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ARTICLE

Anti-Müllerian hormone remains highly expressed in human cumulus cells during the final stages of folliculogenesis

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Abstract This study evaluated whether anti-Müllerian hormone (AMH) was differentially expressed in cumulus (CC) and granulosa (GC) cells from large antral and pre-ovulatory follicles collected from individual follicles in women undergoing in-vitro maturation (IVM) or IVF treatment. Expression studies of AMH, AMH receptor 2, FSH receptor, aromatase and androgen receptor were performed in CC in IVM patients where cumulus–oocyte–complex had expanded, CC in IVM patients where cumulus–oocyte–complex remained compacted, GC from immature follicles and CC and GC from IVF patients. Microarray data on corresponding GC and CC from follicles from IVF patients was included. AMH expression was significantly higher in CC than in GC from both mature and immature follicles and in CC from immature follicles than in CC from pre-ovulatory follicles from IVF patients ($P < 0.05$). AMH expression was significantly higher in CC that remained compacted compared with those that had expanded ($P < 0.008$). AMH was correlated to the expression of FSH receptor, androgen receptor and AMH receptor 2 but not to aromatase expression. The expression pattern of AMH receptor 2 reflected that of AMH. AMH may exert intrafollicular functions even in human large antral and pre-ovulatory follicles and may be related to follicular health.

KEYWORDS: AMH, AMHR2, CYP19, human large antral follicles, steroids

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Introduction

In the female, anti-Müllerian hormone (AMH) is exclusively produced by granulosa cells (GC) of the developing follicle (Donahoe et al., 2003; Knight and Glister, 2006; Matzuk et al., 2002). Expression of AMH follows the developmental pattern of the follicle being expressed from the early stages of follicular development until the large antral stage, where expression becomes low or absent. Based on immunohistochemical studies of AMH in human GC, the highest expression was found in follicles with a diameter of less than 4 mm and that expression decreased in later stages of folliculogenesis (Visser et al., 2006; Weenen et al., 2004). A recent study measured the intrafollicular concentrations of AMH in a large number of normal human small antral follicles (Yding Andersen et al., 2010) and found that AMH concentration is gradually reduced as the diameter of the follicle increases until around 8–10 mm where concentrations undergo a profound reduction and remain low during the rest of follicular development (Yding Andersen et al., 2010). The concentration in human small antral follicles reaches several hundreds ng/ml prior to a diameter of 8–10 mm and is two to three orders of magnitude higher than those observed in circulation and in pre-ovulatory follicular fluid obtained in connection with oocyte collection (Fanchin et al., 2007; Yding Andersen et al., 2008).

Studies of GC and the corresponding follicular fluid (FF) from such human small antral follicles have shown that there is a highly significant inverse correlation between FF AMH concentrations and both FF oestradiol concentrations and the expression of aromatase, which catalyses conversion of androgen to oestrogen (Nielsen et al., 2010). These results confirm and extend earlier studies showing that increasing AMH appears to reduce the expression of aromatase in both humans and animals (ovine: Vigier et al., 1989; bovine: Monniaux et al., 2008; Rico et al., 2009; human: Grossman et al., 2008; Yding Andersen et al., 2008). However, in the rat, especially in large antral follicles, aromatase appears to show a differential expression within the follicle, being more highly expressed in the mural GC as compared with the cumulus cells (CC) (Whitelaw et al., 1992). In addition, immunohistochemical studies in rodents and humans have suggested that AMH appears to be expressed predominantly in CC of small healthy follicles (Visser et al., 2006; Weenen et al., 2004). Thus, this may suggest an interrelationship between AMH and aromatase expression with opposing expression levels of AMH and aromatase within each individual follicle. This could reflect that AMH as well as aromatase demonstrates a differential expression within the follicular compartment during the final stages of human folliculogenesis.

The aim of the present study was to evaluate whether AMH, CYP19 (aromatase) and other follicular-specific genes are expressed differentially in mural GC and in CC in human large antral follicles and pre-ovulatory follicles.

Materials and methods

Patient and sample data are summarized in **Table 1**. The term 'immature' is used for follicles and follicular cells originating from small antral follicles and follicles obtained in connection with oocyte retrieval for the clinical IVM programme.

Patients and collection of immature cumulus cells

Immature cumulus–oocyte–complexes (COC) were obtained from women attending the Biogenesi Reproductive Medicine Centre of Monza, Italy for IVM treatment. The diagnosis of the couples was male factor, tubal factor or unexplained infertility and none suffered from polycystic ovary syndrome. According to Italian legislation, only three oocytes could be used for treatment. Surplus oocytes were considered for research investigation according to a written informed consent from all participating couples.

Prior to oocyte retrieval stimulation of the women with exogenous gonadotrophins included: (i) 150 IU/day recombinant FSH for 3 days, starting from day 3 of the cycle and 10,000 IU human chorionic gonadotrophin (HCG); (ii) 10,000 IU HCG; or (iii) no exogenous stimulation (Fadini et al., 2009). When the leading follicles had a diameter between 10 and 14 mm, oocyte retrieval was performed within 24 h in non-primed women or 36–38 h after HCG administration according to Fadini et al. (2009).

The COC were classified as 'compact cumulus' or 'expanded cumulus' based on the morphology of cumulus oophorus immediately after isolation. CC were enzymatically isolated by using hyaluronidase, washed twice in phosphate-buffered saline (PBS) and transferred with RNase inhibitor (Protector RNase Inhibitor, 5 U/l; Roche Diagnostic, Mannheim, Germany) into a 0.2 ml tube (MicroAmp; Applied Biosystems, Singapore), flash frozen in liquid nitrogen within 30 min after oocyte retrieval and stored at −196°C until RNA extraction.

Patients and collection of immature granulosa cells

Immature GC were collected from 14 women who did not receive any exogenous hormone stimulation attending the University Hospital of Odense, Denmark. The women followed their natural menstrual cycle and the follicular diameter was followed by transvaginal ultrasonography. When the leading follicle reached a diameter of 16–17 mm, a bolus of HCG (Ovitrelle, 6500 IU s.c.; Merck-Serono, Hellerup, Denmark) was given and the oocyte of the mature follicle retrieved 36 h later. This oocyte was used for possible IVF treatment. During the retrieval procedure, small immature follicles (<9 mm in diameter) apparent on ultrasonography were also collected and pooled. The oocytes from the immature follicles were subjected to IVM and the follicular fluid aspirates were centrifuged at 500*g* for 5 min for separation of GC and blood cells from the follicular fluid.

Removal of blood cells from the GC was achieved using a magnetic cell sorting system as previously described (Grøndahl et al., 2009). The GC were re-suspended in 50 μl RNA Later (Ambion, Austin, TX, USA) and stored at 4°C or −20°C until RNA purification.

Patients and collection of mature granulosa and cumulus cells

Mature GC and CC from large pre-ovulatory follicles were collected from 10 women aged 25–36 years undergoing