HOXA10 expression is decreased by testosterone in luteinized granulosa cells *in vitro*

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Abstract. Polycystic ovary syndrome (PCOS) is perhaps the most prevalent endocrine disorder in women of reproductive age, characterized by elevated levels of circulating androgens or clinical manifestations of androgen excess. The specific cytokine profile of PCOS patients is probably related to the lower implantation rate, since follicular fluid appears to function as an embryotrophic agent. For a better understanding of the local regulation of human follicles, the present study investigated the protein expression levels and cellular localization of HOXA10 in granulosa cells (GCs) from women with normal ovarian function undergoing IVF due to their husbands suffering from azoospermia. We demonstrated by immunohistochemical studies that the expression of HOXA10 was mainly localized in the cytoplasm of GCs. Our data indicate that these alterations were associated with changes in the expression of ovarian transcription factors of HOXA10. GC dose-responsive decreases in HOXA10 protein were observed in response to physiological or supraphysiological concentrations (10^{-4} to 10^{-7} M) of testosterone. These data reveal that testosterone may be involved in HOXA10 gene regulation in GCs. Decreased HOXA10 expression in GCs treated with testosterone suggest that this androgen is responsible for the decreased expression of HOXA10 in PCOS patients.

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Introduction

Polycystic ovary syndrome (PCOS) is perhaps the most prevalent endocrine disorder in women of reproductive age, and the most frequent cause of oligo-anovulation and hyperandrogenism (1-3), both of which have substantial psychological, social and economic consequences (2,4). PCOS is associated with 75% of all anovulatory disorders causing infertility (5,6). The excess ovarian androgen production is induced by extraovarian factors, including insulin resistance and hyperinsulinemia (7,8). The clinical manifestations and ramifications of this disorder vary with the degree of hyperandrogenism and include a number of metabolic disorders, including insulin resistance, diabetes mellitus, hypertension, dyslipidemia and cardiovascular disease. However, the lack of a clear etiology associated with these syndromes has led to multiple treatments with few effective therapies for infertility (9,10).

Ovarian follicular maturation represents one of the most complex and clinically important developmental processes that takes place throughout the reproductive life of women. During this process, the oocyte and the granulosa cells (GCs) establish mutual interactions and their growth is regulated by complicated molecular mechanisms (11,12). The transcription factors that control these interactions are poorly defined, but may well include clustered homeobox (HOX) genes, which code for families of transcription factors and act at the top of genetic hierarchies (13).

Homeobox genes are evolutionarily conserved and necessary for body axis patterning during embryogenesis. The expression of HOX genes in defined locations along the paramesonephric duct leads to the development of the adult reproductive tract (14). Specifically, HOXA10 is essential for the development of the uterus during organogenesis (15). The persistent expression of HOXA10 in the adult enables the endometrium to retain a developmental plasticity and allows the sequential differentiation of the endometrium during each menstrual cycle (16). HOXA7 and HOX cofactor expression in normal human ovary is temporally

and spatially specific in GCs and this finding indicates a relationship with GC proliferation (17,18).

However, we are unaware of any study in the literature that has investigated HOXA10 gene expression in granulosa cells. In this study, we examined for the first time the expression patterns of HOXA10 in GCs. In brief, GCs were isolated and identified by immunofluorescence. We evaluated the effect of testosterone on HOX gene expression. Androgen levels are higher in PCOS women with infertility (or recurrent miscarriages) than in normal fertile women (19). Elevated levels of androgens may have a detrimental effect on ovarian function. Androgen receptors are present within the ovarian GCs (20,21). We postulated that hyperandrogenism may result in changes in HOXA10 expression in the GCs. Altered levels of HOXA10 expression, attributable to elevated testosterone, may affect the molecular pathway that leads to ovarian follicular maturation. As a consequence, patients with PCOS may suffer from infertility and early spontaneous miscarriages, despite successful correction of anovulation. Here, we demonstrated that testosterone alters HOXA10 expression in GCs, suggesting further exploration of its effect in PCOS.

Materials and methods

Patients. The study was performed on patients attending the Reproductive Medicine Center of Tongji Hospital in Wuhan, China. Written informed consent was obtained from each patient; consent forms and protocols were approved by the local ethics committee. Individual follicular fluid samples were collected from 42 women who were undergoing IVF treatment. The population consisted of 42 women with normal ovarian function undergoing IVF-ET due to their husbands suffering from azoospermia. The mean age was 27.5±2.8 years (range, 25-34), and mean BMI was 19.11±1.12 kg/m^2 (range, 16.01-23.58). They had regular menstrual cycle lengths between 25-35 days. Ovulation was documented in the luteal phase prior to the study with serum progesterone levels above 10 ng/ml. Clinical and laboratory manifestations showed no hyperandrogenism, and B super-monitoring of ovulation in the ovary was normal. All subjects were healthy, had been taking no medications for at least 3 months prior to the study, and had normal TSH and PRL levels.

Cell culture. The pooled clear follicular fluid (35 ml obtained from 10-18 different follicles) from each individual patient was centrifuged at 2000 rpm for 10 min at room temperature. The GC pellet was suspended in PBS solution (Hyclone Laboratories) (1:1 ratio) and layered by gentle pipetting onto a 0.5-ml Ficoll plaque (Amersham, Arlington Heights, IL, USA) and centrifuged at 2000 rpm for 20 min to remove red blood cells. The luteinized GCs were maintained in DMEM-F12 (Hyclone Laboratories) with EDTA (6.8 mM), HEPES (10 mM), 20% fetal bovine serum, penicillin, streptomycin and ITS. Cells were then treated for 6 or 48 h with hormones or pharmacologic agents. To generate a dose-response curve, testosterone (T) was used at a final concentration of 1x10^{-8} to 1x10^{-4} M. Estradiol (E, 5x10^{-8} M) and progesterone (P, 1x10^{-6} M) were used at approximately maximal physiological concentrations, at which we previously demonstrated alterations in HOXA10 gene expression in endometrium cells (22,23).

Confocal microscopy imaging. For immunofluorescence staining, 1x10^4 cells were seeded on glass coverslips (13 mm diameter). After fixation with 4% paraformaldehyde in PBS, cell membranes were permeabilized with 0.2% Triton X-100 in PBS, and nonspecific binding sites were blocked with 5% BSA in PBS. Treatment with the first antibody was performed at 4˚C overnight (FSH receptor; Santa Cruz, Santa Cruz CA). Afterwards, the cells were washed three times with cold PBS and incubated with Cy3-conjugated IgG diluted 1:50 in PBS for 30 min at room temperature. Nuclei were stained for 5 min at room temperature with DAPI. Cells were rinsed with PBS and were observed by confocal microscopy (Olympus, Japan).

Immunohistochemistry. After a 2-day culture, immunocytochemistry was performed in GCs. Briefly, after fixation, peroxidase treatment and serum blocking, GCs were incubated overnight at 4˚C with a primary rabbit polyclonal antibody against HOXA10 (Abcam). After washing in PBS, GCs were incubated for 30 min at 37˚C with biotinylated goat anti-rabbit IgG antibody and then avidin-biotin-peroxidase complex. The color reaction was performed using stable DAB solution for 5 min, as described previously (24). The nucleus was then stained with haematoxylin or 1 mM Hoechst 33342 (Sigma) for 10 min. The samples were examined by inverted microscopy (Leica, Heerbrugg, Switzerland) with an ultraviolet light filter. The morphology of GCs cultured in different T, P, E, T+P, T+E concentrations was recorded with an Olympus inverted microscope at 48 h.

Western blot analysis. Cells were harvested, and the expression levels of total HOXA10 were analyzed. Cell extracts (50 μg) were electrophoresed on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature using 5% nonfat milk in Tris-buffered saline [10 mM Tris, 150 mM phosphate-buffered saline (PBS), pH 8.0] containing 0.05% Tween 20. Membranes were incubated with diluted primary antibodies (HOXA10, 1:800; β-actin 1:1,000) at 4˚C overnight. They were then incubated with secondary antibodies (1:1000 dilution), and immunoreactive bands were visualized with alkaline phosphatase and BCIP/NBT staining. All blots were repeated three times.

Data analysis. Data are expressed as the means ± SEM. Protein quantification from western immunoblotting was performed via normalization to β-actin for each gel. Statistical analyses were performed using SPSS 13.0 statistical software. Differences between groups were compared using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The FSH receptor was used in this study to isolate GCs, as it is a potent cell marker of GCs. Fig. 1 shows the expression of FSH receptor (FSHR) in GCs. The strongest positive immunostaining of FSHR was observed in GCs. HOXA10 expression is regulated by testosterone in GCs. Endometrial HOX gene expression has been shown to be regulated by

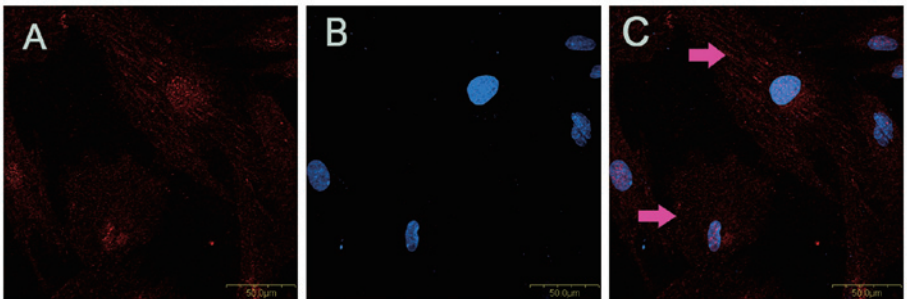

Figure 1. Expression of FSH receptor in GCs. The strongest positive immunostaining of FSHR was observed in GCs (Fig. 1C). GCs, granulosa cells; FSHR, follicle-stimulating hormone receptor.

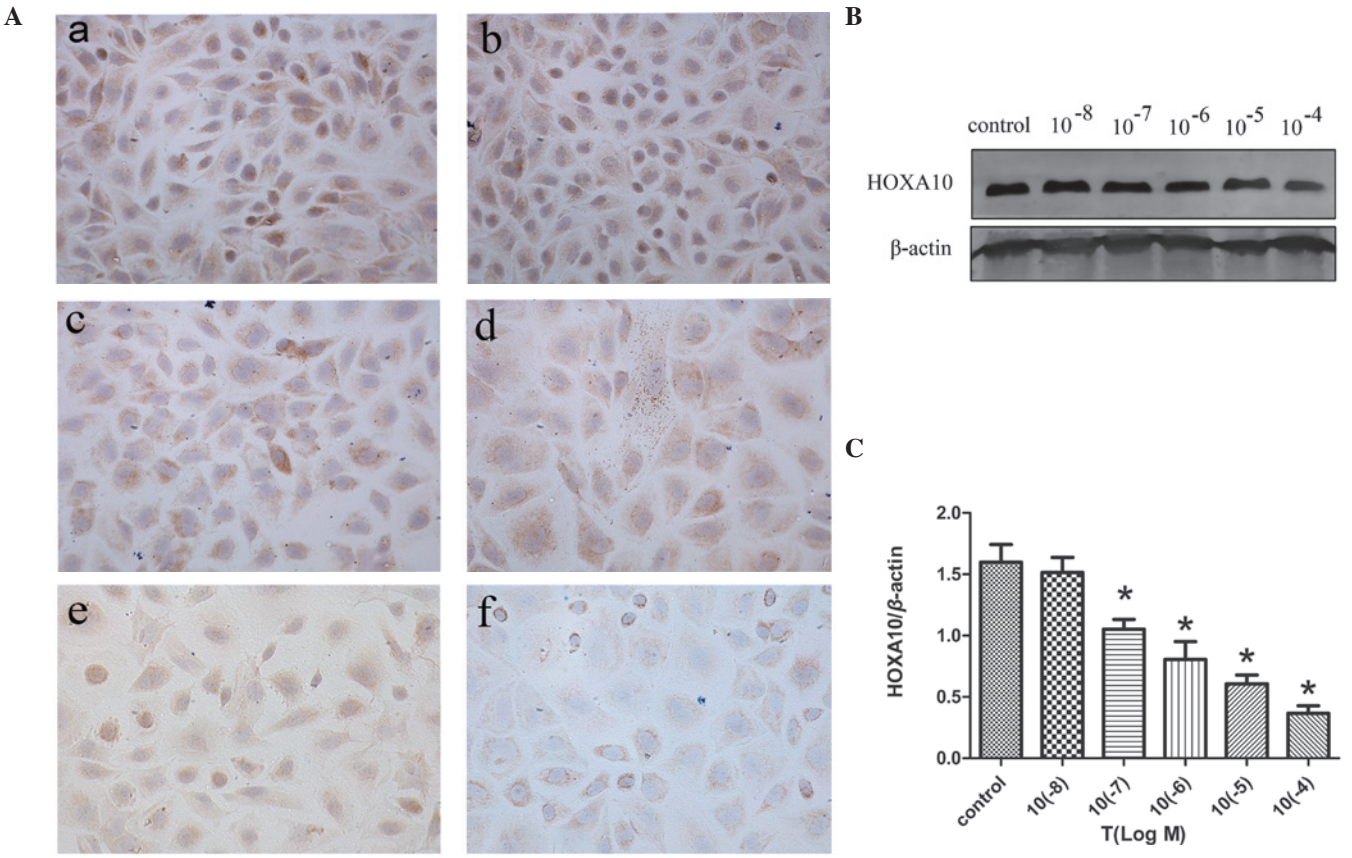

Figure 2. Effect of testosterone on HOXA10 expression. Granulosa cells (GCs) were treated for 24 h with either testosterone or control. (A) Representative immunocytochemical analysis: (a) control; (b) testosterone at 10^{-8} M; (c) 10^{-7} M; (d) 10^{-6} M; (e) 10^{-5} M; (f) 10^{-4} M; the expression of HOXA10 was mainly localized in the cytoplasm of GCs. (B) Representative western blot analysis. HOXA10 expression was decreased after treatment for 6 h with testosterone at 10^{-5} M [T10(-5)], but not at 10^{-8} M [T10(-8)]. At 24 h, HOXA10 expression remained decreased after treatment with 10^{-6} M testosterone. (C) Quantification and normalization of HOXA10 protein levels after testosterone treatment. Testosterone concentration ranged from 10^{-4} to 10^{-8} M. HOXA10 expression is decreased after treatment for 6 h with testosterone at 10^{-5} M [T10(-5)], but not at 10^{-8} M [T10(-8)]. At 24 h, HOXA10 expression remained decreased after treatment with 10^{-6} M [T10(-6)] testosterone. A dose-responsive decrease in normalized HOXA10 protein levels was observed. All experiments were repeated in triplicate. Error bars, SEM; *statistically different from control ($P<0.05$).

estrogen and progesterone (22,23). To determine whether HOXA10 is expressed in GCs and is regulated by androgens, HOXA10 expression was measured in GCs following treatment with testosterone. Fig. 2 shows the expression of HOXA10 was mainly localized in the cytoplasm of GCs. HOXA10 expression decreased following treatment with testosterone in a dose-responsive manner in GCs. Testosterone concentrations at or less than 10^{-8} M produced no discernible effect; however, at 10^{-7} M, a significant decrease in HOXA10 protein expression was observed. A further decrease in HOXA10 expression was observed with supraphysiologic treatments (10^{-6} to 10^{-4} M). The decrease in HOXA10 protein expression persisted 24 h after testosterone (10^{-6} M) treatment. As previously demonstrated, treatment of GCs with estradiol and progesterone leads to increased HOXA10 protein expression. Fig. 3 shows the effect of simultaneous treatment with estradiol ($5x10^{-8}$ M) and testosterone (10^{-6} M). Concomitant use of estradiol and testosterone resulted in decreased HOXA10 expression ($P<0.05$). Fig. 4 demonstrates the effect of simultaneous treatment with progesterone and testosterone (progesterone,

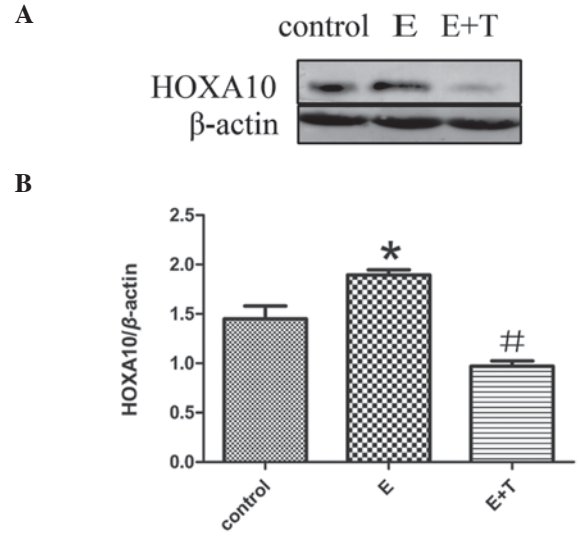

Figure 3. Testosterone reverses the effects of estradiol on HOXA10 expression. Granulosa cells were treated with either control, 17-estradiol ($5x10^{-8}$ M), or the combination of estradiol and testosterone (10^{-6} M) (E+T). Testosterone prevents the estradiol-induced rise in HOXA10 protein. Error bars, SEM; *statistically different from control (P<0.05); #statistically different from estradiol treatment (P<0.05).

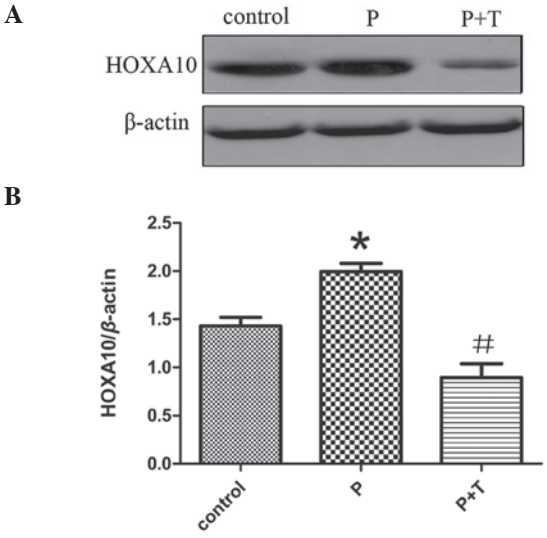

Figure 4. Testosterone reverses the effect of progesterone on HOXA10 expression. Granulosa cells were treated with either control, progesterone (P, 10^{-6} M), or the combination of progesterone and testosterone (P+T). Testosterone prevents the progesterone-induced rise in HOXA10 protein. Error bars, SEM; *statistically different from control (P<0.05); #statistically different from progesterone treatment (P<0.05).

10^{-6} M; testosterone, 10^{-6} M). HOXA10 expression is decreased after simultaneous progesterone and testosterone treatment (P<0.05). Testosterone blocked the expected estradiol- or progesterone-induced increase in HOXA10 protein.

Discussion

The importance of local growth factors for the modulation of follicular cell function, regarding cell proliferation and steroidogenesis (25,26), is becoming increasingly evident. However, there remains a lack of knowledge concerning various growth factors and their interactions in the follicle throughout follicle development. A variety of methods have been used in attempts to develop culture systems for GCs in many species, such as the rat (27), porcine (28), sheep (29) and bovine (30). To the best of our knowledge, no report has been published using human ovarian GCs as an *in vitro* model. For a better understanding of the local regulation of human follicles, the present study investigated the protein expression levels and cellular localization of HOXA10 in GCs from women with normal ovarian function undergoing IVF. In the present study, we confirmed the presence of HOXA10 in mammalian ovaries. We demonstrated by immunohistochemical studies that the expression of HOXA10 was mainly localized in the cytoplasm of GCs. Our data reveal that these alterations are associated with changes in the expression of ovarian transcription factors of HOXA10. The dose-responsive decreases in HOXA10 protein in GCs were observed in response to physiological or supraphysiological concentrations (10^{-4} to 10^{-7} M) of testosterone. These data show that testosterone may be involved in HOXA10 gene regulation in ovarian GCs. Decreased HOXA10 expression in GCs treated with testosterone suggest that this androgen is responsible for the decreased expression of HOXA10 in PCOS patients.

PCOS is perhaps the most common endocrinopathy, affecting approximately 5% of women in developed countries; it is a common cause of anovulation and infertility, characterized by elevated levels of circulating androgens or clinical manifestations of androgen excess (31,32). The chronic anovulation and infertility is often successfully treated in PCOS; however, resultant successful pregnancy rates are lower than expected (33,34). Recurrent miscarriage is also common in women with PCOS (18-20). The mechanism by which hyperandrogenemia could be linked to increased miscarriage risk is not known. The specific cytokine profile of PCOS patients is likely to be related to the lower implantation rate, since follicular fluid appears to function as an embryotrophic agent (35,36). Certain studies have revealed higher levels of testosterone in the follicular fluid of patients with PCOS (37,38). Hyperandrogenism in PCOS may proceed via dysregulated paracrine/endocrine control of androgen synthesis (37), or result from adrenal androgen excess (39,40). Intraovarian androgens have been found to promote GC proliferation and inhibit GC apoptosis in PCOS patients, particularly in small follicles whose GCs are rich in androgen receptors (35,36), indicating that androgens may have a crucial effect on GC development, and intraovarian androgen may play an important role in the pathogenesis of PCOS via regulating early follicle growth. In this study, we observed that excess androgen decreased the expression of the examined HOXA10 genes in GCs, which suggests that androgenic regulation of these genes may specifically contribute to folliculogenesis. Treatment of primary myometrial cell cultures (41) with testosterone decreases HOXA10 expression *in vitro,* paralleling the expression observed in GCs.

Testosterone is a novel negative regulator of ovarian HOXA10 expression. Testosterone-induced ovarian dysfunction may contribute to diminished reproductive success, including a decreased implantation and increased miscarriage

rate. Therapies aimed at correcting hyperandrogenism may be necessary to improve endometrial receptivity and egg quality; ovulation induction alone may not provide optimal treatment. Taken together, these observations may explain the paradox of poor reproductive outcomes in women with PCOS despite correction of ovulatory defects. However, *in vitro*, testosterone at 10^{-6} M significantly decreased the HOXA10 protein in GCs. Several lines of evidence suggest an important role for the homeodomain protein HOXA10 in regulating oogenesis and folliculogenesis. Treatment of primary myometrial cell cultures (41) with testosterone decreases HOXA10 expression *in vitro,* paralleling the expression seen in GCs. Further research is required to explore whether there are significant differences in HOXA10 protein levels between the patients with PCOS and controls.

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