culture the medium was changed to low serum medium (containing 0.5 % FBS) and hormone stimulation was performed. For the assessment of estradiol accumulation, 100 nM androstenedione, a substrate for human aromatase, was added to the medium. Cells were treated for 48 h with medium containing vehicle (control), 10 ng/ml recombinant human AMH (R&D System, Minneapolis, MN) or 0.2 IU/ml recombinant human FSH (Gonal-F, Serono, Ontario, Canada) with/without AMH. The concentration of FSH and AMH used in this study is based on the reports of median serum and follicular fluid levels of these hormones in women with PCOS (255-257).In addition, cells were treated with Forskolin (10μM; Sigma-Aldrich), a direct activator of adenylyl cyclase, or 8-Br-cAMP (1 mM; Sigma-Aldrich), a stable analog of cAMP,

to investigate the mechanism of action of AMH. Human recombinant FSH and 8-Br-cAMP were dissolved in sterile water. Human recombinant AMH was solubilized in PBS containing 0.1% bovine serum albumin. Forskolin was dissolved in DMSO. All groups in each experiment were exposed to all the relevant vehicles for that experiment.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Cells were washed with cold PBS and total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA (2μg) was reversely transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega, Medison, WI). RT-qPCR was performed on the Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 μL RT-qPCR reaction contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were: Aromatase CYP19A, 5'-GAG AAT TCA TGC GAG TCT GGA-3' (sense) and 5'-CAT TAT GTG GAA CAT ACT TGA GGA CT -3' (antisense), FSH receptor (FSHR), 5'- AAC ACC CAT CCA AGG AAT GG -3' (sense) and 5'-GGG CTA AAT GAC TTA GAG GGA CAA -3' (antisense), AMH type II receptor (AMHR2), 5'- TGT GTT TCT CCC AGG TAA TCC G -3' (sense) and 5'- AAT GTG GTC GTG CTG TAG GC -3' (antisense), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'- ATG GAA ATC CCA TCA CCA TCT T -3' (sense) and 5'- CGC CCC ACT TGA TTT TGG -3' (antisense). The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of PCR products. Assay performance was validated by evaluating amplification efficiencies by means of calibration curves, and ensuring that the plot of log input amount *vs.*ΔCq (also known as ΔCt) has a slope < |0.1|. Three separate experiments were performed on

different cultures and each sample was assayed in triplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method with GAPDH as the reference gene and with the formula $2^{-\Delta\Delta Cq}$.

Western blot analysis

Cells were washed with cold PBS and lysed with lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μM aprotinin, 1 μM leupeptin and 1 mM PMSF) (Cell Signaling Technology, Inc., Beverly, MA) containing protease inhibitor cocktail (Sigma-Aldrich). The extract was centrifuged at 20,000 *xg* for 15 min at 4 C to remove cellular debris and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories, Inc, Hercules, CA). Equal amounts of protein (25 μg) were separated by 10% SDS-PAGE and electroransferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 hour in TBS containing 0.05% Tween 20 and 5% nonfat dried milk and incubated overnight at 4 C with the relevant primary antibodies: mouse anti-cytochrome P450 aromatase (1:250) (MCA2077S, Serotec, Oxford, UK), rabbit anti-FSHR (1:1000) (#PAB7975, Abnova, Taiwan), rabbit anti-AMHR2 (1:1000) (#4518, Cell Signaling). After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Bio-Rad) for 1 hour. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL) followed by exposure to CL-X Posure film (Thermo Fisher, Waltham, MA). Membranes were stripped with stripping buffer (50 mmol/L Tris-HCL, pH 7.6, 10 mmol/L β-mercaptoethanol, 1% SDS) at 50 C for 30 min and reprobed with goat anti-Actin (C-11) (1:2000) (sc-1615, Santa Cruz Biotechnology, Inc.) as a loading control. Computerized densitometry was used to quantify

protein levels and normalize to actin levels for the same sample. Graphs consist of combined results (mean ± SEM) from at least three separate experiments performed on different cultures.

Measurement of estradiol and cAMP

Following the specified treatments, cell extracts or culture media were assayed immediately or stored frozen at – 80 C until assayed. Estradiol accumulation in conditioned medium was measured using a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). To assess intracellular cAMP accumulation, hGL cells were pre-incubated for 60 min with incubation buffer consisting of serum-free medium containing 20 mM HEPES (pH 7.5), 0.2% BSA and 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich). Following the pre-incubation period, treatments were applied and the cells were incubated for a further 6 hours, after which they were lysed in 20% perchloric acid and intracellular cAMP accumulation was measured using a cAMP enzyme immunoassay kit (Cayman Chemical). In this study, cAMP was acetylated to improve sensitivity and reduce interference as per the manufacturer's instructions. The detection limits of estradiol and cAMP were 20 pg/ml and 0.1 pmol/ml, respectively. Inter- and intra-assay coefficients of variation for these assays were less than 10%. Each sample was measured in duplicate and secreted estradiol levels were normalized to total cellular protein content.

Small interfering RNA (siRNA) transfection

We performed transient knockdown assays using 75 nM AMHR2-targeting siRNA (ON-TARGET*plus* SMARTpool) or 75 nM control siRNA (ON-TARGET*plus* Non-targeting Pool) purchased from Thermo Fisher Scientific (Lafayette, CO). Cells were pre-cultured for 3 days in

DMEM/F12 medium containing 10% FBS, after which they were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) for 48 h and treated on day 5 as described earlier.

Statistical analysis

PRISM software (GraphPad Software, San Diego, CA) was used to perform one-way ANOVA followed by Tukey's multiple comparison tests on RT-qPCR, Western blot and enzyme immunoassay data from hGL cell cultures. Results are presented as the mean ± SEM of at least three separate experiments performed on different cultures and were considered significantly different from each other if $P < 0.05$.

A.3 Results

AMH inhibits FSH-stimulated estradiol production and aromatase expression

A previous *in vitro* study demonstrated that AMH inhibits FSH-induced estradiol production and aromatase expression in human granulosa-lutein cells (255). We sought to expand on these studies by investigating the specificity and underlying mechanisms of the effects of AMH on estradiol production and aromatase expression *in vitro*. To confirm the effect of AMH on FSH-stimulated estradiol accumulation, hGL cells were cultured for 48 h in the presence of vehicle control, FSH (0.2 IU/ml), AMH (10 ng/ml) or combined FSH and AMH. As expected, treatment of hGL cells with FSH significantly increased estradiol accumulation (Figure A.1A). Treatment with AMH significantly reduced FSH-stimulated estradiol accumulation, whereas it had no effects on basal estradiol accumulation (Figure A.1A). Estradiol concentration in the conditioned medium of control cells ranged from 43.2-93.7 ng/ml.

Next we used RT-qPCR and Western blot analysis to examine the effects of AMH on aromatase, the key regulator of granulosa cell estradiol production. As shown in Figure A.1B and A.1C, treatment with AMH significantly reduced FSH-stimulated aromatase mRNA and protein levels, but did not affect aromatase production in the absence of FSH. Similarly, treatment of hGL cells with different concentrations of AMH (1-100 ng/ml) had no effect on the steady state mRNA levels of aromatase (Figure A.1D).

AMH inhibits FSH-stimulated adenylyl cyclase activity in hGL cells

To investigate the mechanism by which AMH inhibits FSH-stimulated aromatase expression and estradiol production, we first examined the effects of FSH and AMH on FSHR expression. As shown in Figure A.2A and A.2B, treatment with FSH, AMH or combined FSH and AMH did not alter FSHR mRNA and protein levels, suggesting that AMH exerts its effects downstream of FSHR.

Since FSH is known to activate adenylyl cyclase and increase intracellular cAMP levels, we also co-treated the cells with adenylyl cyclase activator (Forskolin, 10 μM) or cAMP analog (8-Br-cAMP, 1 mM) to investigate the site of action of AMH. Consistent with the results for FSH, Forskolin-induced increases in aromatase mRNA and protein levels were reduced by co-treatment with AMH (Figure A.2C and A.2D). In contrast, treatment of hGL cells with AMH did not affect 8-Br-cAMP-stimulated increases in aromatase mRNA and protein levels (Figure A.2E and A.2F).

Given that AMH inhibits the effects of FSH and Forskolin, but not 8-Br-cAMP, this would suggest that its inhibitory effects are exerted at the level of adenylyl cyclase. To test this hypothesis we investigated the effects of AMH on intracellular cAMP accumulation following

treatment with FSH. These experiments were conducted in the presence of the non-selective phosphodiesterase inhibitor IBMX (0.2 mM) in order to exclude potential effects of AMH on phosphodiesterase activity. As shown in Figure A.3, treatment with AMH significantly reduced FSH-stimulated intracellular cAMP accumulation, but did not affect basal intracellular cAMP production. These data support the hypothesis that the inhibitory effects of AMH are exerted at the level of adenylyl cyclase.

AMHR2 is required for the effects of AMH on FSH-stimulated aromatase expression

As a member of the TGF-β superfamily, AMH signals through a complex of its own specific type II receptor, AMHR2, and one of the bone morphogenetic protein (BMP) type I receptors, leading to the phosphorylation and activation of SMADs 1, 5 and 8 (258-260). Knock-out mouse studies and human AMHR2 gene mutations support an essential role for AMHR2 in the functional AMH signaling. Inherited mutations in AMHR2 or AMH lead to persistent Müllerian duct syndrome in human, and similar phenotypes are observed in AMHR2- or AMH-deficient mice (155, 261). Thus, to examine whether AMHR2 is required for the suppressive effects of AMH on FSH-induced aromatase expression, specific siRNA for AMHR2 was used to knockdown endogenous AMHR2 prior to treatment with AMH. RT-qPCR and Western blot analysis showed that AMHR2 siRNA decreased AMHR2 mRNA and protein levels by 45-55 % relative to transfection reagent control (iMAX) and control siRNA (siControl) (Figure A.4A and A.4B). Transfection with siAMHR2 for 48 h abolished the suppressive effects of AMH on FSH-induced aromatase expression (Figure A.4C and A.4E). These data suggest that AMH specifically inhibits FSH-induced aromatase expression via AMHR2.

A.4 Discussion

Estradiol has been shown to play an important role in follicle development and ovulation (262). However in clinical cases, extremely high cumulative estradiol exposure during the follicular phase of IVF cycles may have detrimental effects on implantation and uterine receptivity (263). Moreover, high levels of estradiol put patients at risk of OHSS, a potentially life-threatening iatrogenic complication.

Serum AMH levels have been reported to correlate with ovarian reserve and ovarian response during IVF cycles (242, 264). Baseline serum AMH levels in women with OHSS were six times higher than in women with a normal response to gonadotropins (265). Moreover, baseline AMH levels are a more reliable marker than age and body mass index in predicting OHSS prior to controlled ovarian stimulation (265, 266). In addition to AMH, serum estradiol levels on the day of hCG administration were also predictive of moderate and severe OHSS (265). Serum AMH and estradiol levels are negatively correlated in oligomenorrhic polycystic ovary syndrome patients (253). In the present study, we demonstrate that AMH suppresses estradiol production in primary hGL cells, likely via AMHR2-mediated inhibition of FSH-induced adenylyl cyclase activation and aromatase expression. These results provide further support for the view that AMH may modulate estradiol production and play an important role in folliculogenesis, even in late-stage follicles (periovulatory stage).

Primary cultures of hGL cells were used to study the direct effects of AMH on estradiol production and the mechanism involved. Our results demonstrating the suppressive effects of AMH on FSH-induced estradiol production are consistent with those of previous studies (255, 267). This effect of AMH is different from that of other granulosa cell-derived TGF-β superfamily members, such as activin, inhibin, and BMP6 (108, 268, 269). In cultured rat

granulosa cells, activin enhanced both basal and FSH-stimulated aromatase activity (269), whereas, BMP6 had no effects (108). Likewise, the effects of AMH also differ from those of growth and differentiation factor 9 (GDF9), an oocyte-derived TGF-β superfamily member. Although both AMH and GDF9 inhibit FSH-induced estradiol production, GDF9 enhances basal steroidogenesis in rat granulosa cells (130), whereas treatment of AMH does not alter basal estradiol production in hGL cells. Our results suggest that in addition to its effects on follicle recruitment and the early stage of folliculogenesis, AMH also modulates estradiol synthesis in late-stage follicles and this effect could be important for creating a favorable microenvironment for embryo implantation.

To date, the detailed mechanism by which AMH inhibits FSH-stimulated aromatase expression is not clear.Previous studies demonstrating the inhibitory effects of AMH on FSH-induced aromatase expression and estradiol production in hGL cells did not address potential mechanisms (255). Recently, Pellatt *et al.*demonstrated inhibitory effects of AMH on Forskolin-stimulated aromatase mRNA levels in human granulosa tumor-derived KGN cells (267). While these results suggest effects downstream of FSHR, possible effects of AMH on signaling downstream of adenylyl cyclase were not evaluated. Our results showing that AMH inhibits the effects of FSH and Forskolin, but not 8-Br-cAMP, are important because they suggest that the effects of AMH are exerted at the level of adenylyl cyclase activation. This is supported by our finding that AMH significantly reduces FSH-stimulated intracellular cAMP accumulation in the presence of phosphodiesterase inhibitor IBMX.

Interestingly, Pellatt *et al.* found that treatment of KGN cells with AMH could suppress FSH-induced increases in FSHR, suggesting a second mechanism by which AMH might antagonize FSH action (267). However in our studies, treatment of hGL cells with FSH and/or AMH did not

alter FSHR mRNA and protein levels. This discrepancy could result from differences in the differentiation states of KGN and primary hGL cells. While our results suggest that this mechanism may not be important during the later stages of folliculogenesis, they do not rule out effects of AMH on FSHR localization, folding, trafficking or internalization. Indeed, a potential limitation of our study is that follicular fluid samples were anonymized immediately after collection such that patient diagnosis information is unavailable. Alternatively, KGN cells are known to be heterozygous for the FOXL2 402C>G mutation present in 97% of human adult-type granulosa cell tumors. FOXL2 has been shown to interact with SMADs, downstream mediators of TGF-β superfamily signaling.Thus, it is possible that the observed differences between KGN and primary hGL cells may be attributable to aberrant transcriptional regulation in the tumor cells.

That knockdown of AMHR2 abolished the inhibitory effects of AMH on FSH-induced aromatase expression suggests that the effects are specific to AMHR2 signaling complexes. However, the rapid (< 6 hours) effects of AMH on adenylyl cyclase suggest a mechanism that is not likely to involve transcriptional regulation by canonical SMAD-dependent signaling. SMAD-independent signaling, which can exhibit cross-regulatory effects on other signaling pathways, has been demonstrated for some TGF-β superfamily members (270). In primary hGL cells, oocyte-derived GDF9 stimulates cell proliferation by both SMAD-dependent and SMAD-independent pathways (271). Although it has been shown that AMH can activate SMAD-independent NFκB signaling in prostate and breast cancer cells (272, 273); whether and how such signaling influences hGL cell function is unclear, and there is no data on AMH regulating cAMP synthesis in any cell type. Interestingly, another granulosa cell-derived TGF-β superfamily member, BMP6, has similar inhibitory effects on progesterone, but not estradiol,

production in rat granulosa cells. *In vitro* administration of exogenous BMP6 attenuates FSH-induced StAR and P450 side-chain cleavage (P450scc) enzyme mRNA levels by down-regulating adenylyl cyclase activity and decreasing FSH-induced cAMP production (108). Likewise, treatment with TGF-β1 reduces EGF- and Forskolin-stimulated cAMP accumulation in rat cardiomyocytes *in vitro*(274). In contrast, TGF-β1 inhibits the growth of primary adult rat hepatocyte cultures by stimulating adenylyl cyclase, leading to increased cAMP levels and the activation of protein kinase A (275). Collectively, these studies suggest that TGF-β superfamily members can exert rapid effects on adenylyl cyclase activity in specific cellular contexts. Taken together, it is likely that other signaling pathways are also involved in the AMH-mediated effects; in particular, whether AMHR2 exerts its effects through SMADs or ERK, JNK or p38 MAPK remains to be determined. Future studies aimed at addressing this question will be of great interest and may benefit greatly from the use of immortalized hGL cells as opposed to the primary cultures used in the current study.

In conclusion, our *in vitro* studies demonstrate that AMH inhibits FSH-induced aromatase expression and estradiol production in hGL cells. Moreover, the effects of AMH are dependent on AMHR2 and are likely exerted at the level of adenylyl cyclase activation and cAMP synthesis. These findings contribute to our understanding of the cellular mechanisms underlying the interaction between AMH and FSH in the production of estrogen in both normal and pathological conditions.

A.5 Supplementary materials and methods

Preparation of hGL cells

Granulosa cells were purified by density centrifugation from follicular aspirates collected from women undergoing oocyte retrieval. Individual patient aspirates were centrifuged at 400*g* for 10 min and, after removing the supernatant, the hGL and red blood cells were resuspended in 2 ml of Hanks' solution containing 50 μg/ml deoxyribonuclease I, 0.1% hyaluronidase and 0.1 U/ml Liberase Blendzyme 3. Cell suspensions were shaken at 200 rpm for 20 min at 37℃ and then layered on 8.0 ml Ficoll-Paque Plus and centrifuged at 600 *g* for 20 min.The hGL cell layer was removed from each Ficoll-Paque column and washed three time with 10 ml of DMEM/F-12 supplemented with 10% FBS. Purified hGL cells were seeded in 12-well plates at a density of 2×10^5 cells per well and cultured for five days. Experimental treatments were applied on day 5 at which point the cells were 75-95% confluent. Cell confluency did not change much during treatment because the culture medium was changed to low serum medium (containing 0.5 % FBS) at the time of treatment.

Small interfering RNA (siRNA) transfection

Purified hGL cells were pre-cultured for 3 days in DMEM/F12 medium containing 10% FBS until the cells were 60-80% confluent. Cells were then transfected with 75 nM AMHR2-targeting siRNA (ON-TARGETplus SMARTpool) or 75 nM control siRNA (ON-TARGETplus Non-targeting Pool) using Lipofectamine RNAiMAX (Invitrogen) in antibiotic-free medium (DMEM/F12) for 48 h. Experimental treatments were applied as usual on day 5 at which point the cells were 75-95%. The siRNA transfection procedure was very well tolerated by the cells and survival was well over 90%.

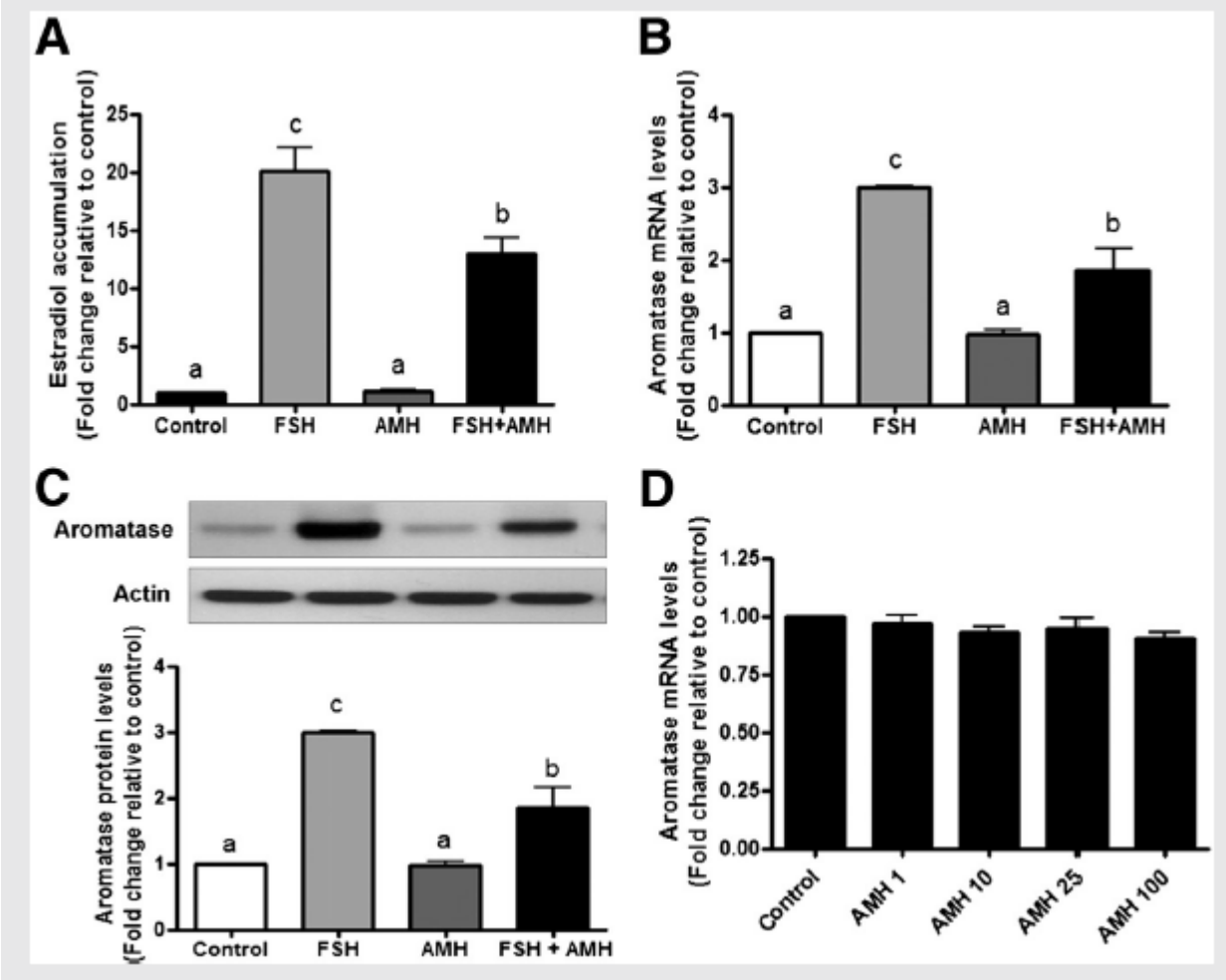

Figure A.1 AMH inhibits FSH-stimulated estradiol accumulation and aromatase expression in hGL cells

Cells were cultured for 48 h in absence or presence of AMH (10 ng/ml) while being co-treated with vehicle control or FSH (0.2 IU/ml). Estradiol accumulation was measured by enzyme immunoassay (N=6) (A) andaromatase mRNA (N=3) (B) and protein (N=4) (C) levels were measured by RT-qPCR and Western blot, respectively.(D) The concentration-dependent effects of AMH (1, 10, 25 or 100 ng/ml) on aromatase mRNA levels were also examined (N=3). Values (mean ± SEM) without a common letter are significantly different ($P < 0.05$).

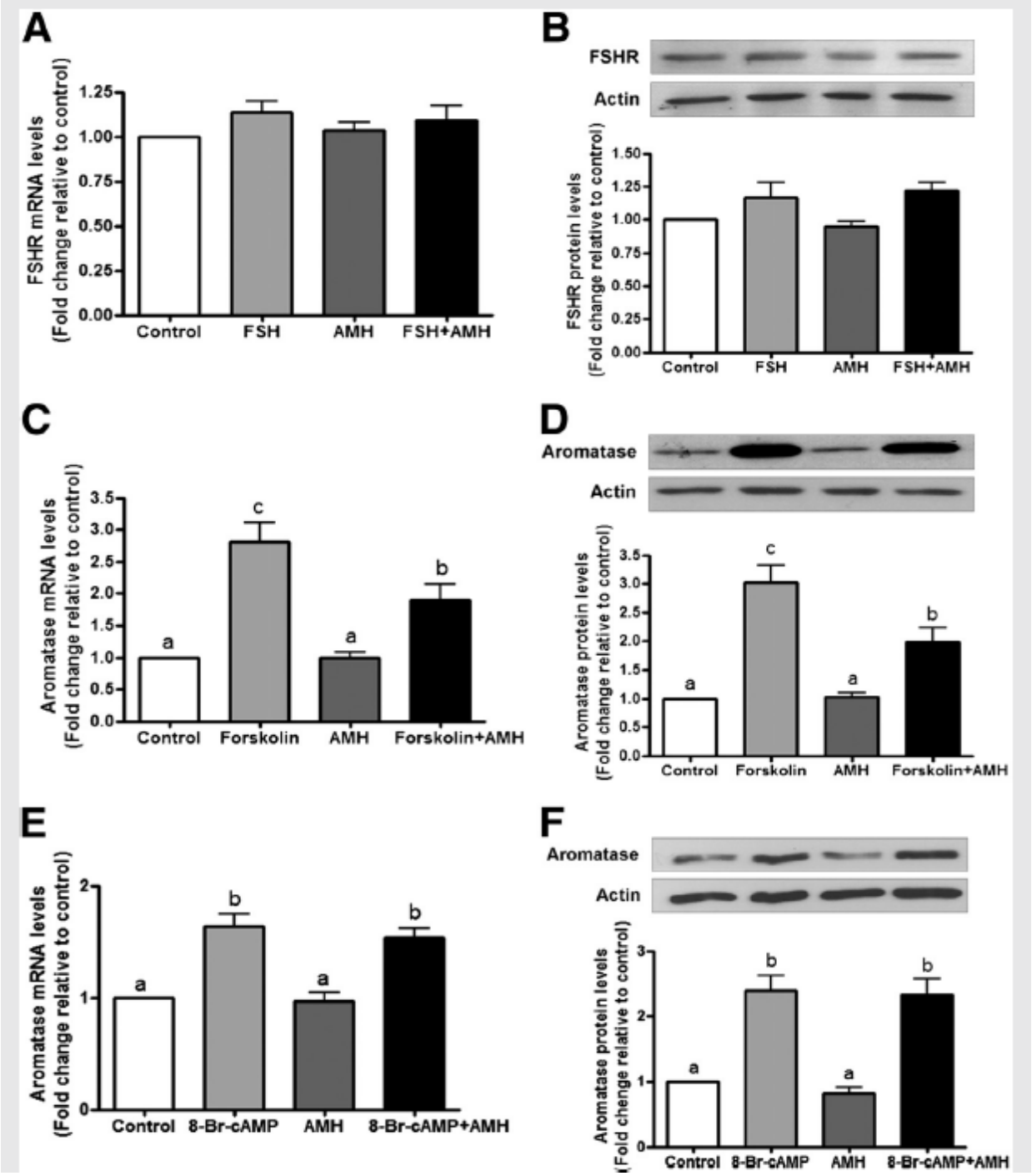

Figure A.2 Effects of AMH on FSHR and Forskolin- and 8-Br-cAMP-stimulated aromatase expression

(A-B) Cultures of hGL cells were incubated for 48 h in the absence or presence of AMH (10 ng/ml) while being co-treated with vehicle control or FSH (0.2 IU/ml), and FSHRmRNA (N=4) (A) and protein (N=3) (B) levels were measured by RT-qPCR and Western blot. (C-F) Cells were cultured for 48 h in the absence or presence of AMH (10 ng/ml) while being co-treated with vehicle control, 10 μM Forskolin (C-D) or 1 mM 8-Br-cAMP (E-F), and changes in aromatase mRNA (N=5) (C, E) and protein (N=3) (D, F) levels were measured by RT-qPCR and Western blot. Values (mean ± SEM) without a common letter are significantly different ($P < 0.05$).

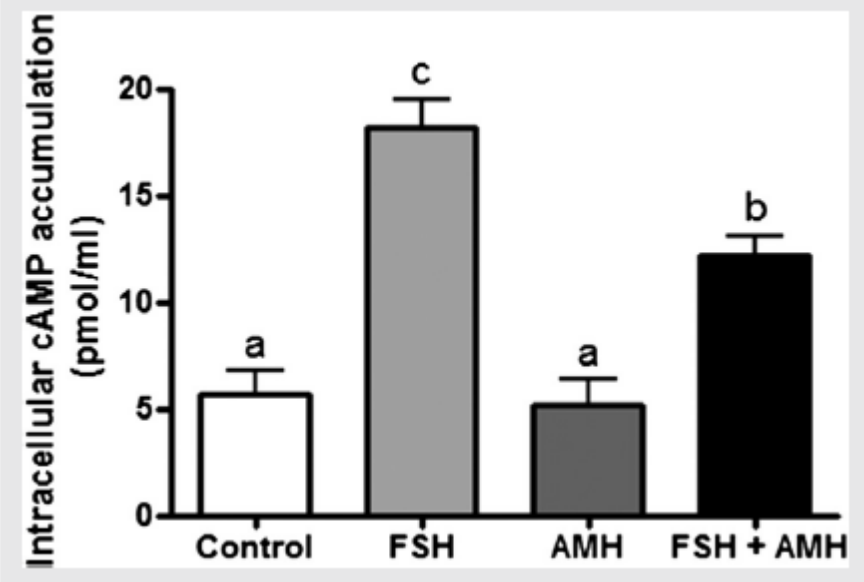

Figure A.3 AMH inhibits FSH-stimulated intracellular cAMP accumulation in hGL cells

Cells were preincubated for 60 min with IBMX and then cultured for 6 h in the absence or presence of AMH (10 ng/ml) while being co-treated with vehicle control or FSH (0.2 IU/ml), and intracellular cAMP accumulation wasmeasured by enzyme immunoassay (N=4). Values (mean± SEM) without a common letter are significantly different ($P < 0.05$).

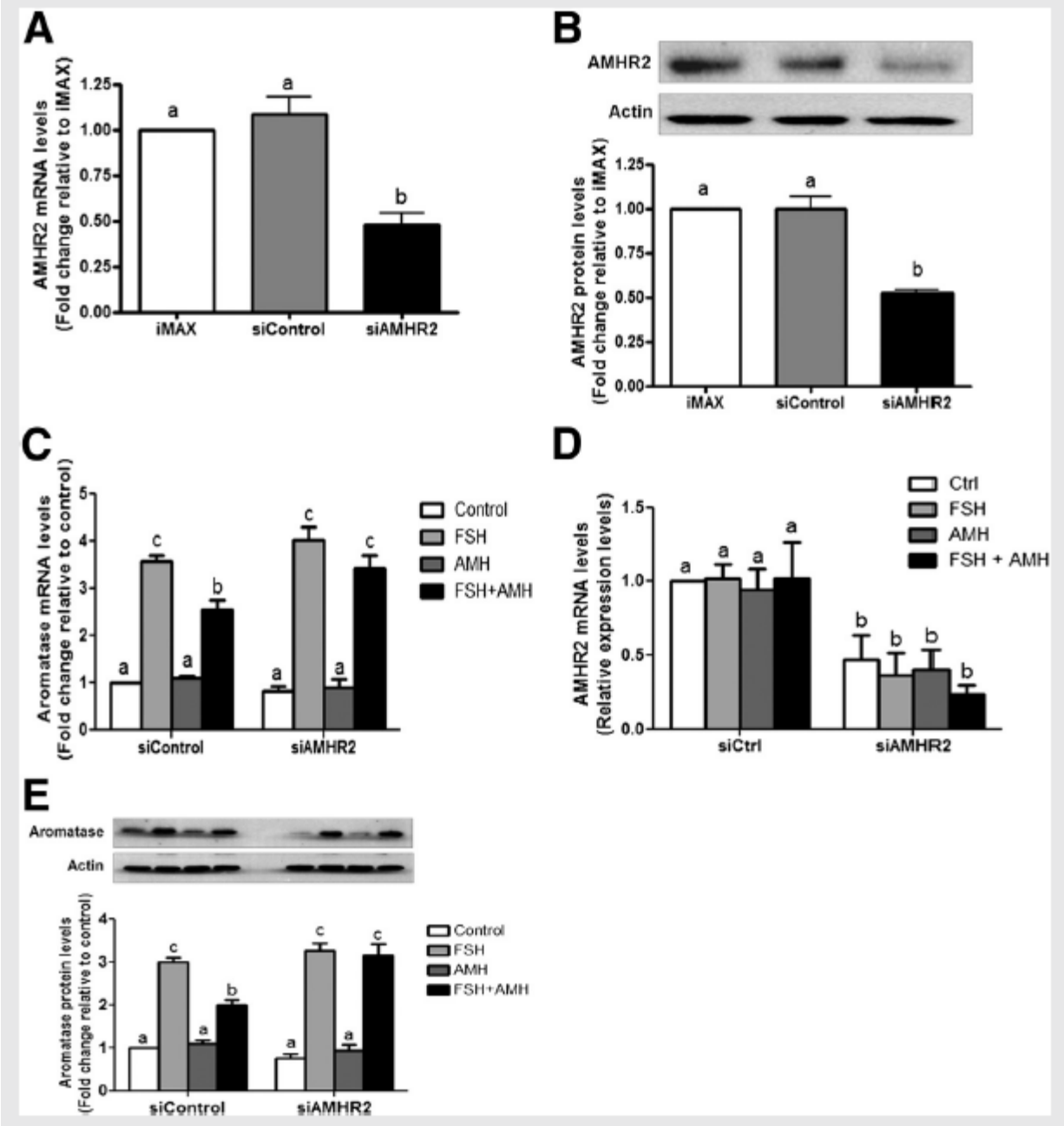

Figure A.4 Knockdown of AMHR2 abolishes the inhibitory effects of AMH on FSH-induced aromatase expression in hGL cells

(A-B) Cells were transfected for 48 h with transfection reagent alone (iMAX), control siRNA (siControl) or AMHR2 siRNA (siAMHR2), and AMHR2 mRNA (N=4) (A) and protein (N=3) (B) levels were measured by RT-qPCR and Western blot. (C-E) Cells were treated for 48 h with

siControl or siAMHR2 prior to being co-treated with vehicle control or FSH (0.2 IU/ml). Changes in aromatase mRNA (N=3) (C) and protein (N=4) (E) levels were measured by RT-qPCR and Western blot. (D) Knockdown effects of AMHR2 after transfection of siControl or siAMHR2. AMHR2 mRNA levels were measured by RT-qPCR. Values (mean ± SEM) without a common letter are significantly different ($P < 0.05$)

Appendix B

B.1 Publications

1. **Chang HM**, Cheng JC, Klausen C, Leung PC. Theca-derived BMP4 and BMP7 increase activin A production by up-regulating inhibin βA and furin expression in human granulosa cells. ***J Clin Endocrinol Metab.*** (Under Revision; JC-14-3026)
2. **Chang HM**, Cheng JC, Fang L, Qiu X, Klausen C, Taylor E, Leung PC. Theca-derived BMP4 and BMP7 down-regulate pentraxin 3 in human granulosa cells. ***J Clin Endocrinol Metab .*** (Under Revision; JC-14-2496)
3. **Chang HM**, Cheng JC, Klausen C, Taylor E, Leung PC. Effects of recombinant activins on steroidogenesis in human granulosa-lutein cells. ***J Clin Endocrinol Metab.*** 2014 Oct;99(10): E1922-32
4. **Chang HM**, Cheng JC, Taylor E, Leung PC. Oocyte-derived BMP15 but not GDF9 down-regulates connexin43 expression and decrease gap junction intercellular communication activity in human granulosa cells. ***Mol Hum Reprod.*** 2014 May;20(5):373-83
5. **Chang HM**, Cheng JC, Klausen C, Leung PC. BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells. ***Mol Endocrinol.*** 27:2093-2104. (***Coverstory; Recommended by Faculty of 1000***).
6. **Chang HM**, Klausen C, Leung PC. Anti-Müllerian hormoneinhibits follicle-stimulating hormone-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells. ***Fertil Steril*** 100(2):585-92.el.

7. **Chang HM**, Cheng JC, Leung PC. Theca-derived BMP4 and BMP7 down-regulate connexin43 expression and decrease gap junction intercellular communication activity in immortalized human granulosa cells. ***J Clin Endocrinol Metab.***2013Mar;98(3):E437-E445.
8. Fang L, **Chang HM**, Cheng JC, Leung PC, Sun YP. TGF-β1 induces COX-2 expressionand PGE2 production in human granulosa cells through Smad signaling pathways. ***J Clin Endocrinol Metab.***2014 July;99(7):E1217-E1226.
9. Qiu X, Cheng JC, **Chang HM**, Leung PC. COX-2 and PGE2 mediate EGF-induced E-cadherin-independent human ovarian cancer cell invasion. ***Endocr Relat Cancer.***2014Aug;21(4):533-43.
10. Cheng JC, **Chang HM**, Qiu X, Fang L, Leung PC. FOXL2-induced follistatin attenuatesactivin A-stimulated cell proliferation in human granulosa cell tumor. ***Biochem Biophys Res Commun.*** 2014 Jan 10;443(2):537-42.
11. Cheng JC, **Chang HM**, Leung PC. Tranforming growth factor-β1 inhibits human trophoblast cell invasion by inducing Snail-mediated down-regulation of vascular endothelial-cadherin protein. ***J Bio Chem.*** 2013 Nov 15;288:33181-33192.
12. Fang L, Cheng JC, **Chang HM**, Sun YP, Leung PC. EGF-like growth factors induce COX-2derivated PGE2 secretion through ERK1/2 in human granulosa cells. ***J Clin Endocrinol Metab.***2013Dec;98(12):4932-4941.
13. Cheng JC, Qiu X, **Chang HM**, Leung PC. HER2 mediates epidermal growth factor-induced down-regulation of E-cadherin in human ovarian cancer cells. ***Biochem Biophys Res Commun.*** 2013 Apr 26;434(1):81-86.

14. Cheng JC, **Chang HM**, Leung PC. Egr-1 mediates epidermal growth factor-induced down-regulation of E-cadherin expression via Slug in human ovarian cancer cells. ***Oncogene.*** 2013 Feb 21;32(8):1041-9.
15. Cheng JC, **Chang HM**, Leung PC. Epidermal growth factor-induced human oviductal epithelial cell invasion by down-regulating E-cadherin expression. ***J Clin Endocrinol Metab.***2012Aug;97(8):E1380-9.
16. Cheng JC, **Chang HM**, Leung PC. Wild-type p53 attenuates cancer cell motility by inducing growth differentiation factor-15 expression. ***Endocrinology.*** 2011 Aug;152(8):2987-95.

B.2 Invited presentation

1. Roles of Bone Morphogenetic Proteins in the Regulation of Ovarian Function. International Guests of The 8th Annual Conference of Obstetrics & Gynecology Department Assiut University. Luxor, Egypt 2014
2. Roles of Bone Morphogenetic Proteins in Human Granulosa Cells. Academic Presentation of Distinguished YoungScientist.Shanghai, China 2014

B.3 Coference proceedings

1. Fang L, **Chang HM**, Cheng JC, Leung PC, Sun YP. (2014) TGF-β1 decreases progesterone production by down-regulating StAR expression through Smad2/3 and ERK1/2 signaling pathways in human granulosa cells. *ESHRE 30th Annual Meeting. Munich, Germany.*

2. Cheng JC, Qiu X, **Chang HM**, Leung PC. (2013) Nuclear HER2 mediates epidermal growth factor-induced down-regulation of E-cadherin in human ovarian cancer cells. *International Ovarian Conference. Taipei, Taiwan.*
3. **Chang HM**, Cheng JC, Leung PC. (2013) Paracrine effects of BMPs on connexin43 expression and gap junction intercellular communication activity in human granulosa cells. *Annual Meeting of Taiwan Society of Reproductive Medicine. Taichung, Taiwan.* **(Oral Presentation)**
4. **Chang HM**, Cheng JC, Klausen C, Leung PC. (2012) Activin down regulate steroidogenic acute regulatory protein (StAR) expression through Smad dependent pathway. *Annual Meeting of Taiwan Society of Reproductive Medicine. Taipei, Taiwan.* **(Oral Presentation)**
5. **Chang HM**, Klausen C, Leung PC. (2011) Antimüllerian hormone inhibits follicle-stimulating hormone-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells. *Annual Meeting of Taiwan Society of Reproductive Medicine. Taipei, Taiwan.* **(Oral Presentation)**
6. **Chang HM**, Klausen C, Leung PC. (2011) Antimüllerian hormone inhibits follicle-stimulating hormone-induced adenylyl cyclase activation, aromatase expression, and estradiol production in human granulosa-lutein cells. *The 44th Annual Meeting of the Society for the Study of Reproduction, Portland, USA.*