Molecular and Cellular Endocrinology 356 (2012) 48–54

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

# Concentration of activin A and follistatin in follicular fluid from human small antral follicles associated to gene expression of the corresponding granulosa cells

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ARTICLE INFO

*Article history:*
Available online 9 August 2011

*Keywords:*
Activin A
Follistatin
Human small antral follicles
Granulosa cells
Follicular fluid

ABSTRACT

The present study correlated concentrations of activin A and follistatin in follicular fluid (FF) from human small antral follicles to FF concentrations of AMH, inhibin B, progesterone, and oestradiol and to the mRNA expression of FSH-receptor (FSHR), LH-receptor (LHR), AMH-receptor2 (AMHR2), CYP19a, and androgen-receptor (AR) in the corresponding granulosa cells (GC). FF from 144 follicles (3–12 mm in diameter) was included whereas mRNA expression profiles were established in GC from 66 of the 144 follicles.

Levels of follistatin remained constant in relation to follicular diameter, whereas activin A levels increased in follicles exceeding 10 mm in diameter. Levels of activin A and inhibin B showed a highly significant inverse association. Follistatin showed highly significant positive associations with AMH and inhibin B levels and with FSHR and AR gene expression in GC.

This study revealed unexpected associations that probably reflect the complicated regulatory mechanisms governing human folliculogenesis.



## 1. Introduction

Human folliculogenesis is a lengthy process lasting several months, it is very complex and requires the involvement of a myriad of substances and signal transduction pathways. The cellular compartments of the follicle and its surrounding theca cells use cell to cell signaling and auto-, para-, and endocrine hormones to synchronize and optimize follicular growth and development. The integrated exquisite communication network between the granulosa cells (GC) and theca cells, and oocyte and granulosa cells/cumulus cells have been shown to be mandatory for follicular development and production of a viable fertilizable oocyte and have only to limited extend been elucidated (Eppig, 2001; Matzuk et al., 2002; Skinner, 2005).

One group of factors which have been shown to be of specific interest *in vivo* and in culture experiments, also of human cells, is the TGF-β superfamily of growth factors, which includes growth differentiation factors (GDF's), bone morphogenetic proteins (BMP's) and inhibins, activins and Anti-Müllerian Hormone (AMH). A number of these growth factors have been shown to be secreted by human GC (Knight and Glister, 2006; Welt et al., 2002) and affect cell function. Culture experiments have shown that gonadotropin stimulated steroidogenesis of human granulosa and theca cells can be synergistically augmented or attenuated by the presence of growth factors like inhibins and activins (Hillier et al., 1991a). Inhibins and activins are now considered classical peptide hormones affecting follicular growth and development although their specific effects are not yet clear. The inhibins and activins form a special class of growth factors with very similar structure but with mostly opposing functions (Welt et al., 2002). There exists three forms of activin, activin A, which consist of two βA subunits, activin AB consisting of an βA and a βB subunit and activin B is formed by dimerization of two βB subunits, while inhibins consists of a unique α-subunit combined with either a βA or a βB to form inhibin A or inhibin B, respectively (Ying, 1988).

Activin stimulates FSH secretion and upregulates FSH-receptor (FSHR) expression, whereas inhibin prevents FSH secretion and prevents FSHR expression (Ling et al., 1986; Welt et al., 2002). In the follicular compartment the activity of activin A is controlled by follistatin, which is a GC protein, structurally unrelated to the TGF-β superfamily (Robertson et al., 1987) that acts to modulate the local follicular activity of activins (Nakamura et al., 1990). In fact, follistatin binds activin with high affinity ($K_d$ = 50 pM) (Krummen et al., 1993; Phillips and de Kretser, 1998; Thompson et al., 2005), nearly irreversible, and prevents activin from being

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doi:10.1016/j.mce.2011.07.051

biologically active (Schneyer et al., 1994). Two follistatin molecules enclose one activin dimer, resulting in blocking of the binding sites for the activin receptor I and II (Thompson et al., 2005). The presence of only one β-chain in inhibins allows follistatin to bind but with lower affinity and the binding does not inhibit the biological activity of inhibins (Krummen et al., 1993; Phillips and de Kretser 1998; Shimonaka et al., 1991).

Although the importance of inhibins and activins in follicular development is well established, their potential para- and autocrine actions in normal human small antral follicles in relation to the gene expression of important GC hormone receptors is not widely studied (Fujiwara et al., 2001). In the present study we have studied normal human small antral follicles and some of the important TBF-β growth factors mentioned above in connection with a program of fertility preservation in which only the ovarian cortex is frozen. The antral follicles cannot sustain freezing and can be aspirated prior to isolating the ovarian cortex. Thus, the aim of the present study was to characterize human small antral follicles by measuring the concentrations of activin A and follistatin in follicular fluid (FF) in human small antral follicles. These concentrations were associated to the intrafollicular levels of inhibin B, AMH and steroids. In addition, the gene expression of the corresponding GC was also performed using quantitative Real-Time PCR (qPCR) and included FSH-receptor (FSHR), LH-receptor (LHR), androgen-receptor (AR), CYP19a (aromatase) and AMH-receptor2 (AMHR2).

## 2. Material and methods

### 2.1. Patients and collection of follicular fluid and granulosa cells from small antral follicles

Isolation of GC and FF samples were performed by aspiration of individual small antral follicles from ovaries surgically removed for fertility preservation. Fertility preservation of the ovarian cortex was offered to women with a disease where the appropriate treatment was gonadotoxic and that posed a risk of rendering her sterile.

A total of 66 follicles from which mRNA was purified were obtained from 32 women aged 13–38 years (median 29 years) at various times during their menstrual cycle. In total mRNA purification was performed in GC from 73 small antral follicles, seven of these had an insufficient mRNA quantity or quality to use in qPCR. Further to the 66 follicles in which mRNA was purified an additional 78 follicles (totally 144), in which only FF hormones were measured, were also available. The remaining 78 follicles derived from a total of 48 women. In total the 144 follicles were collected from 63 women (17 women had follicles from which mRNA was purified and additional FF that were part of the 78 follicles). Diagnosis of the original cancer included: mammary cancer [22], Hodgkin's disease [13], Ewing and other sarcoma [3], lymphoma and leukemia [4], and various others [21], which did not relate to an endocrinological (e.g. polycystic ovarian syndrome) and/or ovarian disease.

Prior to isolation of the ovarian cortex, the ovary was visually inspected for any abnormalities. To further assess the normality of the ovary, a small piece of the cortex was send to histology and was microscopically evaluated. All ovaries used in the present study appeared normal.

All antral follicles exposed on the surface of the ovary or visible during the isolation of the ovarian cortex were collected with a 1 ml syringe with a 26-gauge needle (Becton Dickinson, Brøndby, Denmark). Centrifugation (2000×*g*, 2 min) of each aspirated follicle separated the GC from the FF. An estimation of the follicle volume was made during the isolation of the FF. In the present study the volume ranged from 15 to 1080 μl corresponding to approximately 3 to 12 mm in diameter. GC and FF were each snap-frozen in liquid nitrogen and stored at −80 °C until RNA purification or hormone measurements.

The number of follicles obtained from each ovary varied from 1 to 8: (1 follicle 27 patients; 2 follicles 11 patients; 3 follicles 14 patients; 4 follicles 8 patients; 6 follicles 1 patient; 7 follicles 1 patient and 8 follicles 1 patient). The ethical committee of the municipalities of Copenhagen and Frederiksberg approved the project. Results from a part of this material have been used in previous publications (Nielsen et al., 2010, 2011; Yding Andersen et al., 2010). The ethical committee of the municipalities of Copenhagen and Frederiksberg approved the project (KF)01-170/99 and the patients signed an informed consent form.

### 2.2. RNA purification

Total RNA was purified under RNase-free conditions at room temperature for each GC sample using a combination of Tri Reagent (Sigma–Aldrich, St. Louis, MO, USA) and the RNAeasy Mini Kit (Qiagen, Hilden, Germany).

Each cell sample was lysed by addition of 1 ml Tri Reagent followed by incubation at room temperature for 5 min. 200 μl 1-bromo-3-chloro-propane (Sigma–Aldrich) were added to the lysed cells, shaken vigorously for 15 s and incubated for 5 min at room temperature. Subsequent centrifugation at 15,000×*g* for 15 min at 4 °C separated the solution into an upper aqueous phase containing RNA and a lower phenol-containing organic phase. The upper phase was transferred to a clean, RNase-free Eppendorf tube and RNA purification was continued by following the RNeasy Mini Kit protocol. The final elution step was repeated and the purified total RNA was stored at −80 °C.

The quality and degradation level of the total RNA samples were analysed using an Agilent 2100 Bioanalyzer and a RNA 6000 Pico LabChip, according to the manufacturer's instructions (RNA 6000 Pico assay kit, Agilent Technologies, Waldbronn, Germany). Samples with a RNA integrity number (RIN) higher than the threshold value passed the quality control test, while samples below the threshold value were excluded from the study. Quantity of total RNA in each sample was measured using a Beckman Coulter Du730 life science UV/vis spectrophotometer and the corresponding nanovette.

### 2.3. cDNA synthesis and qPCR analysis

First-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Briefly, a master mix containing 2.0 μl 10× RT Buffer, 0.8 μl 25× dNTP Mix (100 mm), 2.0 μl 10× RT Random Primers, 1.0 μl MultiScribe™ Reverse Transcriptase (50 U/μl) and 3.2 μl nuclease-free (DEPC) water was prepared and for each 20 μl reaction 10 μl master mix was added to an equal volume of total RNA. All steps were performed on ice. Samples were centrifuged briefly at 12,000×*g* and then incubated at room temperature for 10 min, followed by 37 °C for 2 h and finally, 85 °C for 5 s. First-strand cDNA was stored at −80 °C until qPCR analysis.

Evaluation of gene expression levels was achieved by RQ (relative quantification) – PCR analysis using TaqMan® technology (Applied Biosystems). FSHR, LHR, Cyp19a1, AR and AMHR2 TaqMan® Gene Expression Assays (pre-designed) as well as the Endogenous Control Assays for human β-actin and glyceraldehyde 3-phosphatdehydrogenase (GAPDH) were purchased from Applied Biosystems (Assay id-No.: β-actin:#4326315E; GAPDH:#4333764F; FSHR:#Hs00174865_m1; LHR:#Hs00174885_m1; AMHR2:#Hs00179718_m1; CYP19(a1):#Hs00903413_m1; AR: #Hs00171172_m1). Sample duplicates were prepared according to the manufacturer's instructions. A total reaction volume of 10 μl was prepared on ice containing 5.0 μl TaqMan® Universal PCR Master Mix, No AmpErase® UNG(2X), 0.5 μl