Expression of inhibin-activin subunits, follistatin and smads in granulosa-luteal cells collected at oocyte retrieval

Shiuh Young Chang · Hong-Yo Kang ·
Kuo-Chung Lan · Chang-Yi Hseh · Fu-Jen Huang ·
Ko-En Huang

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Abstract *Purpose:* To examine the expression of inhibin-activin subunits, follistatin, and Smads 2, 3 and 4 in granulosa-luteal cells at the time of oocyte retrieval.

Methods: Quantitative reverse transcriptase-polymerase chain reaction was performed to quantify the mRNA expression of the investigated genes: inhibin α -subunit, inhibin-activin β A- and β B-subunits, follistatin, and Smads 2, 3 and 4.

Results: α -, β A- and β B-subunits of inhibin-activin, and follistatin were all well expressed in granulosa-luteal cells obtained from either dominant or cohort follicles. α -subunits strongly correlated with Smad2 ($p < 0.001$). β A-subunit significantly correlated with Smad4, $p < 0.001$. β B-subunit significantly correlated with Smad2, $p = 0.002$, and follistatin expression strongly correlated with the expression of Smads 2 and 4 ($p < 0.001$ and $= 0.007$ respectively).

Conclusions: We found, for the first time that inhibin-activin β B-subunit mRNA was well expressed in human granulosa-luteal cells obtained from either dominant or cohort follicles. α -, β A- and β B-subunits and follistatin cor-

related with Smads to varied degrees, suggesting the active roles of the above genes at the time of oocyte retrieval.

Keywords Activin · Follistatin · Granulosa cells · Inhibin · Smad

Introduction

Within the ovarian follicle, inhibin (alpha/beta) and activin (beta/beta) dimers influence follicular growth and development by modulating thecal and granulosa responses to the gonadotropins. Inhibin is secreted by granulosa cells and exerts a positive paracrine action on LH-induced androgen production by the thecal cells. Activin, also secreted by granulosa cells, has a negative paracrine action on LH-induced androgen production by thecal cells. In addition, activin, either alone or with FSH, has an autocrine action on granulosa cell differentiation and oocyte maturation, leading overall to promotion and maintenance of folliculogenesis [1–3].

Follistatin, a 35-kilodalton monomer secreted by granulosa cells, is a β -subunit-specific high affinity binding protein. Follistatin was originally identified as a specific inhibitor of follicle stimulating hormone secretion, and was later characterized as a binding protein for activin. Follistatin modulates granulosa cell function in favor of luteinization or atresia, and its mechanism of action involves neutralizing the actions of activin and having a direct action on progesterone metabolism by granulosa cells [1].

Activin and inhibin, in addition to bone morphogenetic protein (BMP), growth differentiation factor 9 (GDF-9), and Müllerian-inhibiting substance, belong to the intraovarian TGF- β family [4]. The basal components of the TGF- β family signaling pathway are the type II and type I transmembrane

S. Y. Chang · K.-C. Lan · F.-J. Huang · K.-E. Huang (✉)
Department of Obstetrics and Gynecology, Chang Gung
Memorial Hospital-Kaohsiung Medical Center,
123 Ta-Pei Road, Niao-Sung Hsiang,
Kaohsiung County 833, Taiwan
e-mail: sychang@adm.cgmh.org.tw

S. Y. Chang · H.-Y. Kang · K.-C. Lan · F.-J. Huang · K.-E. Huang
Chang Gung University College of Medicine,
Taoyuan, Taiwan

H.-Y. Kang · C.-Y. Hseh · K.-E. Huang
Center of Reproductive Medicine,
Chang Gung Memorial Hospital,
Kaohsiung, Taiwan

serine and threonine kinase receptors and the cytoplasmic Smad protein [5]. The ligand first binds to the type II receptor. The type I receptor, activated by phosphorylation, acts downstream of the type II receptor. The type I receptors comprise type IA (ActRIA) and type IB (ActRIB). The type II receptors comprise type IIa (ActRIIA) and type IIB (ActRIIB). The inhibin co-receptor, betaglycan, interacts with activin receptors and augments inhibin antagonism of activin action [6].

Once activated, the type I receptor-regulated Smads (R-Smads) dissociate from the receptor complex, bind to the common Smad, namely Smad4, and enter the nucleus as a complex, wherein they participate in the transcription of the target genes [7]. Activated type I receptors propagate the signal to specific intracellular Smad proteins, namely Smad2 and/or Smad3 [7]. The different usage of the type I and type II receptors and Smads may be responsible for the different actions of activin on different tissues and target genes. It has been demonstrated that ActRIB, rather than ActRIA, is the type I receptor that functionally mediates activin signaling in granulosa cells [8].

Follicular development during the physiological menstrual cycle is not directly comparable with the cohort follicles of varied sizes that have previously undergone controlled ovarian hyperstimulation (COH). COH has been reported to alter the intra-follicular hormonal, including the inhibin, milieu [9]. We hypothesized that the cohort follicles of varied sizes under COH may differ from the physiological conditions of the gene expression of the inhibin-activin-follistatin system. The present study, using a quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) technique for the examination of the mRNA expression of the inhibin α -subunit, inhibin-activin βA - and βB - subunits, follistatin, and Smads 2, 3 and 4 in the granulosa-luteal cells obtained at oocyte retrieval, sought to examine the above hypothesis.

Materials and methods

Controlled ovarian hyperstimulation

This study was approved by the institutional review board (IRB) of the hospital. Patients underwent COH for ART [10]. Briefly, subcutaneous luprolide acetate (Lupron; Abbott Australasia PTY, Ltd., Kurnell, New South Wales, Australia) was administered from day 21 of the last menstrual cycle preceding the ovum retrieval. Patients received one of the fixed starting doses (age <35 years: 225 IU/day; $35 \leq \text{age} \leq 40$ years: 300 IU/day) of recombinant FSH (rFSH) after pituitary desensitization had been achieved. Daily doses of rFSH were continued on an individual basis depending upon the extent of follicular growth. Ten thousand

IU of human chorionic gonadotropin (hCG) was administered when the two largest follicles had reached a mean diameter of 17 mm with consistent levels of serum estradiol levels. The ovum retrieval was scheduled 35 to 37 h subsequently.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Preparation of real-time PCR mix

Only the first aspirate without blood contamination from each ovary was used for the study. Hormonal levels were determined by radioimmunoassay (RIA). Collection of granulosa-luteal cells, RNA extraction, and cDNA synthesis followed the protocols we published previously [11]. All of the PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Bio systems). PCR was performed using the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Bio systems). The amplification reactions were performed in a 25- μ l final volume containing 10 X SYBR buffer, 25 mM of $MgCl_2$, 12.5 mM of dNTP, 0.625 units of AmpliTaq Gold and 0.25 units of UNG. The inhibin α -subunit, inhibin-activin βA -, and βB -subunits, follistatin, Smads 2, 3 and 4, and β -actin forward and reverse primers and concentrations are listed in Table 1. To reduce the variability between replicates, PCR premixes that contained all the reagents expected to be used for the cDNA, were prepared and aliquoted into 1.5 ml microfuge tubes.

Real-time RT-PCR time and temperature profiles

The real-time RT-PCR protocol used for inhibin α -subunit, inhibin-activin βA -, and βB -subunits was: denatured program (60°C 10 min, 95°C for 10 min), amplification and quantification program repeated 40 times (95°C for 20 s; 65°C for 30 s; 72°C for 30 s), and melting curve program (95°C for 15 s; 60°C to 95°C with a heating rate of 0.2°C/s).

The real-time RT-PCR protocol used for the Smad family: Smad2, Smad3, and Smad4 was: denatured program (50°C 2 min, 95°C for 10 min), amplification and quantification program, repeated 40 times (95°C for 15 s; 60°C for 1 min; 72°C for 30 s), and a melting curve program (95°C for 15 s; 60°C to 95°C with a heating rate of 0.2°C/s). Specific PCR amplification products were detected using the fluorescent double-stranded DNA-binding dye SYBR Green core reagent kit (Perkin-Elmer Applied Bio systems).

The RT-PCR protocol used for follistatin was: denatured program (50°C 2 min, 95°C for 10 min), amplification and quantification program, repeated 40 times (95°C for 15 s; 60°C for 1 min; 72°C for 30 s), and a melting curve program (95°C for 15 s; 60°C to 95°C with a heating rate of

Table 1 Oligonucleotide primer sequences of inhibin α -subunit, inhibin-activin β A- and β B-subunits, follistatin and Smads 2, 3 and 4 for real-time quantitative reverse transcriptase-polymerase chain reaction

gene	Sequences (5'→3')	Conc
Inhibin	F: ACG CAG CAC TGT GCT TGT ATC T (22mers)	2.5 μ M
α -subunit	R: GGA GTG GGA GCC ATC TAT TCC C (22mers)	
Inhibin-activin	F: GGA GTA TGA TGG CAA GGT CAA (21mers)	5 μ M
β A-subunit	R: GAT AGT CAG AGG GAG CAA TGA T (22mers)	
Inhibin-activin	F: TGC CCT TGT CGA GGG AAA G (19mers)	10 μ M
β B-subunit	R: AGA GGC CCA AGC CAC AAA C (19mers)	
follistatin	F: AAA GCA TCT CAC TGC CAC TCA CA (23mers)	10 μ M
	R: ACA ACA ACA GCG CAG AAG CA (20mers)	
Smad2	F: CCT TCA GTG CGT TGC TCA AG (20mers)	5 μ M
	R: GGA CCA CAC ACA ATG CTA TGA CA (23mers)	
Smad3	F: AGA AGG ACG AGG TCT GCG TG (20mers)	5 μ M
	R: GGA ATG GCT GTA GTC GTC CAG (21mers)	
Smad4	F: CAC CAA GTA ATC GTG CAT CGA (21mers)	5 μ M
	R: GCC ACA GGA ATG CTG GGA AA (20mers)	
β -actin	F: TCA CCC ACA CTG TGC CCA TCT ACG A (25mers)	2.5 μ M
	R: CAG CGG AAC CGC TCA TTG CCA ATG G (25mers)	

0.2°C/s). Another real-time RT-PCR protocol was used for β -actin: denatured program (60°C 5 min, 95°C for 10 min), amplification and quantification program repeated 40 times (95°C for 20 s; 65°C for 30 s; 72°C for 30 s) and the melting curve program (95°C for 15 s; 60°C to 95°C with a heating rate of 0.2°C/s). Specific PCR amplification products were detected using the fluorescent double-stranded DNA-binding dye SYBR Green core reagent kit (Perkin-Elmer Applied Bio systems). Experiments were performed with duplicates for each data point.

Calibration curves

The quantity of mRNA was calculated using the Ct method. Briefly, the fluorescence signal threshold was defined such that each PCR reaction was in the exponential phase. The Ct value was the cycle at which the fluorescence signal of the reaction exceeded this value. Ct values were proportional to the starting copy number. Ct values of each target gene were normalized to the Ct value of a housekeeping gene β -actin. Results are presented as $2^{\Delta C_t}$ ($\Delta C_t = C_t \beta\text{-actin} - C_t \text{target gene}$) [12].

Statistical analysis

For the purpose of this analysis, the correlation of follicular volume, follicular hormones and gene expression levels were examined. A multiple regression analysis with stepwise forward procedure (multivariate analysis) was used to identify independent factors and to test for the interactive effects of the covariates. Follicular volume, follicular hormones and gene expression levels were continuous data. The Sigmas-tat statistical package (Jandel Corporation, San Rafael, CA) was used for data analysis. All *P* values were two-sided,

and a *P* value of less than 0.05 was considered statistically significant.

Results

A total of 240 samples for inhibin α -subunit, 261 samples for inhibin-activin β A-subunit, 264 samples for inhibin-activin β B-subunit, 268 samples for follistatin, 264 samples for Smad2, 221 samples for Smad3, and 267 samples for Smad4 were obtained from 329 patients enrolled in the study. α -, β A- and β B-subunits and follistatin were all well expressed in granulosa-luteal cells obtained either from cohort or dominant follicles (Table 2).

The relative expression levels of inhibin α -subunit, inhibin-activin β A- and β B-subunits, follistatin, Smads 2, 3 and 4 genes were quantified. The expression levels were determined as ratios between the studied genes and the reference gene β -actin. Multivariate and univariate analyses showed α -, β A- and β B-subunits and follistatin gene expression had no correlation with follicular fluid volume (Table 2, Fig. 1).

Multivariate analyses showed: mRNA expression of α -subunit strongly correlated with β A- and β B-subunit ($p < 0.001$ respectively); α -subunits strongly correlated with Smad2 ($p < 0.001$); β A-subunit strongly correlated with β B-subunit, $P < 0.001$ and significantly correlated with Smad4, $p < 0.001$; and β B-subunit significantly correlated with Smad2, $p = 0.002$. Follistatin expression also strongly correlated with the expression of Smads 2 and 4 ($p < 0.001$ and $= 0.007$ respectively). Follistatin negatively correlated with progesterone levels in the follicular fluid ($p < 0.029$), but did not correlate with testosterone or androstenedione levels (Table 2).

Table 2

	B	95%CI of B	P-value
A. Regression analysis of inhibin α-subunit mRNA levels with follicular fluid volume, expression of inhibin-activin βA- and βB-subunits and Smads 2, 3 and 4			
Follicular fluid volume			0.393
Inhibin-activin β A—subunit mRNA	0.225	0.098;0.352	<0.001
Inhibin-activin β B—subunit mRNA	−0.325	−0.413; −0.237	<0.001
Smad2 mRNA	0.581	0.488;0.674	<0.001
Smad3 mRNA			0.053
Smad4 mRNA			0.091
B. Regression analysis of inhibin-activin βA-subunit mRNA levels with follicular fluid volume, expression of inhibin α-subunit, inhibin-activin βB-subunit and Smads 2, 3 and 4			
Follicular fluid volume			0.220
Inhibin α -subunit mRNA	0.299	0.133;0.465	<0.001
Inhibin-activin β B—subunit mRNA	0.353	0.248;0.459	<0.001
Smad2 mRNA			0.928
Smad3 mRNA			0.931
Smad4 mRNA	−0.284	−0.413;0.155	<0.001
C. Regression analysis of inhibin-activin βB-subunit mRNA levels with follicular fluid volume, expression of inhibin α-subunit, inhibin-activin βA-subunit and Smads 2, 3 and 4			
Follicular fluid volume			0.256
Inhibin α -subunit mRNA	−0.748	−0.967; −0.528	<0.001
Inhibin-activin β A—subunit mRNA	0.590	0.406;0.775	<0.001
Smad2 mRNA	0.672	0.241;1.102	0.002
Smad3 mRNA			0.078
Smad4 mRNA			0.073
D. Regression analysis of follistatin mRNA levels with follicular fluid volume, expression of Smads 2, 3 and 4, and follicular progesterone, testosterone, androstenedione levels			
Follicular fluid volume			0.125
Smad2 mRNA	1.233	1.06;1.41	<0.001
Smad3 mRNA			0.143
Smad4 mRNA	−0.223	−0.383; −0.006	0.007
Follicular progesterone levels	−1.04	−1.97; −1.06	0.029
Follicular testosterone levels			0.730
Follicular androstenedione levels			0.814

Findings of the linear regression analyses are shown in Fig. 2. The findings were quite similar to those of multivariate analyses in that: mRNA expression of α -subunit strongly correlated with that of β A- and β B-subunit ($p=0.003$ and 0.004 respectively); α -subunits strongly correlated with Smad2 ($p < 0.001$); β A-subunit strongly correlated with β B-subunit, $P < 0.001$, and also significantly correlated with Smad4, $p = 0.003$; and β B-subunit significantly correlated with Smad2, $p < 0.001$. Follistatin expression also strongly correlated with the expression of Smads 2 and 4 ($p < 0.001$ respectively).

Discussion

Activin production decreases and inhibin production increases as the follicle grows [13–17]. In the early follicular phase, under FSH and estradiol control, inhibin-B secretion

is favored. Late in the follicular phase, when LH controls, inhibin-A secretion is favored [14]. Findings in follicular fluid levels of dimeric inhibins and activin are compatible with findings in their subunits [15–16].

Several studies have been published regarding activin-inhibin system of granulosa cells in animal models, which may not be applicable in human beings [18, 19]. In human, granulosa cells of small antral follicles (1–8 mm) were found to express mRNA for α -, β A-, and β B-subunits [13, 15]. With the development of a dominant follicle, β B-subunit mRNA expression in the granulosa cells becomes no longer or only weakly detectable, while the expression of β A-subunit and follistatin becomes abundant [13, 16, 20]. In the corpus luteum, the inhibin-activin α - and β A-subunits and follistatin mRNA all are expressed in the granulosa-luteal cells [15].

The inhibin α - and β A-subunits, and to a lesser extent β B-subunit, are expressed in cultured human granulosa-luteal

Fig. 1 Linear regression analysis reveals no correlation of follicular fluid volume with the mRNA expression of the inhibin α -subunit (Fig. 1A), inhibin-activin β A-subunit (Fig. 1B), inhibin-activin β B-subunit (Fig. 1C) and follistatin (Fig. 1D)

cells obtained from women undergoing IVF [20, 21]. It has been reported that the differentiated status of granulosa-luteal cells may affect the gene expression [22]. Follicle maturity has been related to follicle size, although the two are not totally dependent [23]. Unfortunately, the follicular size from which granulosa-luteal cells were obtained in the above studies was not mentioned [20, 21]. We found for the first time, and complementary to previous reports [20, 21], that β B-subunit, in addition to α - and β A-subunits, was well expressed in fresh human granulosa-luteal cells obtained either from dominant or cohort follicles under COH.

FSH and IGF-I differentially regulate inhibin A and inhibin B secretion in the granulosa cells of the follicular phase [24]. FSH is the major regulator of inhibin secretion by the granulosa cells. In contrast, in the corpus luteum, inhibin secretion is LH-dependent [25]. Granulosa-luteal cells are half-way between the follicular phase and luteal phase. A previous study demonstrated that granulosa-luteal cells obtained at oocyte retrieval secrete both inhibin A and inhibin B in response to the addition of FSH *in vitro*, although inhibin B stimulation was not maintained at higher FSH doses or a longer incubation time [26]. We therefore speculate that the

constantly high FSH stimulation and the administration of an ovulatory dose of hCG in the process of COH induce abundant co-expression of all three subunits in all the follicles of varied sizes in the present study.

The significance of the abundant mRNA co-expression of all three subunits in the varied sizes of follicles at the time of oocyte retrieval awaits further investigation. Follicular fluid levels of dimeric inhibins and activin were not measured in the present study. Inhibins and activin are transported out of the follicle, as well as metabolized within the follicle [27], it is therefore unlikely that examining concentrations

of inhibins and activin in the corresponding follicular fluid reflects the status of inhibin and activin transcription at the time point of oocyte retrieval. Nevertheless, we demonstrated that the mRNA expression of [1] α -, β A- and β B-subunits are strongly correlated with each other, and [2] α -, β A- and β B-subunits and follistatin are correlated with Smads to varied degrees (Table 2), suggesting the active roles of the above genes at the time of oocyte retrieval. These findings are unprecedented.

Follistatin seems to be a powerful atretic factor for cohort follicles and a luteinizing agent for dominant follicles

Fig. 2 Linear regression reveals a positive correlation between mRNA expression of inhibin α -subunit and inhibin-activin β A-subunit (Fig. 2A), between inhibin α -subunit and inhibin-activin β B-subunit (Fig. 2B), between inhibin α -subunit and Smad2 (Fig. 2C). Inhibin-activin β A- and β B-subunits expression also strongly correlated with

each other (Fig. 2D). Inhibin-activin β A-subunit strongly correlated with Smad4 (Fig. 2E), and inhibin-activin β B-subunit strongly correlated with Smad2 (Fig. 2F). Follistatin strongly correlated with Smads 2 and 4 (Fig. 2G, 2H)

Fig. 2 Continued

[1]. In primates, follistatin levels increase in the follicular fluid with the increasing growth of the follicle, a mechanism for decreasing activin activity [28]. We found follistatin expression strongly correlated with the expression of Smads 2 and 4, suggesting an active role for follistatin at the time of oocyte retrieval. However, follistatin expression negatively correlated with follicular fluid progesterone levels, markers of luteinization, in the present study. In contrast, follistatin expression did not correlate with testosterone (T) or androstenedione (ASD), markers of atresia, levels, and follicular fluid volume in the present study (Table 2).

It has been shown that basal follistatin mRNA levels in rat granulosa cells are regulated by FSH and activin, rather than by LH [29]. In addition, although follistatin increases progesterone secretion in a dose-dependent manner, it does

not augment hCG-stimulated progesterone secretion [30]. We therefore speculate that the follicular volume, and P4, T and ASD levels in the follicular fluid are the cumulative products of high constant FSH stimulation and the ovulatory dose of hCG administered during the process of COH, not necessarily in accordance with the mRNA expression of follistatin at the moment of oocyte retrieval.

In conclusion, the present study demonstrated for the first time that inhibin-activin β B-subunit mRNA expression was well expressed in human granulosa-luteal cells obtained from either dominant or cohort follicles. Expression of α -, β A- and β B-subunits strongly correlated with each other. α -, β A- and β B-subunits and follistatin correlated with Smads to varied degrees, suggesting the active roles of the above genes at the time of oocyte retrieval. These findings are unprecedented.

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