EFFECTS AND MECHANISMS OF GROWTH DIFFERENTIATION FACTOR 9 ON ACTIVIN A-REGULATED INHIBIN B AND PROGESTERONE PRODUCTION IN HUMAN GRANULOSA CELLS

by

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ABSTRACT

Activin A (homodimer of inhibin β_A-subunit) is known to increase inhibin β_B-subunit and inhibin B (heterodimer of inhibin α- and β_B-subunit) levels and decrease progesterone accumulation in human granulosa cells. Growth differentiation factor 9 (GDF9) is a potent paracrine regulator of ovarian function, but its overall effects, particularly relating to activin A actions, are unknown.

We examined the potential crosstalk between activin A and GDF9 in primary cultures of human granulosa-lutein (hGL) cells. Pretreatment of hGL cells with GDF9 for 24 h resulted in an increased expression of activin receptors and Smad2/3, and decreased inhibitory Smad7 activity. These effects were attenuated by BMP type II receptor ectodomain (BMPR2 ECD), a GDF9 antagonist. These GDF9-induced changes, in turn, increased the cellular response to activin A stimulation and resulted in significantly greater production of β_B-subunit mRNA and inhibin B compared to activin A treatment alone.

Interestingly, endogenous GDF9 mRNA and protein were detected in hGL cells. Reduction of endogenous GDF9 by GDF9 siRNA resulted in decreased levels of activin receptors and Smad2/3/4, but increased expression of Smad7. Consequently, GDF9 siRNA treatment significantly attenuated the stimulation of activin A on β_B-subunit mRNA and inhibin B levels.

Additionally, GDF9 suppressed the expression of follistatin (FST) and follistatin-like 3 (FSTL3), which are extracellular inhibitors of activin A. These effects were attenuated by BMPR2 ECD and GDF9 siRNA. Treatment with FST or FSTL3 siRNA augmented activin A-induced β_B-subunit mRNA levels. Conversely, GDF9 enhancement of activin A-induced β_B-subunit mRNA was attenuated by FST.

Activin A decreased expression of StAR but not P450scc and 3βHSD, this effect lead to reduced basal and FSH-induced progesterone accumulation. GDF9 reversed these effects of activin A on StAR and progesterone; these GDF9 effects were attenuated by inhibin α-subunit siRNA.

Together, these findings support a novel hypothesis that GDF9 exerts both paracrine and autocrine control of key components in the activin receptor-signaling pathway and the extracellular inhibition of activin A in hGL cells. As a result, GDF9 may serve to enhance activin A-induced accumulation of inhibin B, which in turn acts to reverse activin A suppression of progesterone accumulation during granulosa cell luteinization.

PREFACE

Refereed papers:

- **Shi FT**, Cheung AP, Huang HF, Leung PC 2010 Growth differentiation factor 9 (GDF9) suppresses follistatin and follistatin-like 3 production in human granulosa-lutein cells. Endocrionolgy, under review. This work is located at chapter 4.

Proportion of Contribution	Shi FT	Cheung AP	Huang HF	Leung PC
Identification and design of the research program	80%	5%	5%	10%
Analysis of the research data	85%	10%	0%	5%
Performance of the various parts of the research	100%	0%	0%	0%
Preparation of manuscripts	75%	20%	0%	5%

- **Shi FT**, Cheung AP, Klausen C, Huang HF, Leung PC 2010 Growth differentiation factor 9 (GDF9) reverses activin A suppression of steroidogenic acute regulatory protein (StAR) expression and progesterone production in human granulosa-lutein cells. The Journal of clinical endocrinology and metabolism, accepted. This work is located at chapter 5.

Proportion of Contribution	Shi FT	Cheung AP	Klausen C	Huang HF	Leung PC
Identification and design of the research program	75%	5%	5%	5%	10%
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Performance of the various parts of the research	100%	0%	0%	0%	0%
Preparation of manuscripts	65%	30%	0%	0%	5%

- **Shi FT**, Cheung AP, Huang HF, Leung PC 2009 Effects of endogenous growth differentiation factor 9 on activin A-induced inhibin B production in human granulosa-lutein cells. The Journal of clinical endocrinology and metabolism 94:5108-5116 (PMID: 19846738). This work is located at chapter 3.

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Identification and design of the research program	80%	5%	5%	10%
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Abstracts and Presentations:

- **Shi FT**, Cheung AP, Huang HF, Leung PC 2010 Growth differentiation factor 9 suppresses follistatin and follistatin-like 3 production in human granulosa-lutein cells. The 92nd Annual Meeting of the Endocrine (ENDO 2010), San Diego, CA, United States (Jun. 19-22, 2010) – Poster presentation
- **Shi FT**, Cheung AP, Leung PC 2010 Mechanisms of growth differentiation factor 9 in enhancing activin A-induced inhibin B and subunits in human granulosa cells. CIHR-Institute of Human Development, Child and Youth Health Scientific Forum on Global Health, Vancouver, Canada (May 4, 2010) – Poster presentation
- **Shi FT**, Cheung AP, Klausen C, Huang HF, Leung PC 2010 Effects of growth differentiation factor 9 (GDF9) and activin A on steroidogenic acute regulatory protein (StAR) and progesterone production in human granulosa-lutein cells. 14th International Congress of Endocrinology, Kyoto, Japan (Mar. 26-30, 2010) – Oral presentation
- **Shi FT**, Cheung AP, Leung PC 2010 Mechanisms of growth differentiation factor 9 in enhancing activin A-induced inhibin B and subunits in human granulosa cells. The 91st Annual Meeting of the Endocrine (ENDO 2009), Washington, DC, United States (Jun. 10-13, 2009) – Poster presentation
- **Shi FT**, Cheung AP, Leung PC 2009 Mechanisms of growth differentiation factor 9 in enhancing activin A-induced inhibin B production in human granulosa cells. OB/GYN Academic day, University of British Columbia, Vancouver, Canada (Mar.12, 2009) – Oral presentation

- **Shi FT**, Cheung AP, Leung PC 2008 Effects of growth differentiation factor 9 on activin A-induced inhibin β_B-subunit expression in human granulosa cells. CFRI Student Research Forum, University of British Columbia, Canada (Jun.19, 2008) – Poster presentation
- **Shi FT**, Cheung AP, Leung PC 2007 Growth differentiation factor 9 (GDF9) and activin A independently and synergistically stimulate inhibin subunits transcription and inhibin B secretion in human granulosa-luteal cells. The Auersperg Symposium on the Etiology of Ovarian Cancers, Vancouver, Canada (Jul. 15, 2007) – Poster presentation

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LIST OF ABBREVIATIONS

3βHSD	3β-Hydroxysteroid dehydrogenase/Δ5-Δ4 isomerases
aa	Amino acid
ACVR	Activin receptor
ALK	Activin receptor-like kinase
AMH	Anti-müllerian hormone
AMHR	AMH receptor
ANOVA	Analysis of variance
ATF2	Activating transcription factor 2
bHLH	Basic helix-loop-helix
BAMBI	BMP and activin membrane-bound inhibitor
BMP	Bone morphogenetic protein
BMPR	BMP receptor
BMPR2 ECD	BMP type II receptor extracellular domain
bp	Base pair
Ca^{2+}	Calcium
CamKII	Ca^{2+}/Calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid homolog
CL	Corpus luteum
Co-Smad	Common Smad
COC	Cumulusoocyte complexe
COX2	Cyclo-oxygenase 2, prostaglandin-endoperoxide synthase 2

CR-1	Cripto-1
CRE	cAMP-response elements
CREB	CRE-binding protein
Dab2	Disabled2
dNTP	Deoxynucleoside 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
EGF-CFC	Epidermal growth factor–Cripto/FRL-1/Cryptic
ELISA	Enzyme-lined immunosorbant assay
EMT	Epithelial to mesenchymal transition
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FHA	Forkhead-associated
Fig-α	Factor in the germline alpha
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
GCNF	Germ cell nuclear factor
GCT	Granulosa cell tumor
GDF	Growth differentiation factor
GDNF	Glial cell-derived neurotrophic factor
GH	Growth hormone
GIPC	GAIP-interacting protein C terminus
GnRH	Gonadotropin-releasing hormone
GnRH-II	Gonadotropin-releasing hormone type II
GnRHR	Gonadotropin-releasing hormone receptor
GS-domain	Glycine-serine rich domain
h	Hour
HAS2	Hyaluronan synthase 2
hCG	Human chorionic gonadotropin
HECT	Homologous to the E6-accessory protein C-terminus
hGL	Human granulosa-lutein
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein

IL	Interleukin
I-Smad	Inhibitory Smad
IU	International unit
IVF	*In vitro* fertilization
JNK	c-Jun N-terminal kinase
Kb	Kilobase
kDa	Kilodaltons
KL	Kit ligand
LH	Luteinizing hormone
LHR	Follicle stimulating hormone receptor
LRH-1	liver receptor homolog-1
μ	Micro
MAP	Mitogen-activated protein
MAPK	MAP kinases
MADH	Mad-homologues
MEKK1	MAPK/Erk kinase kinase 1
MH	Mad-homology
ml	Mililiters
min	Minutes
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
n (as in nM)	Nono

NES	Nuclear export signal
NFκB	Nuclear factor kappa B
NLS	Nuclear localization sequence
OOX	Cumulus-oocyte complexe
P (as in pM)	Pico
P450scc	P450 cholesterol side-chain cleavage enzyme
P450c17α	cytochrome P450 17α-hydroxylase/17,20-lyase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered solution
PCO	Polycystic ovaries
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PKA	Protein kinase A
PKC	Protein kinase C
PI3K	Phosphatidylinositol-3-kinase
PR	Progesterone receptor
RNA	Ribonucleic acid
R-Smad	Receptor-activated Smad
RT-PCR	Reverse transcription polymerase chain reaction
SAD	Smad activation domain

SAPK	Stress-activated protein kinase
SARA	Smad anchor for receptor activation
SBE	Smad binding element
SCF	Stem cell factor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sec	Seconds
Ser	Serine
SF-1	Steroidogenic factor 1
siRNA	Small interfering RNA
Smad	Son of mothers against decapentaplegia
Smurf	Smad ubiquitination-related factor
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TβR	TGFβ receptor
Taq	*Thermus acuaticus*, source of a DNA polymerase
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethlenediamine
TGF	Transforming growth factor
Thr	Threonine
TNFα	Tumor necrosis factor α
Tris	Tris(hydroxyl methyl) aminomethane
YY1	Transcription factor Yin Yang 1

uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor

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1. LITERATURE REVIEW

1.1. Introduction

In the past, an incredible amount of effort has been made to understand the development of ovary, a part of the reproductive system of all female mammals. Ovary is not essential to individual life but very important to the perpetuation of species. The major function of the ovary is to produce a well-differentiated and fertilizable oocyte for successful propagation. In addition, the ovary secretes steroid hormones into the reproductive tract and establishes a supportive environment for fertilization and pregnancy to occur. Both activities are included in the continuous cycling through follicle growth, ovulation and corpus luteum formation and degeneration. The ovary is consisted of numerous follicles which are at the different developmental stages. An oocyte is centered in the follicle and enclosed by inner layers of granulosa cells and outer layers of theca cells (1).

Numerous neural, neuroendocrine, paracrine and endocrine cell-cell communication pathways are involved in a complicated system that controls follicular development. The roles of the gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle stimulating hormone (FSH) and gonadal steroids such as progesterone and estrogen are well-known, but there is increasing evidence that various cytokines and growth factors are also involved in this process (review in (1)). These include activin, inhibin, growth differentiation factor (GDF) 9 and bone morphogenetic protein (BMP) 15, which are to the members of the transforming growth factor β (TGFβ) superfamily, and follistatin (FST), which is a structurally distinct molecule that is functionally related to the members of the TGFβ superfamily (Fig. 1.1) (2-4). At the different stages of folliculogenesis, not only many other molecules regulate the

signaling of these molecules but also they can interact with each other. The absence or genetic alterations of these factors have been found to have profound effects on fertility (4).

The first part of this thesis, the Literature Review section, focuses on the TGFβ signaling pathway molecules and signaling mechanisms. The courses of ovarian folliculogenesis and steroidogenesis are briefly reviewed, and the biological roles of activins/inhibins, follistatin, follistatin-like (FSTL) 3 and the oocyte-secreted factors (GDF9 and BMP15) are described. The second part, including four chapters of research results, focuses on the interactions between activin A and GDF9 on inhibin B and progesterone accumulation in human granulosa-lutein (hGL) cells. The last section discusses the outcome of the study and offers some suggestions for further study.

1.2. Folliculogenesis

The primary function of the follicle is to support the oocyte. In human beings, the ovaries contain about one million immature, primordial follicles, which contain an oocyte suspended in prophase of meiosis surrounded by one layer of pregranulosa cells that is enveloped by a basement membrane. After the first menstruation in puberty, a group of follicles begin folliculogenesis, initiating a growth pattern that ends in atresia or ovulation. Folliculogenesis is a long process and only occurs during the reproductive lifespan. It takes about 375 days for a primordial follicle to develop and grow to the ovulatory stage, which includes two periods. The first period is called preantral stage, where the follicle grows and differentiates independently from FSH and LH but is driven by growth factors. In the second period, the antral stage, a few of the follicles stimulated by gonadotropins and growth factors grow large and become dominant antral follicles, finally reaching the preovulatory stage, while most other follicles

undergo atresia. During each reproductive cycle, the dominant antral follicle enlarges due to the effects of preovulatory FSH and LH, surges and ruptures, releasing the mature oocyte for fertilization, while the corpus luteum is established by the remaining granulosa and theca cells.

1.3. Steroidogenesis in Ovary

1.3.1. Overview of steroidogenesis

Steroidogenesis involves the synthesis of steroids from cholesterol and their subsequent transformation into alternative types of steroids. It is the basis of essential physiologic functions such as reproduction, metabolism and the immune response. The steroid hormones are generated from subtle modifications in the four-fused ring of the sterol skeleton (three 6-carbon rings and one 5-carbon ring) and the side chain. Cholesterol is the basic building block of steroidogenesis. During steroidogenesis, the number of carbon atoms in cholesterol or any other steroid molecule can be reduced but never increased. Steroid production starts with the transformation of cholesterol (27 carbons) to pregnenolone (21 carbons) by the cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme (5). The transformation of pregnenolone to progesterone (21 carbons) is catalyzed by 3β-hydroxysteroid dehydrogenase (3βHSD) (6). In some tissues, progesterone is hydroxylated, and the side chain is subsequently cleaved by cytochrome P450 17α-hydroxylase/17, 20-lyase (P450c17) to form androstenedione (19 carbons). Androstenedione can be reduced to testosterone (19 carbons) by 17β-hydroxysteroid dehydrogenase (17βHSD). Both androstenedione and testosterone are converted to estrogens (estrone and estradiol, all 18 carbons) by an aromatization reaction that is mediated by cytochrome P450 aromatase (P450arom) in the endoplasmic reticulum (7).

The steroidogenic acute regulatory protein (StAR) principally regulated the rate-limiting

step of steroid production, that means the translocation of cholesterol from the outer to the inner mitochondrial membrane where fully active cytochrome P450scc locates (8). In the ovary, StAR expression is primarily present in steroid-producing cells, including the luteal and theca cells. The StAR mRNA and protein is rapidly stimulated by hormones and catalyzes the intermembrane transfer of cholesterol to P450scc to initiate steroidogenesis.

In the ovary, granulosa cells are the cellular source of estradiol and progesterone, which are the most important steroids involved in ovarian function. Progesterone production only needs granulosa cells, but estrogen synthesis requires collaboration between granulosa and theca cells. The cooperation of these two cell types and of the two gonadotropins (FSH and LH) in estrogen synthesis can be logically explained by the concept of the two cell/two gonadotropin system, first proposed by Falck in 1959 (9).

In human preantral and antral follicles, the LH receptor (LHR) is only expressed on the theca cells and the FSH receptor (FSHR) only on the granulosa cells. Androgens produced by LH-stimulated theca cells are the main substrates for estrogen synthesis by FSH-stimulated granulosa cells. FSH increases granulosa cell aromatase activity, which is absent in theca cells. The interaction between the granulosa and theca compartments causes accelerated estrogen production. At the later follicular phase, the LH surge that occurs during ovulation causes a remarkable stimulation of progesterone production and a sharp decline in estradiol production by the remaining luteinizing granulosa and theca cells (10). Together with terminal differentiation and luteinization, progesterone production of granulosa cells depends on an increase in the number of LHR just before the LH surge. The dramatic rise in LH leads to StAR expression in granulosa cells, thereby endowing granulosa cells with the ability to make progesterone from cholesterol (11).

After ovulation, it is believed the two cell types continue to function as a two-cell system; theca-lutein cells produce androgens for aromatization into estrogens by granulosa-lutein cells. The newly formed corpus luteum produces progesterone predominantly to prepare the uterine endometrium for implantation and to maintain gestation. Luteal cells express high levels of StAR, P450scc and 3βHSD to produce large amounts of progesterone (11). If implantation occurs, the lifespan of the corpus luteum can be prolonged by the emergence of a new, rapidly propagated hormone, human chorionic gonadotropin (hCG), which maintains luteal function until placental steroidogenesis is well established. Otherwise, the corpus luteum would regress, allowing for another reproductive cycle. The demise of the corpus luteum may involve luteolytic activity against its own estrogen production, which is regulated by prostaglandin and endothelin-1.

1.3.2. Regulation of the StAR gene

Many agents have been shown to be able to modulate StAR expression positively or/and negatively by acting on its promoter. Studies have been performed to investigate which transcription factors and other proteins interact with the regulatory elements in the StAR promoter. Intracellular cyclic adenosine monophosphate (cAMP) is thought to be a positive modulator that can cause a rapid increase in StAR expression (12). LH, hCG, insulin and growth hormone (GH) stimulate StAR transcription in the theca, luteinized granulosa and luteal cells.

Several locally produced growth factors, such as insulin, insulin-like growth factor (IGF)-I and IGF-II, also directly induce or modify gonadotropin-stimulated steroidogenesis by regulating the mRNA expression of StAR. In pig granulosa cells, IGF-I induces StAR mRNA

production and enhances the effects of FSH, LH and cAMP in steroid production (13-15). Low concentrations (10 ng/ml) of leptin stimulate, while high concentrations (1000 ng/ml) reduce StAR mRNA in porcine granulosa cells (16). In luteal cells, StAR production is stimulated by the luteotroph prostaglandin E2 (PGE2) (17).

Various peptides secreted by the oocyte, granulosa or theca cells suppress the basal or gonadotropin-stimulated StAR expression and ovarian steroidogenesis through autocrine/paracrine pathways. These negative regulators include TGFβ in the theca cells (18), leptin (16), TGFβ (19), BMP2 (20), BMP4 (20, 21), BMP5 (22), BMP6 (20), BMP7 (20), BMP15 (23) and epidermal growth factor (EGF) (15) in the granulosa cells, tumor necrosis factor α (TNFα) (24) in the luteal cells and cholesterol sulfate in a granulosa tumor cell line (25).

Oocyte-secreted GDF9 modulates StAR expression in a species-specific manner, increasing basal StAR expression in cultured mice granulosa cells but not human granulosa-lutein cells (26). GDF9 further reduces cAMP-induced StAR expression in human granulosa-lutein cells (26). This protein also inhibits basal StAR protein levels and steroidogenesis in human theca cells.

StAR promoter activation can be regulated by numerous transcription factors, including steroid factor-1 (SF-1), GATA4, GATA6, C/EBP and CREB. SF-1 controls the basal and cAMP-induced transcription of the human StAR (27). GATA4 and GATA6 play important roles in StAR transcription. Overexpression of both GATA4 and GATA6 increase the activity of StAR promoter in porcine granulosa cells (28). The StAR promoter is stimulated by the concerted effects of C/EBP and GATA4 in porcine granulosa cells (29). Basal and cAMP-induced murine StAR promoter activation can be increased by overexpression of CREB

in MA-10 and Y1 cells (30).

1.3.3. Regulation of the P450scc gene

The level of P450scc is mainly regulated by two pituitary-secreted gonadotropins, LH and FSH, in the granulosa, theca and luteal cells. The regulation of P450scc in folliculogenesis has similarities with that of StAR. Compared with StAR, P450scc mRNA expression is first detected in the granulosa cells of the preovulatory follicles of most species and it increases following follicular development (31-33). Similar to StAR, P450scc levels are stimulated by LH in the theca cells of developing follicles (34). Before ovulation, P450scc expression is elevated by gonadotropin surges and is retained at high levels in luteal cells (33). Consistent with the requirement of LH to induce luteal P450scc mRNA expression *in vivo*, P450scc is induced by FSH, LH and hCG in cultured rat, porcine and human granulosa cells (33, 35-38). The continued expression of P450scc during the luteal phase is species-specific: it either becomes constitutive, as observed in rats, or remains reliant on LH, as observed in primates (33, 39). In bovine luteal cells, P450scc mRNA accumulation is also increased by estradiol (E2) (17). The corpus luteum rescued by hCG in humans (40) or prolactin in rodents (41) during gestation keeps the expression of P450scc continuing. The stimulating effects of gonadotropin on P450scc expression can be further enhanced by growth factors, such as IGF-I (42) in rats, IGF-II (43) in humans and EGF (15) in porcine granulosa cells. P450scc expression can also be increased by estradiol (33), progesterone (44), GH (45), insulin (37, 46) in granulosa cells, amphiregulin (47) in cumulus-oocyte complexes and insulin (46) in theca cells.

The effect of TGFβ on mRNA expression is species dependent: it induces P450scc protein synthesis in rat granulosa cells (48) but suppresses P450scc mRNA in cultured bovine

granulosa cells (19). Several BMP members, such as BMP4, BMP6 and BMP15, inhibit FSH-induced P450scc mRNA or protein levels in granulosa cells by impairing FSH signaling. BMP4/6/7 also reduce basal P450scc mRNA in theca cells (49). Other negative regulators of P450scc expression include PGF2α (50) and TNFα (51) in granulosa cells and PGF2α (52) in luteal cells.

P450scc promoter activation involves the same transcription factors as StAR, but also includes its own regulatory factors. FSH or forskolin stimulates P450scc gene transcription via transcription factors, such as SF-1 (53) and GATA4 (54) in rat granulosa cells. P450scc expression in human granulosa cells is regulated by liver receptor homolog-1 (LRH-1) (55). The transcription of P450scc can be regulated by the activating protein 2 (AP-2) in the human placenta (56). The activation of the human P450scc promoter, which is induced by LRH-1, is inhibited remarkably by the nuclear receptor DAX-1 in human granulosa cells (55). Unlike StAR, the roles of C/EBP and NR4A members in the regulation of P450scc transcription remain unclear.

1.3.4. Regulation of 3βHSD genes

There are two isoforms of the 3βHSD gene in humans: type I (3βHSD1) and type II (3βHSD2). 3βHSD2 is expressed in the adrenal gland, ovary and testis whereas 3βHSD1 is expressed in other tissues, such as the prostate, mammary gland and placenta.

The expression of 3βHSD is stimulated by the FSH and LH surges that occur before ovulation and 3βHSD is expressed in porcine, bovine and sheep luteal cells (57, 58). *In vitro*, FSH, LH and hCG increase 3βHSD expression in the hypophysectomized rat ovary and monkey corpus luteum (39, 59). Growth factors, such as IGF-I and insulin, also induce 3βHSD

expression in granulosa cells, but their enhanced effects on FSH-stimulated 3βHSD mRNA are species-specific: IGF-I cannot work synergistically with FSH to increase 3βHSD mRNA expression in rat granulosa cells (42), while insulin is able to cooperate with FSH to augment its mRNA levels in human granulosa cells (60). IGF-I can also increase 3βHSD mRNA levels in cultured theca cells from hypophysectomized immature rats (61). EGF enhances the action of FSH on 3βHSD expression, and this interaction is further augmented by TGFβ in cultured granulosa cells isolated from the ovaries of immature rats primed with diethylstilbestrol (62). cAMP-stimulated 3βHSD activity is enhanced by fibroblast growth factor (FGF) in cultured human theca and granulosa-lutein cells (63).

Similar to P450scc, BMP2 (64), BMP4 (21), BMP6 (64) and BMP15 (23) can impair the FSH signaling pathway, thereby decreasing the FSH-augmented 3βHSD expression in granulosa cells. BMP4, BMP6 and BMP7 also reduce basal 3βHSD mRNA in bovine theca cells (49). TGFβ negatively affects 3βHSD mRNA in cultured bovine granulosa cells (19). Other inhibitors of 3βHSD expression include PGF2α in cultured porcine granulosa and luteal cells (50) and prolactin in the rat ovary (59, 65).

Like StAR and P450scc, human 3βHSD promoter activity can be driven by SF-1 or LRH-1 and inhibited by DAX-1 in human granulosa tumor cells (66); additionally, the co-expression of GATA4 or GATA6 can interact with SF-1 and LRH-1 to synergistically activate the promoter of human 3βHSD in both heterologous cells (CV-1 fibroblasts) and steroidogenic cells (MA-10 and H295R) (67). One difference of 3βHSD as compared to StAR and P450scc in granulosa cells is the reaction of the promoter to Nur77, which also regulates StAR and P450scc. Exogenous expression of Nur77 augments the 3βHSD promoter in human granulosa tumor cells but is not synergistic with SF-1 (68). Another difference is that FXR in human

adrenocortical cells (69) and Stat5 in Hela cells (70) modulate 3βHSD transcription.

For more details about the regulation of StAR, P450scc and 3βHSD genes, please refer to review (71).

1.4. Overview of TGFβ Superfamily

1.4.1. Ligands

The first member of the TGFβ superfamily, TGFβ1, was discovered in 1981 (72). Today, there are more than 45 members found in this superfamily, which includes the TGFβ subfamily (comprising TGFβ1, TGFβ2, TGFβ3), the activin/inhibin subfamily (including activin A, AB, B, C, D, E and inhibin A, B), the GDF subfamily (with at least 9 members), the BMP subfamily (with more than 20 members), the glial cell-derived neurotrophic factor (GDNF) subfamily (including GDNF, artemin and neuturin) and some other members, such as nodal, lefty and anti-Müllerian hormone (AMH; also known as Müllerian-inhibiting substance, MIS) (1).

These peptides are multifunctional growth factors that control proliferation, differentiation, motility, migration, apoptosis, adhesion, matrix synthesis and other functions in many cell types. They express in complicated temporal and tissue-specific patterns and consequently play an important role in the development of almost all tissues in many organisms, from insect to humans. Collectively, these factors account for a substantial portion of the intercellular signaling pathway controlling cell fate.

There are several distinctive features shared by the TGFβ superfamily members. Firstly, the pre-proprotein of the TGFβ superfamily members consist of a short N-terminal signal peptide, a propeptide that consists of 200-300 amino acids (aa) pro-region and a C-terminal

mature region (Fig. 1.2A) (73). The signal peptide directs the protein to the endoplasmic reticulum (ER) and through the secretory pathway. The variations in the pro-regions may relate to the folding and dimerization of proteins and ultimately determine the different structures of different TGFβ superfamily members (74).

Six to nine evolutionarily conserved cysteine residues are found in the mature region of the C-terminus. Six cysteines engage in an intramolecular cystine knot configuration that consists of three covalent disulfide bonds (Fig. 1.2B) (75). In addition, this knot fastens the base of several β-sheets together and it seems to help to drive molecular dimerization and increase the stability of the TGFβ superfamily members with butterfly-shaped structures.

Most proteins from the TGFβ superfamily are only functional as disulfide bond-linked homodimers or heterodimers, consisting of two polypeptide chains, known as monomers. The seventh conserved cysteine residue (the fourth consecutive cysteine from the N-terminus) present in most TGFβ superfamily members is involved in forming this disulfide bond (76). Nevertheless, some superfamily members, such as GDF3, GDF9 and BMP15, cannot form covalent dimers due to a lack of this cysteine residue (77-81). Instead, these ligand monomers may be linked by noncovalent bonds.

1.4.2. The signaling pathway

Except inhibin and the GDNF subfamily, most ligands of the TGFβ superfamily firstly bind to the transmembrane components of both type I and type II receptor and then induce the formation of a ligand-receptors complex. This complex initiates the autophosphorylation of the type I receptor and then actives the second message system by phosphorylating cytoplasmic Smads (Sons of mothers against decapentaplegia), which go to the nucleus and bind to the

specific DNA sequence to modulate the transcription of the targeted gene.

1.4.2.1. Signaling receptors

Based on their structures and functions, the receptors of the TGFβ superfamily can be separated into two subfamilies: type I and type II receptors. There are five type II receptors (TβR2, ACVR2A, ACVR2B, BMPR2 and AMHR2) and seven type I receptors [activin receptor-like kinase (ALK) 1-7] recognized in mammals (see Table 1.1 and Table 1.2) (82, 83). Proper signaling of the TGFβ superfamily ligands requires the involvement of both type I and type II receptor. These receptors are similar in structure, but their kinase domains are different in function. The receptor peptide includes an N-terminal extracellular domain, a transmembrane domain and a C-terminal intracellular serine/threonine (Ser/Thr) kinase domain.

1.4.2.2. The interaction between ligand and receptors

The activative peptides of the TGFβ superfamily are homo- or heterodimers. Their structures suggest that they function to bring the dimers of the type I and type II receptors together, forming heterotetrameric receptor complexes. The TGFβ superfamily members bind differently to their receptors, and there are two binding patterns that have been observed for these interactions. TGFβ and activin do not interact with the isolated type I receptors and have high affinities for type II receptors (84). In contrast, some BMP ligands, such as BMP2 and BMP7, bind first to type I receptors, then recruit the type II receptor to the complex of ligand and type I receptor (84). This ligand-type I-type II receptor combination causes the type II receptor to phosphorylate specific serine residues in the 30-amino glycine-serine-rich domain (GS-domain) located upstream of the type I receptor kinase domain. The phosphorylated type I

receptor then further activates a second messenger system, Smads, by phosphorylation, to regulate the downstream signaling pathway (Fig. 1.3).

1.4.2.3. The Smads family

Smads were initially recognized as the products of the *Drosophila Mad* and *C. elegans Sma* genes, and their name is a result of the combination of the *Sma* and *Mad* genes (85, 86). In vertebrates, eight Smad proteins are identified: Smad 1 through 8. They are universally expressed in all adult tissues and play diverse roles in intracellular signaling pathways (87, 88).

Based on the structural and functional features, there are three groups in the Smads family (Fig. 1.4): receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5 and Smad8), which can be phosphorylated by the type I receptors; the common Smad (Co-Smad: Smad4), which can oligomerize with the dimer of phosphorylated R-Smads; and inhibitory Smads (I-Smads: Smad6 and Smad7), which can be stimulated by the TGFβ superfamily ligands. I-Smads can compete with R-Smad receptor binding or co-operate with Smurfs (Smad ubiquitination-related factor) to stimulate receptor degradation via ubiquitination and consequently inhibit the signaling pathway (Fig. 1.3). In addition, Smurfs can mediate R-Smads degradation through the ubiquitination.

The differences in the structures of the ligands lead to diverse combinations of type I and type II receptors and the activation of various R-Smads. Phosphorylated TGFβ/activin/ nodal/myostatin (also called GDF8) type I receptors (ALK4, ALK5 and ALK7) can activate Smad2 and Smad3, while BMP type I receptors (ALK2, ALK3 and ALK6) can activate Smad1, Smad5 and Smad8 (Table 1.3). Phosphorylated R-Smads combine with Smad4 before entering the nucleus and at there, they cooperate with diverse DNA-binding proteins, transcription

activators and transcription repressors, such as FoxH1 (89), AML (90) and p300/CBP (91) and bind to specific DNA binding sequences to modulate target gene transcription in a cell type-specific manner. Different transcription factors cooperate with Smads, and cross-talking with other signaling pathways can cause various responses to ligands in different cells. Smad6 inhibits the TGFβ superfamily signaling by competing with phosphorylated R-Smads to bind to Smad4. In contrast, Smad7 can bind to the type I receptors to block the signaling. Smad6 can specifically block the BMP signaling pathway, while Smad7 can disrupt both the TGFβ/activin and BMP signaling pathways by preventing R-Smads from phosphorylation (review in (92)).

In the unstimulated cells, R-Smads remain in the cytoplasm, mainly as monomers that do not form complexes with Smad4, which shuttles between the cytoplasm and nucleus, while I-Smads are localized to the nucleus. Cytoskeletal proteins such as filamin also play roles in the localization and signaling of Smads (93).

1.4.2.3.1. The conserved structure of Smad family

There are two kinds of conserved domains in the Smad family: the N-terminal MH1 (Mad homology 1) domain and the C-terminal MH2 (Mad homology 2) domain. In contrast with MH1, the similarity of MH2 domain sequence in all Smads is high. The linker segment between these two domains is variable in sequence and length (Fig. 1.4 and Table 1.4) (review in (94)). Smad4 and R-Smads have both MH1 and MH2 domains. I-Smads only contain the MH2 domain, but N-terminal sequences of them are mildly similar to the MH1 domain. The MH1 domains of Smad4 and R-Smads are able to bind to a specific DNA sequence (Smad binding element, SBE) of the target gene. However, Smad2 has an extra residue in exon 3, leading to a loss in the ability to bind to the DNA sequence (Fig. 1.4) (95). MH1 and MH2 domains can co-operate with the transcription factors, transcription co-activators and

co-repressors to modulate specific gene transcription (Table 1.4). The linker region is a multifunctional region, which includes the sites for regulatory phosphorylation, cytoplasmic adaptors (filamin), ubiquitination adaptors (Smurf1 and Smurf2) and other transcription regulation proteins (Table 1.4).

1.4.2.3.2. The phosphorylation of Smad family

Activation of the Smad signaling pathway is achieved through the phosphorylation of its SSXS motif in the C-terminus by phosphorylated type I receptors. The structures of L3 loop in the MH2 domain and the type I receptor of R-Smads determine the specificity of the receptor substrate, R-Smads (Fig. 1.4) (94). TGF*β*/activin/nodal/GDF8 signaling activates Smad2 and Smad3 by phosphorylation, while BMPs/GDFs signaling activates Smad1, Smad5 and Smad8. In addition, other signaling pathways such as Ca^{2+}/Calmodulin-dependent protein kinase II (CamKII) (96), extracellular signal-regulated kinase (Erk)-family MAP (mitogen-activated protein) kinases (97) and protein kinase C (PKC) (98) can interact with the Smad signaling pathway and then phosphorylate Smads (review in (94)).

1.4.2.3.3. Smad-independent signaling pathway

In addition to the general Smad-dependent signaling pathway, the TGF*β* superfamily ligand-induced cellular signaling can be modulated by Smad crosstalk with other signaling pathways, adding further complexity to TGF*β* superfamily signaling. The crosstalk can be achieved through other signal molecules that directly alter the function of the Smad by alternative phosphorylation, or the Smad can modify the action of other signal molecules. The interaction between these signaling pathways enables the reduction or magnification of other growth factor effects.

MAPK/Erk kinase kinase 1 (MEKK1) activation can phosphorylate the MAP kinase sites in the Smad2 linker region and hence stimulate the interaction of TGFβ-induced Smad2 with Smad4 and the nuclear translocation and transcription of Smad2 in cultured bovine aortic endothelial cells (99). The C-terminal SSXS motif of Smad2 is not required in this step, although it is the site of TGFβ type I receptor-mediated phosphorylation (99). In addition, the overexpression of Smad7 can attenuate the MEKK1-stimulated transcriptional activity of Smad2 (99). The MKK3/p38 pathway, activated by TGFβ, can phosphorylate the activating transcription factor 2 (ATF2, also called CRE-BP1), enhancing ATF2-Smad4 complex formation to regulate target gene transcription (100). PKC directly phosphorylates Smad3, abrogating its DNA binding ability, causing the disruption of the transcriptional regulation and finally leading to the down-regulation of TGFβ-induced growth inhibition and apoptosis (98).

CamKII activation induces Smad2 and Smad3 phosphorylation to a lesser extent, blocking the import of Smad2 into the nucleus, thereby stimulating the heteromerization of Smad2-Smad4 and inhibiting TGFβ-induced Smad2-dependent transcription (96).

TGFβ can induce the process of epithelial to mesenchymal transition (EMT), although it can also inhibit tumorigenesis. The Rho GTPase signaling pathways play an important role in this process. In epithelial cells, TGFβ induces stress fiber formation and mesenchymal characteristics by rapidly activating the RhoA-dependent signaling pathways, while the expression of the dominant-negative mutants blocks RhoA signaling, inhibiting TGFβ-induced EMT (101). The studies has shown that the long-term treatment of human prostate carcinoma cells with TGFβ can cause the assembly of stress fibers via a collaboration between the Smad and Rho GTPase signaling pathways (102).

The PI3K/Akt pathway is another Smad-independent pathway. PI3K involves in

TGFβ-regulated the proliferation of fibroblast. This TGFβ-induced response occurs through the p21-activated kinase-2 kinase, but it is not Smad2 or Smad3 dependent (103). In liver cells, the PI3K/Akt pathway activated by insulin interferes with TGFβ-induced apoptosis by blocking TGFβ-induced caspase-3 activity rather than through the suppression of the heteromerization of Smad2-Smad4 or nuclear translocation (104).

Compared to the phosphorylation of the receptor-regulated Smads, the phosphorylation of Smad7, which is independent of TGFβ stimulation, cannot affect TGFβ signaling while regulating the transcription caused by TGFβ-independent Smad7 activation (105).

1.4.2.4. Extracellular regulation of TGFβ superfamily signaling

Several cell membrane-bound proteins, such as betaglycan, cripto, endoglin and BAMBI (BMP and activin membrane-bound inhibitor), can interact with the TGFβ superfamily ligands and facilitate ligand-receptor binding (see Table 1.5).

Betaglycan is reported to be the first such molecule and is identified as a TGFβ type III receptor (106), while it is now also recognized to be a co-receptor for inhibin signaling (107). Betaglycan is a membrane-anchored, glycosylated, transmembrane protein that can regulate the signaling pathway of TGFβ, inhibin and activin. It can restore the autocrine function of TGFβ through facilitating TGFβ binding to its receptors in human breast cancer cells (108). In contrast, betaglycan inhibits the tumor-promoting activity of TGFβ and therefore suppresses the malignant properties of human carcinoma cells (109). Betaglycan also inhibits activin binding to its own receptors (ACVRs) by promoting betaglycan/inhibin/ACVRs complex formation in human embryonic kidney (HEK) 293 cells (107). In addition, betaglycan enables inhibin to compete with BMPs in the human liver carcinoma cell line, HepG2, for binding to

the BMP type II receptor (BMPR2), which does not bind inhibin in the absence of betaglycan, and thus blocks BMP signaling (110). In human granulosa-lutein cells, the expression of betaglycan mRNA is stimulated by FSH, LH and PGE2, probably through the PKA pathway (111).

Human cripto (CR-1) is a member of EGF-Cripto/FRL-1/Cryptic (EGF-CFC) family which has only been identified in vertebrates, and the main members of this family include cripto in humans, cryptic in mice and FRL1 in *Xenopus* (112, 113). The study already shows that CR-1 is an important signaling co-receptor for nodal during early embryonic development (114). CR-1 interacts with ALK4 via its CFC motif and nodal via its EGF-like motif to form a complex consisting of ACVR2B-ALK4-nodal-CR-1, which allows nodal to induce the activation of Smad2 by phosphorylation (115). In contrast to its enhancing effect on nodal, CR-1 can block activin signaling by combining with activin and ACVR2A/2B, thereby inhibiting ALK4 phosphorylation and activation (116). CR-1 is also found to be critical in cancers and can stimulate the growth and spread of tumors. The stable overexpression of human CR-1 in the mammary glands of mice can induce the formation of mammary hyperplasias and papillary adenocarcinomas (117).

Endoglin is a membrane glycoprotein primarily expressed in human vascular endothelial cells and is shown to bind TGFβ by forming a stable ligand-receptor complex (118). Endoglin cannot bind ligands on its own but facilitates TGFβ1 and TGFβ3 binding to their receptors through an association with TβR2 (119). Endoglin specifically enhances the Smad 1/5/8 phosphorylation induced by TGFβ1 and suppresses the migration of endothelial cells dependent on the crosstalk between endoglin and the scaffolding protein GIPC (GAIP-interacting protein C terminus) (120). Endoglin shares corresponding regions of

sequence identity (71% amino acid sequence similarly with 63% identity) with betaglycan (121). The complex formation between endoglin and betaglycan may be a important modulator in TGFβ signaling regulation in chondrocytes (122). With the exception of TGFβ itself, endoglin can bind members of the TGFβ superfamily, such as activin A, BMP2 and BMP7 (123).

BAMBI is a transmembrane glycoprotein that is evolutionarily conserved in vertebrates and close to the TGFβ superfamily type I receptors in the extracellular domain (124). It can act as a general antagonist of TGFβ/BMP/activin signaling. BAMBI is a pseudo receptor and has a shorter intracellular domain that does not encode the Ser/Thr kinase domain required for signaling. It can form a constant association with type I and type II receptors independent of the ligand to block TGFβ superfamily ligand signaling (124). In addition, BAMBI cooperates with Smad7 to form a ternary association with ALK5 to inhibit the interaction of ALK5 with Smad3, thus impairing Smad3 activation and consequently blocking TGFβ signaling (125). BAMBI transcription can be activated by BMP4 (126) and Wnt/β-catenin signaling (127). TGFβ signaling also can directly increase BAMBI expression through the three tandem repeats of 13 bp sequences containing SBE in the BAMBI promoter (128).

Other proteins, including follistatin (FST), follistatin-like 3 (FSTL3), inhibin, latency-associated protein (LAP), noggin, chordin and the related factors caronte, cerberus and gremlin, are recognized to antagonize TGFβ superfamily signaling by binding the TGFβ superfamily ligands and preventing them from binding to the receptors.

FST, a glycosylated single-chain protein, is functionally linked to but structurally different from ligands of the TGFβ superfamily. It is characterized by its inhibition of pituitary cells FSH production (129). FST has been shown to be able to bind with activin and prevent activin

binding to its receptor, neutralizing most but not all of the actions of activin in the rat ovary (130). In addition, FST also binds and regulates the actions of other ligands of the TGFβ superfamily, such as BMP2, BMP4, BMP7 and BMP15, by forming a trimeric complex (131). FSTL3 is also a member of the follistatin-related protein family and contains a highly conserved follistatin domain which is cysteine-rich. Like FST, FSTL3 binds activin with high affinity and prevents activin from binding to its receptors, neutralizing its biological activities (132).

Inhibin is a member of the TGFβ superfamily and a naturally occurring antagonist of activin. Inhibin antagonizes activin action in Chinese hamster ovary cells by combining with the activin type II receptors ACVR2A and ACVR2B, thereby blocking activin binding (133). Inhibin has also been shown to suppress cellular responses to various BMP family members in several BMP-responsive cell types (110).

Mature TGFβ is proteolytically derived from the C terminus of its propeptide. Unlike most other hormones, the mature TGFβ remains associated with its propeptide after secretion. LAP, the N-terminal remnant of the TGFβ prepeptide, is able to interact with all isoforms of TGFβ (TGFβ1, TGFβ2, and TGFβ3), binding and neutralizing their activities (134).

Noggin and chordin are secreted proteins expressed in the Spermann's organizer (SO) that induce the ventral mesoderm to become the lateral mesoderm. Noggin is a small glycoprotein while Chordin is a large protein. Although Noggin and Chordin do not have structural similarities, neither of them can bind to activin and TGFβ, but they can specifically bind to BMPs, such as BMP2, BMP4 and BMP7, suppressing BMPs signaling by antagonizing BMPs interactions with their receptors (135-138).

1.4.2.5. Intracellular regulation of TGFβ superfamily signaling

Smads are the main intracellular regulators of TGFβ superfamily signaling, and they are regulated by many factors, such as their access to receptors, protein concentrations, phosphorylation by activated type I receptors, formation of receptors-Smads complexes and nuclear accumulation.

The binding of R-Smads to activated TGFβ superfamily type I receptors can be assisted by the SARA protein (139). Smad2 and Smad3 can bind to its centurial FYVE domain and adjacent Smad binding domain, while Smad1 cannot. Overexpression of SARA in COS-1 cells leads to the clustering of Smad2/3 and increases Smad2/3 phosphorylation as mediated by the activated type I receptor (139). The regulation of SARA protein expression or interactions with Smad2/3 could regulate the signaling of the TGFβ superfamily ligands. The loss of the TGFβ response by the interruption of SARA function could cause tumor formation.

The intracellular concentration of Smads can be modulated by Smurf1/2 through protein ubiquitination and consequent proteasomal degradation. Smurf1/2 are E3 ubiquitin ligases that can promote the degradation of R-Smads by binding to and blocking TGFβ superfamily signaling (140, 141). In addition to Smurfs, other ubiquitin ligases also play a role in the degradation of Smads (142).

The activated type I receptor phosphorylating R-Smads is a vital process in TGFβ superfamily signaling. After activation, phosphorylated R-Smad forms an oligomeric complex with Smad4. Except for type I receptors, which are activated by TGFβ superfamily ligands, R-Smads can also be phosphorylated by other signaling molecules as mentioned above, such as MAPK, PKC and CamKII. In contrast, Smad-mediated signaling can be blocked by specific phosphatases through dephosphorylating TGFβ-activated Smad2 and Smad3 and the

promoting their nuclear export (143). Therefore, Smads activity is not only induced but also regulated by phosphorylation, providing mechanisms for the interaction of Smad signaling with other signaling pathways.

The combining of receptors and Smads can be interrupted by some mechanisms, and inhibitory Smads and Smurfs are involved in these processes (review in (92)). The combination of Smad7 with Smurf1/2 can bind to the TGFβ type I receptors, resulting in accelerated receptor turnover through the polyubiquitination pathway (144). In contrast with the R-Smads, the expressions of both Smad6 and Smad7 are induced by TGFβ, activin and BMPs and then are used as negative feedback to block further signaling (145, 146). Smad7 expression can also be stimulated by the pathways that inhibit TGFβ signaling, such as interferon-γ, through the JAK1 tyrosine kinase and STAT1 transcription factor in immune cells (147) and the proinflammatory cytokines TNFα and interleukin-1β through actions of the NFκB/RelA transcription factor (148).

The phosphorylated R-Smads combine with Smad4 and enter the nucleus to regulate the transcription of targeted gene. The nuclear accumulation of Smads can be modulated by other signaling pathways. Ras signaling can inhibit ligand-induced Smad1/2/3 nuclear accumulation, directly interfering with Smad-dependent responses, while this effect is regulated through the Smad1/2/3 phosphorylation by Erk1 and Erk2 protein kinases which are activated by Ras (149). Ubiquitin/proteasome-mediated degradation is involved in the decrease in the nuclear concentration of phosphorylated Smad2, which is activated by TGFβ (150). The exact function of the ubiquitin-dependent degradation of Smad2 remains unclear. It may rapidly eliminate the signal of TGFβ or remove extra phosphorylated Smads from the nucleus by targeting Smads that do not bind to the DNA sequence of the targeted gene.

1.4.3. The roles of TGFβ superfamily members in folliculogenesis

In addition to the pituitary gonadotropins (FSH and LH) and GH, steroids, growth factors and other cytokines act in on the autocrine/paracrine systems and play important roles in ovarian follicle growth (review in (92)). Before the small antral stage, the development of the follicle is thought to be gonadotropin-independent. At these early stages, signals from the oocyte and the surrounding granulosa and theca cells are considered to promote the progression of follicular development. Five different classes of growth factors within human ovarian follicles have been described: IGF, TGFβ, TGFα, FGF and cytokines.

The role of TGFβ superfamily members in ovarian organogenesis and folliculogenesis has been studied widely in animals. These studies show that the oocyte, granulosa and theca cells express various TGFβ superfamily ligands in a developmental-stage related manner, and these ligands play important roles in folliculogenesis, including the initiation of the primordial follicles, the proliferation/atresia of the somatic cells, the expression of the gonadotropin receptors, the production of steroids, oocyte maturation, ovulation, luteinization and the formation of the corpus luteum (reviewed in (1, 151)).

1.5. Inhibin and Activin

1.5.1. The gene and protein structures of inhibin and activin

Inhibins are heterodimers of two inhibin subunits (α- and either β_A- or β_B-subunit), from which two isoforms are associated: inhibin A ($\alpha\beta_A$) and inhibin B ($\alpha\beta_B$) (Fig. 1.5). The homology of amino acid sequence between the α subunit and β-subunits is 23 to 27%, while the homology between β subunits is 64% (74).

Activins are homodimers of the β-subunit. The primary isoforms of activin are activin A

($\beta_A\beta_A$), AB ($\beta_A\beta_B$) and B ($\beta_B\beta_B$). There are other isoforms of activin (C, D, E) have also been recognized, however their biological significance remains unclear (152). Activin C is highly expressed in the mouse liver (153) and human prostate and liver (154), while the β_C-subunit is disable to dimerize with the α subunit to make inhibin C ($\alpha\beta_C$) (154).

Activins and inhibins are primarily identified as they can increase or decrease pituitary cells FSH production *in vitro*. The effect of activin is thought to be antagonized by inhibin. Whether inhibin A shows qualitative and quantitative similarities with inhibin B in biological activities still remains unclear. Inhibin A and B exhibit similar bioactivities, inhibiting rat pituitary FSH secretion *in vitro* (155), while inhibin B activity shows 15 to 20% similarity with that of inhibin A in ovine pituitary cell cultures (156). In addition, activin A suppresses EGF-stimulated DNA synthesis in rat hepatocyte primary cultures, while activin B is inactive (157). It is uncertain whether the activins or inhibins exhibit similar behavior in humans.

1.5.2. Signaling pathways

The activin signaling pathway is well characterized, while the inhibin signaling pathway remains unclear. Firstly activin combines with the its type II receptors (ACVR2A and ACVR2B) specifically. After combining, the ligand-type II receptor dimer binds type I receptors (ACVR1A or ACVR1B) to create a complex. This union promotes the type I receptor auto-phosphorylation, which then activates the downstream Smads system. Activin causes Smad 2/3 to associate with Smad 4 and then go to the nucleus together. Interacting with other transcription factors, the complex binds to specific DNA sequence and leads to gene stimulation or suppression.

Compared to activin, it remains unclear about inhibin signaling pathway. There are no

specific inhibin receptor identified in genome searches (158). Based on the inhibin-induced suppression of the activin stimulatory effects on FSH production in pituitary and in other cells where the action of activin is antagonized by inhibin, that the authors speculate that inhibin may not act through a particular inhibin receptor but through binding to activin receptor and then blocking activin binding to its receptor (159). High dose of inhibin can bind to ACVR2A/B and block activin signaling (133). The study has been shown that betaglycan can binds with inhibin and promotes the binding of inhibin to ACVR2A/B and thus it is a very potent antagonist of activin signaling pathway (107). However, while betaglycan appears to play an important role in inhibin signaling, additional findings propose other mechanisms may be involved. The inhibin-binding protein (InhBP/p120) does not bind inhibin, but it facilitates the inhibin-induced suppression of activin signaling (160).

It is yet to be established whether inhibin can bind to its particular receptor or whether it competes with other TGFβ superfamily members. Both inhibin and activin are shown to have a similar action in some systems and this is hard to clarify based on the theory that inhibin inhibits activin actions. However, inhibin has recently been shown to also be able to antagonize BMP signaling, suggesting that it has a wider mode of action (110). This observation may explain the activin-independent stimulation of inhibin mentioned above, and inhibin may regulate BMP signaling. Other binding proteins, such as follistatin and FSTL3, may also play important roles in this process.

1.5.3. Expression profiles of inhibins and activin A in the menstrual cycle

In the human menstrual cycle, inhibin A, inhibin B and activin A have different expression profiles. Inhibin A begins to increase in the late follicular phase and has one peak at ovulation

and another in the middle luteal phase, while inhibin B increases in the later luteal phase, peaking in the middle follicular phase and one day after LH surge (Fig. 1.6) (review in (161)). Activin exists in both a free form and a follistatin-bound form, in which it is nearly irreversibly bound (162). In the human menstrual cycle, free activin A levels do not fluctuate (163), whereas total activin A shows small peaks before the beginning of menses, in ovulation and in the later luteal phase (164).

1.5.4. Regulation of inhibins

The productions of inhibin A and inhibin B are under different regulation. Several studies have already shown that FSH and cAMP induce inhibin A, but not inhibin B, inhibin A levels increase while inhibin B levels remain constant in follicular fluid following follicle maturation (165-168). In contrast to the promoters of the inhibin α- and β_A-subunits (169-171), the promoter of the inhibin β_B-subunit lacks cAMP-response element (169, 172); thus, inhibin B secretion cannot be induced by FSH and cAMP in granulosa cells. Other studies have shown that activin, TGFβ and BMP2 can regulate inhibin β_B-subunit expression (173-175) and that inhibin B secretion requires IGF-I, BMP2, GDF9 and/or the stimulation of the PKC pathways (168, 175-177).

1.5.5. The functions of inhibins and activin A in the ovary

The regulation of FSH levels is very important for follicular development. Inhibins have been reported to suppresses FSH secretion in pituitary culture (178); however, the relative function of inhibin A and inhibin B in humans is still being established.

In the late luteal phase, following the corpus luteum demise, estradiol levels decrease and

consequently FSH levels increase because of the removal of the negative feedback. Inhibin A decreases in a similar manner and it suggests that inhibin A may play a role in this process. Some studies provide indirect evidence that inhibin B functions in FSH modulation because FSH increases selectively following the inhibin B decline in the early follicular phase (167). Serum activin A levels show a small rise following the increase of FSH in the menstrual cycle, but in physiologic situations, activin A levels do not fluctuate along with largely varied FSH levels (179, 180). Therefore, activin A may not play an endocrine-related role in regulating the secretion of FSH. In addition to their potential endocrine-related roles in FSH control, some studies have showed that inhibin and activin play vital roles in regulating follicle development (181).

1.6. FST and FSTL3

FST is a single chain protein that was first isolated from the follicular fluids of both porcine (182) and bovine (183) and found to possess pituitary FSH release-inhibitory activity. FST has a similar biological activity as inhibin, but its structure is totally different, because it is a monomeric protein, while inhibin is a dimeric protein. FST neutralizes most activin activities by binding activin with a very high affinity (K_d=50-500 pM) and preventing it from binding to its receptors (184, 185). These activin activities include promoting pituitary FSH release (186), enhancing FSH-stimulated ovarian granulosa cell differentiation (187) and inducing the formation of *Xenopus* embryo mesodermal tissue (188). It also binds other ligands of the TGFβ superfamily such as BMP2, BMP4, BMP7, BMP15 and GDF11, but its affinity is lower than activin (130, 131, 189-191). FSTL3 is a secreted glycoprotein that was first identified from a B-cell leukemia line; because of its major sequence homology to FST, it was firstly called

FST-related gene (FLRG) (192). FSTL3 has been detected in the cultured media of HeLa, JAR and LOVO cells (192). In addition, a recombined mouse FSTL3 protein is shown to bind activin and antagonize activin-regulated gene transcription *in vitro* (132). Compared to FST, FSTL3 cannot bind to BMP2, BMP4 and BMP7 (193).

1.6.1. The gene and protein structures of FST and FSTL3

FST is present in the follicular fluid in several forms with molecular weights ranging from 31 to 39 kDa, according to several protein studies (182, 183). The amino acid homology of FST protein is high among different species. The FST gene consists of six exons, and its length is about 6 kb. There are two major preproteins of FST that are generated at an alternative splicing site. One preprotein is full-length and has 344 aa, and the another has a shortened C-terminus, missing exon 6, and has 317 aa (194). The mature peptides of FST are evolutionarily conserved in structure. They consist of a signal peptide that is followed by an N-terminal domain, three successive follistatin domains (FS domains) and a C-terminal domain (195). Although encoded by separate exons, each FS domain consists of 73-77 aa and is distinguished by 10 conserved cysteine residues. With the exception of FST, a number of secreted proteins are also found to have these FS domains, including follistatin-like (FSTL) proteins such as FSTL3 (192), SPARC (for secreted protein acidic and rich in cysteine (196), agrin (197) and matrix glycoprotein SC1 (198). Apart from follistatin and FSTL3, which are major regulators of activin action, most members do not have the activity like follistatin.

The three main domains of FST have different functions. Residing in the N-terminal domain of FST, two specific tryptophan residues at positions 4 and 36 have been suggested to be the important sites accountable for the binding of FST with activin (199). FS domains are

presumed to serve as growth factor binding motifs (200). In addition, residues on the second FS domain are found to be important for activin binding (201). The number and sequence of FS domains are essential for full FST activation because the ability of FST to bind activin can be attenuated or destroyed by the replacement or rearrangement of the FS domains (201). Two FST molecules largely surround one activin dimer (202) and completely block the binding sites for both activin type I and type II receptors. Furthermore, the C-terminal domain is able to stabilize the FST-activin complex, as a study has shown that one FST molecule appears to contact the N-terminus of the other FST molecule, so that they become fastened in place (202), providing a potential explanation for the almost irreversible dynamics mentioned above (162).

The removal of the signal peptide from the preprotein reveals the mature peptides, and three main isoforms of follistatin (FST315, 315 aa; FST300, 300 aa; FST288, 288 aa) form (review in (203)). After different proteolytic processing, the C-terminal sequences of these isoforms are different. Less than 5% of follistatin mRNA produces FST288, while the longer isoform FST315 is the main product (204). The ability to bind heparin-sulfated proteoglycans located in the cellular surface is the main difference between these isoforms functions. The main binding sites for heparin are located in residues 72–86 of the first FS domain (182). The studies have already shown that the peptide fragments from this region have a high heparin binding affinity (205). This structural characteristic allows the shortest isoform, FST288, to have a higher affinity for heparin, while the longest isoform, FST315, has a reduced heparin affinity possibly caused by its C-terminal acidic tail that covers the sites for heparin binding, which FST288 does not possess (205, 206). This difference in heparin binding allows FST288 to bind to the cell surface, bringing activin for degradation and consequently blocking its autocrine-, paracrine- and endocrine-related actions (207), while FST315 localizes mainly in

circulation, consistent with its small or absent affinity to heparin (208). FST315 can act as storage for follistatin in the circulation, carry activin to target cells and inhibit activin from binding to FST288 for degradation. FST303, which is produced by the proteolytically cleavage of the C-terminal acidic tail, is distributed predominantly in the fluids and extracts of the gonadotrope (209).

FSTL3 has several structural characteristics in common with FST. Like FST, FSTL3 can also bind to activin A with a high affinity to form a complex. Structural studies of the complex revealed that two FSTL3 molecules envelop one activin molecule, while FSTL3 has a more general contact with activin than FST, promoting its activin binding affinity despite its lack of the third FS domain (210). The specificity of ligand binding may be affected by this extensive activin binding pattern (210) and also may be the potential explanation for the difference in the binding of BMP to FST and FSTL3, as previously mentioned (193). Like FST315, FSTL3 also does not have heparin binding sites, and therefore, under normal conditions, it does not have the ability to bind cell-surface proteoglycans. These biochemical distinctions also affect their biosynthesis and secretion. FST315 is the most rapidly produced and released, while FST288 is released more slowly and FSTL3 is the slowest to be released, with newly produced peptides being both released and translocated to the nucleus (211). Taken together, these results indicate that FSTL3 and different FST isoforms have different biological actions *in vivo*.

1.6.2. The actions of FST and FSTL3

When FST was first purified from follicular fluid, there was little information about its signaling mechanism, but later the concept of FST as an activin binding protein allowed for a breakthrough in the research (130). FST can bind activin with a high affinity, and the K_d can be

up to 500 pM. In addition because the binding of activin to FST is almost irreversible (162), FST can be looked at as another kind of activin receptor that can strongly inhibit activin actions. However, the ratio of the neutralization ability of FST to activin is varied, from about equal to sevenfold in different cell systems, which may be caused by the amounts and types of activin receptors expressed in various cell and the different binding affinities to FST.

Generally, two FST molecules contact one activin dimer to form the complex, and thus one β-subunit of activin binds to one FST molecule (212). Based on this characteristic, activin B or activin AB can bind FST with similar affinities as that observed with activin A (146), while inhibin binds FST with a markedly lower affinity because it only has one β-subunit (212). It remains unclear whether other activins such as activin C, activin D and activin E can interact with FST.

How does FST antagonize activin actions at the cellular level? Some studies already provide some answers to this question. As mentioned above, FST288 binds to activin, and this complex is able to bind to the heparin-sulfated proteoglycans and be endocytosed quickly by lysosomal enzymes (207). Although FST315 binds activin with a similar affinity to FST288, it has a lower affinity for heparin, and the binding of activin to FST315 can allow it to escape degradation (213). In certain situations, activin can be released from its bond with FST315 and interact with its receptor to activate downstream signaling. The role of fate in the FST315-activin complex is not well understood and need to be further investigated.

In addition to the main function of FST, which is to antagonize the actions of activin, compelling evidence suggests that it is not exclusively an activin binding protein and can also bind to other TGFβ superfamily members, particularly BMPs (131). The affinity of FST for these proteins remains unclear, but it is supposed to be lower than that for activin, and the

physiological significance of these findings has not yet been described in detail.

Even though the interaction between FST and GDF9 or BMP6 was not reported, FST can bind BMP15 and inhibit BMP15-induced the proliferation of rat granulosa cells and -reduced FSH receptor expression in rat granulosa cells (191). These results suggest the roles of FST in the ovary are wide. Because BMP15 is the homologue to GDF9, these results also suggest that FST is possible to bind to GDF9 and modulates its function, but this hypothesis needs to be investigated further. FST is also shown that it can bind with BMP2, BMP4, BMP7 and BMP4/BMP7 heterodimers and neutralize their actions in *Xenopus* embryos, however no reports are obtainable in mammalian (189).

Like FST, FSTL3 can also bind to GDF8 (also called myostatin) (193). However, unlike FST, BMP2, BMP4, BMP6 and BMP7 did not compete with activin to bind to FSTL3 (193). The relative abilities of the FST isoforms or FSTL3 to bind cell-surface proteoglycans are correlated with neutralization of exogenous GDF8 or BMPs bioactivity. These results indicate that the differential biological actions among the FST isoforms and FSTL3 are principally reliant on their relative cell-surface binding abilities and ligand specificities.

1.7. GDF9 and BMP15

1.7.1. The gene and protein structure of GDF9 and BMP15

GDF9 was first recognized as a TGFβ superfamily member from mouse genomic DNA in 1993 (77). Human GDF9 was obtained from a complementary DNA library prepared from mRNA that was isolated from human adult ovaries (214). Five years after the discovery of GDF9, a close homolog named BMP15 (or GDF9B) was discovered simultaneously by two research groups using homology-based cloning approaches (78, 79).

GDF9 and BMP15 have some common traits, and their mature protein regions are quite small. Both of them are created as precursor proteins, with the mature proteins locating in their C-terminus. Human GDF9 and BMP15 precursor protein consists of 454 and 392 aa respectively. The human GDF9 precursor protein contains a hydrophobic stretch of 24 aa at the N-terminus as a signal peptide for secretion, followed by a 295 aa propeptide with has a putative RXXR cleavage site and a 135 aa C-terminal mature protein that is high homologous to other TGFβ superfamily members (214), while the BMP15 precursor protein consists of a 18 aa signal peptide, 249 aa propeptide and a 125 aa mature protein (79). The sequence of the mature GDF9 protein is relatively conserved among different species, while that of the BMP15 mature protein varies a lot. In the C-terminus of the mature protein, the identity between GDF9 and BMP15 is 52.4% in human, 53.2% in sheep, 47.6% in mouse and 44.8% in rat.

The human GDF9 propeptide contains six potential N-glycosylation sites at 106, 163, 236, 255, 268 and 338. Unlike GDF9, there are only five potential N-glycosylation sites at 87, 147, 237, 277 and 373 for BMP15. Most members of the TGFβ superfamily can form homodimers or heterodimers with other members of TGFβ superfamily. In general, TGFβ superfamily member mature proteins contain seven cystines. Six of them form a specific cystine knot and the remaining one involves in the formation of a dimer that is linked by a disulfide bond (74). In contrast, GDF9 and BMP15 lack the cystine involved in dimer formation and the missing cysteine residue is replaced by a serine residue (76, 78, 79, 215). Therefore, they may only form dimers through noncovalent interactions. Although the biological activities of other members of TGFβ superfamily require the dimer formation, whether it is also important for GDF9 and BMP15 remains unclear. However, recent results show that it is possible for the formation of biologically active heterodimers of GDF9/BMP15 because when GDF9 and

BMP15 are co-expressed in transfected HEK293T cells, both heterodimers and homodimers can be formed (216). Because combining conditioned media containing GDF9 and BMP15 and adding it to the granulosa cell culture is enough to observe their co-operative effects, it is thought that these proteins eliciting their effects may not need to be produced by the same cell (217). Therefore, these growth factors could be present as homodimers, heterodimers or even as monomers to affect granulosa cell development.

1.7.2. The expression and regulation of GDF9 and BMP15

The GDF9 mRNA and protein are expressed in the growing oocytes at all stages of follicular development but not in primordial follicles (218-221). Nevertheless, GDF9 mRNA can be detected in primordial follicles of bovine and ovine ovaries (222). GDF9 mRNA continues to be expressed in mouse oocytes through ovulation until 1.5 days after fertilization (214). BMP15 mRNA is also expressed in the mouse oocyte from the primary follicle stage until fertilization (78).

GDF9 is originally thought to be an oocyte-specific factor; however, GDF9 is found recently to express in monkey granulosa cells (223), goat (224) and pig (225) ovaries and granulosa and cumulus cells from human ovaries (226, 227). GDF9 mRNA has also been found in the human uterus, placenta and testis, in non-reproductive tissues such as the bone marrow, adrenal gland, pituitary gland and thymus (228), in rodent testis and hypothalamus (228), in ovine cortical slices (229) and in the brushtail possum pituitary gland (230). In addition to the oocyte, which is the main site of expression, BMP15 and its receptors BMPR1B and BMPR2 are also detected during the *in vitro* culturing of ovine cortical slices (229). In summary, these data propose that the action of GDF9 and BMP15 may be not exclusive in the

ovary.

The function of this low-level expression of GDF9 in other tissues outside of the ovary is unclear, but the characteristic high-level expression of GDF9 in the ovary and the regulatory elements in its promoter are of interest to researchers. Using transgenic mice, in which regions of the GDF9 locus are fused to the reporter genes, Yan *et al*. report that a conserved E-box sequence (CAGCTG) is a vital regulatory sequence for GDF9 expression in the ovary (231). However, the factors and mechanisms involved in the commencement of GDF9 expression in the oocyte have still not been identified.

Cho *et al.* report that the mRNA levels of the Kit ligand and Kit itself, but not GDF9 and BMP15, are induced in a culture of cumulus-oocyte complexes and granulosa cells by FGF7, which may play an important role in regulating the stimulation of oocyte growth (232).

The germ cell nuclear factor (GCNF) which is a transcription factor, can repress the transcription of GDF9 and BMP15 by binding to a AGGTCA repeat with 0 base pair (bp) spacing between the half sites (DR0) in their promoter (233). This repression directly regulates oocyte-somatic cell paracrine communication and hence affects the fertility of female mice. The results of oocyte-specific GCNF knockout female mice has also shown that the absence of GCNF repression may cause increased expression of GDF9 and BMP15 and consequently the formation of abnormal double-oocyte follicles (233).

1.7.3. GDF9 and BMP15 Signaling pathway

Recent studies have been shown that GDF9 signaling is regulated by the TGFβ type I receptor (TβRI) (234) and that its type II receptor is a BMP type II receptor (BMPR2) (235); therefore, it mediates signaling by phosphorylated Smad2 and Smad3 (176, 236). This is a

particular type I-type II receptors complex and is the first report of a TGFβ superfamily member utilizing both a TGFβ-type receptor (TβRI) and a BMP-type receptor (BMPR2) for its signaling.

BMP15 probably utilizes the same type II receptor as GDF9 (i.e. the BMPR2) (235), while its type I receptor is suggested to be ALK6 (BMP1B) (79); thus, it can activate Smad1, Smad3 and Smad5 to regulate downstream signaling (237). In addition, mouse BMP15 is reported to cooperate with GDF9 via the BMPR2 and ACVR1B/TGFβR1/ ACVR1C-mediated pathways to stimulate rat granulosa cell proliferation (238).

1.7.4. Biological functions of GDF9 and BMP15 in follicle development

GDF9 and BMP15 are reported to be the important regulators of follicular development, including the initiation of the primordial follicles and the further development of follicles. Both GDF9 and BMP15 are considered to be related to follicle formation because the primordial follicle population is not altered in GDF9 knock-out mice, and the follicle number in sheep is not changed in sheep missing BMP15 (219). These results are consistent with the lack of GDF9 expression in murine follicles and the lack of BMP15 expression in ovine follicles until the follicles start growing. The follicular development of the GDF9 null mutant female mice are arrested at the primary stage and are infertile, while male mice are fertile, demonstrating that GDF9 plays an important role in stimulating early follicle growth (219, 239). The results of Vitt *et al.* show that GDF9 *in vivo* treatment promotes the primary-preantral follicle transition (240). Furthermore, GDF9 stimulates the survival and development of human primordial follicles (241). The *in vitro* exposure of rodent ovarian tissues to GDF9 has been shown to stimulate the growth of the primary follicle (242). In contrast with GDF9 knockout

mice, null BMP15 mutations have little effect on folliculogenesis and fertility, and the mice show weak ovarian phenotypes (243). Taken together, GDF9, but not BMP15, may be critical to the beginning of the growth of primordial follicle.

Following the follicle growth, the oocyte within the antral follicle continues to produce GDF9, BMP15 and BMP6 to affect the cell development of the surrounding granulosa cells and consequently regulates antral follicle growth (244, 245). The immunoneutralization of GDF9 and BMP15 by their corresponding peptides in sheep causes anovulation, and these results suggest that they are necessary for follicle development before ovulation (246).

Following the preovulatory LH surge, cumulus cells lose intercellular connections with the oocyte, but they undergo cumulus expansion, which is regulated by some proteins, such as cyclo-oxygenase 2 (COX2), hyaluronan synthase 2 (HAS2), urokinase plasminogen activator (uPA), tumor necrosis factor-induced protein 6 and pentraxin 3. Studies on mice have shown that recombinant GDF9 or BMP15 can mimic the actions of oocytes, and they are critical in regulating the proteins involved in cumulus expansion (26, 247). In addition, GDF9 can regulate StAR and LHR transcription in granulosa cells from antral follicles, which are also related to cumulus expansion in mouse (26).

1.7.5. Roles of GDF9 and BMP15 in granulosa cell functions

Both GDF9 and BMP15 have been reported to be vital in regulating granulosa cell functions. First, GDF9 (248) and BMP15 (249) can stimulate granulosa cell mitosis and proliferation. Also, GDF9 can increase inhibin α-subunit mRNA levels in explants of rat neonatal ovaries (220) and inhibin A and B production in cultured rat granulosa cells (250). The effects of GDF9 obtained from different species on inhibin production in ovine granulosa

cells are different: mouse GDF9 inhibits while ovine GDF9 stimulates inhibin production (251). In contrast, BMP15 does not have any effect on the inhibin production in bovine or ovine granulosa cells (251). In addition, GDF9 (252) and BMP15 (249) can be modulators of steroidogenesis in granulosa cells. GDF9 and BMP15 can also attenuate the stimulated effects of FSH in cAMP, estradiol and progesterone production and LHR expression in rat granulosa cells, possibly through inhibiting FSHR expression and/or FSHR binding to the Gs protein (248). Additionally, the expression of the Kit ligand, which is a critical regulator in female reproduction, can be modulated by GDF9 and BMP15. Kit ligand expression is reduced by GDF9 in mouse granulosa cells (253) and induced by BMP15 in rat granulosa cells (254). This difference may be caused by the species difference between mouse and rat.

1.8. Objectives

As mentioned above, activins, inhibins and GDF9 have been shown to play important roles in follicle development. During the human menstrual cycle, plasma inhibin B levels fluctuate in a typical, cyclical pattern distinct from that of inhibin A (255, 256). Gonadotropins stimulate steady-state inhibin α- and β_A-subunits mRNA levels in hGL cell cultures (257), but do not stimulate β_B-subunit mRNA levels which are up-regulated by TGFβ (174), activin A (173) and BMP2 instead (177). Treatment with either activin A (173) or GDF9 (176) alone has been shown to increase inhibin β_B-subunit mRNA and inhibin B levels in hGL cells. However, previous studies have only focused on the effects of activin A or GDF9 alone, while the interactions between activin A and GDF9 on inhibin B accumulation remain unknown. Furthermore, activin A is a well-known inhibitor of luteinization but whether GDF9 plays a role in the intracellular and extracellular regulation of activin A is unclear. The objectives of this thesis were to investigate the following:

- To investigate the interaction between GDF9 and activin A on inhibin subunit (α, β_A, β_B) mRNA levels, inhibin A and B accumulation, and related intracellular regulating signaling mechanisms in hGL cells;
- To determine if GDF9 is expressed in hGL cells, and if it so, whether it plays a role in regulating activin A-induced inhibin subunit (α, β_A, β_B) mRNA levels, inhibin A and B accumulation, and the mechanisms involved;
- To characterize the role of GDF9 in the expression of follistatin and follistatin-like 3 protein, well-known extracellular inhibitors of activin A, and whether they are involved in regulating GDF9 and hence, its interaction with activin A;

- To study whether the progesterone production, an important function of hGL cells, is affected by any interactions between GDF9 and activin A.

To accomplish this, we used human granulosa-lutein cells obtained from women undergoing *in vitro* fertilization (IVF) as our research model. Because these cells had been exposed to pharmacological doses of exogenous gonadotropins and in the process of luteinization from hCG stimulation, we recognize the limitations of extrapolating our results to normal ovarian physiology. However, compared to other *in vitro* and *in vivo* research models, our hGL cell primary culture model does provide a homogenous population of granulosa cells which can be used to study the cell-type specific spatio-temporal roles of, and interactions between, selected growth factors for research by eliminating the impacts from others. In addition, our *in vitro* culture model allows for detailed studies involving multiple manipulations and endpoints, which would be difficult or impossible to do *in vivo*. Thus, in the absence of granulosa cells from the unstimulated, normal human ovaries that are easily accessible and of sufficient amount for research, findings from our cell model do provide interesting hypotheses for further exploration particularly during the follicular-luteal transition.

1.9. Tables

Table 1.1. The type II receptors of the TGF*β* superfamily and the ligands known to bind with the type II receptor.

Type II receptors	Alternative names	Ligands binding to the receptor
T*β*R2	AAT3, FAA3, LDS1B, LDS2B, MFS2, RIIC, TAAD2, MFS2	TGF*β*
ACVR2A	ACVR2, ActRII, ActRIIA, ActR-IIA	Activin A, Inhibin A/B, GDF5/6/7, GDF8 (Myostatin), GDF11 (BMP11), BMP2, BMP3, BMP6/7, BMP10, BMP15
ACVR2B	ActRIIB, ActR-IIB, MGC116908	Activin A, Inhibin A/B, Nodal, GDF5, GDF8 (Myostatin), GDF1, GDF11, (BMP11), BMP2, BMP6/7
BMPR2	BMPR-2, BMPR-IIBRK-3, FLJ41585, FLJ76945, PPH1, T-ALK	Inhibin A (with T*β*R3) , GDF5/6, GDF9, BMP2/4, BMP6/7, BMP15
AMHR2	AMHR, MISR2, MISRII, MRII	AMH (MIS)

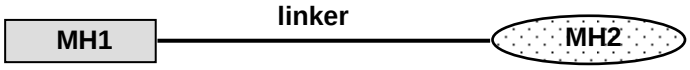
Table 1.2. The type I receptors of the TGF*β* superfamily and the ligands known to bind with the type I receptor.

Type I receptors	Alternative names	Ligands binding to the receptor
ALK1	ACVRL1, TSR1, SKR3, HHT, HHT2, ORW2, ACVRLK1	TGF*β*1, Activin A
ALK2	ACVR1A, ActRIA, TSK7L, SKR1, FOP, TSR1, ACVRL2	TGF*β*, Activin A, AMH (MIS), BMP6/7
ALK3	BMPR1A, BMPR1, BRK1, Tfrl1, ACVRLK3, CD292, SKR5	BMP2/4, BMP6/7, BMP10, GDF5/6/7, GDF8 (Myostatin), AMH
ALK4	ACVR1B, ActRIB, SKR2, ACVRLK4	Activin A, activin B, GDF1 and Nodal (with Cripto), BMP3, GDF8 (Myostatin), GDF11 (BMP11)
ALK5	T*β*R1, SKR4, AAT5, LDS1A, LDS2A, ACVRLK5	TGF*β*, GDF8 (Myostatin), GDF9
ALK6	BMPR1B, BRK2, CDw293, ACVRLK6	BMP2/4, BMP6/7, BMP10, GDF5/6/7, BMP15, AMH
ALK7	ACVR1C, ACTR-IC, ACVRLK7	Nodal, Activin B

Table 1.3. Combinational interaction of TGFβ superfamily receptors and Smads

Type II receptors	Type I receptors	Activated Smads
TβR2	ALK5, ALK1 ALK2	Smad2, Smad3, Smad1, Smad5
ACVR2A, ACVR2B	ALK4	Smad2
ACVR2B	ALK7	Smad2
BMPR2	ALK2, ALK3, ALK6	Smad1, Smad5, Smad8
AMHR	ALK3, ALK2, ALK6	Smad1, Smad5

Table 1.4. The proteins interacted with Smads

			
Function	Nuclear import Cytoplasmic anchoring DNA-binding transcription	Ubiquitination	Oligomerisation Cytoplasmic anchoring Transcription
Regulatory phosphorylation	CamKII (–) (S2) PKC (–) (S2, S3)	CamKII (–) (S2) Erk (–) (S1-3)	Type I receptors (+) (S1-3, S5, S8)
Receptors			ALK1-7
Oligomerisation			R-Smads, Co-Smad
Cytoplasmic adaptors-effectors	Calmodulin (S1-4) Filamin (S1-6) Importin-b 1 (S3)	Filamin (S1-6)	Axin, Axil (S2, S3) Dab2 (S2, S3) SARA, Hrs/Hgs (S2, S3)
		ARIP (S3) β-catenin (S4) Microtubules (S2-4) TAK1 (S6)	STRAP (S2, S3, S6, S7)
Ubiquitination adaptors-substrates	HEF1 (N-ter) (S3)	Smurf1 (S1, S5, S7) Smurf2 (S2, S3, S7)	HEF1 (C-ter) (S3) SCF subunits (S3) APC subunits (S3)
Transcription factors	ATF2 (S3, 4) Jun, JunB, JunD (S3, S4) Lef1/Tcf (S2, S3)		AR (S3) BF-1 (S1-4) E1A (S1-3)

	MH1	linker	MH2
Transcription factors	Sp1, Sp3 (S2-4) TFE3 (m E3) (S3, S4) VDR (S3) YY1 (S1, S3, S4)	Gli3 D C-ter (S1-4) HNF4 (S3) p52 (NFκB) (S3)	ERα (S2-4) Evi-1 (S3) FAST (FoXH1) (S2, S3) Fos (S3) GR (S3) Lef1/Tcf (S2, S3) Menin (S2, S3) Milk (S2) Mixer (S2) OAZ (S1, S4) Runx/CBFα
Transcriptional co-activators	pX HBV (S4)	Swift (S1, S2)	MSG1 (S4) p300/CBP (S1-4) P/CAF (S1-4)
Transcriptional co-repressors	HDAC (?) (S3) Hoxc-8 (S1)	Hoxc-8 (S1) SNIP1 (S1, S2, S4)	SIP1 (S1-3, S5) Ski (S2-4) SnoN (S2-4) TGIF (S2) Tob (S1, S4, S5, S8)

A simplified diagram of the three Smad domains is followed by a table of the Smad post-translational modifications and protein-protein interactions known to occur in each domain. The symbols (+ and –) indicate regulatory phosphorylation of Smads that results in functional activation or inhibition, respectively. Entries in more than one domain indicate interactions with or modifications by the same factor at multiple domains. The specific Smad members that are known to exhibit the listed modifications or interactions are shown in parenthesis and are abbreviated as S1-S8 for Smad1 to Smad8, respectively. Proteins, for which the specific Smad domain that they interact with is not yet determined, are listed in the centre in stippled boxes. A question mark (?) indicates that HDAC activity but not physical protein interaction has been found to associate with the MH1 domain of Smad3. The names of factors not discussed in the text are: TAK1 (TGF-activated kinase 1), pX HBV (pX oncoprotein of hepatitis B virus), Swift (*Xenopus* BRCA1 C-terminal domain nuclear protein), MSG1 (melanocyte specific gene 1, transcriptional co-activator), Hoxc-8 (homeobox c-8 transcriptional repressor), SNIP1 (Smad nuclear interacting protein 1, Smad- and p300-associating transcriptional corepressor), SIP1 (Smad interacting protein 1, zinc-finger/homeodomain repressor), Tob (transducer of ErbB2, APRO/Btg family of anti-proliferative factors), ATF2 (activating transcription factor 2), Lef1/TCF (lymphoid enhancer-binding factor 1/T cell-specific transcription factor 1), Sp1, Sp3 (Specificity protein 1, zinc finger transcription factor), TFE3 (transcription factor recognising the immunoglobulin enhancer motif E3), VDR (vitamin D receptor, nuclear hormone receptor), YY1 (yin yang 1, zinc finger transcription factor), AR (androgen receptor, nuclear hormone receptor), BF-1 (brain factor 1 oncoprotein), E1A (early region of adenovirus binding transcription factor 1A), ER (estrogen receptor), Evi-1 (Evi-1 oncoprotein), FAST (Forkhead activin signal transducer), GR (glucocorticoid receptor, nuclear hormone receptor), Menin (multiple endocrine neoplasia-type 1 tumour suppressor protein), Milk (Mix 1-related homeobox transcription factor), Mixer (homeobox transcription factor), OAZ (olfactory factor O/E-1-associated zinc finger protein), Runx (runt

domain transcription factor), Gli3 C-ter (glioblastoma Kruppel zinc finger transcription factor-3 with deletion of the C-terminal domain), HNF4 (hepatocyte nuclear factor 4, nuclear hormone receptor), NFκB (B cell-specific nuclear factor binding to the intronic light chain enhancer). For references see review (94). (Moustakas A, Souchelnytskyi S, Heldin CH 2001. Journal of cell science 114:4359-4369. Adapted with permission)

Table 1.5. Non-signalling binding proteins of TGF*β* superfamily

Name	Alternative names	Ligands binding to the receptor
Betaglycan	T*β*R3, BGCAN	TGF*β*1-3, inhibin A (with ActRIIA)
BAMBI	NMA	BMPs, activins
Cripto	FRL-1	Nodal
Endoglin	CD105, END, FLJ41744, HHT1, ORW, ORW1	TGF*β*1/3, activin A, BMP2/7
InhBP (Inhibin binding protein)	Inhibin coreceptor, IGCD1, IGDC1, IgSF1, KIAA0364, MGC75490, p120, PGSF2	Inhibin A/B (with ALK4)

1.10. Figures

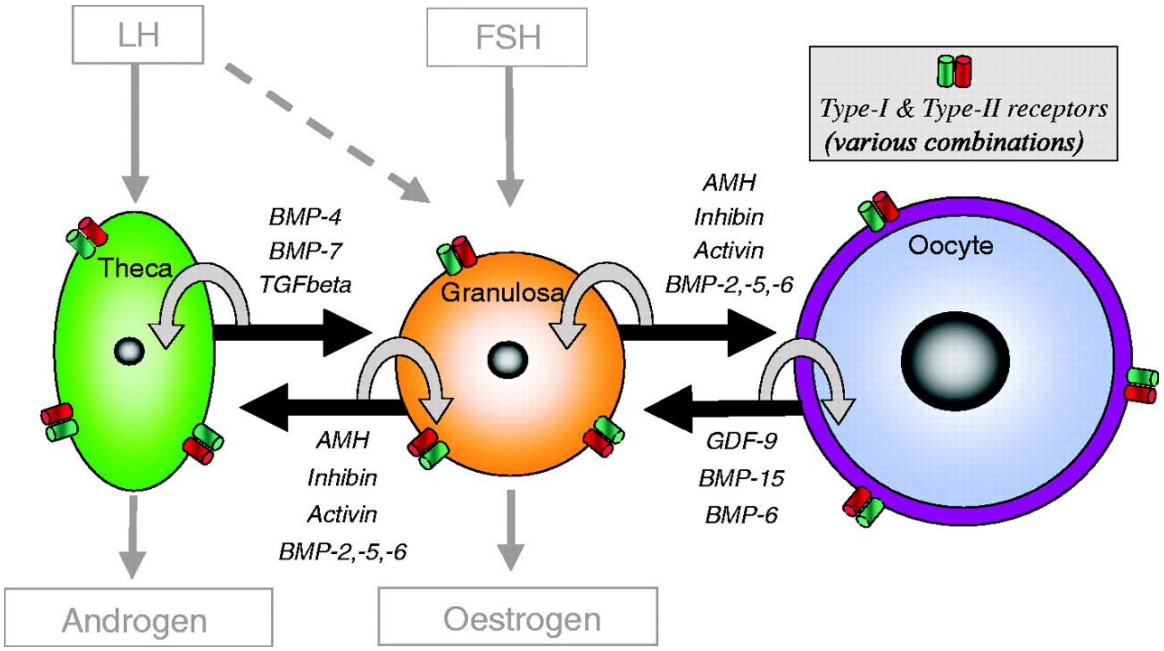


FIG. 1.1 Members of the TGFβ superfamily play important roles in the bi-directional communication between oocyte and granulosa cells, and granulosa and theca cells. Both autocrine (thick grey arrows) and paracrine (thick black arrows) signalling events are likely, depending on the expression of appropriate combinations of type-I and type-II receptors on the cell surface. Abbreviations: AMH, Anti-müllerian hormone; BMP, Bone morphogenetic protein; GDF, Growth differentiation factor; TGF, Transforming growth factor. (Knight PG, Glister C 2006. Reproduction 132:191-206. (c) Society for Reproduction and Fertility (2010). Reproduced with permission)

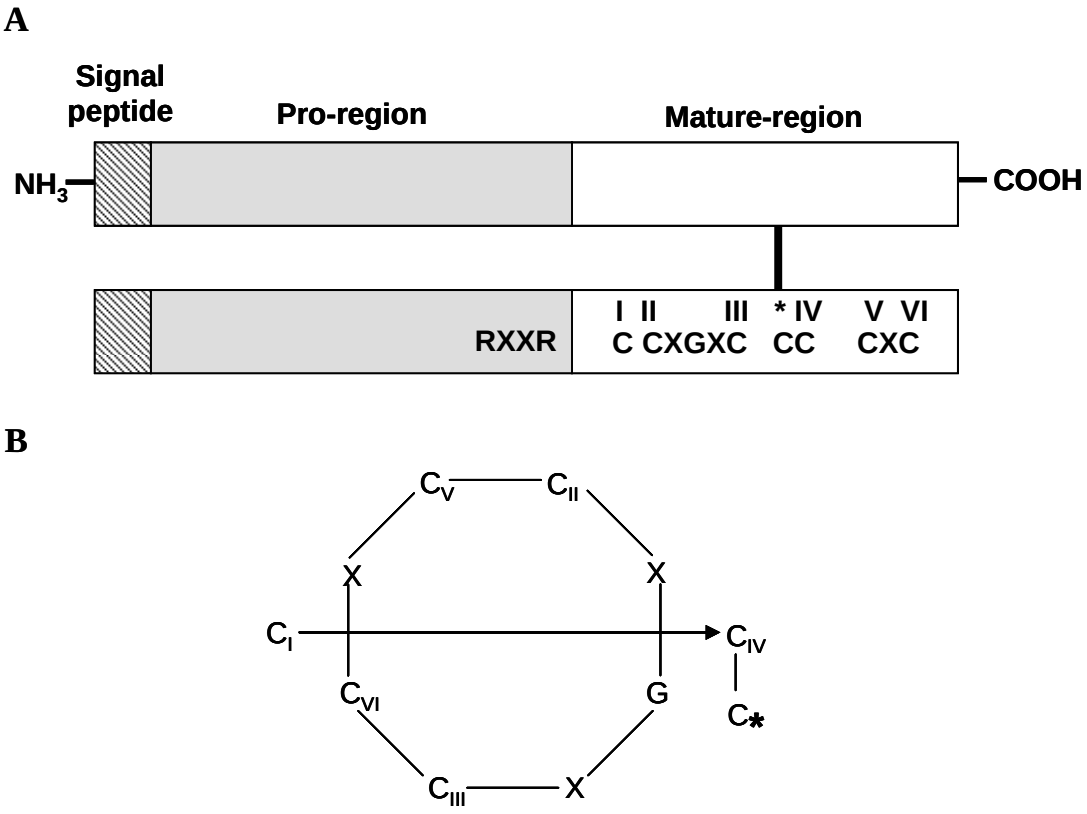


FIG. 1.2 The protein structure of TGFβ superfamily members. (A) A pre-proprotein of TGFβ (Transforming growth factor) superfamily members is composed of a signal peptide, pro-region and a mature region. Cleavage at a dibasic site (RXXR motifs) releases a mature region that contains seven highly conserved cysteine residues, forming three intramonomeric disulphide bonds (C_I–C_{IV}, C_{II}–C_V and C_{III}–C_{VI}). NH3, amino-terminus; COOH, carboxyterminus; black line, intermolecular disulfide bond; "C" labelled with "*", the fourth cysteine residue lacking in GDF9 (Growth differentiation factor 9) and BMP15 (Bone morphogenetic protein 15). (B) The cystine knot motif arises from the three intra-monomeric disulphide bonds by two (C_{II}–C_V and C_{III}–C_{VI}) of them building an eight-membered ring structure, through which the third (C_I–C_{IV}) passes. The remaining cysteine (labelled with *) forms an inter-monomeric disulphide bond with that of the other monomer into a dimmer. (Lin SY, Morrison JR, Phillips DJ, de Kretser DM 2003. Reproduction 126: 133–148. (c) Society for Reproduction and Fertility (2010). Adapted with permission)

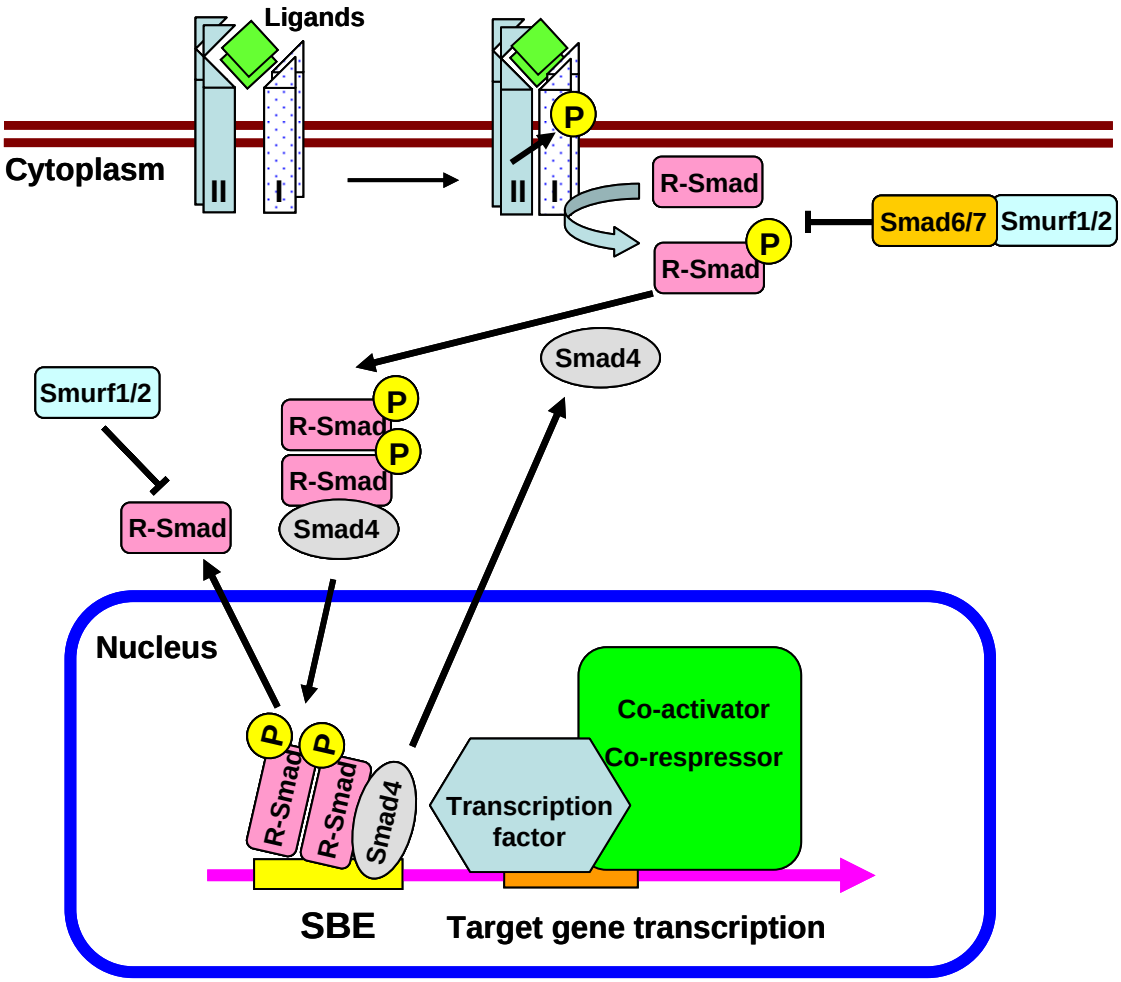


FIG. 1.3 Diagrammatic representation of the TGFβ superfamily signaling pathway. Abbreviations: Smad, Son of mothers against decapentaplegia; R-Smad, Receptor-activated Smad; SBE, Smad binding element; Smurf, Smad ubiquitination-related factor; Ⓟ : Phosphorylation.

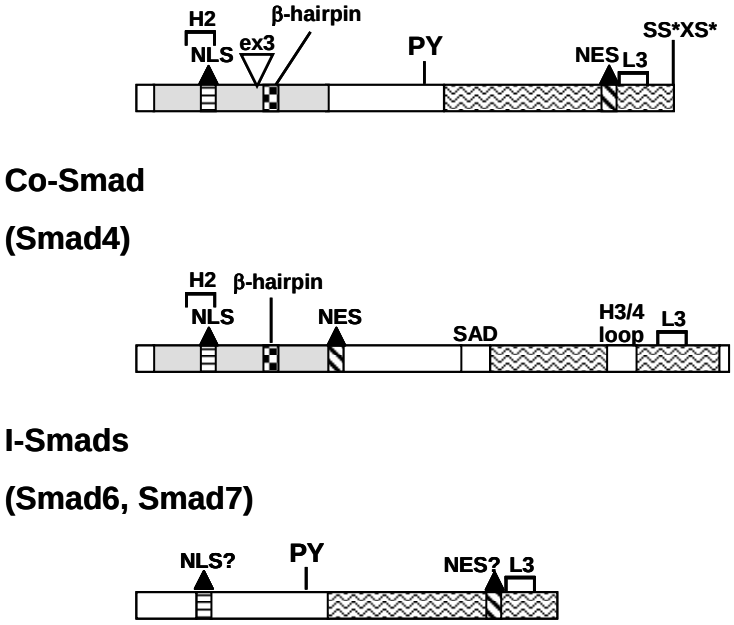


FIG. 1.4 The protein structures of the three subfamilies of Smads. The protein diagrams are arbitrarily aligned relative to their C-terminus. The MH1 (Mad homology 1) domain is coloured in grey and the MH2 (Mad homology 2) domain in wave pattern. Selected domains and sequence motifs are indicated as follows: -helix H2, L3 and H3/4 loops, *β*-hairpin, the unique exon 3 of Smad2 (ex3), NLS (nuclear localization signal) and NES (nuclear export signal) motifs or putative (?) such motifs, the proline-tyrosine (PY) motif of the linker that is recognised by the Hect domain of Smurfs (Smad ubiquitination-related factors), the unique SAD (Smad activation domain) domain of Smad4 and the SSXS motif of R-Smads with asterisks indicating the phosphorylated serine residues. Abbreviations: Smad, Son of mothers against decapentaplegia; R-Smad, Receptor-activated Smad; Co-Smad, Common Smad; I-Smad, Inhibitory Smad. (Moustakas A, Souchelnytskyi S, Heldin CH 2001. Journal of Cell Science 114:4359-4369. Adapted with permission)

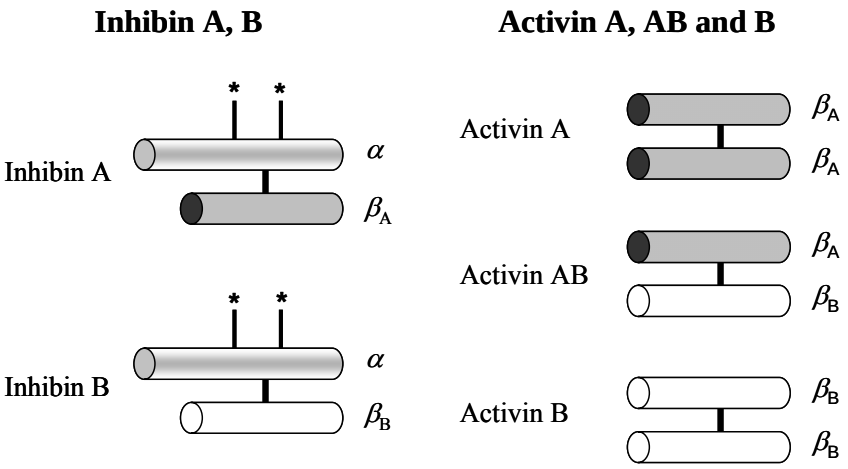


FIG. 1.5 Schematic diagram of different isoforms of inhibin and activin.

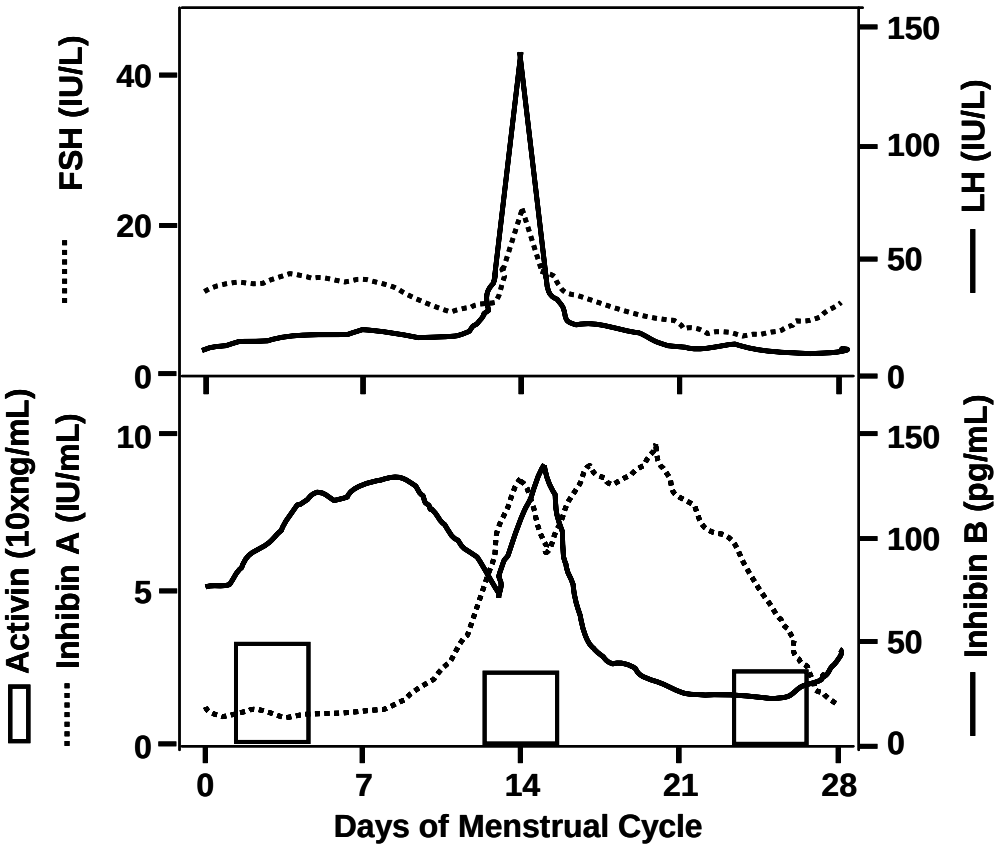


FIG. 1.6 Levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), inhibin A, inhibin B and activin across the menstrual cycle. Total activin A levels only at the time of menses, the midcycle, and the midluteal phase were measured while other times in the menstrual cycle were not measured.

2. GDF9 ENHANCES ACTIVIN A-INDUCED INHIBIN B ACCUMULATION IN HUMAN GRANULOSA-LUTEIN CELLS[1]

2.1. Introduction

Activin A or GDF9 alone has been shown to increase inhibin β_B-subunit mRNA levels in hGL cells (173, 176), but little is known about their interactions in regulating inhibin subunits and, if so, the underlying cellular mechanisms involved. The objectives of the present work were to further examine GDF9 on basal and activin A-induced inhibin subunit mRNA levels and inhibin B accumulations and activation of relevant receptors and Smads in the activin signaling pathway in hGL cells.

2.2. Materials and Methods

We first conducted concentration-response studies of activin A (1-50 ng/ml) or GDF9 (1-200 ng/ml) alone on α-, β_A-, and β_B-subunit mRNA levels in cultured hGL cells to identify the respective concentration for corresponding time-dependent and cotreatment experiments. Free plasma activin A levels have been estimated to be 30-45 ng/ml across the menstrual cycle (161). In our concentration-response experiments, activin A induced increases in β_B-subunit mRNA levels in a concentration-related manner from 10 to 50 ng/ml. At 25 ng/ml, β_B-subunit mRNA levels were significantly greater than levels for control (0 ng/ml) and the 1 ng/ml concentration; at 50 ng/ml, levels were significantly greater than all lower concentrations of activin A (Fig. 2.1E). In our GDF9 concentration-response experiments, an increasing trend in

[1] A version of this chapter has been published. Shi FT, Cheung AP, Leung PC 2009 Growth differentiation factor 9 enhances activin A-induced inhibin B production in human granulosa cells. Endocrinology 150:3540-3546

β_B-subunit mRNA levels from baseline (0 ng/ml) or the 10 ng/ml concentration was first observed at the 100 ng/ml concentration (a common concentration chosen in reported studies) but reached statistical significance only at the 200 ng/ml concentration. Guided by these findings, the concentration of 25 ng/ml of activin A and 100 ng/ml of GDF9 were selected to assess the corresponding time-dependent changes and on whether cotreatment of recombinant GDF9 and activin A could affect levels of inhibin subunits, inhibin A and B, and activin A receptors and Smads involved in downstream cell signaling.

Preparation of hGL cells

The study was approved by the Research Ethics Board of the University of British Columbia. hGL cells were obtained from women undergoing IVF treatment. For each patient, cells from multiple follicles and consequently follicular fluid were pooled respectively. The extraction procedure of granulosa cells from each patient were modified from that described previously (258). Follicular fluid from each subject was divided equally into 15-ml disposable, sterile tubes and centrifuged at 400 × g for 10 min. After removing the supernatant, the layers of hGL cells with the red blood cell pellet were re-suspended in 2 ml of Hanks' solution (GIBCO BRL Life Technologies, Grand Island, NY) containing 50 µg/ml deoxyribonuclease I (Worthington Biochemical Inc., Freehold, NJ), 0.1% hyaluronidase (Sigma, St. Louis, MO), 50 U/ml Heparin (Sigma), and 0.1 U/ml Blendzyme 3 (Roche, Inc., Indianapolis, IN) in a sterile 50-ml centrifuge tube. The cell suspensions were shaken at 200 r.p.m. for 20 min at 37 C and then layered on 8.0 ml Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) in 15-ml sterile tubes and centrifuged at 600 × g for 20 min. The cell layer was removed from each Ficoll-Paque column and washed three time with 10 ml of DMEM/nutrient mixture F-12 Ham (DMEM/F-12; Sigma) supplemented with 10% fetal bovine serum (FBS; HyClone

Laboratories, Logan, UT), 100 U/ml penicillin (GIBCO), 100 µg/ml streptomycin sulphate (GIBCO) and 1 × GlutaMAX^{TM} (GIBCO), and the cells were suspended in 5 ml of medium, counted on a hemocytometer, and brought to a final concentration of 2 × 10^5 cells/ml. Cell viability was determined by 0.04% Trypan Blue dye (GIBCO), and 1 × 10^5 viable cells were seeded per well in 24-well culture plates and cultured in a humidified atmosphere of 5% CO_2-95% air at 37 C. After 48 h, the above medium containing only 0.5% FBS ("low-serum medium") was added to each well, and the cell culture was now designated as time 0 for all subsequent experiments described below. Culture media were collected after treatment and stored frozen until assays for inhibin A and B.

Activin A and GDF9 experiments

hGL cells were seeded to each well of a 24-well plate for mRNA study and inhibin A and B assays (1 × 10^5 cells/well) and to each well of a 12-well plate for Smad experiments (2 × 10^5 cells/well). Cells were stimulated with 1-50 ng/ml activin A (Sigma), or 1-200 ng/ml GDF9 (Peprotech Inc., Rocky Hill, NJ) for 24 h in concentration-response studies. For time-dependent experiments, cells were treated with the concentration of 25 ng/ml of activin A or 100 ng/ml of GDF9 as mentioned earlier, and for 3, 12, and 24 h. For experiments with both GDF9 and activin A, cells were preincubated with 100 ng/ml of GDF9 in low-serum medium for 24 h before stimulation with 25 ng/ml of activin A. In neutralization experiments to render GDF9 inactive, 2 μg/ml of recombinant extracellular domain (ECD) fused to the Fc region of human IgG (receptor-ECD/Fc chimera) of human BMPR2 (BMPR2 ECD; R&D Systems, Minneapolis, MN) and 100 ng/ml of GDF9 were preincubated in low-serum medium for 30 min before adding to cultured hGL cells.

RNA extraction and Real-time RT-PCR

At the end of the treatment period, medium was removed from the culture plate and RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Total RNA (800 ng) was reverse-transcribed into first-strand cDNA according to the protocol of the ThermoScript amplification System (Invitrogen). The primers used for SYBR Green real-time PCR were designed using the Primer Express Software (Applied Biosystems, Foster City, CA) (See Table S1 at Appendices) and tested with the intron-spanning assay. Real-time PCR was performed on the ABI PRISM 7300 sequence detection system according to the manufacturer's protocol (Applied Biosystems). Amplification specificity using the melting curve and analysis and quantification of the relative mRNA levels using the comparative cycle threshold method were carried out on the ABI Prism 7300 Sequence Detection Software version 1.3 (Applied Biosystems). Relative expression levels were quantified using the comparative Ct method with normalization to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and changes following treatments were recorded as fold differences from values in untreated controls at each time point as appropriate.

Smad activation experiments and Western blot analysis

After treatment, cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) containing protease inhibitors (Sigma). The extract was centrifuged at 14,000 r.p.m for 15 min at 4 C to remove cellular debris; protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA). Twenty micrograms of protein samples

were heated to 95 C for 5 min, run in 12% SDS-PAGE gels, and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked for 1 h in Tris-buffered saline containing 0.01% Tween 20 with 5% non-fat dried milk, and incubated overnight at 4 C with the relevant antibodies (Cell Signaling Technology, Inc., Beverly, MA): anti-phospho-Smad2 ($Ser^{245/250/255}$), anti-phospho-Smad2 ($Ser^{465/467}$), anti-phospho-Smad3 ($Ser^{423/425}$), anti-Smad2, and anti-Smad3. After washing, the membranes were incubated with the secondary antibody, a peroxidase-conjugated anti-IgG (Bio-Rad Laboratories) for 1 h. Immunoreactive proteins were detected using enhanced chemiluminescence reagents (Amersham Bioscience) followed by exposure to Thermo CL-X Posure film (Thermo Fisher Scientific Inc., Waltham, MA). Antiserum to total β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as the internal control.

Inhibin A and B assays

Dimeric inhibin A and B levels in the spent culture media were quantified with specific ELISA kits (Diagnostic Systems Laboratories, Webster, TX). The lowest limits of detection for human inhibin A and B were 1 pg/ml and 7 pg/ml, respectively. Corresponding intrassay coefficients of variation were 3.0 and 3.5%; interassay coefficients of variation were 6.1% and 6.2%. All hormone measurements were performed in duplicate. Secreted hormone levels were normalized to total cellular protein content.

Statistical analysis

Each set of experiments consisted of granulosa cells from at least three sets of experiments (each from a separate patient). Real-time PCR samples were assayed in triplicate and ELISA samples were assayed in duplicate. Data were analyzed by one-way ANOVA, followed by

Tukey's multiple comparison tests if the overall *P* values were significant using the computer software PRISM (GraphPad Software Inc., San Diego, CA). Results were reported as means ± SEM and were considered significantly different from each other at $P < 0.05$.

2.3. Results

Dose- and time-dependent effects of activin A or GDF9 on inhibin subunit mRNA levels

Activin A, but not GDF9, increased inhibin α-subunit mRNA levels which reached statistical significance at the 50 ng/ml concentration (Fig. 2.1, A and B). Activin A could also induce inhibin β_A-mRNA levels, but the small increases (Fig. 2.1C) were not significantly greater than control (0 ng/ml) value; whereas GDF9, at 200 ng/ml, increased inhibin β_A-subunit mRNA to levels that were significantly greater than those for all lower concentrations ($P < 0.05$) (Fig. 2.1D). Activin A induced inhibin β_B-subunit mRNA levels in a concentration-dependent manner with the maximal level 58-fold of control at the 50 ng/ml concentration ($P < 0.001$) (Fig. 2.1E); in contrast, GDF9 increased inhibin β_B-subunit mRNA level only at 200 ng/ml and to only 1.5-fold of control ($P < 0.05$) (Fig. 2.1F). For activin A, there were no significant time-dependent changes in inhibin α- and β_A-subunit mRNA levels (Fig. 2.2, A and C) but statistically significant time-dependent increases in inhibin β_B-subunit mRNA levels (Fig. 2.2E). For GDF9, significant increases were not observed for inhibin α- and β_A-subunit mRNA (Fig. 2.2, B and D) but were observed for inhibin β_B-subunit mRNA at 24 h ($P < 0.05$) (Fig. 2.2F).

Effects of GDF9 pretreatment on activin A-induced inhibin β_A- and β_B-subunit mRNA levels and dimeric inhibin A and B secretion

Neither concurrent treatment with activin A and GDF9 nor pretreatment with activin A for

24 h before adding both activin A and GDF9 enhanced inhibin β_A- or β_B-subunit mRNAs at 3, 6, 12, and 24 h (data not shown). In contrast, GDF9 pretreatment for 24 h amplified activin A-induced inhibin β_A- and β_B-subunit mRNAs in a time-dependent manner, reaching a peak at 6 and 12 h for inhibin β_A- and β_B-subunit mRNA, respectively, before leveling off (Fig. 2.3, A and C, *P* values all < 0.05). BMPR2 is the type 2 receptor for GDF9 and the ECD of BMPR2 is known to antagonize recombinant GDF9 bioactivity (235, 259) and the mitogenic effects of GDF9 on granulosa cells (260). When GDF9 preincubated with BMPR2 ECD for 30 min was added to the cell culture before activin A treatment, the enhancing effects of GDF9 on activin A-induced inhibin β_A- and β_B-subunit mRNA levels were attenuated at both 12 and 24 h (Fig. 2.3, B and D, *P* values all < 0.05). In contrast, there were no significant changes with respect to control when BMPR2 ECD alone was added (Fig. 2.3, B and D). Protein concentrations of inhibin A and B in the corresponding culture media showed that activin A induced only a small rise in inhibin A in cells pretreated with GDF9 at 24 h, and as a result, no significant decrease was observed when GDF9 was first neutralized with BMPR2 ECD. In contrast, Activin A increased inhibin B secretion by 2-fold of control value ($P < 0.01$) and 4-fold in cells pretreated with GDF9 ($P < 0.001$), and the latter was attenuated when GDF9 was first neutralized with BMPR2 ECD (Fig. 2.4).

Effects of GDF9 treatment on activin receptors and Smads and cell response to activin A

GDF9 enhanced the activin receptors ACVR2B/1B and Smad2/3 mRNAs and reduced Smad7 mRNAs in a time-dependent manner but had no effect on ACVR2A and Smad4 mRNAs (Fig. 2.5). (Baseline ACVR1A mRNA level was too low for further study). Corresponding cell lysates analyzed 5, 15, 30, and 45 min after activin A stimulation showed

that GDF9 pretreatment enhanced activin A-induced Smad3 (Fig. 2.6A) and Smad2 ($Ser^{465/467}$) phosphorylation (Fig. 2.6B), effects that were attenuated by BMPR2 ECD. In contrast, there was little change from control in Smad2 ($Ser^{245/250/255}$) phosphorylation irrespective of GDF9 treatment and consequently BMPR2 ECD (Fig. 2.6C). The stimulatory effects of activin A, with and without GDF9 pretreatment, on Smad phosphorylation were evident by 30 min after adding activin A because there was little change at 5 and 15 min (results not shown). Thus, GDF9 enhances cell response to activin A by increasing mRNA levels of facilitating components and simultaneously reducing that of inhibitory components of the activin signaling pathway.

2.4. Discussion

As reported by some investigators (173), our study confirmed that activin A had only limited stimulatory effects on inhibin α- and β_A-subunit mRNA levels but marked stimulatory effects on inhibin β_B-subunit mRNA levels in hGL cells. We also confirmed that GDF9 alone had no effects on inhibin α-subunit mRNA levels and modestly induced inhibin β_A-subunit mRNA levels in these cells only at the higher concentration of 200 ng/ml. Whereas GDF9 increased inhibin β_B-subunit mRNA and corresponding inhibin B levels as observed in a previous study (176), the effects were much lower than those induced by activin A. Differences in species and follicle development are important consideration in studying the effects of GDF9. For example, GDF9 can stimulate not only inhibin β_A- and β_B-subunit mRNA levels, but also inhibin α-subunit promoter activity in granulosa cells obtained from small antral follicles of estrogen-treated immature rats (250). In contrast to the effects of GDF9 alone, our study demonstrates for the first time that GDF9 enhances activin A-induced inhibin β_B-subunit

mRNAs and inhibin B secretion in hGL cells and the underlying mechanisms involved.

Pretreatment with GDF9 before activin A stimulation is required to elicit the enhancing effects of GDF9 on activin A-induced inhibin β_B-subunit mRNA and inhibin B levels from hGL cells because concurrent activin A and GDF9 cotreatment or activin A pretreatment followed by GDF9 stimulation have revealed no enhancing actions. Using a number of approaches, we further characterized that these enhancing effects were specifically related to GDF9 actions.

First, the enhancing effects of GDF9 are neutralized by BMPR2 ECD, a well known GDF9 antagonist (Fig. 2.3 and 2.4); hence, inhibin β_A- and β_B-subunit mRNA level and inhibin B secretion were not significantly different from those for activin A alone when GDF9 was preincubated with BMPR2 ECD. Second, mRNA levels of activin receptors and Smads in the activin signaling pathway are altered by GDF9. GDF9 increased mRNA level of the receptors, ACVR2B and ACVR1B, and activating Smads, Smad2/3, but decreased mRNA level of the inhibitory Smad, Smad7. Furthermore, GDF9 enhanced activin A-induced Smad2/3 phosphorylation, effects that were attenuated by BMPR2 ECD. Smad2 has two different phosphorylation sites ($Ser^{465/467}$ and $Ser^{245/250/255}$). Our results showed that activin A and GDF9 had effect on $Ser^{465/467}$, located in the carboxy-terminal SSXS sequence of Smad2 but not $Ser^{245/250/255}$, located in the linker region of Smad2. Phosphorylation of Smad2 on Ser^{465} and Ser^{467} is required for oligomerization with Smad4 and translocation into the nucleus in mammalian cells (261). In contrast, $Ser^{245/250/255}$ serves as phosphorylation sites for proline-directed protein kinases including ERK (262). Thus, whereas our data show GDF9 can activate $Ser^{465/467}$, other ovarian regulators are required to activate $Ser^{245/250/255}$.

Inhibin A and inhibin B exhibit distinct patterns of secretion across the menstrual cycle.

Serum inhibin B levels increase across the luteal-follicular transition, reaching a peak in the midfollicular phase and a second peak on the day after the LH surge. In contrast, inhibin A levels begin to rise in the late follicular phase, reaching a peak in the midcycle and another peak in the midluteal phase (161). Whereas activin A may be present as a free form, it is almost irreversibly bound to follistatin, and there is little variation in free activin A levels (30-45 ng/ml in plasma) across the menstrual cycle (161). hGL cells likely have the capacity to express the various inhibin subunits, but the expression of a specific subunit, hence secretion of a particular inhibin, is influenced, among other factors, by the prevailing facilitating and/or inhibiting autocrine or paracrine factors. Of particular interest is that GDF9 mRNA is expressed in hGL cells and together with activin A, can significantly influence the inhibin β_B-subunit mRNA levels and consequently inhibin B secretion.

We cannot rule out the possibility that increased levels of inhibin β_B-subunit mRNA lead to an increased synthesis of activin B ($\beta_B\beta_B$) or activin AB ($\beta_A\beta_B$), but specific activin B and activin AB assays are currently unavailable. Although GDF9, activin A, or both have limited effects on inhibin α-subunit mRNA levels, it is expressed in theca interna in addition to granulosa cells, and its levels are higher than those of inhibin β_B-subunit mRNA in the ovary (263-265). Because the inhibin α-subunit is available in abundance, a selective rise in inhibin β_B-subunit mRNA levels is sufficient to cause a clear increase in the amount of inhibin B produced by hGL cells when stimulated by GDF9, activin A or both.

In the *Gdf9* null female mouse model (266), inhibin α- and β_A-subunits are expressed in *Gdf9* null ovaries at similar levels to controls, whereas inhibin β_B-subunit is dramatically decreased. However, this may also be related to a lack of antral follicles in *Gdf9* null mice. Pan *et al.* (267) found that mouse GDF9 mRNA was markedly up-regulated between the primordial

and primary follicle stage and reached a maximum in the oocyte of the secondary follicle, but its level was still higher in the large antral follicle than in the primordial follicle. Correspondingly, inhibin β_B-subunit mRNA was up-regulated during follicular development and reached a peak in the large antral follicle. Teixeira *et al.* (268) reported that oocyte GDF9 mRNA expression increased progressively during follicle development of the human ovary, with near maximum amounts observed in oocytes of fully grown secondary follicles. In addition, GDF9 mRNA expression remained high in oocytes of healthy small Graafian follicles. These studies and our findings suggest that inhibin β_B-subunit mRNA levels in both the human and the mouse ovary are related to those of GDF9.

We acknowledge the limitations of extrapolating our results from human granulosa-lutein cells obtained from women undergoing IVF treatment to normal ovarian physiology as these cells have been exposed to pharmacological concentrations of exogenous gonadotropins and in the process of luteinization from hCG stimulation. Nevertheless, in the absence of granulosa cells from the unstimulated, normal ovaries that are easily accessible for research, findings from our cell culture model do provide interesting hypotheses for further evaluation of the role of GDF9 and related mechanisms involved in regulating inhibin subunit and protein production during the periovulatory transition.

This notwithstanding, our study suggests that GDF9 increases hGL cell response to activin A by acting on its receptors, BMPR2/TβR1, which then activates facilitating Smad2/3 downstream to form complexes with Smad4. These complexes then activate transcription factors in the nucleus to induce target genes that increase ACVR2B/1B and Smad2/3 and reduce Smad7 activities. These changes, in turn, increase cellular response to activin A stimulation. It is tempting to speculate that increasing GDF9 expression (268) during

folliculogenesis enhances human granulosa cell response to activin A, which leads to rising inhibin B levels in the follicular phase. With release of the oocyte after ovulation, a main source of GDF9 is removed; hence, cell response to activin A with respect to inhibin β_B-subunit is withdrawn, which may explain the decline in inhibin B secretion after ovulation. However, further studies will be required to characterize the roles of endogenous GDF9 activities in human granulosa cells and its interactions with activin A in modulating levels of inhibin and its subunits.

2.5 Figures

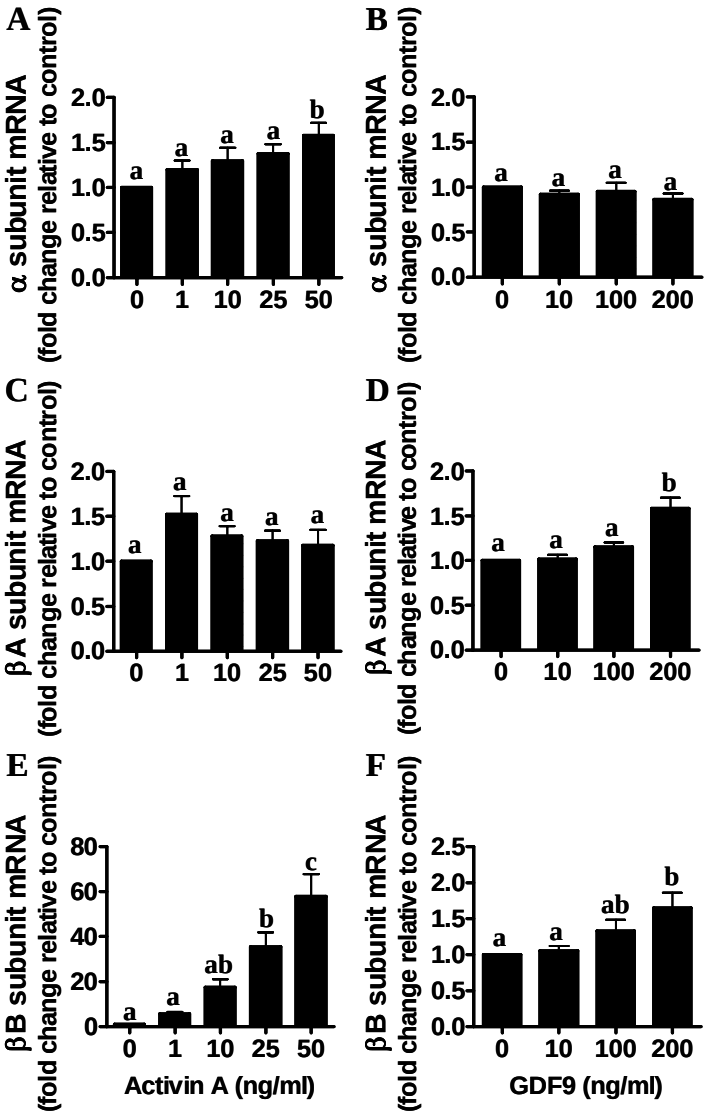


FIG. 2.1. Concentration-dependent effect of activin A (A, C, E) or GDF9 (B, D, F) on inhibin subunit mRNA level in hGL cells. After 48 h preculture, hGL cells were cultured in low-serum media (containing only 0.5% FBS) and treated with different concentration of activin A (0-50 ng/ml) or GDF9 (0-200 ng/ml) for up to 24 h. RNA of hGL cells were isolated and mRNA contents were assessed by real-time PCR. Results were the means ± SEM from at least three sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate. *Means without a common letter* are significantly different ($P < 0.05$).

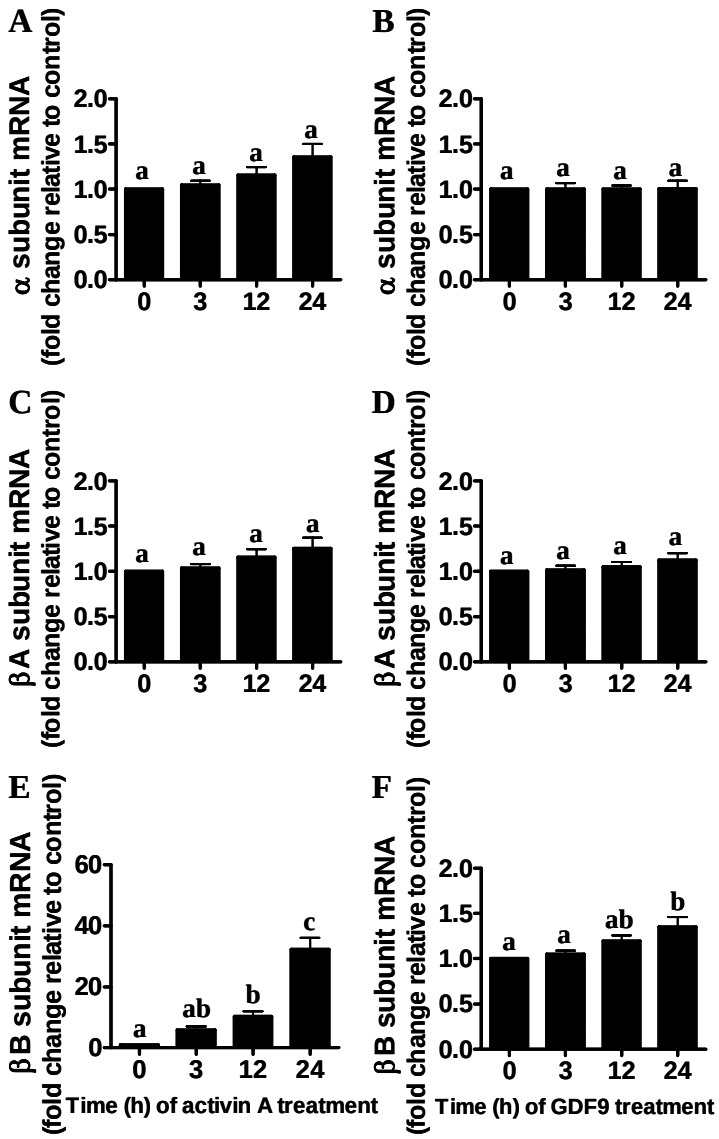


FIG. 2.2. Time-dependent effect of activin A (A, C, E) or GDF9 (B, D, F) on inhibin subunit mRNA level in hGL cells. After 48 h preculture, hGL cells were cultured in low-serum media (containing only 0.5% FBS) and treated with 25 ng/ml activin A or 100 ng/ml GDF9 for up to 24 h. RNA of hGL cells were isolated and mRNA contents were assessed by Real-time PCR. Results were the means ± SEM from at least three sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate. *Means without a common letter* are significantly different ($P < 0.05$).

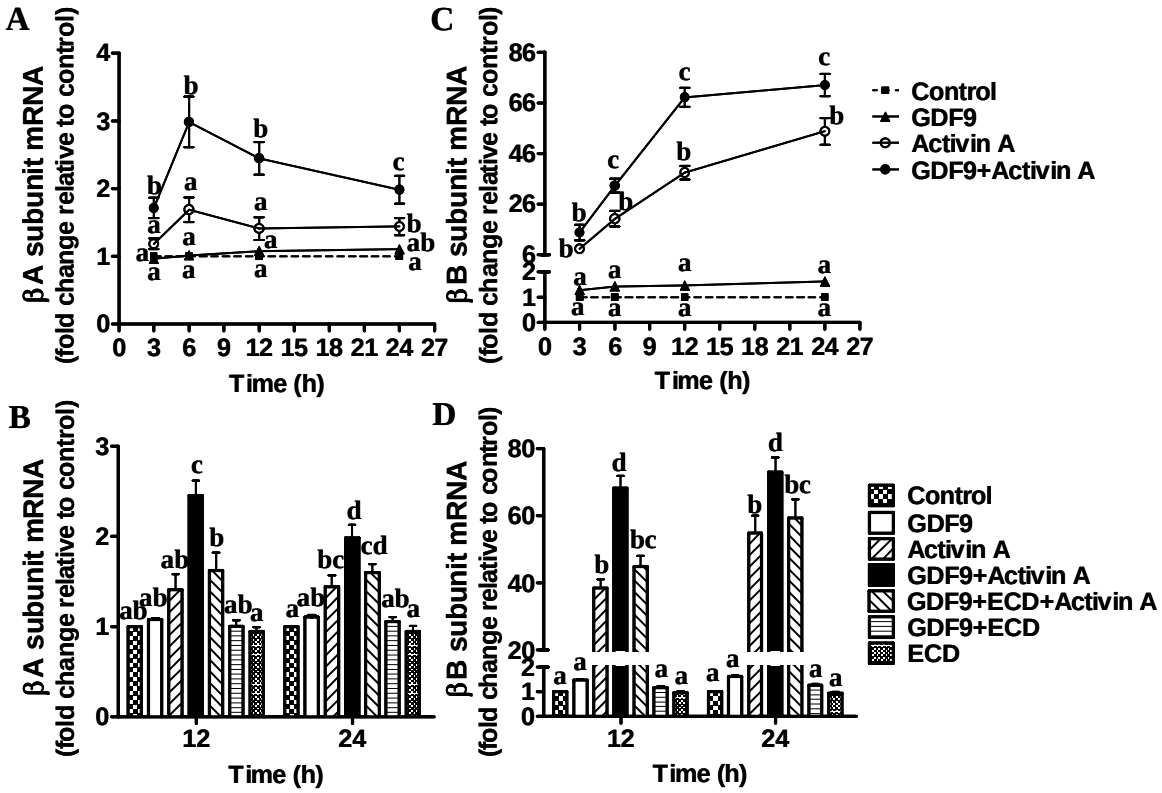


FIG. 2.3. GDF9 pretreatment enhanced activin A-induced β_A- (A, B) and β_B- (C, D) subunit mRNA level in hGL cells, effects attenuated in the presence of BMPR2 ECD, a GDF9 antagonist. After 48 h preculture, hGL cells were cultured in low-serum media (containing only 0.5% FBS). hGL cells were preincubated with 100 ng/ml of GDF9 for 24 h in low-serum media before stimulation with 25 ng/ml of activin A. In neutralization experiments to render GDF9 inactive, 2 μg/ml of BMPR2 ECD and 100 ng/ml of GDF9 were preincubated in low-serum media for 30 min before adding to cultured hGL cells. RNA of hGL cells were then isolated and mRNA contents were assessed by real-time PCR. Results were the means ± SEM from at least three sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

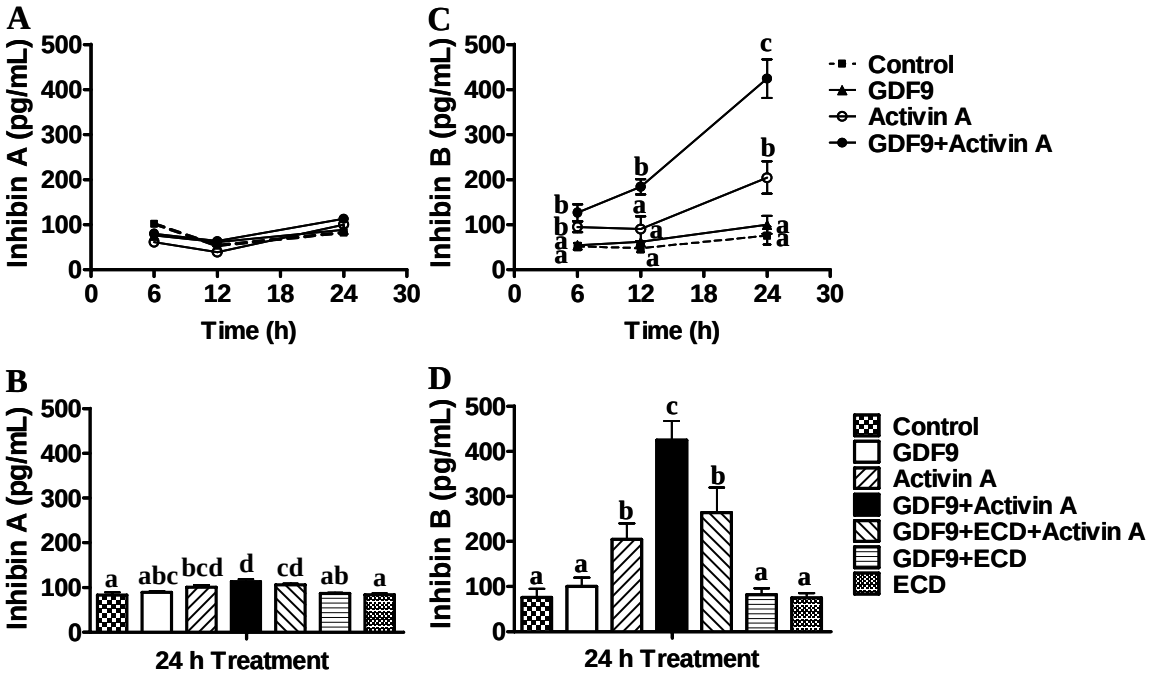


FIG. 2.4. GDF9 pretreatment enhanced activin A-induced inhibin B accumulation in culture media. The culture conditions were similar to those in Fig. 2.3. After treatments, media were collected and assayed for inhibin A (panels A and B) and B (panels C and D) levels by ELISA. Results were the means ± SEM from at least three sets of experiments (each from a separate patient), and in each set, measurements were made in duplicate. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

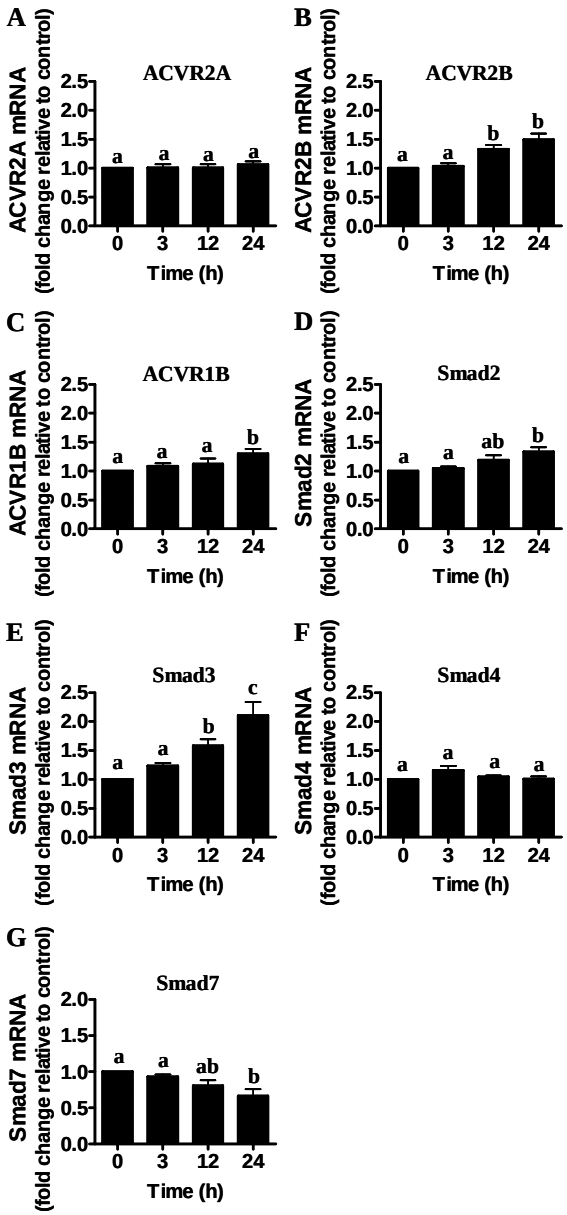


FIG. 2.5. GDF9 pretreatment for 24 h increased cell response to activin A by regulating mRNA levels of ACVR2B/1B (B and C), Smad2/3 (D and E), and Smad7 (G). After 48 h preculture, hGL cells were cultured in low-serum media (containing only 0.5% FBS) and treated with 100 ng/ml GDF9 for up to 24 h. RNA of hGL cells were isolated and activin receptors and Smad mRNA levels were assessed by real-time PCR. Results were the means ± SEM from at least three sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate. *Means without a common letter* are significantly different ($P < 0.05$).

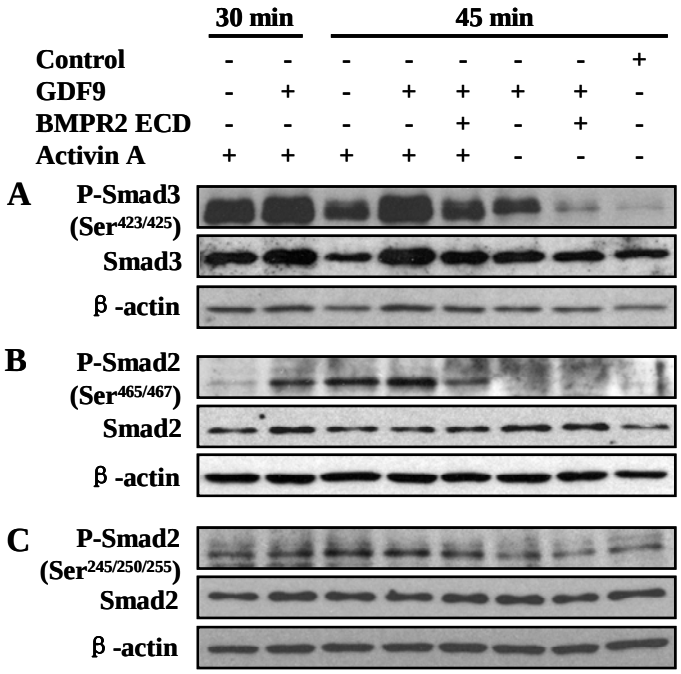


FIG. 2.6. GDF9 pretreatment for 24 h transiently enhanced phosphorylation of Smad3 ($Ser^{423/425}$) and Smad2 ($Ser^{465/467}$) induced by activin A, effects that were neutralized by BMPR2 ECD. The culture and treatment conditions were similar to those in Fig. 2.3 except for the time scale of activin A treatment. Cell lysates were collected and assessed by Western blot analysis for the expression of phosphorylated Smad3 ($Ser^{423/425}$) (A), phosphorylated Smad2 ($Ser^{465/467}$) (B), and phosphorylated Smad2 ($Ser^{245/250/255}$) (C). β-actin was used as the internal control. Results are representative of at least three sets of experiments (each from a separate patient).

3. EFFECTS OF ENDOGENOUS GDF9 ON ACTIVIN A-INDUCED INHIBIN B ACCUMULATION IN HUMAN GRANULOSA-LUTEIN CELLS[2]

3.1. Introduction

GDF9 was first identified in the early 1990s as a member of the TGFβ family (77). In mice, GDF9 is expressed mainly in the oocytes of ovarian follicles (214) but is also expressed in testicular and hypophyseal tissues (228). Female mice deficient in GDF9 are infertile and fail to demonstrate any normal follicles beyond the primary one-layer follicle stage, whereas GDF9-deficient male mice are fertile (219). Recent studies have reported GDF9 mRNA expression in human cumulus (227, 269) and granulosa cells (226, 270) and not just in the oocyte, suggesting that GDF9 may have autocrine effects on granulosa cells as well. Treatment with activin A or GDF9 alone has been shown to induce β_B subunit mRNA levels and inhibin B accumulation in hGL cells (173, 176). We have demonstrated recently that GDF9 pretreatment for 24 h can significantly enhance activin A-induced β_B mRNA levels, inhibin B accumulation and Smad2/3 phosphorylation and can induce ACVR2B/1B and Smad2/3 but reduce Smad7 (an inhibitory Smad) mRNA levels (271). However, it was unknown whether endogenous GDF9 could exert similar autocrine effects.

To characterize the effects of endogenous GDF9, we first confirmed the presence of GDF9 mRNA and protein expression in cultured hGL cells in addition to follicular fluid by real-time PCR and Western blot analysis. We then transfected hGL cells with GDF9-targeting small

[2] A version of this chapter has been published. Shi FT, Cheung AP, Huang HF, Leung PC 2009 Effects of endogenous growth differentiation factor 9 on activin A-induced inhibin B production in human granulosa-lutein cells. The Journal of Clinical Endocrinology and Metabolism 94:5108-5116

interfering RNA (siRNA) to reduce endogenous GDF9 expression and evaluated corresponding changes, with and without activin A treatment, in inhibin β_B-subunit mRNA and inhibin B levels and key components of the activin signaling pathway. Our study showed for the first time that reduced expression of endogenous GDF9 by specific siRNA in hGL cells can attenuate the stimulatory effects of activin A on inhibin β_B-subunit mRNA levels and inhibin B secretion by modulating key components of the activin signaling pathway. Hence, GDF9 may be a critical autocrine regulator of inhibin subunit mRNA levels and inhibin B accumulation by the granulosa cells during the menstrual cycle.

3.2. Materials and Methods

Preparation of hGL cells

hGL cells from each patient were extracted as described in Section 2.2. Two milliliters of the corresponding follicular fluid supernatant were stored frozen for GDF9 protein detection with Western blot analysis; $2x10^5$ viable cells were seeded per well in 12-well culture plates and cultured in the same conditions as described in Section 2.2. After 48 h, the culture media were removed, and the hGL cells were collected for RNA or protein extraction. Human GDF9 mRNA and protein levels were assessed by real-time PCR and Western blot analysis.

Knockdown analysis for human GDF9

We performed transient knockdown assays using 80 nM of GDF9-targeting siRNA or control nontargeting siRNA (ON-TARGETplus SMARTpool, Dharmacon Research, Inc., Lafayette, CO). After preculture of hGL cells for 48 h, the media were replaced by fresh culture media but without antibiotics and GDF9-targeting siRNA with Lipofectamine

RNAiMAX (Invitrogen, Carlsbad, CA), non-targeting siRNA ("control siRNA") with Lipofectamine RNAiMAX, or RNAiMAX alone ("RNAiMAX") was then added to the culture media. The cell culture, immediately after adding transfection reagents, was now designated as "Time 0 h" for all subsequent experiments described below. After 24 h ("Time 24 h"), RNAiMAX with or without siRNA in the spent media were replaced with fresh antibiotic-free culture media, and the hGL cells were cultured for another 24 hours ("Time 48 h"). The spent media were then replaced with the original culture media but containing only 0.5% FBS ("low-serum media"), and hGL cells were incubated for an additional 24 h ("Time 72 h") to starve the cells under conditions identical to those in Section 2.2. mRNA levels of GDF9, and receptors and Smads of the activin signaling pathway in harvested cells were quantified with real-time PCR at 48 and 72 h after adding the transfection reagents. Corresponding protein levels were quantified with Western blotting at 72 h. In separate experiments, 25 ng/ml activin A (Sigma) was added to the media 72 h after transfection, and hGL cells were then collected to assess inhibin β_B-subunit mRNA levels at 6, 12, and 24 h, or Smad2 and Smad3 phosphorylation at 30 and 45 min.

RNA extraction and Real-time PCR

Total RNA extraction, first-strand cDNA synthesis and real-time PCR were performed as described in Section 2.2 using primers listed in Table S1 of the Appendices.

Western blot analysis

Western blot analysis was performed as described in Section 2.2 with the following antibodies: antiphospho-Smad2 ($Ser^{245/250/255}$), antiphospho-Smad2 ($Ser^{465/467}$), antiphospho-Smad3 ($Ser^{423/425}$), anti-Smad2, and anti-Smad3 (Cell Signaling Technology); anti-ACVR2B

and anti-ACVR1B (R&D Systems); anti-GDF9, anti-Smad4, anti-Smad7 and anti-*β*-actin (Santa Cruz Biotechnology Inc.). Particularly, we used the SuperSignal Femto West (Pierce Chemical Co., Rockford, IL) reagent which is more sensitive than normal ECL reagent to detect the signal of antibody bound to GDF9. In addition, Scion Image Analysis software (Scion Co., Frederick, MD) was used to determine protein density levels.

Inhibin B assays

In experiments in which hGL cells were cultured for an additional 24 hours with or without 25 ng/ml of activin A, 72 h after siRNA transfection, the culture media were collected at 12 and 24 h and stored frozen until assay for inhibin B as described in Section 2.2.

Statistical analysis

The methods used for data analysis and presentation of results were as described in Section 2.2.

3.3. Results

GDF9 mRNA and protein levels and effects of GDF9-targeting siRNA transfection

To determine whether cultured hGL cells were capable of expressing GDF9 mRNA, we used a set of real-time PCR primers to verify the identity of the amplicon by sequencing. The PCR primers spanned the single 1577-bp intron in the GDF9 gene and yielded a single PCR fragment of the appropriate size and sequence (data not shown). GDF9 mRNA and protein were detected in cultured hGL cells from all 11 patients tested (Fig. 3.1A, *top* and *middle* panels). GDF9 protein was also detected in all follicular fluid samples on Western blot analysis (Fig. 3.1A, *bottom* panel). Transfection with siRNA specific for human GDF9 significantly

decreased GDF9 mRNA levels at 24 h and 48 h (Fig. 3.1B) and GDF9 protein at 72 h (Fig. 3.1C) in hGL cells; as expected, transfection with control siRNA or RNAiMAX (transfection reagent only) showed no changes.

Effects of GDF9-targeting siRNA transfection on activin A-induced inhibin β_B-subunit mRNA levels and inhibin B accumulations in hGL cells

After GDF9-targeting siRNA transfection, inhibin α-, and β_A-subunit mRNA levels showed no changes with RNAiMAX or control siRNA treatment at all time points (Fig. 3.2, A and B). Although activin A could induce inhibin α- and β_A-subunit mRNA levels, changes reached statistical significance only at 24 h, and the increases were small. Although these levels were lower when cells were first transfected with GDF9-targeting siRNA before activin A treatment, the observed changes were not significantly different. In contrast, activin A induced significant increases in inhibin β_B-subunit mRNA levels at 6, 12 and 24 h, effects that were significantly attenuated at all time points ($P < 0.01$; Fig. 3.2) when cells were first transfected with GDF9-targeting siRNA. Furthermore, the effects of GDF9-targeting siRNA were reversed by 100 ng/ml exogenous GDF9 (Fig. 3.3). As a comparison, RNAiMAX, control siRNA, or GDF9 siRNA alone did not induce inhibin β_B-subunit mRNA levels. Correspondingly, activin A increased inhibin B protein concentration in the culture media by 3-fold of control value ($P < 0.001$), and its effect was attenuated significantly (by 32% at 24 h) with GDF9-targeting siRNA transfection (Fig. 3.4).

Effects of GDF9-targeting siRNA transfection on mRNA and protein levels of components involved in the activin signaling pathway in hGL cells

When endogenous GDF9 activities were reduced following GDF9-targeting siRNA

transfection, levels of mRNA for ACVR2B/1B and Smad2/3 decreased, whereas those for Smad7 increased, with changes significantly different from cells treated with RNAiMAX or control siRNA at 72 h. A small decrease was also noted in Smad4 mRNA level which reached statistical significance at 72 h. These data affirm that endogenous GDF9 enhances cell response to activin A by increasing mRNA for facilitating components and simultaneously reducing that for inhibitory components of the activin A pathway (Fig. 3.5A) as demonstrated by our results from experiments with exogenous GDF9 (271). Corresponding changes in protein levels of these components were similarly observed (Fig. 3.5B).

Effects of GDF9-targeting siRNA transfection on activation of activin A-induced Smads

Transfection with GDF9-targeting siRNA reduced the levels of phosphorylated Smad3 ($Ser^{423/425}$) and Smad2 ($Ser^{465/467}$) following treatment with activin A for 30 or 45 minutes (Fig. 3.6, A and B). However, because GDF9 siRNA also reduced total Smad3 and total Smad2 levels, the ratios of phosphorylated Smad to total Smad were either lower as for Smad3 ($Ser^{423/425}$) or remained unchanged as for Smad2 ($Ser^{465/467}$) (Fig. 3.6, A and B). In contrast, there were no changes in phosphorylation of Smad2 ($Ser^{245/250/255}$) irrespective of activin A treatment and/or GDF9-targeting siRNA transfection (data not shown).

3.4. Discussion

GDF9 was originally thought to be an oocyte-specific growth factor. However, recent studies have shown that GDF9 is present in granulosa cells from monkey (223), goat (224) and pig (225) ovaries, and granulosa and cumulus cells from human ovaries (226, 227, 269, 270). Our study confirmed that GDF9 mRNA and protein were indeed present in hGL cells from all 11 patients examined (Fig. 3.1A, *top* and *middle* panels). The availability of more sensitive

real-time PCR and Western blot analysis in current studies might explain why previous studies failed to identify its presence in granulosa cells (218, 228). In addition, GDF9 protein was also detected in all our follicular fluid samples by Western blot analysis (Fig. 3.1A, *bottom* panel), findings consistent with those recently reported by Huang *et al.* (270). Taken together, these findings raise the possibility of important paracrine and autocrine roles for GDF9 in regulating ovarian functions.

Indeed, our study provided evidence for the first time of an autocrine role for endogenous GDF9 in regulating inhibin β_B-subunit mRNA expression, and hence, inhibin B accumulation by human granulosa-lutein cells. When endogenous GDF9 activities in these cells were reduced following GDF9-targeting siRNA transfection, the mRNA level of GDF9 decreased significantly at 24 h and decreased further at 48 h (Fig. 3.1B), and protein level was reduced significantly at 72 h (Fig. 3.1C). Correspondingly, the effects of activin A on inhibin β_B-subunit mRNA levels and inhibin B accumulations were diminished (Fig. 3.2-3.4). The lower inhibin β_B-subunit mRNA levels and inhibin B accumulations induced by activin A in our present experiments relative to those observed in experiments with exogenous GDF9 pretreatment in our previous study (271) were caused by the additional transfection time of 48 h before activin A treatment: ~19.3-fold *vs*. ~54.8-fold increase, respectively at 24 h for inhibin β_B-subunit; ~131.9 pg/ml *vs*. ~207 pg/ml, respectively, at 24 h for inhibin B.

We have further characterized that heightened cell response to activin A in regulating inhibin β_B-subunit mRNA expression is directly related to endogenous GDF9 mRNA expression. Thus, decreased endogenous GDF9 mRNA levels were associated with decreased ACVR2B/1B and Smad2/3/4 and increased Smad7 mRNA and protein levels in the activin signaling pathway (Fig. 3.5). Furthermore, decreased endogenous GDF9 activity was

associated with decreased activin A-induced phosphorylation of Smad3 ($Ser^{423/425}$) and Smad2 ($Ser^{465/467}$) (Fig. 3.6) but not Smad2 ($Ser^{245/250/255}$) in hGL cells.

Our findings with endogenous GDF9 mirror those reported in our previous study with exogenous GDF9 (271) in which GDF9 pretreatment for 24 h significantly enhanced activin A-induced inhibin β_B-subunit mRNA levels, inhibin B accumulation, and Smad2/3 phosphorylation [effects attenuated by BMPR2 ECD, a well-known GDF9 antagonist (235, 259, 260)] and induced ACVR2B/1B and Smad2/3 but reduced Smad7 mRNA levels. The results of our current work demonstrate similar roles for endogenous GDF9 in regulating inhibin β_B-subunit mRNA level and inhibin B accumulation and the cell signaling pathway involved and further affirm that GDF9 can activate phosphorylation of Smad2 on Ser^{465} and Ser^{467}, while other ovarian regulators are required to activate $Ser^{245/250/255}$.

The synthesis and secretion of inhibin and activin dimers in the human ovary is dependent on the regulation of the three known inhibin/activin subunits that are controlled not only by endocrine hormones but also by local factors. Although circulating inhibin B profiles (256) and inhibin B concentrations in human follicular fluid have been determined (272), the regulation of inhibin B secretion in human granulosa cells at different stages in the menstrual cycle is unclear. Some studies have shown that TGFβ family ligands such as TGFβ, activin A, and BMP2 (173, 174, 177, 273) can stimulate mRNA levels of inhibin β_B-subunit in cultured hGL cells but not inhibin α- and β_A-subunits, whereas gonadotropins can up-regulate those of inhibin α- and β_A-subunits (257). Indeed, our study shows that GDF9 can only influence activin A-induced inhibin β_B-subunit mRNA levels and inhibin B accumulations but not inhibin α- and β_A-subunit mRNA levels. Our study further affirms that Smad-dependent signaling pathway is involved in the regulation of inhibin β_B-subunit mRNA levels in hGL

cells, which is consistent with the results of Bondestam *et al*. (274) and ours on the roles of exogenous GDF9 (271).

Our current study with endogenous GDF9 further supports our proposal that increasing GDF9 expression during folliculogenesis (268) enhances human granulosa cell response to activin A, which leads to rising inhibin B levels in the follicular phase. Although it is tempting to suggest that with release of the oocyte after ovulation, a main source of GDF9 is removed (hence, the cell response to activin A with respect to inhibin β_B-subunit mRNA is withdrawn) to explain the decline in inhibin B secretion after ovulation, the contribution of GDF9 in granulosa cells in this regard remains unknown. Because GDF9 mRNA levels in granulosa cells have been positively correlated with the number of dominant follicles observed but not the number of oocytes retrieved during ovarian stimulation for IVF treatment (270), the authors suggest that GDF9 in granulosa cells may play a role in FSH-dependent follicle maturation. This may explain, in part, the higher serum inhibin B levels observed during gonadotropin treatment in women with polycystic ovary syndrome (PCOS) than women with normal ovulatory function despite similar basal inhibin B levels (Cheung AP, University of British Columbia, Vancouver, Canada; unpublished data) because women with PCOS have a predilection for developing a large number of codominant follicles during ovarian stimulation. A previous study has found similar inhibin B levels in size-matched small antral follicles from unstimulated ovaries in women with PCOS and normal ovulatory women (275). In contrast, higher circulating basal inhibin B levels, but similar inhibin B levels during monofollicular ovarian response to FSH stimulation, in women with PCOS than normal ovulatory women have been reported (276). Thus, these temporal associations are far from clear. A recent study has found decreased GDF9 mRNA levels in developing oocytes from women with PCOS or

PCO (polycystic ovaries) compared with normal ovaries; the decreased levels are evident throughout folliculogenesis, particularly at the primary and secondary stages (268). During IVF/intracytoplasmic sperm injection treatment, women with PCOS have lower GDF9 mRNA levels in cumulus cells than women with normal menstrual cycles (227). However, changes in endogenous GDF9 activities in granulosa cells and its interactions with activin A and FSH in regulating inhibin β_B-subunits and inhibin B at different stages of follicle development have not been characterized. To what extent results from granulosa-lutein cells obtained from women undergoing IVF treatment reflect normal ovarian physiology needs to be further evaluated because these cells have been exposed to pharmacological concentrations of exogenous gonadotropins and are in the process of luteinization from hCG stimulation. Further studies will be needed to determine more precisely when granulosa cell GDF9 expression begins, what regulates its expression, and what are the relative autocrine/paracrine contributions of granulosa cell *vs*. oocyte GDF9 from unstimulated ovaries. Finally, GDF9 mRNA has been detected in the human testis, uterus, and placenta and non-reproductive tissues such as bone marrow, adrenal gland, pituitary gland, and thymus (228), the rodent testis and hypothalamus (228), and the brushtail possum pituitary gland (230), suggesting that GDF9 may have actions not exclusive to the ovary.

In summary, our work indicates that the endogenous GDF9 can regulate the mRNA and protein levels of activin receptors ACVR2B/1B and downstream signaling molecules Smad 2/3/4/7. These changes, in turn, increase cellular response to activin A stimulation in inhibin β_B-subunit mRNA levels and inhibin B accumulations.

3.5. Figures

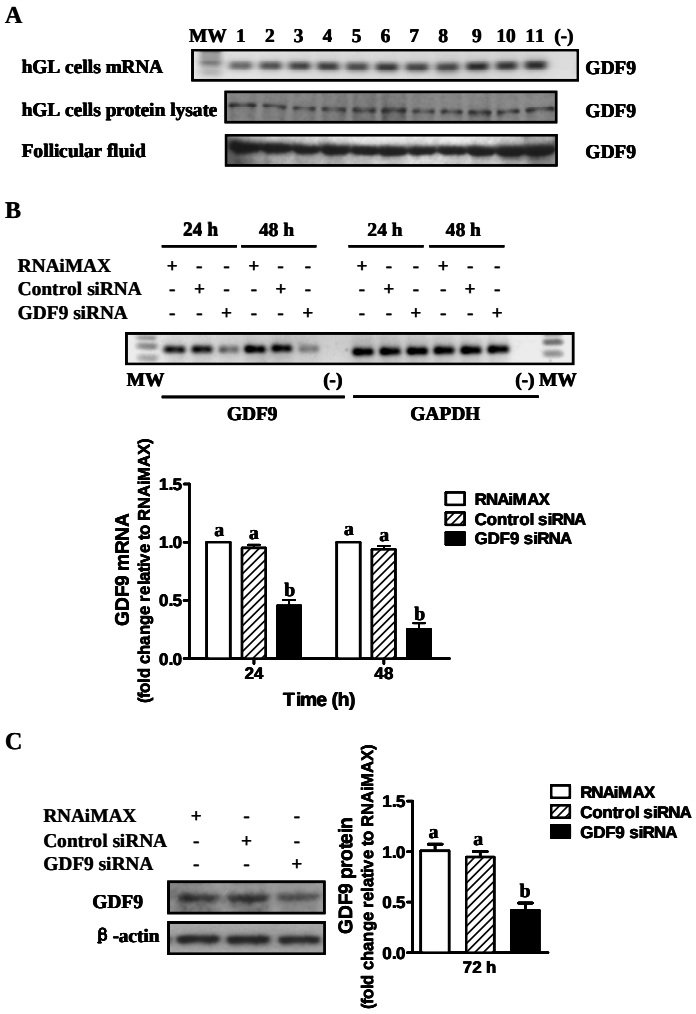


FIG. 3.1. GDF9-targeting siRNA reduced the GDF9 expression in hGL cells. A, GDF9 levels in hGL cells and follicular fluid. GDF9 mRNA was expressed in hGL cells from 11 patients by real-time PCR (*top* panel). GDF9 protein in hGL cells was represented by a major band at 51 kD on Western blot analysis, which was detected in all 11 patients tested (*middle* panel). GDF9 protein (51 kD) was similarly detected in follicular fluid from the same 11 patients (*bottom* panel). B, After preculture for 48h, hGL cells were transfected with 80 nM GDF9-targeting siRNA for 24 h and 48 h, and RNA from hGL cells was isolated, and GDF9 mRNA levels were assessed by real-time PCR. The *upper* panel shows the gel electrophoresis from one patient; the *lower* panel shows the effects of GDF9-targeting siRNA on GDF9 mRNA levels (mean ± SEM) from hGL cells of three patients. C, Forty-eight hours after adding GDF9-targeting siRNA, the culture media were replaced by low-serum media (containing 0.5% FBS), and hGL cells were cultured for one more day. hGL cell protein was isolated, and GDF9

protein was assessed by Western blot analysis. The *left* panel shows the immunoblots from one patient; the *right* panel shows the effects of GDF9-targeting siRNA on GDF9 protein levels (mean ± SEM) from hGL cells of the same three patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$). MW, molecular weight standards; (-), no cDNA control.

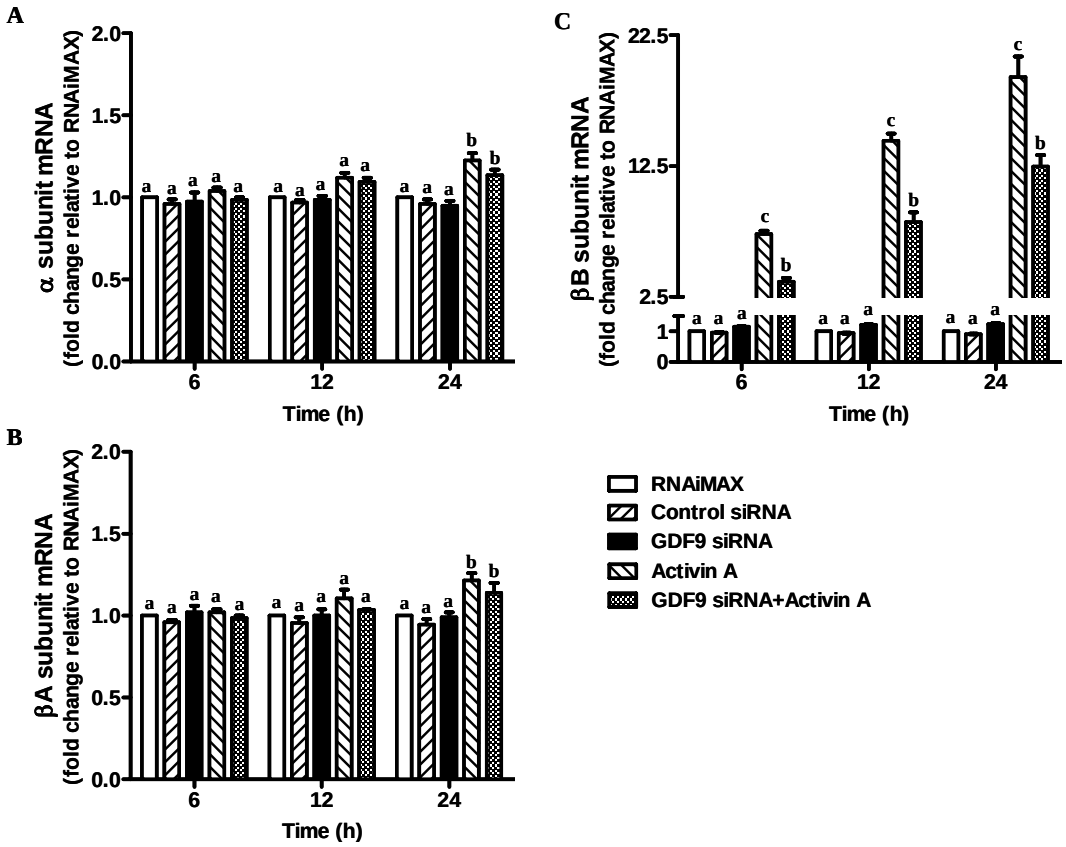


FIG. 3.2. GDF9-targeting siRNA diminished the effects of activin A on levels of inhibin β_B-subunit mRNA in hGL cells. After preculture for 48h, hGL cells were transfected with 80 nM GDF9-targeting siRNA for 48h. The culture media were replaced by low-serum media (0.5% FBS), and hGL cells were cultured for one more day before treatment with 25 ng/ml activin A for 6, 12, and 24 h. RNA was isolated from hGL cells, and inhibin subunit mRNA levels were assessed by real-time PCR. Results are the means ± SEM from hGL cells of four patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

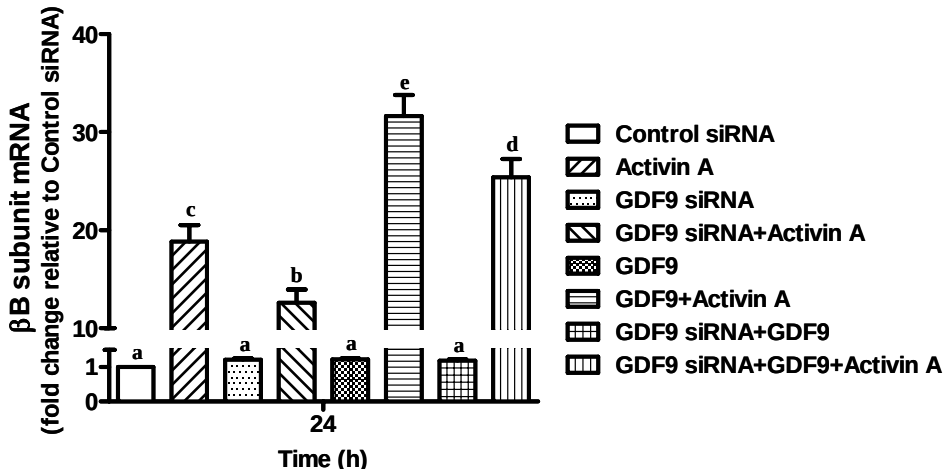


FIG. 3.3. GDF9-targeting siRNA diminished the effects of activin A on levels of inhibin β_B-subunit mRNA in hGL cells, and the effects of siRNA were reversed by exogenous GDF9 treatment. After preculture for 48 h, hGL cells were transfected with 80 nM GDF9-targeting siRNA for 48 h. The culture media were then replaced by low-serum media (0.5% FBS), and hGL cells were cultured with and without 100 ng/ml of GDF9 for another 24 h before incubating with 25 ng/ml activin A for a further 24 h period. RNA was then extracted from cells, and inhibin β_B-subunit mRNA levels in the cells were measured by real-time PCR. Results are the means ± SEM from hGL cells of three patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

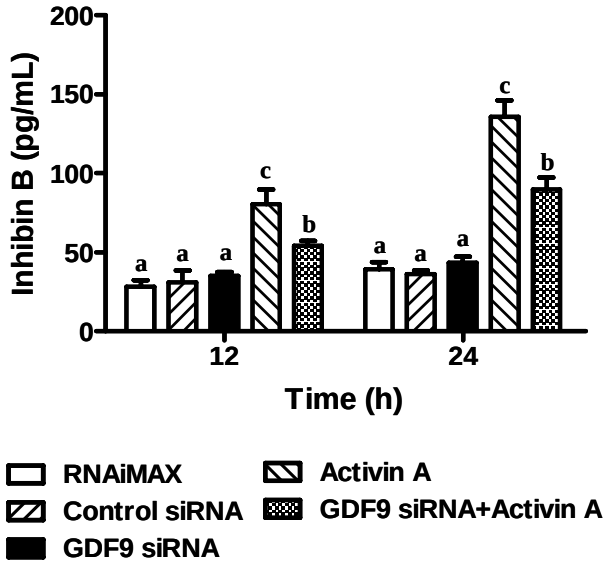


FIG. 3.4. GDF9-targeting siRNA reduced activin A-induced inhibin B accumulations in culture media. The culture conditions were same as those in Fig. 3.2. After treatments, media were collected and assayed for inhibin B levels by ELISA. Results are the means ± SEM from hGL cells of four patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

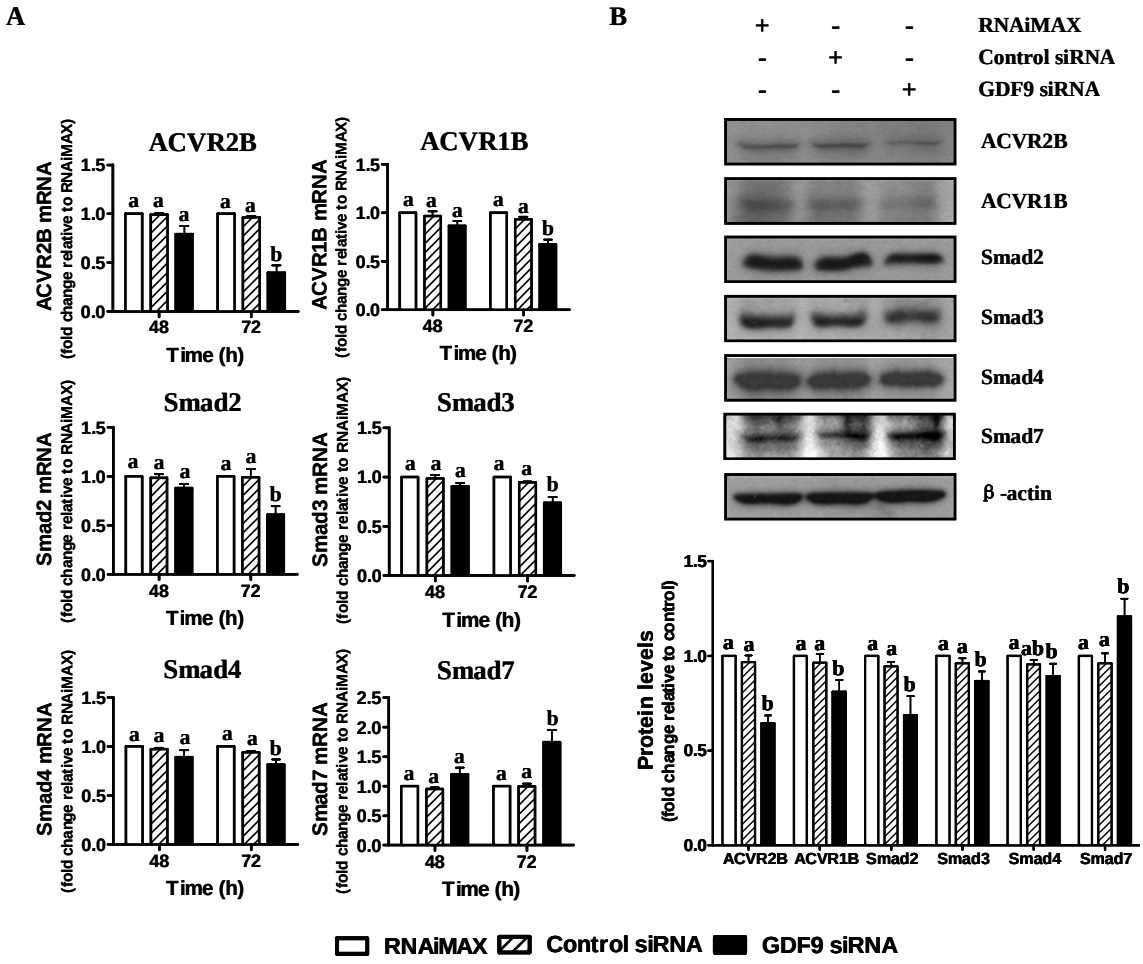


FIG. 3.5. GDF9-targeting siRNA decreased cell response to activin A by regulating mRNA levels for ACVR2B/1B, Smad2/3, and Smad7 in the activin signaling pathway. After preculture for 48 h, hGL cells were transfected with 80 nM GDF9-targeting siRNA for 48 h. The culture media were replaced by low-serum media (0.5% FBS), and hGL cells were cultured for one more day. A, mRNA levels of the relevant receptors and Smads of the activin signaling pathway were measured by real-time PCR after transfection with GDF9-targeting siRNA for 48 and 72 h. Results are the means ± SEM from hGL cells of four patients. B, Protein levels of the relevant receptors and Smads of the activin signaling pathway were measured by Western blot analysis after transfection with GDF9-targeting siRNA for 72 h. The *upper* panel shows the immunoblots from one patient; the *lower* panel shows the effects of GDF9-targeting siRNA on the protein levels of the relevant receptors and Smads of the activin signaling pathway. Results are the means ± SEM from hGL cells of three patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

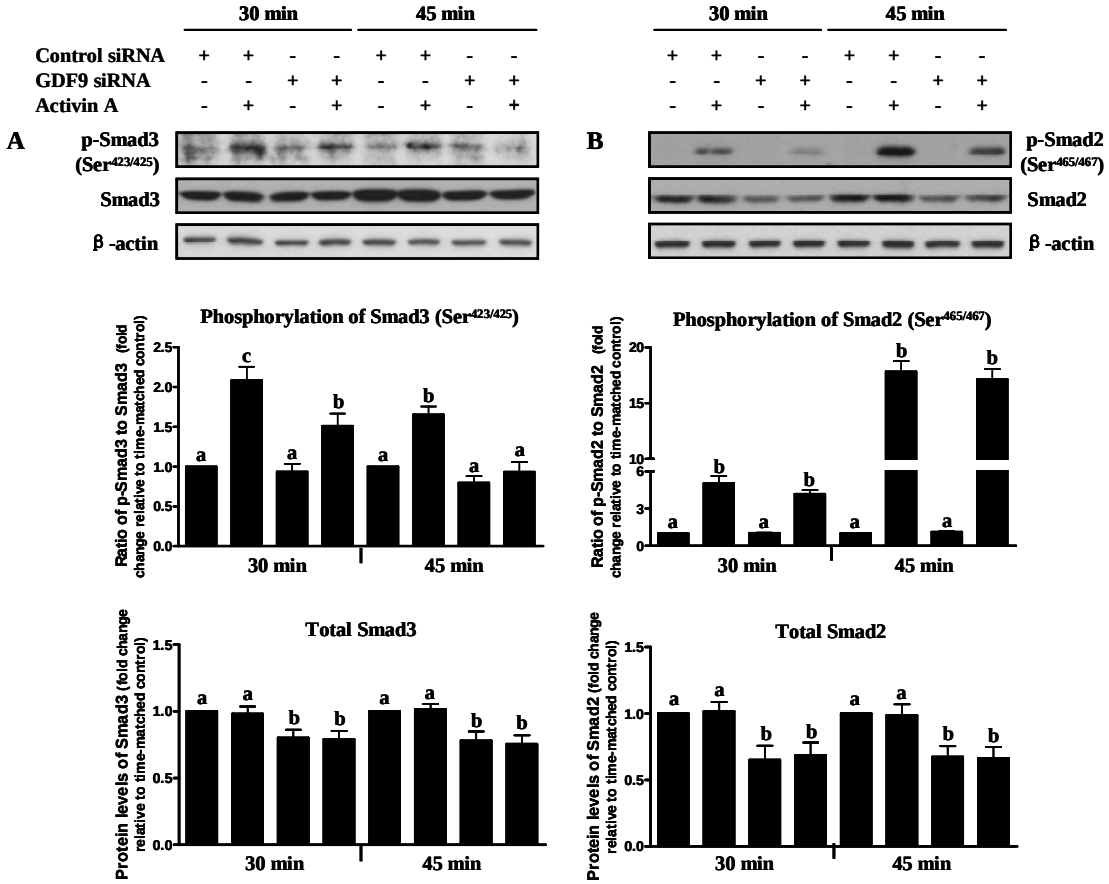


FIG. 3.6. GDF9-targeting siRNA decreased phosphorylation of Smad3 ($Ser^{423/425}$) and Smad2 ($Ser^{465/467}$) in hGL cells induced by activin A (25 ng/ml). After preculture for 48 h, hGL cells were transfected with 80 nM GDF9-targeting siRNA for 48 h, the culture media were replaced by low-serum media (0.5% FBS), and hGL cells were cultured for one more day before treatment with 25 ng/ml activin A for 30 and 45 min. Cell lysates were collected, and levels of Smad3 ($Ser^{423/425}$) (A) and Smad2 ($Ser^{465/467}$) phosphorylation were assessed by Western blot analysis (B). *β*-actin was used as the internal control. The *top* panel shows the immunoblots from one patient, and the *middle* panel shows the effects of GDF9-targeting siRNA on activin A-induced phosphorylated Smad3 (pSmad3) and phosphorylated Smad2 (pSmad2). Levels of pSmad3 and pSmad3 were normalized to the levels of total Smad3 (tSmad3) and total Smad2 (tSmad2), respectively, and are expressed as fold change relative to time-matched controls (control siRNA). The *bottom* panel shows tSmad3 and tSmad2 levels normalized to *β*-actin levels. Results are the means ± SEM from hGL cells of three patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

4. GDF9 SUPPRESSES FOLLISTATIN AND FOLLISTATIN -LIKE 3 PROTEIN ACCUMULATION IN HUMAN GRANULOSA -LUTEIN CELLS[3]

4.1. Introduction

We have recently reported (271, 277) that exogenous and endogenous GDF9 can significantly enhance activin A-induced inhibin β_B-subunit mRNA levels by inducing activin receptors (ACVR2B/1B) and Smad2/3 but reducing Smad7 (an inhibitory Smad) mRNA levels in hGL cells. We have also confirmed GDF9 expression in hGL cells (277). However, its effects on extracellular regulators of activin such as FST and FSTL3 remain unknown.

Activin A can promote FST production in undifferentiated and partially differentiated rat granulosa cells, but suppress FST production in fully differentiated granulosa cells (278). Activin A can cause a 1.8-fold rise in FST release in rat anterior pituitary cells, suggesting an autocrine/paracrine role of activin and FST in the pituitary (279). Activin A can also increase FST and FSTL3 mRNA and protein levels in the human hepatoma cell line HepG2 (280) but whether the same occurs in human granulosa cells is unknown. The objectives of the present work were to examine the effects of GDF9 on FST and FSTL3 expression, with and without activin A treatment, as potential mechanisms on its enhancing action on activin A-induced inhibin β_B-subunit in hGL cells.

[3] A version of this chapter will be submitted for publication. Shi FT, Cheung AP, Huang HF, Leung PC 2010 Growth differentiation factor 9 (GDF9) suppresses follistatin and follistatin-like 3 protein production in human granulosa-lutein cells.

4.2. Materials and Methods

Firstly, we compared FST and FSTL3 mRNA in hGL cells and protein in culture media with and without GDF9 treatment in time- and concentration-dependent experiments. Secondly, we explored the effects of GDF9 on activin A-induced FST and FSTL3 mRNA and protein levels. Thirdly, we transfected hGL cells with GDF9 siRNA to assess changes in basal and activin A-induced FST and FSTL3 mRNA and protein levels. Fourthly, we compared inhibin β_B-subunit mRNA levels after activin A treatment with and without FST or FSTL3 siRNA. Finally, to further evaluate if the enhancing effect of GDF9 on activin A-induced inhibin β_B-subunit mRNA is related to FST or FSTL3 expression, we measured these changes in activin A-treated hGL cells (with and without GDF9) at different concentrations of FST or FSTL3.

Preparation of hGL cells

hGL cells from each patient were extracted and cultured as described in Section 2.2, and seeded as described in Section 3.2.

Activin A, GDF9, FST and FSTL3 experiments

After preculture of hGL cells for 48 h, the low-serum medium containing 0.5% FBS instead of 10% FBS was added to each well and the cell culture was now designated as "Time 0 h" for all subsequent experiments described below. For time-dependent experiments, cells were treated with the concentration of 100 ng/ml of GDF9 for 12, 24 and 48 h. Cells were stimulated with 1-200 ng/ml GDF9 for 24 h in concentration-response studies. For experiments with both GDF9 and activin A, cells were preincubated with 100 ng/ml of GDF9 in low-serum media for 24 h before stimulation with 25 ng/ml of activin A. In neutralization experiments to

render GDF9 inactive, 2 μg/ml of BMPR2 ECD (R&D Systems) and 100 ng/ml of GDF9 were preincubated in low-serum media for 30 min before adding to cultured hGL cells. To test the effects of recombinant FST (288-amino acid FST; Peprotech) or FSTL3 (R&D Systems) on activin A-induced inhibin β_B-subunit mRNA expression, different concentrations of FST or FSTL3 were preincubated with 25 ng/ml activin A at 37 C for 1 h in PBS containing 0.1% BSA before adding to hGL cells which were then cultured for 24 h.

Knockdown analysis for human GDF9, FST or FSTL3

We performed transient knockdown assays with 80 nM of GDF9, FST or FSTL3 siRNA using non-targeting siRNA (ON-TARGETplus SMARTpool; Dharmacon Research Inc.) as control. The conditions of siRNA transfection were same as described in Section 3.2. mRNA levels of FST or FSTL3 were then quantified with real-time PCR at 48 and 72 h after adding the transfection reagents. Corresponding protein levels were quantified with ELISA at 72 h after siRNA transfection. In separate experiments, the spent media at "Time 48 h" were replaced with low-serum media and hGL cells were incubated with and without 100 ng/ml GDF9 for one more day ("Time 72 h"), and FST or FSTL3 mRNA in cells and protein levels in culture media were quantified with real-time PCR and ELISA respectively.

RNA extraction and Real-time RT-PCR

Total RNA extraction, first-strand cDNA synthesis and real-time PCR were performed as described in Section 2.2 using primers listed in Table S1 of the Appendices.

FST and FSTL3 assays

After treatment, culture media were collected and stored frozen until assay with specific

ELISA kits (FST, Peprotech; FSTL3, R&D Systems); corresponding hGL cells were lysed with lysis buffer, and total cellular protein content determined as described earlier. The lowest limits of detection for FST and FSTL3 were 23 pg/ml, and 312.5 pg/ml, respectively. All hormone measurements were performed in duplicate. Secreted hormone levels were normalized to total cellular protein content.

Statistical analysis

The methods used for data analysis and the descriptions of results were as described in Section 2.2.

4.3. Results

Effects of GDF9 on FST and FSTL3 mRNA and protein levels

GDF9 significantly decreased FST and FSTL3 mRNA levels in a time-dependent manner with maximum effects at 48 h (Fig. 4.1, A and B). GDF9 also reduced mRNA levels of FST and FSTL3 in a concentration-dependent manner which reached statistical significance at both the 100 and 200 ng/ml concentrations (P values all < 0.01) (Fig. 4.1, C and D). BMPR2 is the type 2 receptor for GDF9 and the ECD of BMPR2 is a well known GDF9 antagonist (235, 259, 260, 271, 277). When 100 ng/ml of GDF9 was preincubated with BMPR2 ECD for 30 min before adding to the cell culture, the inhibitory effects of GDF9 on FST and FSTL3 mRNA levels were attenuated (Fig. 4.1, C and D; P values all < 0.05). Correspondingly, GDF9 decreased FST and FSTL3 protein levels in a concentration-dependent manner and reached statistical significance at the 100 ng/ml concentration (P values all < 0.05); as a result, no significant decreases were observed when GDF9 was first neutralized with BMPR2 ECD (Fig.

4.1, E and F; *P* values all < 0.05). As expected, there were no significant changes in FST and FSTL3 mRNA levels relative to controls when BMPR2 ECD alone was added. Basal protein levels of FST in culture media were significantly higher than FSTL3 (Fig. 4.1, E and F, 4450 *vs*. 548 pg/ml, $P < 0.001$; Fig. 4.2, 4586 *vs*. 573 pg/ml, $P < 0.001$); correspondingly, the absolute decreases in FST accumulations following GDF9 treatment were greater than those of FSTL3 (Fig. 4.1, E and F, 1440 *vs.* 136 pg/ml at the GDF9 concentration of 200 ng/ml; $P < 0.001$).

Effects of GDF9 on activin A-induced FST and FSTL3 mRNA and protein levels

Activin A increased both FST and FSTL3 mRNA levels (Fig. 4.3A; *P* values all < 0.001). In contrast, GDF9 suppressed basal and activin A-induced FST and FSTL3 mRNA, effects that were attenuated by BMPR2 ECD (Fig. 4.3A; *P* values all < 0.05). As noted earlier, BMPR2 ECD alone had no effects on FST and FSTL3 mRNA levels. Changes in FST and FSTL3 protein levels in culture media followed an identical pattern to changes in mRNA levels (Fig. 4.3B). However, FST mRNA peaked at 12 h while FSTL3 peaked at 24 h in response to activin A or activin A with GDF9 (Fig. 4.3A).

Effects of GDF9-targeting siRNA transfection on basal and GDF9-reduced FST and FSTL3 mRNA and protein levels

When endogenous GDF9 levels decreased following GDF9 siRNA transfection (see Fig. 3.1B for details), there were significant increases in mRNA levels of FST and FSTL3 at 48 h and 72 h (Fig. 4.4A; *P* values all < 0.001), and corresponding proteins levels (Fig. 4.4B; *P* values all < 0.001). Furthermore, these effects of GDF9 siRNA were attenuated at 24 h after 100 ng/ml GDF9 was added to the culture ("Time 72 h" in Fig. 4.4; *P* values all < 0.001). As a

comparison, transfection with control siRNA showed no changes relative to transfection reagent only ("RNAiMAX").

Effects of GDF9-targeting siRNA transfection on activin A-induced FST and FSTL3 mRNA and protein levels

GDF9 siRNA transfection increased basal and activin A-induced FST mRNA levels at 12 h (*P* values all < 0.05) and 24 h in hGL cells (*upper* panel), and basal and activin A-induced FSTL3 mRNA at 12 and 24 h (*lower* panel; *P* values all < 0.05), effects that were attenuated after 100 ng/ml GDF9 was added to the culture (*P* values all < 0.05) (Fig. 4.5A). Corresponding changes in accumulations of FST and FSTL3 in the culture media showed a similar pattern to mRNA levels (Fig. 4.5B).

FST or FSTL3-targeting siRNA enhanced activin A-induced inhibin β_B-subunit mRNA levels

As expected, control siRNA, GDF9-, FST- or FSTL3-targeting siRNA in the absence of activin A had no effect on the basal inhibin β_B-subunit mRNA levels. In the presence of activin A and consistent with our previous study (277), GDF9 treatment increased while GDF9 siRNA transfection decreased inhibin β_B-subunit mRNA levels (Fig. 4.6B; *P* values all < 0.05). With reduced endogenous FST or FSTL3 expression (Fig. 4.6A; *P* values all < 0.001) after targeting siRNA transfection, activin A-induced inhibin β_B-subunit mRNA levels increased from 15.7 to 27.7 fold for FST and 21 fold for FSTL3 (Fig. 4.6B; *P* values all < 0.05).

FST or FSTL3 reversed GDF9 enhanced effect in activin A-induced inhibin β_B-subunit mRNA level

To further confirm that GDF9 enhanced activin A-induced inhibin β_B-subunit mRNA

levels by reducing FST or FSTL3 expression, we compared these changes in the presence of different concentrations of FST and FSTL3. We chose FST concentrations of 1, 2, 4, and 50 ng/ml and FSTL3 concentrations of 0.1, 0.2, 0.4, 4 and 50 ng/ml based on changes in FSH or FSTL3 protein levels following GDF9 treatment (Fig. 4.1E and F) and that FST or FSTL3 binds to activin in a 2:1 molar ratio (210, 212, 281, 282). Increasing concentrations of FST (1-4 ng/ml) or FSTL3 (0.1-0.4 ng/ml) attenuated activin A-induced inhibin β_B-subunit mRNA levels with levels completely suppressed at the saturated concentration of 50 ng/ml for both (Fig. 4.7). In the presence of GDF9, inhibin β_B-subunit mRNA levels decreased in a concentration-dependent manner for both FST and FSTL3 (Fig. 4.7).

4.4. Discussion

We have recently reported that exogenous and endogenous GDF9 enhances activin A-induced expression of inhibin β_B-subunit mRNA in hGL cells through modulation of activin receptors and key components of the intracellular signaling pathway (271, 277). Using the same hGL cell culture system in our current study, we have demonstrated for the first time that GDF9 can decrease not only basal but also activin A-induced mRNA and protein levels of FST and FSTL3, known extracellular inhibitors of activin (132, 186). These actions of GDF9 were supported by our experiments when GDF9 effects were neutralized by BMPR2 ECD or when endogenous GDF9 levels were reduced by targeting siRNA transfection. We therefore hypothesize that GDF9 decreases FST or FSTL3 expression which allows more activin A to bind to its receptors and hence, enhances activin A-induced inhibin β_B-subunit mRNA levels. Our hypothesis is further supported by results from two additional experimental approaches. First, reduced endogenous FST or FSTL3 mRNA and protein levels after targeting siRNA

transfection augmented activin A-induced inhibin β_B-subunit mRNA levels similar to those observed with GDF9 treatment (Fig. 4.6B). Second, the enhancing effects of GDF9 on activin A-induced inhibin β_B-subunit mRNA levels were attenuated by exogenous FST or FSTL3 in a concentration-dependent manner (Fig. 4.7).

Alternative precursor mRNA splicing produces two main isoforms of mature mammalian follistatin, a core protein of 315 amino acids (FST315) and a carboxy-truncated variant of 288 amino acids (FST288) (195, 204, 283). Although both isoforms have a similar binding affinity for activin (206), FST288 also has a high affinity for heparin (205, 206). In rat pituitary cells, complexes of activin and FST288 bind to cell surface proteoglycans via the heparin binding site of FST288 and is a mechanism by which activin is targeted for degradation (207). Although FST315 has the same affinity for activin as FST288, it is primarily present in the human circulation (213) and does not bind to heparin. Instead, FST315 acts as a storage for follistatin in the circulation, which delivers activin to target cells and prevents activin from binding to FST288, and hence, degradation. However, the actual function of FST315 is yet to be elucidated. We could not differentiate the roles of these isoforms because anti-human FST antiserum used in our follistatin assay was raised against a mixture of FST isoforms. The relative ratio of activin-free to activin-bound FST is essential in determining the bioavailability of activin and estimating the potential endocrine function of circulating FST but free follistatin assays are not commercially available at present. Because FST binds activins and inhibins through the common β-subunit (212), we cannot exclude the possibility that FST may also interact with inhibins. However, FST has a 500 to 1000 fold higher affinity for activins than inhibins (162).

FSTL3, which shares several structural features with FST, does not have a heparin binding

sequence and therefore, does not bind cell-surface proteoglycans under normal conditions (284). Isoforms of FST are secreted faster than FSTL3 by stable, transfected CHO cells and the amount of newly synthesized FSTL3 localized in the nucleus is still substantial for up to 8 h, which is significantly longer than that for FST (211). This may explain partly why the protein concentration of FST in the media of our cultured hGL cells was about 8 fold higher than that of FSTL3 (Fig. 4.2, 4586 *vs*. 573 pg/ml, $P < 0.001$). Despite a similar decrease of FST or FSTL3 mRNA and protein levels 48 h after targeting siRNA transfection (Fig. 4.6A), activin A-induced inhibin β_B-subunit mRNA levels were higher following FST siRNA (Fig. 4.6B; $P < 0.05$). Furthermore, 4 ng/ml FST had a stronger effect than the same concentration of FSTL3 in reversing activin A-induced inhibin β_B-subunit mRNA in the presence of GDF9 although this difference did not reach statistical significance (Fig. 4.7). Whether this indicates that FST may play a more dominant role than FSTL3 in regulating activin A action requires further studies.

FST protein levels were lower following FST siRNA transfection (from 4601 to 2544 pg/ml, Fig. 4.6A *lower* panel) than 100 ng/ml GDF9 treatment (from 4451 to 3266 pg/ml, Fig. 4.1E), but corresponding activin A-induced inhibin β_B-subunit mRNA levels were lower after FST siRNA transfection than GDF9 pre-treatment (Fig. 4.6B). This difference in activin A-induced inhibin β_B-subunit mRNA response may suggest that GDF9 acts through not just the extracellular (FST and FSTL3) but the intracellular (activin receptors and Smads) mechanisms as reported in our previous studies (271, 277). However, we cannot exclude the additional, although smaller effect of FSTL3 accounting for this difference. In addition, we also cannot rule out the possibility that GDF9 may affect other extracellular inhibitors such as the BMP and activin membrane-bound inhibitor (BAMBI). BAMBI is a transmembrane protein related to the TGFβ superfamily type I receptors. However, BAMBI lacks an intracellular kinase

domain and can block activin signaling by forming stable associations with activin type IB receptor (ACVR1B, also known as ALK4) but not activin type IA receptor (ACVR1A, also known as ALK2) (124).

Higher FST levels observed in some women with polycystic ovary syndrome (PCOS) have led to the suggestion that altered FST function may contribute to the PCOS phenotype (285). However, an updated study on allelic variants of the follistatin gene in PCOS suggests that the contribution of the follistatin gene to the etiology of PCOS is small (286). In mouse models, over-expression of FST has also been shown to result in a PCOS-like phenotype (287). Decreased GDF9 mRNA levels have been found in developing oocytes from women with PCOS or polycystic ovaries compared to women with normal ovaries; the decreased levels are evident throughout folliculogenesis, beginning at recruitment initiation and continuing through the small, Graafian follicle stage (268). Although increased FST or FSTL3 expression with decreased endogenous GDF9 levels after targeting siRNA transfection (Fig. 4.4) may provide a mechanism by which altered GDF9 expression can affect follicle development, our granulosa cells were not specifically obtained from women with PCOS. Future studies comparing the interactions of FST or FSTL3 and GDF9 in granulosa cells from women with and without PCOS may shed new insight on the pathophysiology of this condition.

While our granulosa cell culture systems had provided a convenient model to study the inter-relationships of FST or FSTL3 and GDF9, these cells were exposed to pharmacological concentrations of exogenous gonadotropins and were undergoing luteinization following hCG administration. However, in the absence of human granulosa cells from normal ovaries for research, findings from our cell culture systems do provide interesting hypotheses on the potential role of GDF9 in granulosa-lutein cell functions.

In summary, our previous studies suggest that GDF9 enhances activin A-induced inhibin β_B-subunit mRNA levels in hGL cells by regulating receptors and crucial intracellular components of the activin signaling pathway. Our current study shows that GDF9 can decrease FST and FSTL3 expression in addition, which then allows more free activin A to bind to its receptors and activate the signaling pathway downstream. Whether this extracellular mechanism is instrumental to explain our previous findings or whether GDF9 also has an intracellular regulating role requires further research.

4.5. Figures

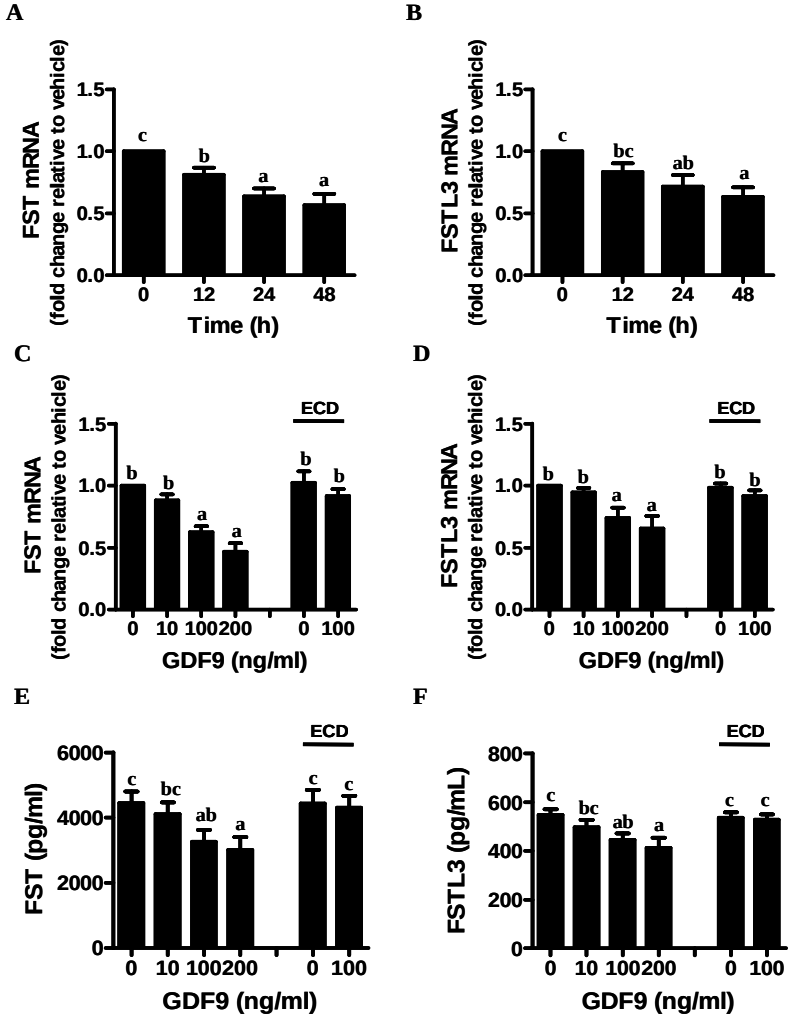


FIG. 4.1. Time- (A and B) and concentration- (C and D) dependent effect of GDF9 on FST and FSTL3 mRNA levels in hGL cells and corresponding concentration-response in protein levels (E and F). After 48 h preculture, the culture media were replaced with low-serum media (0.5% FBS); hGL cells were then treated with 100 ng/ml GDF9 for 12 h ("Time 12 h"), 24 h ("Time 24 h") and 48 h ("Time 48 h") in time-dependent experiments (A and B), or with different concentrations of GDF9 for 24 h in concentration-dependent experiments (C, D, E and F). In neutralization experiments to render GDF9 inactive, 2 μg/ml of BMPR2 ECD ("ECD") and 100 ng/ml of GDF9 were preincubated in low-serum media for 30 min before adding to cultured hGL cells. FST and FSTL3 mRNA levels in hGL cells and protein concentrations in culture media were assessed by real-time PCR and ELISA, respectively. Results were the means ± SEM from at least four sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate for real-time PCR or duplicate for ELISA. *Means without a common letter* are significantly different ($P < 0.05$).

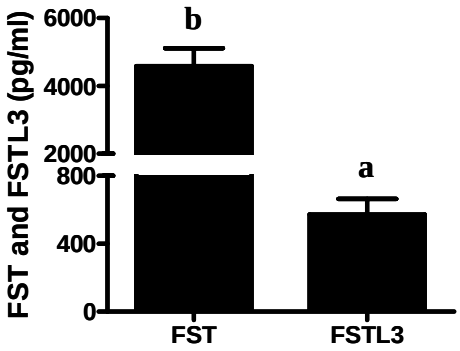


FIG. 4.2. Comparison of FST and FSTL3 protein levels in culture media of hGL cells. After 48 h preculture, hGL cells were cultured in low-serum media (0.5% FBS) for another 24 h. Culture media were then collected and protein concentrations of FST and FSTL3 were assessed by ELISA. Results were the means ± SEM from at least six sets of experiments (each from a separate patient), and in each set, measurements were made in duplicate for ELISA. *Means without a common letter* are significantly different ($P < 0.05$).

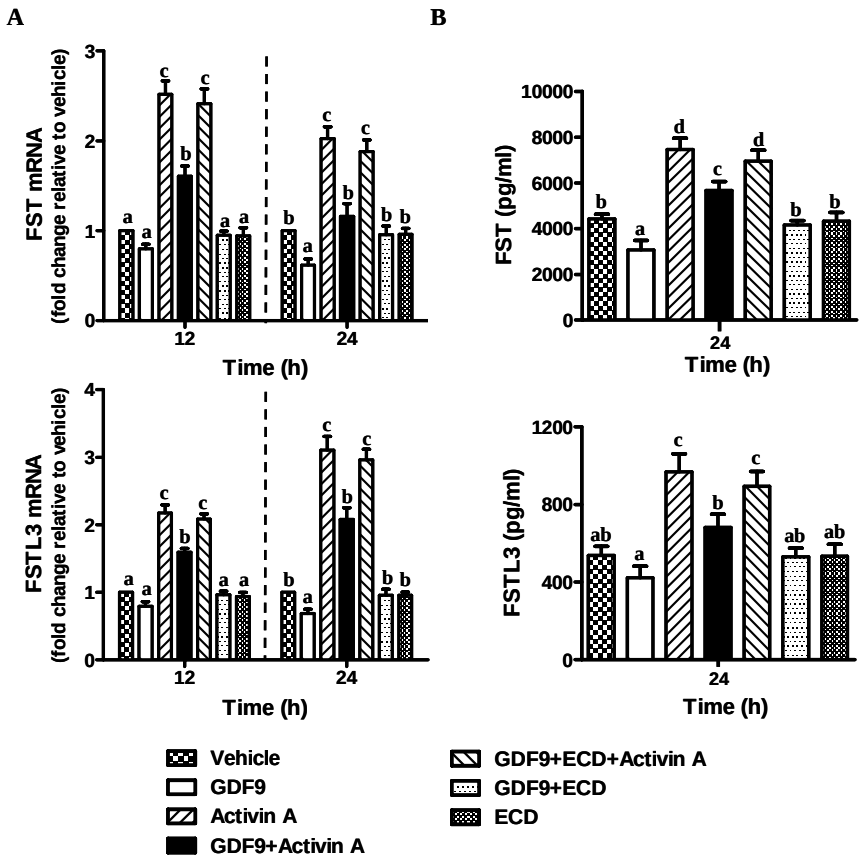


FIG. 4.3. GDF9 reversed activin A-induced FST and FSTL3 mRNA (A) and protein (B) levels, effects attenuated by BMPR2 ECD ("ECD"). After 48 h preculture, hGL cells were incubated in low-serum media (0.5% FBS) with and without 100 ng/ml of GDF9 for another 24 h before stimulation with 25 ng/ml of activin A for 12 h ("Time 12 h") and 24 h ("Time 24 h"). The neutralization experiments with BMPR2 ECD and GDF9 were as described in Fig. 4.1. FST and FSTL3 mRNA levels in hGL cells and protein concentrations in culture media were assessed by real-time PCR and ELISA, respectively. Results were the means ± SEM from at least four sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate for real-time PCR or duplicate for ELISA. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

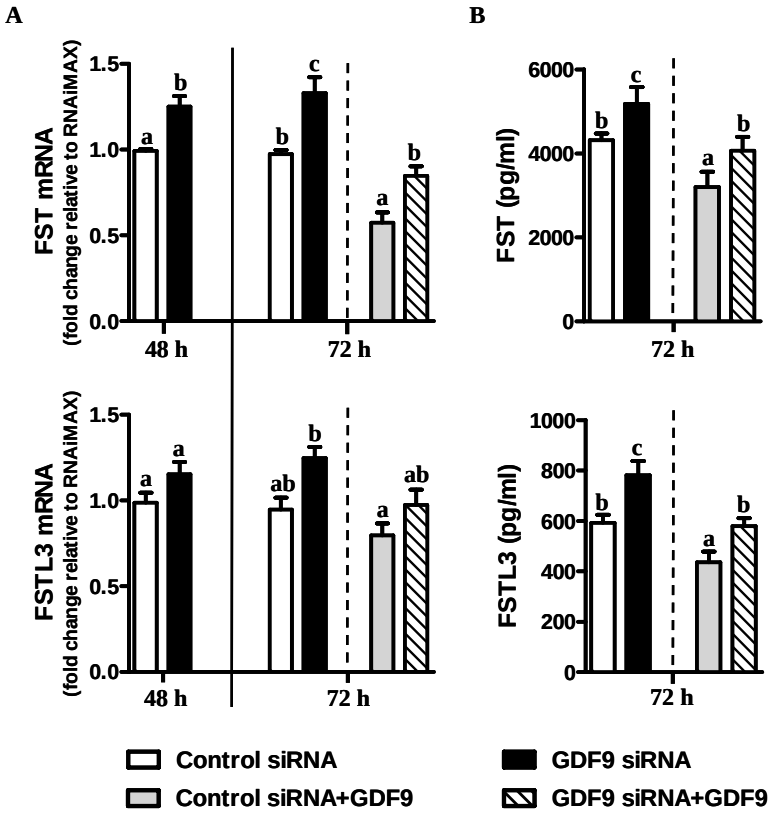


FIG. 4.4. GDF9-targeting siRNA increased mRNA (A) and protein (B) levels of FST and FSTL3, effects that were reversed by adding exogenous GDF9. After preculture for 48 h, hGL cells were transfected with 80 nM GDF9 targeting siRNA for 48 h ("Time 48 h"). The culture media were then replaced by low-serum media (0.5% FBS), and hGL cells were cultured with and without 100 ng/ml GDF9 for another 24 h ("Time 72 h"). FST and FSTL3 mRNA levels in hGL cells and protein concentrations in culture media were assessed by real-time PCR and ELISA, respectively. Results were the means ± SEM from at least four sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate for real-time PCR or duplicate for ELISA. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

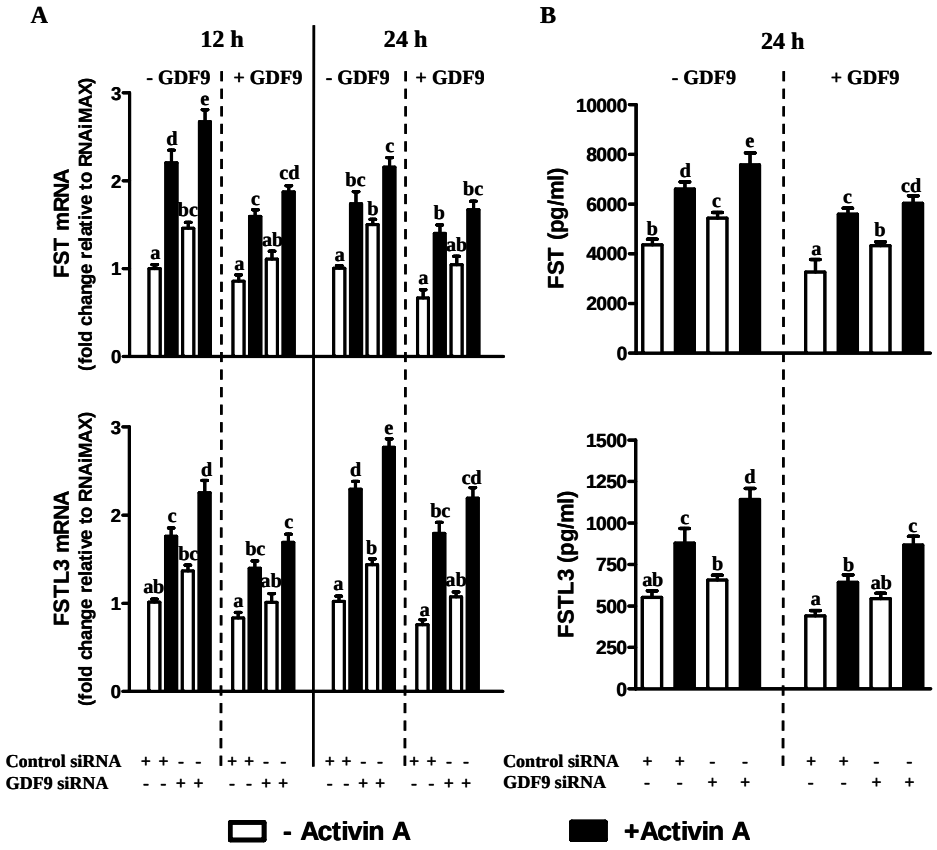


FIG. 4.5. GDF9-targeting siRNA increased activin A-induced mRNA (A) and protein (B) levels of FST and FSTL3, effects that were reversed by adding exogenous GDF9. After preculture for 48 h, hGL cells were transfected with 80 nM GDF9 targeting siRNA for 48 h. The culture media were then replaced by low-serum media (0.5% FBS), and hGL cells were cultured with and without 100 ng/ml GDF9 for another 24 h period before stimulation with 25 ng/ml of activin A for 12 h ("Time 12 h") and 24 h ("Time 24 h"). FST and FSTL3 mRNA levels in hGL cells and protein concentrations in culture media were assessed by real-time PCR and ELISA, respectively. Results were the means ± SEM from at least four sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate for real-time PCR or duplicate for ELISA. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

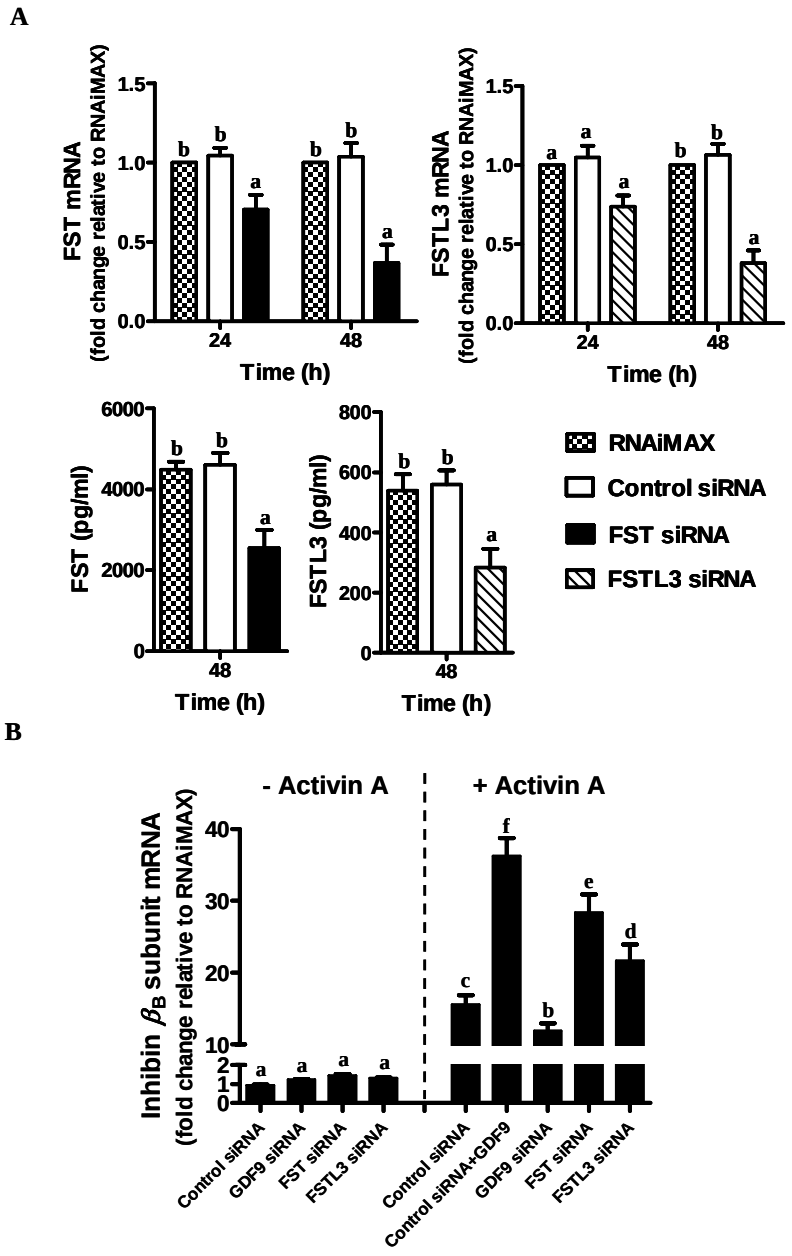


FIG. 4.6. GDF9-targeting siRNA transfection attenuated while FST or FSTL3-targeting siRNA transfection enhanced activin A-induced inhibin β_B-subunit mRNA levels. A, after preculture for 48 h, hGL cells were transfected with 80 nM of non targeting siRNA ("Control siRNA") and FST- or FSTL3-targeting siRNA for 24 h ("Time 24 h") and 48 h ("Time 48 h"), FST and FSTL3 mRNA levels in hGL cells (*upper* panel) and protein concentrations in culture media (*lower* panel) were assessed by real-time PCR and ELISA, respectively. Results were the means ± SEM from at least four sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate for real-time PCR or duplicate for ELISA. At each time point, *means without a common letter* are

significantly different ($P < 0.05$). B, after preculture for 48 h, hGL cells were transfected with 80 nM of control siRNA and GDF9-, FST- or FSTL3-targeting siRNA for 48 h. The culture media were then replaced by low-serum media (0.5% FBS), and hGL cells were cultured with and without 100 ng/ml GDF9 for another 24 h before treatment with 25 ng/ml activin A for 24 h. Inhibin β_B-subunit mRNA levels in hGL cells were measured by real-time PCR. Results were the means ± SEM from at least four sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate for real-time PCR. *Means without a common letter* are significantly different ($P < 0.05$).

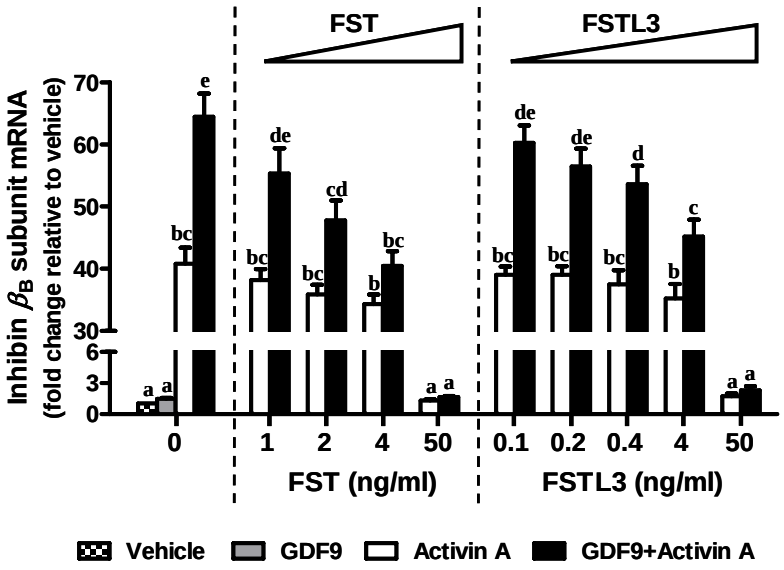


FIG. 4.7. GDF9 pretreatment enhanced activin A-induced inhibin β_B-subunit mRNA levels in hGL cells, effects that were attenuated by FST or FSTL3 in a concentration-dependent manner. After 48 h preculture, the culture media were replaced by low-serum media (0.5% FBS), and hGL cells were cultured with and without 100 ng/ml GDF9 for another 24 h in low-serum media. Different concentration of FST or FSTL3 were preincubated with 25 ng activin A at 37 C for 1 h in 50 μl PBS containing 0.1% BSA before adding to the cells and cultured for another 24 h. Inhibin β_B-subunit mRNA levels in hGL cells were measured by real-time PCR. Results were the means ± SEM from at least four sets of experiments (each from a separate patient), and in each set, measurements were made in triplicate for real-time PCR. *Means without a common letter* are significantly different ($P < 0.05$).

5. GDF9 REVERSES ACTIVIN A SUPPRESSION OF STEROIDOGENIC ACUTE REGULATORY PROTEIN (STAR) EXPRESSION AND PROGESTERONE ACCUMULATION IN HUMAN GRANULOSA-LUTEIN CELLS[4]

5.1. Introduction

Activin A has been shown to decrease progesterone production in human granulosa cells of follicles ≤ 5 mm from women undergoing oophorectomy (effects attenuated by inhibin A) (288) and inhibit hCG-induced progesterone secretion in hGL cells (289). In contrast, GDF9 can induce StAR expression and progesterone production in mouse mural granulosa cells with (26, 252) and without FSH (25). GDF9 can also increase basal but decrease FSH-induced progesterone in rat granulosa cells from small antral and preovulatory follicles (248). Unlike in rodents, GDF9 inhibits basal and 8-Br-cAMP-stimulated StAR and P450scc expression and progesterone release in hGL cells (226).

We have reported recently (271, 277) that exogenous and endogenous GDF9 can enhance activin A-induced inhibin B secretion in hGL cells. However, the interactions of GDF9 with activin A on StAR and ovarian steroidogenic enzyme expressions and progesterone accumulation are unknown, and there is little information on the effect of inhibin B on progesterone accumulation in granulosa cells. We hypothesized that GDF9, by increasing inhibin B levels, with inhibin B acting as an activin A competitor, could reverse activin A

[4] A version of this chapter has been accepted and will be published. Shi FT, Cheung AP, Christian Klausen, Huang HF, Leung PC 2010 Growth differentiation factor 9 (GDF9) reverses activin A suppression of steroidogenic acute regulatory protein (StAR) expression and progesterone production in human granulosa-lutein cells. The Journal of Clinical Endocrinology and Metabolism. Authors (Shi FT and Cheung AP) have equal contributions at this chapter.

suppression of progesterone accumulation. We therefore undertook the following study to further evaluate GDF9 in this regard.

5.2. Materials and Methods

We first compared mRNA and protein levels of StAR, P450scc, and 3βHSD in hGL cells and progesterone accumulation in culture media with and without GDF9 and/or activin A treatment. Second, we transfected hGL cells with inhibin α-subunit siRNA to assess changes in activin A, inhibin A and B levels, StAR, P450scc and 3βHSD expression, and progesterone accumulation following activin A treatment with and without GDF9. Finally, we tranfected hGL cells with GDF9 siRNA to evaluate the effects of activin A on StAR, P450scc, and 3βHSD expression and progesterone accumulation after endogenous GDF9 expression was reduced.

Preparation of hGL cells

hGL cells from each patient were extracted and cultured as described in Section 2.2, and seeded as described in Section 3.2.

Activin A and GDF9 experiments

The experimental conditions were same as described in Section 2.2 except for experiments with both GDF9 and activin A, cells were preincubated with 100 ng/ml of GDF9 in low-serum media for 24 h before activin A treatment with or without 0.2 IU/ml of recombinant human FSH (Gonal-F; Industria Farmaceutica Serono SPA, Bari, Italy) for 12, 24, 36 and 48 h.

Knockdown analysis for human inhibin α-subunit or GDF9

We performed transient knockdown assays with 80 nM of inhibin *α*-subunit or GDF9 siRNA using non-targeting siRNA (ON-TARGETplus SMARTpool; Dharmacon Research Inc.) as control. The conditions of siRNA transfection were same as described in Section 3.2. mRNA levels of inhibin *α*-subunit or GDF9 were then quantified with real-time PCR at 24 and 48 h after adding the transfection reagents. In separate experiments, the spent media at "Time 48 h" were replaced with low serum media and hGL cells were incubated with and without 100 ng/ml GDF9 for one more day ("Time 72 h") before incubating with 25 ng/ml activin A for 24 and 48 h. Corresponding activin A, inhibin A and inhibin B levels in culture media were quantified with ELISA at 48 h after activin A treatment.

RNA extraction and Real-time RT-PCR

Total RNA extraction, first-strand cDNA synthesis and real-time PCR were performed as described in Section 2.2 using primers listed in Table S1 of the Appendices.

Western blot analysis

Western blot analysis was performed as described in Section 2.2 with the following antibodies: anti-StAR, anti-P450scc, anti-3*β*HSD and anti-*β*-actin (Santa Cruz Biotechnology Inc.). Scion Image Analysis software (Scion Co., Frederick, MD) was used to determine protein density levels.

Hormone assays

After treatment, culture media were collected and stored frozen until hormone assays. The ELISA kits specific for progesterone (Cayman Chemical Co., Ann Arbor, MI), activin A (R&D

Systems), and inhibin A and B (Diagnostic Systems Laboratories) were used to measure the corresponding hormone levels in the culture media. The assays for inhibin A and inhibin B ELISA were as described in Section 2.2. The lowest limits of detection for progesterone was 7.8 pg/ml. Corresponding intrassay and interassay coefficients of variation were 7.3% and 7.7%, respectively. All progesterone measurements were performed in duplicate. Secreted progesterone levels were normalized to total cellular protein content.

Statistical analysis

The methods used for data analysis and the descriptions of results were as described in Section 2.2.

5.3. Results

Interactions of GDF9 and activin A on StAR expression and progesterone accumulation

Activin A but not GDF9 treatment alone significantly reduced mRNA of StAR in a time-dependent manner (Fig. 5.1A). Pretreatment with GDF9 for 24 h reduced these suppressive effects of activin A in a time-dependent manner with maximum reversal at 48 h – from 0.33 fold with activin A treatment alone to 0.68 fold with GDF9 pretreatment (Fig. 5.1A; $P < 0.01$). As expected, when GDF9 was preincubated with BMPR2 ECD, a GDF9 antagonist (235, 259, 260, 271), the reversing effects of GDF9 on activin A suppression of StAR mRNA were attenuated and were statistically significant at 36 and 48 h (Fig. 5.1B, *left* panel; $P < 0.01$). In contrast, there were no significant changes in StAR mRNA levels with BMPR2 ECD alone or GDF9 and BMPR2 ECD (Fig. 5.1B, *right* panel). Compared to StAR, all the above treatments had no significant effects on corresponding mRNA levels of P450scc and 3βHSD

(Fig. 5.2).

In parallel with changes in StAR mRNA levels, GDF9 pretreatment also attenuated activin A suppression of StAR protein levels from 0.58 fold (activin A alone) to 0.86 fold (activin A with GDF9) at 48 h ($P < 0.05$), effects that were neutralized by BMPR2 ECD ($P < 0.05$) (Fig. 5.3B). The effects of GDF9 on activin A effect were also assessed in the presence of FSH (Fig. 5.3). As expected, FSH, a known enhancer of StAR expression in granulosa cells, increased basal StAR protein levels almost 2-fold (Fig. 5.3B; $P < 0.001$). Activin A decreased FSH-stimulated StAR protein levels ($P < 0.001$), and again, its effects were attenuated by GDF9 pretreatment ($P < 0.001$) – 1.9 fold, 1.08 fold, and 1.76 fold for FSH alone, FSH with activin A, and FSH with activin A plus GDF9, respectively (Fig. 5.3B). FSH also induced a small rise in P450scc protein levels ($P < 0.05$) but had no effects on 3βHSD protein levels; neither Activin A, GDF9 nor combination had any effects on P450scc and 3βHSD protein expression (Fig. 5.4).

Corresponding to the changes in StAR mRNA and protein levels, activin A but not GDF9 significantly decreased basal and FSH-stimulated progesterone accumulation (Fig. 5.5; P values all < 0.001). GDF9 pretreatment reduced the suppressive effects of activin A on basal and FSH-stimulated progesterone accumulation by 0.18 fold and 0.47 fold respectively but reached statistical significance only for the latter (Fig. 5.5; $P < 0.001$). As expected, these GDF9 effects were neutralized by BMPR2 ECD and the effect was statistically significant when progesterone accumulation was amplified by FSH ($P < 0.001$).

Effects of GDF9 and activin A on StAR expression and progesterone accumulation following inhibin α-subunit siRNA

Because inhibin is a heterodimer of α- and β-subunits, and activin A a homodimer of

β-subunits, inhibin and activin production can be affected by the ratio of α to β subunits. After inhibin α-subunit siRNA transfection, endogenous inhibin α-subunit mRNA levels decreased as expected (Fig. 5.6A; $P < 0.001$); activin A levels in culture media increased by 0.9 fold from 16.4 to 31.5 pg/ml (Fig. 5.6B *top panel*; $P < 0.001$); inhibin A levels, basal or induced by activin A with and without GDF9 decreased but changes were not statistically significant (Fig. 5.6B *middle* panel); and, inhibin B levels induced by activin A alone or activin A with GDF9 decreased (Fig. 5.6B *bottom* panel; *P* values all < 0.05).

Correspondingly, FSH-stimulated StAR mRNA levels decreased (columns 2 *vs.* 6 at 24 h and at 48 h, Fig. 5.7A) but reached statistical significance at 48 h only ($P < 0.05$); activin A suppression of FSH-stimulated StAR mRNA levels was enhanced but changes were not statistically significant (columns 3 *vs.* 7 at 24 h and at 48 h, Fig. 5.7A); the reversing effects of GDF9 on activin A suppression of FSH-induced StAR mRNA levels were attenuated, with maximum effect at 48 h (columns 5 *vs.* 9 at 24 h and at 48 h, Fig. 5.7A; $P < 0.01$). After inhibin α-subunit siRNA transfection, corresponding changes in StAR protein levels (Fig. 5.7B) and progesterone accumulations in the culture media (Fig. 5.7C) showed a similar pattern to StAR mRNA levels. The small increase in P450scc mRNA expression induced by FSH at 48 h (Fig. 5.8A *upper* panel; $P < 0.05$) was not affected by inhibin α-subunit siRNA transfection. None of the above treatments altered 3βHSD mRNA levels (Fig. 5.8A *lower* panel). Changes in P450scc and 3βHSD protein levels followed the same pattern as their corresponding mRNA (Fig. 5.8B).

Effects of activin A on StAR expression and progesterone accumulation after GDF9 siRNA

With reduced endogenous GDF9 expression (see Fig. 3.1B of Charpter 3) after GDF9

siRNA transfection, the suppressive effects of activin A on StAR mRNA and protein levels and progesterone accumulations in the culture media were enhanced and reached statistical significance at 48 h (Fig. 5.9). The reversing effects of GDF9 on these changes were also attenuated but results were not statistically significant (Fig. 5.9). GDF9 siRNA transfection had no effects on P450scc and 3βHSD mRNA and protein levels (Fig. 5.10).

5.4. Discussion

Our results showed that GDF9 reversed activin A suppression of both basal and FSH-stimulated StAR mRNA and protein expression, and FSH-stimulated progesterone secretion. We also demonstrated increased activin A levels and decreased inhibin B levels with decreased FSH-stimulated progesterone accumulation after inhibin α-subunit siRNA transfection (Fig. 5.6B). Increased activin A levels have been likewise observed in inhibin α-subunit knockout mice (290). The effects of GDF9 in reversing activin A suppression of FSH-induced StAR expression and progesterone accumulation were also attenuated after inhibin α-subunit siRNA transfection (Fig. 5.7). Similarly, there was increased suppression of StAR expression and progesterone accumulation by activin A following decreased endogenous GDF9 activities with GDF9 siRNA transfection (Fig. 5.9). This paralleled the reduced suppression by activin A after exogenous GDF9 pretreatment described earlier, and was consistent with unchanged plasma progesterone levels throughout the estrus cycle in GDF9 mutant sheep (291). Taken together with our recent observations (271, 277) that GDF9 can enhance activin A-induced inhibin B accumulation in hGL cells, we hypothesize that inhibin B is involved in facilitating these changes.

Activin A is a known inhibitor of luteinization as reported in human (292, 293), bovine

(294, 295), and rat (296) ovary studies. In the menstrual cycle, serum activin A levels are higher in the early follicular phase, midcycle and late luteal phase (164) while serum inhibin B levels peak in the midfollicular phase and just after ovulation (161). During ovarian stimulation for IVF, activin A levels remained unchanged after 7-8 days of FSH treatment but rose significantly with follicle maturity (297). Based on these observations and findings of our current and previous studies (271, 277), it is tempting to speculate that this increased activin A expression in the preovulatory follicle in the presence of GDF9 further enhances inhibin B expression to overcome the inhibitory effect of activin A on luteinization. To what extent this plays a physiological role remains unknown given the dominant effects of LH on luteinization.

While expression of StAR was tightly coupled with changes with progesterone accumulation in all our experiments, corresponding changes were not observed in P450scc and 3βHSD activities. Despite the known effects of FSH in stimulating P450scc and 3βHSD activities (38, 60), such changes were not observed. One explanation is that P450scc and 3βHSD activities in these hGL cells obtained from women undergoing IVF treatment are already primed for progesterone production and unlike StAR protein, are not the rate-limiting steps in our study model. Indeed, these cells have been exposed to pharmacological concentrations of exogenous gonadotropins and in the process of luteinization. However, in the absence of easily accessible granulosa cells from unstimulated ovaries, they do provide a convenient model to generate hypothesis for further study during the follicular-luteal transition. Alternatively, while the StAR, P450scc and 3βHSD genes share several common promoter elements such as SF-1 (298), each also possesses unique elements (see review in (71)) that may respond to Activin A and/or GDF9 with different sensitivities and time courses.

FSH and LH are known to stimulate StAR expression in the mammalian ovary

predominantly via PKA signaling (299), but factors such as estradiol (300) and IGF-I (301) can potentiate, while TGFβ1 (18, 302), BMP15 (23), and BMP 2/4/6/7 (20) can suppress the actions of gonadotropins. Indeed, our current study has confirmed activin A suppression of basal and FSH-induced StAR expression and progesterone accumulation as reported previously in hGL cells (288) and porcine granulosa cells (303). We have also confirmed that GDF9 alone has no effects on basal StAR expression and progesterone accumulation as reported previously in hGL cells (226) and rat granulosa cells (248). However, species differences exist as GDF9 induces basal progesterone production in cultured granulosa cells from preovulatory follicle of mice (26, 252).

We cannot rule out that decreased levels of inhibin α-subunit mRNA after targeting siRNA transfection may lead to increased synthesis of activin B ($\beta_B\beta_B$) and/or activin AB ($\beta_A\beta_B$) until sensitive and specific activin B and activin AB assays are commercially available. In addition, follistatin is known to bind activin A with high affinity which prevents activin A from binding to its receptor (130). Because granulosa cells and luteal cells are the main source of follistatin in the ovary, we also cannot exclude a regulatory role for GDF9 on follistatin activities and are currently conducting studies in this regard. The regulation of granulosa cells in the follicular–luteal transition involves complex endocrine, paracrine and autocrine interactions of multiple factors which have not been completely mapped out. Although the physiological implications of our findings remain to be tested in granulosa cells from unstimulated ovaries, they, nevertheless, suggest that local interactions between GDF9, activin A, and inhibin B can influence the functional evolution of a mature follicle to a corpus luteum and provide potential targets for future studies to further characterize the physiological process of the ovarian and menstrual cycle, and treatment manipulation in reproductive medicine.

In conclusion, our results indicate that GDF9 has no direct effects on basal or FSH-stimulated StAR activity and progesterone accumulation in hGL cells. Rather, GDF9 reverses the suppressive effects of activin A on StAR and progesterone accumulation by enhancing the levels of inhibin B, which in turn, acts as an inhibitor of activin A. This is corroborated by an increase in activin A suppression of StAR expression and progesterone accumulation when endogenous GDF9 levels are reduced after GDF9 siRNA transfection, and a corresponding decrease in activin A-induced (with or without GDF9 treatment) inhibin B accumulations after inhibin α-subunit siRNA transfection. While we would like to hypothesize a feedback loop between activin A and inhibin B in granulosa cells, further studies are required to establish if inhibin B can indeed directly inhibit activin A accumulation and other mechanisms involved in antagonizing activin A action.

5.5. Figures

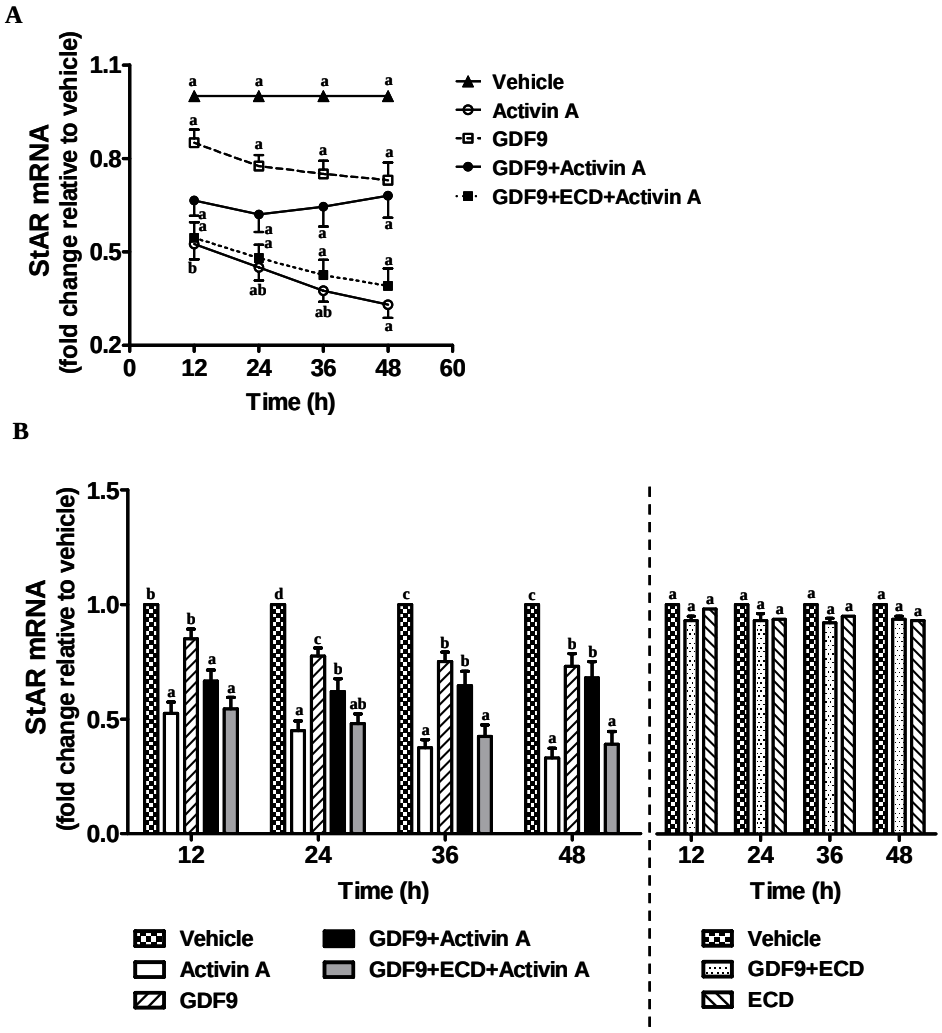


FIG. 5.1. GDF9 pretreatment reduced activin A suppression of StAR mRNA levels in hGL cells, effects that were attenuated by BMPR2 ECD ("ECD"), a GDF9 antagonist. After preculture for 48 h, the culture media were replaced with low-serum media (0.5% FBS) and hGL cells were preincubated with 100 ng/ml of GDF9 for 24 h in low-serum media before stimulation with 25 ng/ml of activin A for 12, 24, 36 and 48 h. In neutralization experiments to render GDF9 inactive, 2 μg/ml of BMPR2 ECD and 100 ng/ml of GDF9 were preincubated together for 30 min before adding to cultured hGL cells. RNA was isolated from hGL cells, and mRNA levels of StAR were assessed by real-time PCR. Results are the means ± SEM from hGL cells of five patients. Panel A shows the time-dependent changes of StAR mRNA levels for each treatment group. Panel B shows StAR mRNA level changes for the various treatments at each time point. In both panels, *means without a common letter* are significantly different ($P < 0.05$).

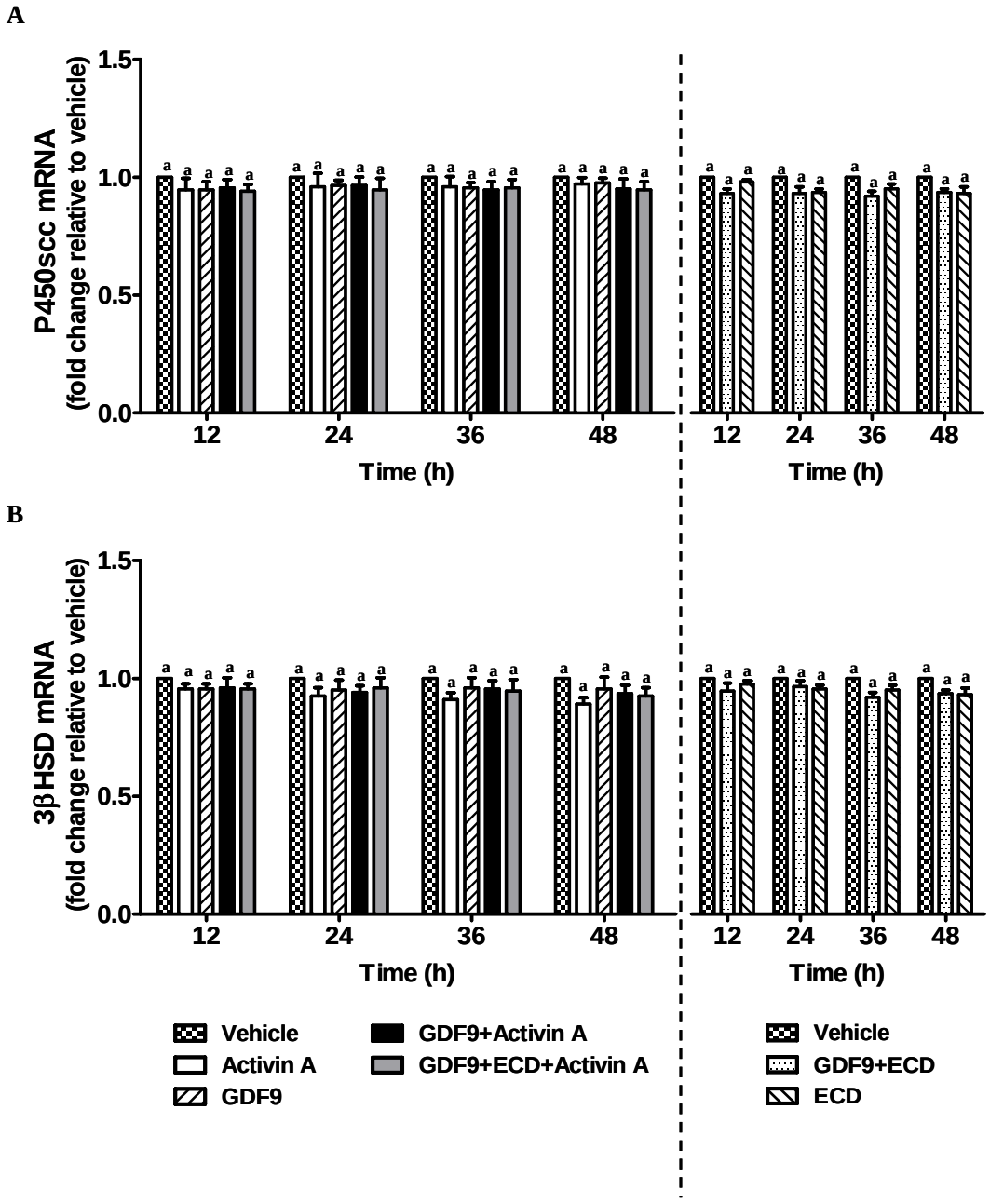


FIG. 5.2. The effects of GDF9 and activin A on P450scc and 3βHSD mRNA levels in hGL cells. The culture and treatment conditions were same as those in Fig. 5.1. After treatment, RNA was isolated from hGL cells, and mRNA levels of P450scc (A) and 3βHSD (B) were assessed by real-time PCR. Results are the means ± SEM from hGL cells of five patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

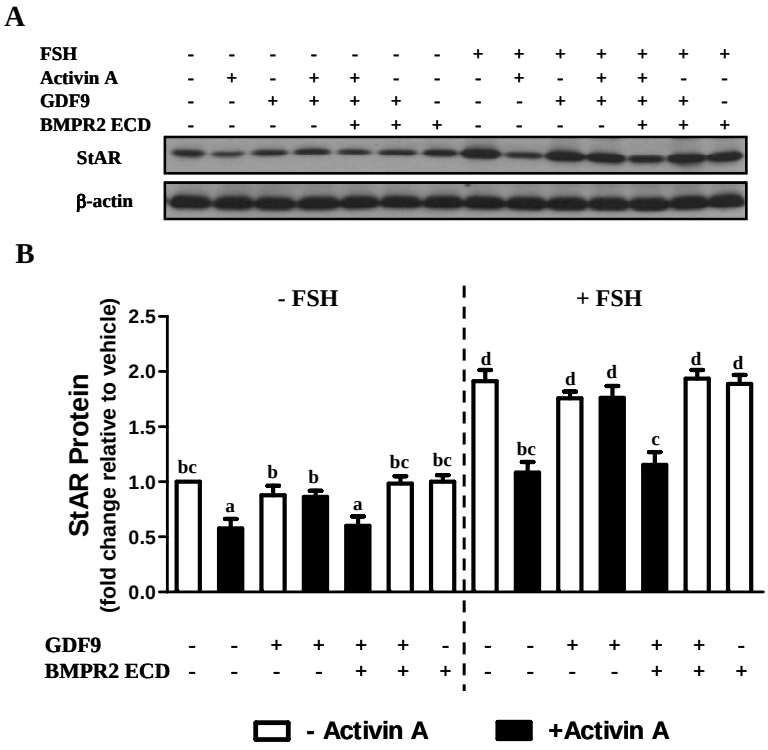


FIG. 5.3. GDF9 pretreatment reduced activin A suppression of basal and FSH-induced StAR protein levels in hGL cells, effects that were attenuated by BMPR2 ECD. The culture and treatment conditions were as described in Fig. 5.1 except that after GDF9 pretreatment, hGL cells were stimulated with activin A for 48 h with and without 0.2 IU/ml FSH. Cell lysates were then collected and assessed by Western blot analysis for protein levels of StAR. Panel A shows the immunoblots from one patient representative of similar results from other patients; panel B shows the protein levels of StAR normalized to those of β-actin. Results are the means ± SEM from hGL cells of five patients. *Means without a common letter* are significantly different ($P < 0.05$).

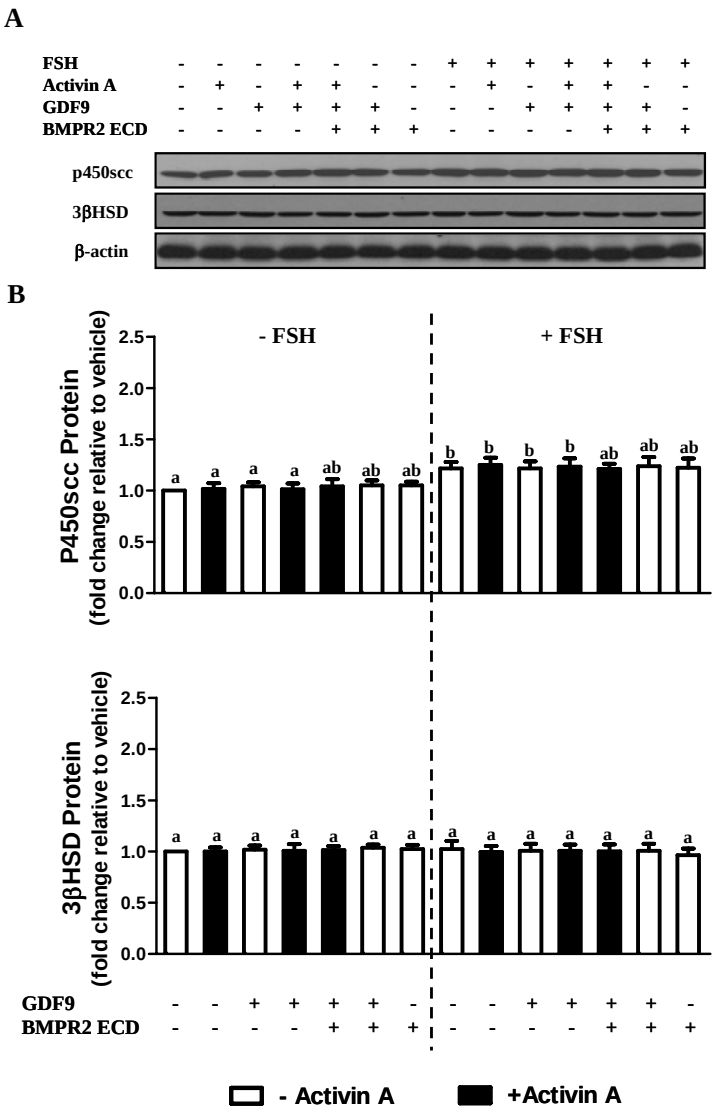


FIG. 5.4. The effects of GDF9 and activin A on P450scc and 3βHSD protein levels in hGL cells. The culture and treatment conditions were same as those in Fig. 5.3. After treatment, cell lysates were then collected and assessed by Western blot analysis for protein levels of P450scc and 3βHSD. Panel A shows the immunoblots from one patient representative of similar results from other patients; panel B shows the protein levels of P450scc and 3βHSD normalized to those of β-actin. Results are the means ± SEM from hGL cells of five patients. *Means without a common letter* are significantly different ($P <$ 0.05).

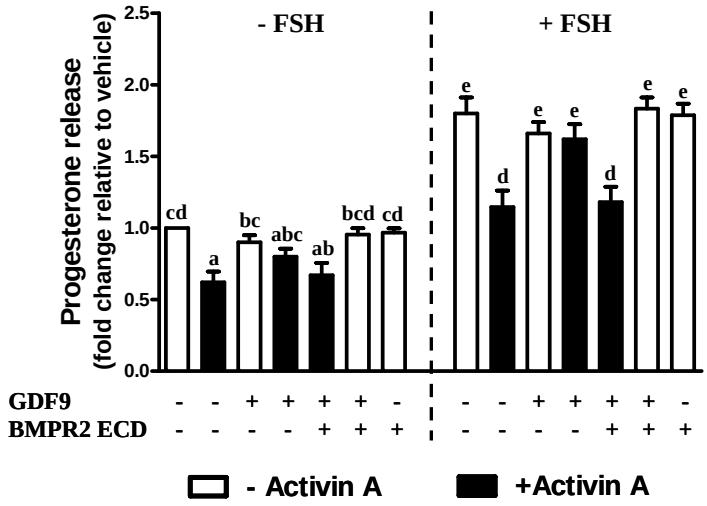


FIG. 5.5. GDF9 pretreatment reduced activin A-suppressed progesterone accumulations in culture media, effects that were attenuated by BMPR2 ECD. The culture and treatment conditions were as described in Fig. 5.3. Results are the means ± SEM from hGL cells of five patients. *Means without a common letter* are significantly different ($P < 0.05$).

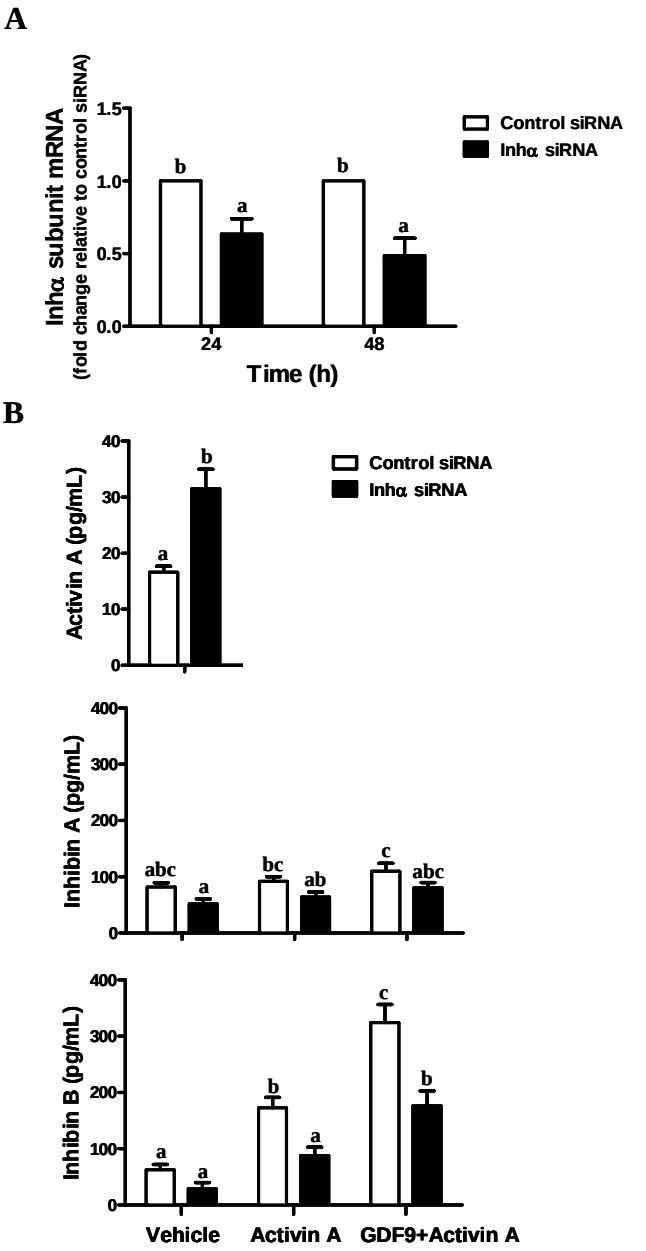


FIG. 5.6. After transfection of hGL cells with inhibin α-subunit siRNA, accumulations of activin A increased; accumulations of inhibin A and inhibin B decreased following treatment with activin A alone or activin A with GDF9 pretreatment. After preculture for 48 h, hGL cells were transfected with 80 nM inhibin α-subunit siRNA for 48 h. The culture media were replaced by low serum media (0.5% FBS), and hGL cells were cultured with and without 100 ng/ml of GDF9 for another 24 h before incubating with 25 ng/ml of activin A for a further 48 h period. Panel A shows inhibin α-subunit mRNA levels 24 and 48 h after adding the transfection reagents; panel B shows the levels of activin A, inhibin A and inhibin B in culture media 48 h after adding activin A. Results are the means ± SEM from hGL cells of four patients. *Means without a common letter* are significantly different ($P < 0.05$).

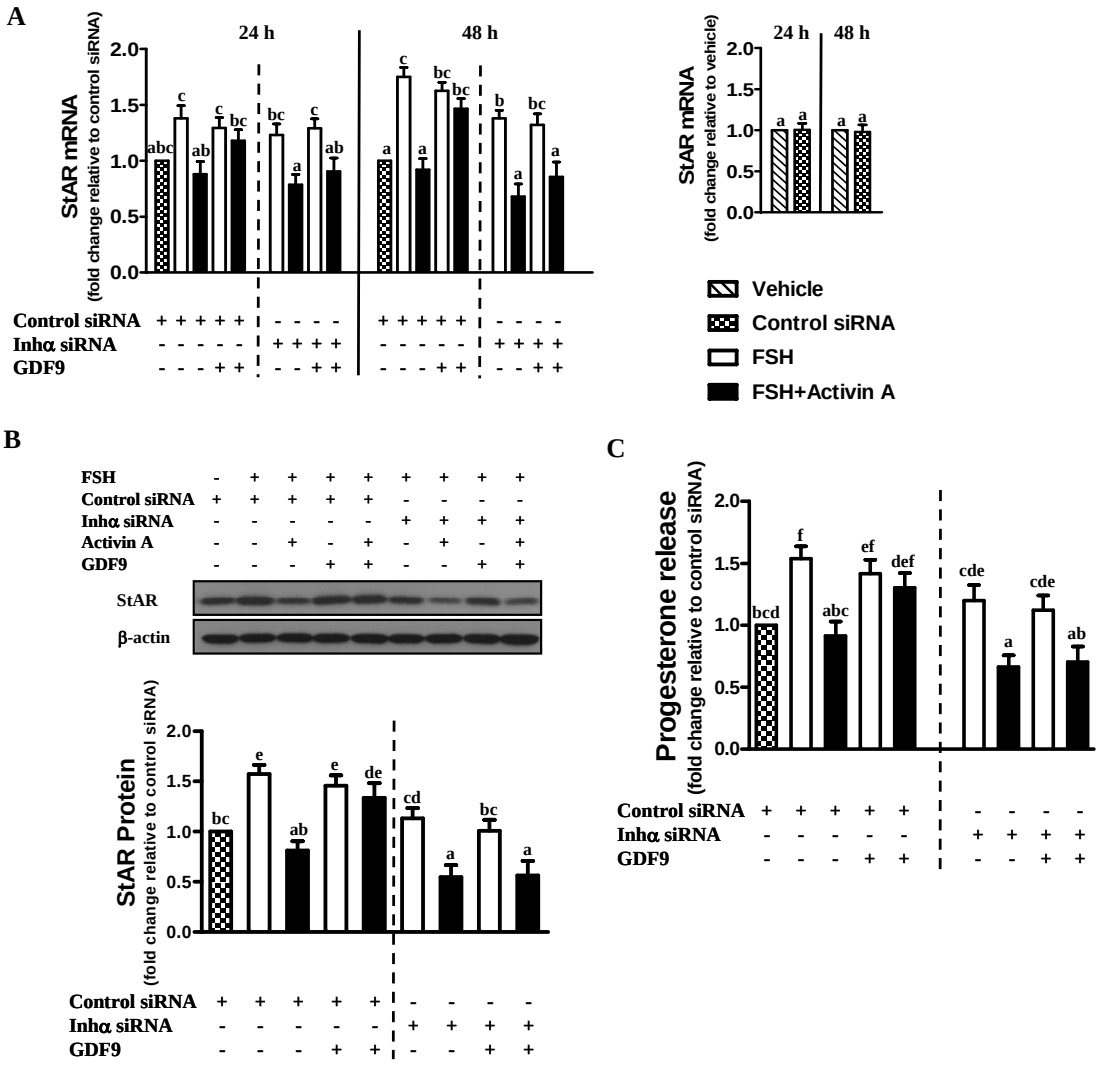


FIG. 5.7. GDF9 reduced the suppressive effects of activin A on StAR expression and progesterone accumulation, effects that were attenuated when cells were transfected with inhibin α-subunit siRNA. The culture and treatment conditions were as described in Fig. 5.6 except that after GDF9 pretreatment, hGL cells were treated with activin A for 24 and 48 h with 0.2 IU/ml FSH in addition. Fig. 5.7A shows mRNA levels of StAR 24 and 48 h after adding activin A with and without FSH. The "inset" confirmed control siRNA had no effects on StAR mRNA levels when compared with transfection reagent alone ("Vehicle"). Fig. 5.7B shows the corresponding protein expression at 48 h: the *upper* panel shows the immunoblots from one patient representative of similar results from other patients; the *lower* panel shows the protein levels of StAR normalized to those of β-actin. Fig. 5.7C shows the progesterone accumulations in culture media 48 h after adding activin A. Results are the means ± SEM from hGL cells of six patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

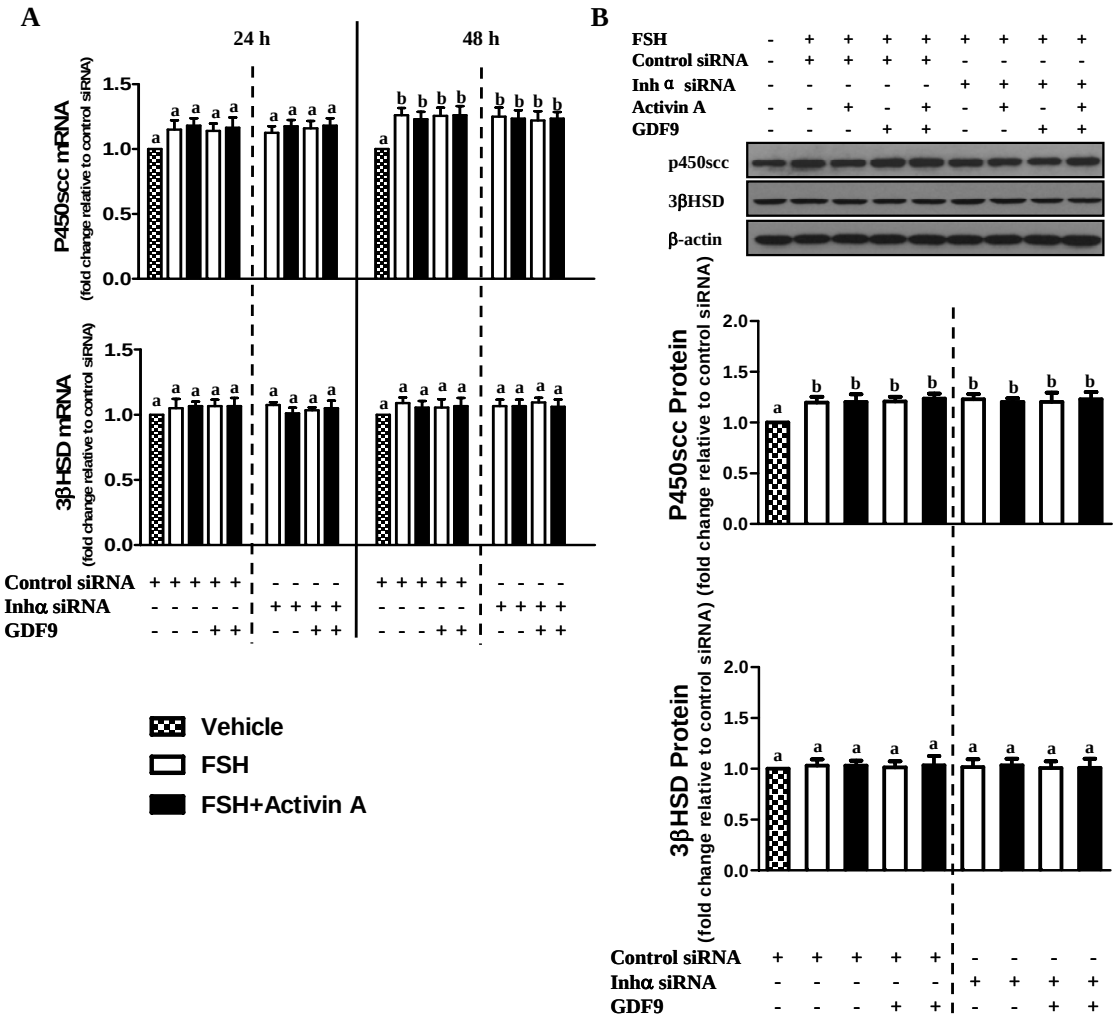


FIG. 5.8. The role of inhibin *α*-subunit siRNA in the interaction of GDF9 and activin A on P450scc and 3*β*HSD expression and progesterone accumulation in hGL cells. The culture and treatment conditions were as described in Fig. 5.7. Fig. 5.8A shows the corresponding mRNA levels of P450scc and 3*β*HSD 24 h and 48 h after activin A treatment. Fig. 5.8B shows the protein levels of P450scc and 3*β*HSD 48 h after activin A treatment: the *upper* panel shows the immunoblots from one patient representative of similar results from other patients; the *lower* panel shows the protein levels of P450scc and 3*β*HSD normalized to those of *β*-actin. Fig. 5.8C shows the progesterone levels in culture media 48 h after adding activin A. Results are the means ± SEM from hGL cells of six patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

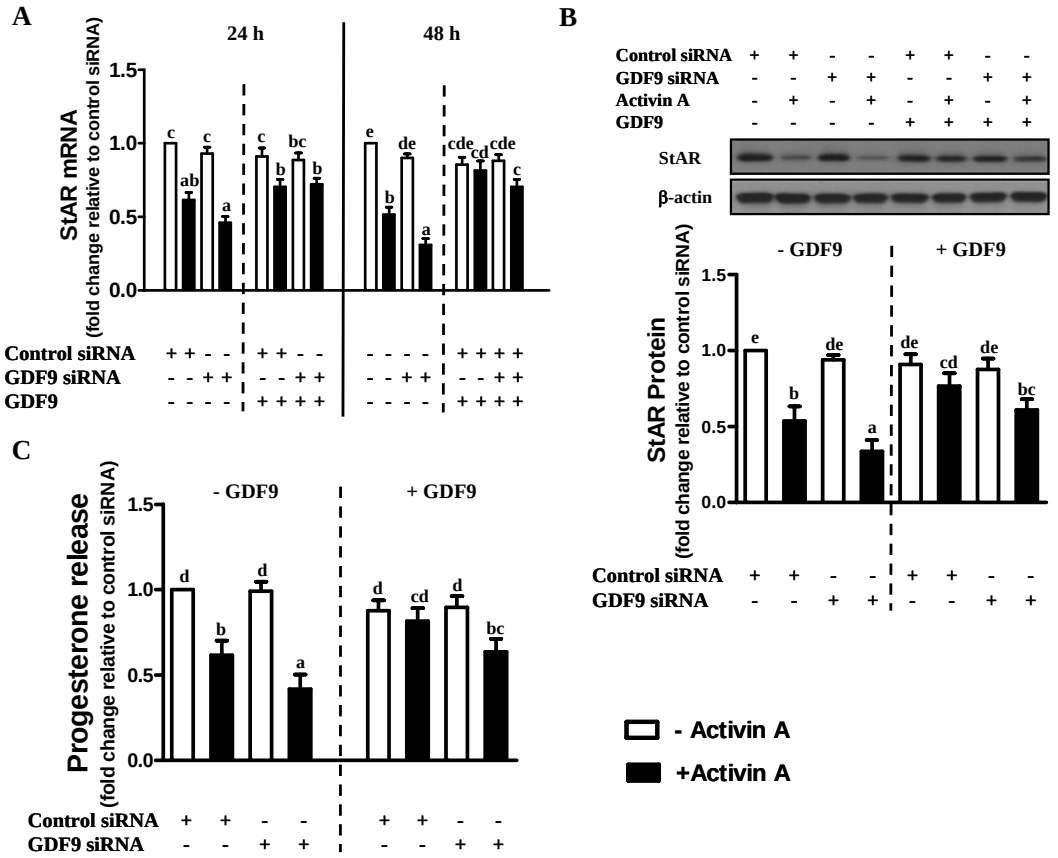


FIG. 5.9. Transfection of hGL cells with GDF9 siRNA enhanced the suppressive effects of activin A on StAR expression and progesterone accumulation. The culture and treatment conditions were as described in Fig. 5.6. Fig. 5.9A shows mRNA levels of StAR 24 and 48 h after adding activin A. Fig. 5.9B shows the corresponding protein expression at 48 h: the *upper* panel shows the immunoblots from one patient representative of similar results from other patients; the *lower* panel shows the protein levels of StAR normalized to those of *β*-actin. Fig. 5.9C shows the progesterone levels in culture media 48 h after adding activin A. Results are the means ± SEM from hGL cells of six patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

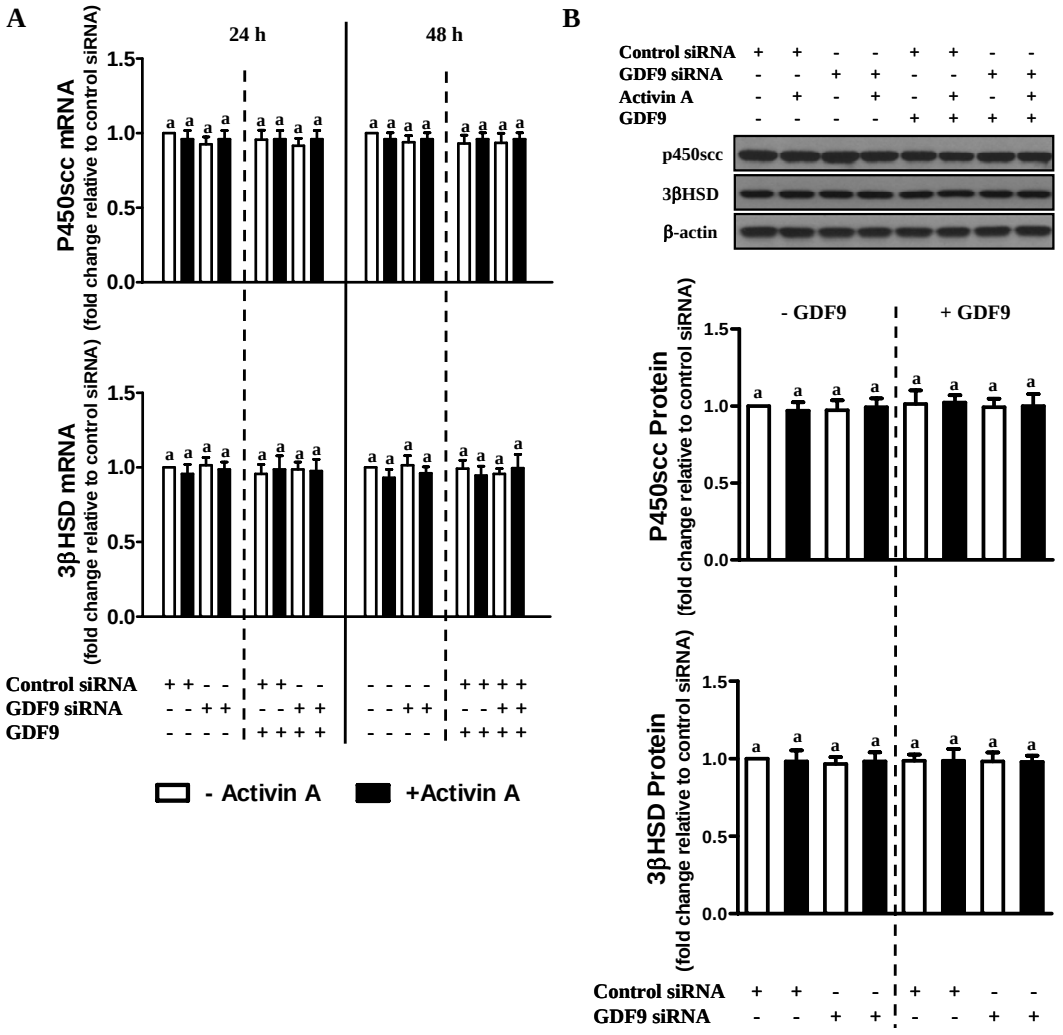


FIG. 5.10. The role of GDF9 siRNA in the interaction of GDF9 and activin A on P450scc and 3*β*HSD expression and progesterone accumulation in hGL cells. The culture and treatment conditions were as described in Fig. 5.9. Fig. 5.10A shows the corresponding mRNA levels of P450scc and 3*β*HSD 24 h and 48 h after activin A treatment. Fig. 5.10B shows the protein levels of P450scc and 3*β*HSD 48 h after activin A treatment: the *upper* panel shows the immunoblots from one patient representative of similar results from other patients; the *lower* panel shows the protein levels of P450scc and 3*β*HSD normalized to those of *β*-actin. Fig. 5.10C shows the progesterone accumulations in culture media 48 h after adding activin A. Results are the means ± SEM from hGL cells of six patients. At each time point, *means without a common letter* are significantly different ($P < 0.05$).

6. CONCLUSION AND RECOMMENDATIONS FOR FUTURE WORK

6.1. Conclusion

The main objective of this thesis was to explore the relationship between GDF9 and activin A as it relates to the expression of inhibin β_B-subunit mRNA, the accumulations of inhibin B and progesterone in human granulosa-lutein cells from women undergoing IVF and the signaling mechanisms involved. Several results provide information about these objectives.

First, activin A and GDF9 alone had little stimulatory effect on inhibin α- and β_A-subunit mRNA levels. In contrast, GDF9 could stimulate inhibin β_B-subunit levels but to a lesser degree than could activin A. GDF9 pretreatment for 24 h significantly enhanced activin A-induced inhibin β_B-subunit mRNA levels, inhibin B accumulation, and Smad2/3 phosphorylation; these effects were attenuated by BMPR2 ECD. GDF9 pretreatment also induced activin receptors (ACVR2B/1B) and Smad2/3 but reduced inhibitory Smad7 mRNA levels.

Second, GDF9 was detected as mRNA and protein in hGL cells. Reduced endogenous GDF9 expression following targeting siRNA transfection was associated with decreased levels of ACVR2B/1B and Smad2/3/4 but increased Smad7 mRNA and protein and consequently, reduced levels of activin A-induced inhibin β_B-subunit mRNA and inhibin B. These results further confirm the effects of exogenous GDF9 treatment mentioned previously.

Third, GDF9 was found to suppress basal and activin A-induced expression of FST and FSTL3, which are extracellular inhibitors of activin A. These effects were attenuated by BMPR2 ECD and GDF9 siRNA transfection. FST or FSTL3 siRNA transfection significantly

augmented levels of activin A-induced inhibin β_B-subunit mRNA. Furthermore, the enhancing effects of GDF9 in activin A-induced inhibin β_B-subunit mRNA were attenuated by FST. This finding may explain why GDF9 can enhance expression of activin A-induced inhibin β_B-subunit mRNA extracellularly; this result further supports the role of GDF9 in the regulation of inhibin β_B-subunit mRNA level.

Fourth, GDF9 reverses the suppressive effects of activin A on StAR expression and progesterone accumulation by enhancing the expression of inhibin B, which in turn, acts as an inhibitor of activin A. This relationship is corroborated by the following. GDF9 siRNA transfection reduces endogenous GDF9 level, thus increasing acitivin A suppression of StAR expression and progesterone accumulation. Furthermore, we detected a corresponding decrease when endogenous inhibin B accumulation was reduced after inhibin α-subunit siRNA transfection.

In summary, these results describe a pathway (Fig. 6.1) in which GDF9 increases hGL cell response to activin A by acting on GDF9 receptors, BMPR2/TGFβR1, which then activate Smad2/3 to form complexes with Smad4. These complexes then activate transcription factors in the nucleus to target genes that increase ACVR2B/1B and Smad2/3 expression and reduce Smad7 activity. These changes, in turn, allow more activin A to bind to its receptors and thus increase the cellular response to activin A stimulation in inhibin B accumulation intracellularly. Additionally, GDF9 inhibits FST and FSTL3 gene transcription and then FST and FSTL3 secretions into the culture media. More activin A can avoid binding with its extracellular inhibitors, FST and FSTL3, and then activate activin receptors and a downstream signaling cascade that stimulates more inhibin B accumulation. Thus, GDF9 enhances activin A-induced inhibin B accumulation via not only intracellular mechanism but also extracellular regulating

mechanism, however, which mechanism plays more important role in this process must be further elucidated. Increasing inhibin B is subsequently as an inhibitor of activin A to remove its suppression in the basal and FSH-induced progesterone accumulation via reducing the expression of StAR, the rate-limiting step in steroidogenesis of hGL cells. Although we would like to hypothesize a feedback loop exists between activin A and inhibin B in granulosa cells, further studies are required to establish if inhibin B can indeed directly inhibit activin A accumulation and and other mechanisms involved in antagonizing activin A action.

Inhibin A and inhibin B exhibit distinct patterns of secretion throughout the menstrual cycle (166, 255, 256, 304, 305). Serum inhibin B levels increase during luteal-follicular transition and peak in the mid-follicular phase and again the day following the LH surge. In contrast, inhibin A levels begin to rise in the late follicular phase and peak in the mid-cycle and again in the mid-luteal phase. Whereas activin A may be present in an unbound form, it is almost irreversibly bound to follistatin and there is little variation in levels of free activin A throughout the menstrual cycle (163). Conversely, the level of activin A peaks before the onset of menses, in the luteal phase and mid-cycle (164).

We describe for the first time the close relationship between GDF9 and activin A as it relates to inhibin B accumulation in hGL cells. One may speculate that increasing GDF9 expression (268) during folliculogenesis enhances human granulosa cell response to activin A and leads to increased inhibin B in the follicular phase. With release of the oocyte after ovulation, a main source of GDF9 is removed; hence, cell response to activin A with respect to β_B-subunit is withdrawn, which may explain the decline in inhibin B secretion after ovulation. This finding is also consistent with the marked decreased in the inhibin β_B-subunit whereas α- and β_A-subunits are expressed in *Gdf9* null mouse ovaries (266).

PCOS is one of the most common causes of infertility in women and can affect 5-10% of women of reproductive age worldwide (306). Studies have shown that ovary dysfunction is involved in the pathology of PCOS (307), in particular the number of growing follicles in PCOS ovaries are double that of normal ovaries (308). This important finding proposes that the process of follicle development may be abnormal in PCOS patients.

Results from animal studies have shown that GDF9 has a close relationship to folliculogenesis and female fertility (309-311). Many abnormalities emerge in GDF9-deficient female mice (219, 239, 266). Decreased levels of GDF9 mRNA are found in developing oocytes from women with PCOS or PCO compared to women with normal ovaries; the decreased levels are evident throughout folliculogenesis, particularly during the primary and secondary stages (268). These data suggest that GDF9 transcription is postponed and inhibited in PCOS and PCO oocytes throughout the enlargement and differentiation phase. During IVF and intracytoplasmic sperm injection treatments, women with PCOS have lower GDF9 mRNA levels in cumulus cells than do women with normal menstrual cycles (227).

Additionally, FST levels are significantly higher in women with PCOS (285); the authors suggest that altered FST function may contribute to the PCOS phenotype. Important characteristics of PCOS such as induced ovarian androgen production, decreased serum FSH level, and impaired ovarian follicle development could be caused by increased expression and function of FST (286). A PCOS-like phenotype has also been reported in an FST transgenetic mouse model (287).

The roles of inhibin and activin in the pathology of PCOS remain unclear. Studies have shown that serum levels of inhibin α-subunit, pro-αC, inhibin A, and inhibin B are increased in women with PCOS (276, 312-315). One study describe that compared to size-matched follicles

from normal ovaries, levels of inhibin α- and β_A-subunit mRNA decline in PCOS follicles while inhibin β_B-subunit mRNA levels do not change (316). However, several groups have also reported that inhibin B levels in follicular fluid from PCOS follicles are not different with that of nomal follicles, even if inhibin A levels are lower in PCOS follicles (275, 304, 317). It is not very clear about the significance of these results; nevertheless, we cannot exclude the role of inhibins in the irregular inhibition of pituitary FSH secrection in women with PCOS.

The results from this thesis demonstrate that GDF9 can enhance activin A-induced inhibin B accumulation in hGL cells partly via suppressed expression of FST and FSTL3. These results help define connections between these important factors involved in the pathogenesis of PCOS and further assist us to develop effective methods for curing patients.

Previously, GDF9 was primarily considered to be an oocyte-specific factor. However, recent studies show that GDF9 is present in granulosa cells from monkey (223), goat (224) and pig (225) ovaries, and granulosa and cumulus cells from human ovaries (226, 227, 269, 270). Our study confirmed that GDF9 was also present in hGL cells. Indeed, our study demonstrates an autocrine role of endogenous GDF9 for the first time and further supports the enhancing action of GDF9 secreted by the oocyte in activin A-induced inhibin B accumulation. Although it is tempting to suggest that with release of the oocyte after ovulation, a main source of GDF9 is removed (hence, the cell response to activin A with respect to β_B-subunit is withdrawn) to explain the decline in inhibin B secretion after ovulation, the contribution of GDF9 secreted by granulosa cells in this regard remains unknown.

Progesterone production is an important function of granulosa cells. Activin A is a known inhibitor of luteinization as reported in human (292, 293), bovine (294, 295), and rat (296) ovary studies. In the menstrual cycle, serum activin A levels are higher in the early follicular,

mid-cycle and late luteal phases (164) while serum inhibin B levels peak in the mid-follicular phase and immediately following ovulation (161). During ovarian stimulation for IVF, activin A levels remained unchanged after 7-8 days of FSH treatment but rose significantly with follicle maturity (297). Based on these observations and findings of our studies, we speculate that increased expression of activin A in the pre-ovulatory follicle in the presence of GDF9 further enhances inhibin B expression and overcomes the inhibitory effect of activin A on luteinization. This hypothesis suggests that local activin activity is detrimental to luteal function and structure; and removal of activin from the system is critical for successful luteal function. To what extent this plays a physiological role remains unknown given the dominant effects of LH on luteinization.

We acknowledge the limitations of extrapolating our results from granulosa-lutein cells obtained from women undergoing IVF treatment to normal ovarian physiology because these cells have been exposed to pharmacological concentrations of exogenous gonadotropins in the process of luteinization and hCG stimulation. Nevertheless, in the absence of granulosa cells from the unstimulated, normal ovaries that are easily accessible for research, findings from our cell culture model provide interesting hypotheses for further evaluation with regards to the role of GDF9 and related mechanisms involved in regulating inhibin B production during the peri-ovulatory transition. Furthermore, because of the limitation, we cannot link our results from the *in vitro* model system with other clinic parameters of IVF patients to build up the information network. Whether these conclusions can be applied to normal or PCOS patients remains to be determined.

IVF is an accepted clinical procedure for treatment of specific fertility problems not amenable to other forms of therapy. In summary, our results raise interesting questions about

the GDF9, inhibin B, FST, and progesterone expression profiles in IVF patients. If GDP9 is clearly shown to play a crucial role in human follicular development, it will introduce new targets for IVF treatment in infertile couples, potential biochemical markers for successful fertilization and embryo development and successful pregnancy.

6.2. Recommendations for Future Work

The results advance our understanding of the regulatory roles of GDF9 in activin-induced inhibin B production in hGL cells. However, the complete relationship between GDF9 and activin A in follicular development is still far from understood. Further studies are needed to improve our knowledge with regards to the development of hGL cells. These questions include:

1) Progesterone production is one important aspect of steroidogenesis of hGL cells. Whether interaction of GDF9, activin A and inhibin B play a role in other aspects of steroidogenesis like estradiol accumulation needs to be investigated.
2) Although our knowledge of GDF9 and BMP15 has increased, many questions about the biological roles of these proteins within the ovary remain. Our studies demonstrate that GDF9 is crucial for regulation of activin A in the accumulation of inhibin B and progesterone in hGL cells. The role of its close homologue BMP15 in this system remains unknown. Additionally, whether GDF9 and BMP15 actually function as homodimers, heterodimers or both, and whether the formation of the putative heterodimer is temporarily regulated must still be studied.
3) Based on our i*n vitro* results, further studies are needed to test the hypothesis that the GDF9/activin/inhibin/FST/FSTL3 system is closely related with the pathogenesis of PCOS.

Compared to normal patients, PCOS patients have different inhibin B, FST and steroid expression profiles as mentioned previously. Additionally, very recently a number of reports have suggested that variations in GDF9 may cause abnormal ovary functioning in conditions like PCOS. Human granulosa-lutein cells from healthy women and women with PCOS need to be cultured and tested. The results can be considered in conjunction with IVF clinic data to contribute to the literature on this topic to provide important insight into the roles of these autocrine and paracrine regulators in PCOS.

4) BMP3 (318), BMP5 (22) and BMP6 (319) (also secreted by oocytes) have been shown to be expressed in granulosa cells. Whether they have similar functions as GDF9 in the regulation of activin-induced inhibin B and progesterone production needs to be further examined.

5) GDF9 enhanced activin A-induced inhibin β_B-subunit mRNA level and thus inhibin B accumulation as well. Yet we cannot rule out the possibility that increased levels of β_B-subunit mRNA lead to increased synthesis of activin B ($\beta_B\beta_B$) or activin AB ($\beta_A\beta_B$). Additionally, we cannot dismiss the possibility that decreased levels of inhibin α-subunit mRNA after targeted siRNA transfection may lead to increased synthesis of activin B and activin AB. Unfortunately, specific and sensitive activin B and activin AB assays are commercially unavailable. Furthermore, whether activin B and activin AB can behave similarly to activin A in inhibin B and progesterone accumulation, and whether their actions can be regulated by GDF9 remains to be determined.

6) Aside from FST and FSTL3, other extracellular regulators like BAMBI (124), Cripto (116), betaglycan (107) and InhBP/p120 (160) can antagonize while endoglin (123) can facilitate activin signaling. The role of GDF9 in the regulation of these factors needs to be further

investigated.

7) FST isoforms (FST288, FST303 and FST315) have different locations in the cell culture. The shortest isoform (FST288) has a higher affinity for heparin-sulfated proteoglycans, allowing it to be concentrated in the outer surface of the plasma membrane (320). This localization could prevent the autocrine effects of activin as well as the paracrine or endocrine effects. Conversely, the longest isoform (FST315) is found primarily in the circulation, a finding that is consistent with its reduced affinity for heparin (208). The intermediate isoform (FST303) is found primarily in gonadal extracts and fluids (209). The real-time PCR primers and ELISA used in this thesis can only detect the mRNA and proteins of all isoforms and thus cannot distinguish the different roles of these three isoforms. More studies must be undertaken in this regard. Furthermore, because FSTL3 could be localized to the nucleus and secrete more slowly than other FST isoforms, additional studies are needed to test the hypothesis that GDF9 not only decreases the synthesis and secretion of FST isoforms and FSTL3, but also modulates their cellular location.

8) The results from this thesis are from *in vitro* cell models. Whether these conclusions can be applied *in vivo* remains to be determined.

6.3. Figures

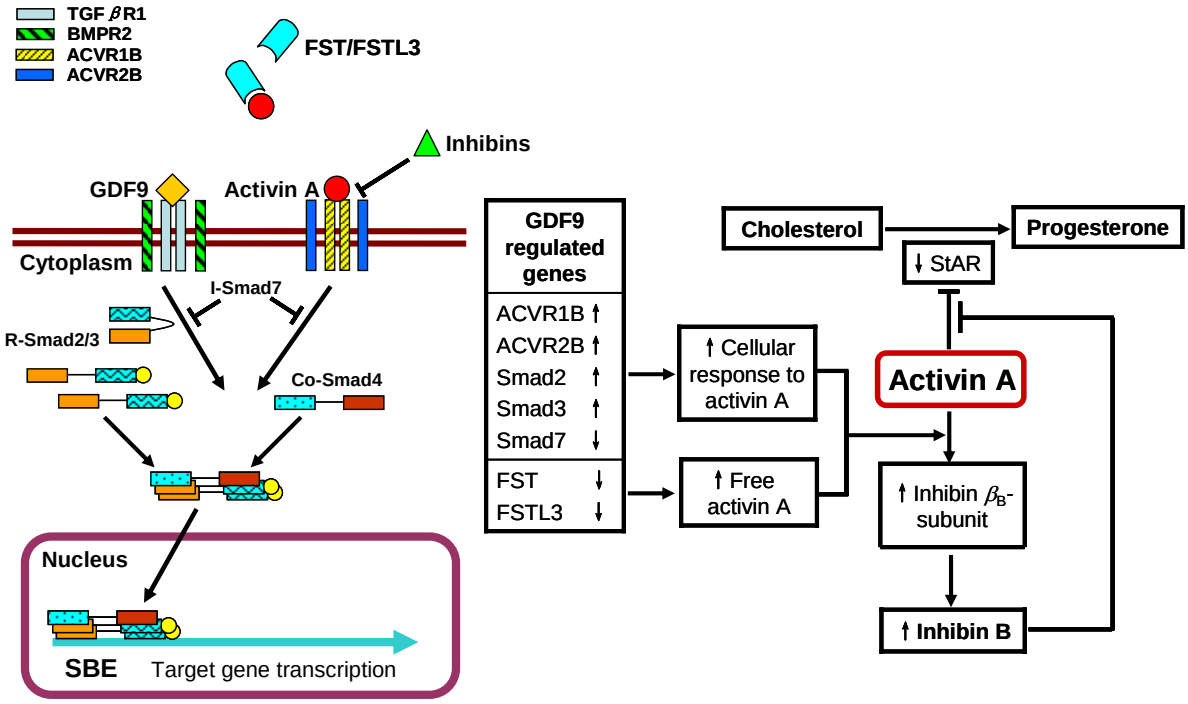


Fig. 6.1 A model, suggested by the results, for the interactions between GDF9 and activin A in the regulation of inhibin B and progesterone accumulation by human granulosa-lutein cells. Abbreviations: ACVR, Activin receptor; BMPR, Bone morphogenetic protein receptor; FST, Follistatin; FSTL3, Follistatin-like 3; GDF9, Growth differentiation factor 9; Smad, Son of mothers against decapentaplegia; SBE, Smad binding element; Co-Smad, Common Smad; I-Smad, Inhibitory Smad; R-Smad, Receptor-activated Smad; StAR, Steroidogenic acute regulatory protein; TGFβR, Transforming growth factor β receptor.

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APPENDICES

Table S1. Nucleotide sequences of primers used for quantitative real-time PCR

Gene Name	Forward primer sequence	Reverse primer sequence
Inhibin α-subunit	5'-GTCTCCCAAGCCATCCTTTT-3'	5'-TGGCAGCTGACTTGTCCTC-3'
Inhibin β_A-subunit	5'-CTCGGAGATCATCACGTTTG-3'	5'-CCTTGGAAATCTCGAAGTGC-3'
Inhibin β_B-subunit	5'-ATCAGCTTCGCCGAGACA-3'	5'-GCCTTCGTTGGAGATGAAGA-3'
ACVR1A	5'-TCATGAATTTGGCTTTTGGA-3'	5'-TTTGGCAGTGTGACGCTTAC-3'
ACVR1B	5'-ATATTGGGAGATTGCTCGAAGA-3'	5'-GGCAGCTGATATTCTTCATGG-3'
ACVR2A	5'-AAAGCCCAGTTGCTTAACGA-3'	5'-TGCCATGACTGTTTGTCCTG-3'
ACVR2B	5'-TGTCAAGATCTTCCCACTCCA-3'	5'-CATGCCAGGTGTGCTGAA-3'
Smad2	5'-GCCTTTACAGCTTCTCTGAACAA-3'	5'-ATGTGGCAATCCTTTTCGAT-3'
Smad3	5'-CCCCAGCACATAATAACTTGG-3'	5'-AGGAGATGGAGCACCAGAAG-3'
Smad4	5'-TGGCCCAGGATCAGTAGGT-3'	5'-CATCAACACCAATTCCAGCA-3'
Smad7	5'-CGATGGATTTTCTCAAACCAA-3'	5'-ATTCGTTCCCCCTGTTTCA-3'
GDF9	5'-CTCTTCACCCCCTGTACCC-3'	5'-CAGTTCCACTGATGGAAGGAT-3'
FST	5'-TGCTCTGCCAGTTCATGG-3'	5'-CTTGACGGAGCCAGCAGT-3'
FSTL3	5'-CTACATCTCCTCGTGCCACA-3'	5'-TCTTCTGCAGACTCACCACCT-3'
StAR	5'-AAACTTACGTGGCTACTCAGCATC-3'	5'-GACCTGGTTGATGATGCTCTTG-3'
P450scc	5'-CAGGAGGGGTGGACACGAC-3'	5'-AGGTTGCGTGCCATCTCATAC-3'
3βHSD	5'-GCCTTCAGACCAGAATTGAGAGA-3'	5'-TCCTTCAAGTACAGTCAGCTTGGT-3'
GAPDH	5'-ATGGAAATCCCATCACCATCTT-3'	5'-CGCCCCACTTGATTTTGG-3'

CHILDREN'S & WOMEN'S HEALTH CENTRE OF BRITISH COLUMBIA
AN AGENCY OF THE PROVINCIAL HEALTH SERVICES AUTHORITY

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ETHICS CERTIFICATE OF MINIMAL RISK APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC C&W NUMBER:
Peter C.K. Leung		H90-70337

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Children's and Women's Health Centre of BC (incl. Sunny Hill)	Child & Family Research Institute

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
In-Sun Hong
Junling Chen
Anthony P. Cheung
Fengtao Shi
Song Ling L. Poon

SPONSORING AGENCIES:
- British Columbia Health Research Foundation - "Immortalization of human ovarian granulosa cells"
- Canadian Institutes of Health Research (CIHR) - "Endocrine Control of Reproduction"
- Canadian Institutes of Health Research (CIHR) - "Role of oocyte-derived growth factors in human ovarian follicle development"

PROJECT TITLE:
Autocrine and paracrine control of ovarian granulosa cell function

REMINDER: The current UBC Children's and Women's approval for this study expires: May 19, 2010

APPROVAL DATE: May 19, 2009

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Children's and Women's Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Children's and Women's Research Ethics Board.

Approved by one of:

Dr. Marc Levine, Chair Dr. Mason Bond, Associate Chair