Relative expression of genes encoding SMAD signal transduction factors in human granulosa cells is correlated with oocyte quality

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Abstract

Purpose To determine the expression of SMAD transcripts in human granulosa cells.

Methods Luteinized mural granulosa cells were harvested from forty women undergoing oocyte retrieval, and RNAs were isolated. SMAD expression levels were determined by polymerase chain reaction (PCR) and quantitative real-time PCR (q-RT-PCR).

Results *SMAD1-7* and *9* are expressed in human granulosa cells, with *SMAD2*, *3* and *4* showing the highest expression levels. Peak estradiol (E2) levels correlated with the number of oocytes retrieved during IVF. Oocyte number showed no correlation with *SMAD* expression levels or ratios. Fertilization rates also did not correlate with the expression levels of individual *SMADs*, but did correlate with higher *SMAD4:SMAD3* ratios ($p=0.0062$) and trended with *SMAD4:SMAD2* ($p=0.0698$).

Conclusions SMAD transcripts are differently expressed in human granulosa cells, where they may mediate TGF-beta superfamily signaling during folliculogenesis and ovulation. Further, the relative expression ratios of *SMAD2*, *3* and *4* may differentially affect fertilization rate.

Capsule Relative expression levels of SMAD transcripts in human granulosa cells correlate with fertilization rate.

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Introduction

Granulosa cells play important roles in follicular differentiation, influencing the optimal conditions for oocyte development, ovulation, fertilization and implantation [1]. The events surrounding follicle activation, the development of the preovulatory follicle, and follicular atresia are controlled by both ovarian factors such as IGF-1 and estrogen, and by the gonadotropins LH and FSH [2]. This process involves many regulatory factors, including the pituitary gonadotropins FSH and LH, and various transcription factors, growth factors and cytokines. In addition, members of the transforming growth factor (TGF)- β superfamily, which includes TGF- β , nodal, activins, bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) [3–6], are expressed in the ovary and are involved in the control of normal folliculogenesis and ovulation [7, 8]. Understanding the pathways underlying these events is crucial for understanding the causes of infertility, which affects approximately 10% of women of reproductive age in the U.S. (6.1 million) [9], and for understanding the factors which may influence the outcome of in vitro fertilization (IVF).

The effects of TGF- β superfamily members are known to be mediated by SMAD intracellular signal transduction proteins [10–13]. The SMAD proteins are classified into three subclasses based on their intracellular functions: receptor-activated (R-) SMADs (SMAD1, 2, 3, 5, and 9), the common-partner (Co-) SMAD (SMAD 4), and inhibitory (I-) SMADs (SMAD6 and 7) [14, 15]. Of the R-SMADs, SMAD2 and 3 (AR-SMADs) act downstream of

activated TGF- β /activin/nodal type I receptors, and SMAD1, 5, and 8 (BR-SMADs) act downstream of activated BMP type I receptors [13, 16, 17]. These R-SMADs interact with and form a complex with the co-SMAD (SMAD4) [18, 19], which then enters the nucleus and regulates target gene transcription by directly binding to the SMAD-binding DNA element [16]. I-SMADs negatively regulate intracellular SMAD pathways: SMAD6 specifically inhibits the activation of BMP pathways and interferes with the binding of R-SMADs with Co-SMAD4, whereas SMAD7 inhibits the activation of TGF- β /activin and BMP pathways [20–22]. I-SMADs have also been shown to regulate apoptosis in the absence of involvement of other SMAD signaling pathways [23].

All *Smads* are expressed in the rodent ovary, where they have been shown to be important regulators of viability and fertility [24], and *Smad2* and *Smad3* have been shown to exhibit stage-specific expression patterns and differential ligand sensitivities in rodent granulosa cells during folliculogenesis [25]. In addition, ovarian- and granulosa-specific *Smad* knockout and transgenic mouse models exhibit a variety of defects in folliculogenesis, coupled with reduced fertility [26–30]. In humans, transcripts for *SMAD2*, 3 and 4 have been identified in granulosa cells collected at oocyte retrieval [31], which suggested that these and other SMADs might also be involved in human ovarian function and fertility. In this study, we examined the expression of *SMAD1*–7 and 9 in human luteinized mural granulosa cells from patients undergoing IVF, and analyzed the relationships between SMAD transcript expression levels and the number and fertilization rates of oocytes produced.

Materials and methods

Human granulosa cell isolation and complementary DNA (cDNA) production

We obtained luteinized mural granulosa cells from 40 consecutive patients undergoing in vitro fertilization (IVF). The study protocol was approved by the Cedars-Sinai Institutional Review Board (IRB), and informed consent to participate in the study was obtained from each patient. The cumulus-oocyte complexes were removed and granulosa cells were obtained from the follicular aspirates. The granulosa cells were separated from red blood cells by a previously-described protocol using Percoll gradients [32]. Briefly, the follicle aspirates were centrifuged at 1800 rpm for 10 min, the supernatant was removed and the cells were resuspended in 10 ml of 1 $\times$ phosphate-buffered serum (PBS) and spun at 1500 rpm for 10 min. The supernatant was aspirated and the cells were resus-

pended in 4 ml of 1 $\times$ PBS, and layered on Percoll. After centrifugation at 2000 rpm for 30 min, the luteinized mural granulosa cells were washed in 1 $\times$ PBS and spun at 1500 rpm for 10 min.

The cell pellet was then lysed in RLT buffer (Qiagen, Valencia, CA) and total RNA was extracted using the RNeasy Mini Kit as described by the manufacturer (Qiagen). Complementary DNA (cDNA) was synthesized from 3 μ g total RNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories).

Amplification of SMAD transcripts by Polymerase Chain Reaction (PCR)

Human ovarian cDNA (Ambion, Austin, TX) was first used to amplify SMAD transcripts in the ovary. Human Ovary PCR-Ready cDNA (2.5 ng) was used as a template in 50 μ l PCR reactions performed using the HotStar Taq DNA Polymerase Kit (Qiagen). The primers used to amplify *SMAD* cDNA fragments were as follows: *SMAD1* (5'-ACCTGCTTACCTGCCTCCTG-3' and 5'-CATAAGCAACCGCTGAACA-3'); *SMAD2* (5'-ACCGAAATGCACGGTAGAA-3' and 5'-TGGGGCTCTGCACAAA GAT-3'); *SMAD3* (5'-GCCTGTGCTGGAACATCATC-3' and 5'-TTGCCCTCATGTGTGCTCTT-3'); *SMAD4* (5'-CATCCTGCTCCTGAGTATTGG-3' and 5'-GGGTCCACGTATCCATCAAC-3'); *SMAD5* (5'-CTGGGATTACAGGACTTGACC-3' and 5'-AAGTTC CAATTA AAAAGGGAGGA-3'); *SMAD6* (5'-CTGCAACCCCTACCACTTCA-3' and 5'-TTGGTAGCCTCCGTTTCAGT-3'); *SMAD7* (5'-AGAGGCTGTGTTGCTGTGAA-3' and 5'-AAATC CATCGGGTATCTGGA-3'); and *SMAD9* (5'-AGCTCTTCGCTCAGCTCCTG-3' and 5'-CTGCTATC CAGTACCAGCA-3'). The PCR cycling profile consisted of an initial denaturation step at 95°C for 15 min, followed by 35 cycles of 94°C for 60 s; 55°C for 60 s; and 72°C for 60 s. After PCR, the amplified products were subjected to electrophoresis on 1.5% agarose gels, which were then stained with ethidium bromide to visualize the anticipated fragments. As a control, β -actin was amplified in a similar fashion. Bands of the anticipated sizes were obtained, and a band for each gene was eluted, purified using a QIAquick gel extraction kit (Qiagen) and the identity confirmed by sequencing.

We then used these primers to amplify SMAD 1–5 transcripts from granulosa cells obtained from 10 IVF patients. We used granulosa cells from 10 subsequent patients to amplify SMAD 6, 7 and 9. Five (5) μ l of the reverse transcription reaction was used in a 50 μ l PCR performed using ChromaTaq™ DNA Polymerase (Denville Scientific Inc.). Positive controls (human ovary cDNA (Ambion)) and negative controls (sterile distilled water)

Fig. 1 Expression of *SMAD1-7* and *9* in human luteinized mural granulosa cells. Total RNAs were isolated from human granulosa cells obtained from patients undergoing IVF treatment, and were then reverse-transcribed and PCR-amplified with gene-specific primer pairs. Human luteinized mural granulosa cells from patients were positive for the products of *SMAD1-7* and *9*. +: positive control (Human Ovary PCR-Ready cDNA, Ambion); -: negative control (water)

were used for each primer. PCR primers and the PCR cycling profiles were as described above. After PCR, the amplified products were subjected to electrophoresis on 1.5% agarose gels and stained with ethidium bromide to visualize the anticipated fragments. As a control, β -actin was amplified in a similar fashion. A band for each gene was gel-eluted, purified and confirmed by sequencing.

Quantitative real-time PCR (q-RT-PCR)

Total RNA was extracted from 22 additional IVF patient samples, and cDNA was synthesized from 1 μ g total RNA as described above. To quantify the amounts of the SMAD transcripts present, 1 μ l of the reverse transcription reactions were used in 25 μ l q-RT-PCR reactions. Q-RT-PCR was performed on a MyiQ Thermal Cycler (Bio-Rad Laboratories) with iQ SYBR green supermix (Bio-Rad Laboratories) for 40 cycles in two step reactions: 95°C for

10 s and 60°C for 45 s. The primers for SMADs were as described for PCR above, and the primers for β -actin were 5'-CATTGCTGACAGGATGCAGAAGGAG-3' (forward) and 5'-CCTGCTTGCTGATCCACATCTGCTG-3' (reverse). SMAD gene expression levels were calculated by normalizing the SMAD cycle numbers to those of β -actin.

Statistical analyses

For quantitative real-time PCR, experiments were carried out in triplicate and the mean and standard deviation was determined. Prior to statistical analysis, all data were confirmed to follow a normal distribution by way of the Kolmogorov-Smirnov test. Linear regression analysis was used to examine the relationships between the expression ratios of different SMAD family members and peak estradiol (E2) levels obtained one day prior to HCG, oocyte number, and fertilization rate (number of bipronuclear oocytes (2PN) retrieved vs. the number of oocytes for IVF). Influential data (e.g. outliers) were determined as data >2 standard deviations beyond the mean and all linear regression were repeated without any influential values to evaluate goodness of fit. For each linear regression, the R^2 value was used to express the correlation efficiency. P-values less than 0.05 were considered significant.

Results

Transcripts for *SMAD2*, *3* and *4* have previously been identified in human granulosa cells [31]. In order to determine whether other *SMAD* transcripts are also expressed in the human ovary, we tested for the presence of human *SMAD1-7* and *9* transcripts in commercially available human ovarian cDNA by PCR amplification

Fig. 2 Example of the relative expression patterns of *SMADs* in human luteinized mural granulosa cells. Q-RT-PCR was performed on RNAs isolated from additional patients undergoing IVF treatment, using gene-specific primers for *SMAD1-7* and *9* and β -actin, and *SMAD* gene expression levels were normalized to those of β -actin. The expression level of *SMAD1* was designated as 1. Expression levels of *SMAD2*, *3*, and *4* were 10 times greater than the expression level of *SMAD1*, whereas the expression levels of *SMAD5*, *6*, *7*, and *9* were relatively low

Fig. 3 Correlations of E2 levels and oocyte numbers in patients undergoing IVF. The number of oocytes retrieved was found to correlate significantly with E2 levels ($R^2=0.5107$)

(Fig. 1). We also tested for the presence of *SMAD1-5* transcripts in luteinized mural granulosa cells obtained from ten consecutive patients undergoing IVF and *SMAD 5-7* and 9 in ten additional patients. *SMAD1-7* and 9 were found

to be expressed in luteinized mural granulosa cells from the patients tested, as shown in Fig. 1.

We then compared the relative expression levels of *SMAD1-7* and 9 in luteinized mural granulosa cells, using RNAs isolated from nine patients undergoing IVF. In all nine patients, designating the expression level of *SMAD1* as 1, we found that the expression levels of *SMAD2*, 3, and 4 were up to 10 times greater than the expression level of *SMAD1*, whereas the expression levels of *SMAD5*, 6, 7, and 9 were relatively low. A sample expression pattern is shown in Fig. 2.

We next tested for relationships between the expression levels of *SMAD2*, 3, and 4, as well as their relative expression ratios, and IVF protocol type, peak E2 level, and number of oocytes retrieved in 22 patients. Patient characteristics were as follows: age 35.11 ± 5.49 years; estradiol (E2) levels 2245.78 ± 1109.94 ; number of oocytes retrieved 10.22 ± 5.70 ; number of oocytes for IVF 7.22 ± 5.19 ; number of bipronuclear oocytes (2PN) retrieved 5.50

Fig. 4 Correlations of oocyte number to the individual expression levels of *SMAD2*, 3 and 4 and the relative expression ratios of *SMAD4:SMAD3*, *SMAD3:SMAD2*, and *SMAD4:SMAD2* in human luteinized mural granulosa cells in patients undergoing IVF. Oocyte number did not show any correlation to individual *SMAD2* (a), *SMAD3* (b) or *SMAD4* (c) levels, or to the expression ratios of *SMAD4:SMAD3* (d), *SMAD3:SMAD2* (e), and *SMAD4:SMAD2* (f)

± 4.04 ; fertilization rates 0.67 ± 0.26 . The number of oocytes retrieved was, as expected, found to correlate significantly with E2 levels (Fig. 3). Oocyte number did not correlate to individual *SMAD2*, 3, and 4 or expression ratios of *SMAD4:SMAD3*, *SMAD3:SMAD2*, and *SMAD4:SMAD2* (Fig. 4).

We also analyzed the relationship between the expression levels and relative expression ratios of *SMAD2*, 3, and 4, and the fertilization rates of oocytes retrieved from these patients. Fertilization rates were determined only for the oocytes that were exposed to conventional IVF. Fertilization rates also did not correlate with the expression levels of these individual *SMADs* (Fig. 5). However, fertilization rates did correlate positively with the expression ratios of *SMAD4:SMAD3* ($R^2=0.3323$, $p=0.0062$, Fig. 5d), but not with *SMAD3:SMAD2* (Fig. 5e). However, there was a trend towards positive correlation between fertilization rate and the expression ratio of *SMAD4:SMAD2* ($R^2=0.1627$, $p=0.0698$, Fig. 5f).

Discussion

Although the use of assisted reproductive technology (ART) has risen to the point where more than 1% of live births in the US results from IVF, the success rates for IVF remains low—2 out of 3 IVF cycles fail to result in pregnancy, and more than 8 out of 10 transferred embryos fail to implant [33]. Thus, understanding the mechanisms and pathways underlying fertility and fecundity remains of crucial importance. As the *SMAD* signal transduction proteins are known to mediate signaling by members of the *TGF- β* superfamily, which is involved in the control of normal folliculogenesis and ovulation [7, 8], and they associate with members of the Forkhead family of transcription factors, the *FOXO* class [34], thought to play important roles in oocyte maturation, ovulation, and possibly luteinization [34–39], with expression in human granulosa cells [40], it is possible that *SMAD* expression levels may also affect ovarian folliculogenesis and fertility.

Fig. 5 Correlations of fertilization rates to the individual expression levels of *SMAD2*, 3 and 4 and the relative expression ratios of *SMAD4:SMAD3*, *SMAD3:SMAD2*, and *SMAD4:SMAD2* in human luteinized mural granulosa cells in patients undergoing IVF. Fertilization rate did not show any correlation to individual *SMAD2* (a), *SMAD3* (b) and *SMAD4* (c) levels, but correlated with the expression ratio of *SMAD4:SMAD3* ($R^2=0.3323$) (d), but not *SMAD3:SMAD2* (e), and a positive trend with *SMAD4:SMAD2* ($R^2=0.1627$) (f)

Female *Smad3*^{-/-} mice exhibit impaired folliculogenesis and reduced fertility [28, 30] and *Smad4* ovarian-specific knockout mice exhibit multiple defects in folliculogenesis and decreased fertility over time [27]. Female mice in which dominant negative *Smad2* is expressed in granulosa cells exhibit impaired folliculogenesis and the females are subfertile [26]. Granulosa cell-specific *Smad2/Smad3* double knockouts exhibit defects in follicular development, ovulation and cumulus cell expansion [41]. These data, coupled with the identification of *SMAD2*, *3* and *4* transcripts in human granulosa cells [31], support a potential role for SMAD signal transduction in the regulation of human folliculogenesis and fertility.

In this manuscript, we have shown that all currently identified SMADs, *SMAD1* to *7* and *SMAD9*, are expressed in human luteinized mural granulosa cells obtained at oocyte retrieval. Moreover, we found that the expression levels of *SMAD2*, *3*, and *4* in these human granulosa cells were higher than those of other *SMADs*, supporting a potential role for these *SMADs* in mediating TGF- β superfamily signaling in the human ovary, where *Smad2* is more responsive to activin and *Smad3* more responsive to TGF β [25]. We found that the individual *SMADs* and expression ratios of *SMAD4:SMAD3*, *SMAD4:SMAD2* and *SMAD3:SMAD2* did not correlate with the number of oocytes retrieved. However, we found that fertilization rates for these oocytes did show correlations to the relative expression ratios of *SMAD4:SMAD3* and a trend with *SMAD4:SMAD2* (Fig. 5). This may be consistent with the cumulus cell defects, premature luteinization of granulosa cells and premature ovarian failure seen in *Smad4* ovarian-specific knockout mice [27]. Moreover, these findings illustrate that oocyte number alone may not be predictive of oocyte quality and subsequent IVF outcomes.

Various markers of ovarian quality have been assessed for their usefulness in predicting the success of IVF treatments. These have included: early-follicular-phase blood values of FSH and estradiol, antral follicle counts (AFCs); inhibin B levels in serum and follicular fluid, and anti-Müllerian hormone (AMH) levels [42, 43]. Although some studies have demonstrated these factors to be associated with oocyte quantity [44, 45], some but not all have been associated with oocyte quality and fertilization potential [44, 45]. For example, serum AMH levels have been associated with ovarian response to stimulation, but not embryo quality in some studies [44, 45] whereas other studies found serum AMH comparable to AFC when looking at number of oocytes retrieved and oocytes fertilized [46]. Further, Lekamge et al. [47] did find higher pregnancy rates per IVF cycle in patients with higher serum AMH levels and follicular levels of AMH have also been associated with fertilization potential and hence oocyte quality [48].

Evaluation of cumulus or granulosa cells to identify additional factors associated with oocyte/embryo quality and positive IVF outcomes has been performed [49, 50]. For example, large-scale microarray analyses have identified a correlations between ferridoxin (*FDX1*), aromatase (*CYP19A1*), and *CDC42* levels in follicular cells and oocyte competence [51], and between *BCL2L1*, *PCK1* and *NFIB* levels in cumulus cells and embryo potential and the likelihood of a successful pregnancy [52]. However, large scale microarray studies are currently not feasible for oocyte quality assessment.

Chang et al. [31] found that the expression levels of *SMAD2*, *3* and *4* in granulosa cells obtained at oocyte retrieval correlated differently with the expression levels of follistatin and subunits of inhibin-activin: α -subunits and β B-subunits correlated with *SMAD2*, β A-subunits correlated with *SMAD4*, and follistatin correlated with the expression of both *SMAD2* and *SMAD4*, but did not relate their findings to oocyte quantity or quality, or to resulting pregnancy outcomes [31]. In this study, we found that *SMAD4:SMAD3* and *SMAD4:SMAD2* expression ratios in granulosa cells obtained at oocyte retrieval may be used as markers of fertilization rate, and thus of the likelihood of successful IVF outcomes. Further analyses in larger numbers of patients will reveal whether *SMAD4:SMAD3* and *SMAD4:SMAD2* expression ratios in conjunction with other factors, perhaps the FOXO class of transcription factors, which have been recently identified in human granulosa cells [40], may prove useful as predictive markers for oocyte fertilization rates and IVF success.

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