J Assist Reprod Genet (2017) 34:1121–1129
DOI 10.1007/s10815-017-0930-6

REPRODUCTIVE PHYSIOLOGY AND DISEASE

Age-related expression of TGF beta family receptors in human cumulus oophorus cells

A. Ribeiro[1,2,3] · C. Freitas[4] · L. Matos[2,3,5] · A. Gouveia[2,3,5] · F. Gomes[1,7] · J. L. Silva Carvalho[1,2,6,7] · H. Almeida[2,3,7]

Received: 25 October 2016 / Accepted: 19 April 2017 / Published online: 2 May 2017

Abstract
Purpose During ovarian follicle growth, local cellular interactions are essential for oocyte quality acquisition and successful fertilization. While cumulus cells (CCs) nurture oocytes, they also deliver oocyte-secreted factors (OSFs) that activate receptors on CCs. We hypothesized that disturbance of those interactions contributes to age-related lower reproductive success in women submitted to assisted reproductive technology treatments.
Methods Women aged 27–48, without recognized personal reproductive disorder, were enrolled in the study and divided in <35- and ≥35-year-old groups. CCs collected upon follicle aspiration were processed for immunocytochemistry and RNA extraction. The expression patterns of OSF receptors BMPR2, ALK 4, ALK5, and activin receptor-like kinase (ALK6) were studied.
Results Independently of age, receptors were found mostly in the cell periphery. The quantitative assay revealed that in older women, BMPR2, ALK 4, and ALK6 were all significantly decreased, whereas ALK5 was slightly increased.
Conclusions Female age imparts an effect on the expression of OSF receptors in CCs. The findings indicate that reproductive aging affects the local regulation of signaling pathways mediated by BMPR2, ALK6, and ALK4 receptor activation, suggesting their joint involvement.

Keywords Aging · Cumulus cells · Female infertility · Gene expression · Growth factors

✉ A. Ribeiro
angela.ribeiro@ceti.pt

1 Centro de Estudo e Tratamento da Infertilidade (CETI), 4100-118 Porto, Portugal

2 IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Instituto de Investigação e Inovação em Saúde - i3S, Universidade do Porto, 4200-135 Porto, Portugal

3 Biomedicine Department, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal

4 Reprodutive Medicine, Obstetrics and Gynecology, Hospital Dr. Nélio Mendonça, SESARAM, 9004-541 Funchal, Portugal; Obstetrics-Gynecology Department, University of Porto, Porto, Portugal

5 Faculty of Nutrition and Food Sciences, University of Porto, 4200-465 Porto, Portugal

6 Obstetrics-Gynecology Department, University of Porto, 4200-319 Porto, Portugal

7 Obstetrics-Gynecology, Hospital CUF Porto, 4100-180 Porto, Portugal

Introduction

In the course of the ovarian follicle development, oocytes progress to metaphase II stage and acquire the biological properties to be fertilized [1]. They are surrounded by a cellular and liquid environment, composed of differentiated, granulosa-derived cumulus oophorus cells (CCs) and the follicular fluid (FF) compartment. The diversity of FF biomolecules and the peculiarities of the intercellular contacts make this environment unique.

The FF is a rich solution of peptides and steroids derived from the oocyte, the CCs, the granulosa cells, and the circulation [2]. In contrast to FF, which may function as a pool of compounds, CCs interact permanently with the oocyte and acquire new structural and functional properties, distinct from their granulosa cell ancestors. The mechanism behind this differentiation is likely a morphogen gradient, by which similar cells are

directed into different structural and functional destinations as a consequence of the concentration of specific molecular signals [3]. In the follicle, while mural cells remain actively steroidogenic, human CCs form cytoplasmic extensions that penetrate the zona pellucida and establish intimate contacts with the oocyte membrane by specialized features as gap junctions [4]. Before fertilization, this morpho-functional organization will enable a bidirectional communication between the oocyte and granulosa cells, crucial for the next developmental processes such as embryogenesis [5].

Oocyte-secreted factors (OSFs) have growth factor properties when acting on granulosa cells. They include members of the transforming growth factor beta (TGFB) superfamily of which the growth differentiation factor 9, GDF9, and the structurally related bone morphogenetic protein 15, BMP15, are very important for a variety of species [6]. In the *Gdf9* knockout mice, ovaries maintain the normal structure but follicles do not evolve beyond the primary follicle stage [7] and oocytes evidence secretory disturbances [8]. *Bmp15* knockout female mice also have reproductive alterations, albeit minor. Their ovulatory response to pharmacological stimulation, litter size, and litter frequency is reduced; moreover, the number of CCs enveloping oocytes is also smaller, suggesting that, in contrast to GDF9, the intervention of BMP15 occurs later in mouse folliculogenesis [9].

Considerable evidence on the importance of OSFs in fertility was gathered over the last years [5–16]; however, less consistent is the structure and dynamics of their receptors, mainly due to the lack of in vivo studies on account of the lethality of some genetic modifications [13].

OSFs form dimers that signal through membrane receptors. In vitro studies have identified receptor arrangements capable of interacting with GDF9:BMP15 heterodimers, whose biological activity is stronger, compared to OSFs homodimers [14, 15].

Receptors are composed of two types of serine/threonine kinases, members of the TGFB receptor superfamily, that include type 1 (ALK 1–7) and type 2 (BMPR2 and TGFBR2) receptors [6]. It has been considered that they assemble as different tetramers that, when interacting with the OSF heterodimers, are composed of one type 2 (BMPR2) homodimer, an ALK4/5/7 type 1 receptor (likely ALK4), and an ALK6 type 1 co-receptor, apparently involved in receptor organization stability [14].

Upon ligand interaction, type 2 receptor transphosphorylates type 1, which promotes the activation of the cytoplasmic SMAD protein cascade [6, 12], in which the proteins translocate into the nucleus to function as transcription factors [11]. Despite their structural similarities, BMP15 and GDF9 activate, in a species-related way, different downstream signaling cascades, respectively SMAD 1/5/8 and 2/3 [12–15].

The peculiar structural organization of CCs around the oocyte favors their continuous interaction. In fact, CCs provide nutrients to the oocytes [16, 17], protect them from oxidative stress [18, 19], and endow the oocyte with additional conditions for fertilization [20]. To accomplish those tasks, CCs actively express a large number of genes involved in a varied number of cell processes [21, 22], whose study may be an important tool in the evaluation of oocyte quality [23]. CCs are also capable of expressing OSFs, and apparently their transcription is inhibited by women's physiological status including overweight or aging [24].

The cumulus cells/oocyte crosstalk is likely to be disturbed in some fertility disorders and in the course of natural reproductive aging, a major risk factor for female fertility [25]. Structural, unbiased ovarian studies evidenced age-related decrement in non-growing follicles [26], supporting previous epidemiological data showing age-related decreased pregnancy rates in groups not using contraception [27] and women submitting to artificial insemination [28]. Furthermore, in assisted reproductive technology (ART) procedures, employing previously selected oocytes, the trends were not different. In European countries, the overall pregnancy rate following intracytoplasmic sperm injection (ICSI) was 35.2% in women aged ≤34 years and decreased to 14.0% at ≥40 years; in in vitro fertilization (IVF), the rates were 35.4 and 14.7% at the same age ranges [29]. Other similar trends were found in the USA [30], Australia, and New Zealand [31].

There is thus compelling evidence for the age-related reduction in women's fertility. Beyond follicle loss, additional focus has been put on the decrement in oocyte quality consequent to enhanced meiotic non-disjunction, oocyte damage, and changes in the quality of the surrounding cells [32], further emphasizing the importance of oocyte environment. As CCs are part of such environment, we hypothesized that aging imparts functional disturbance on them that jeopardize their interaction with the oocyte. To address it, we verified the expression of main OSF receptors in human cumulus oophorus cells along reproductive aging.

Material and methods

Women who participated in this study were observed at Centro de Estudo e Tratamento da Infertilidade (CETI), Porto, Portugal, during the assessment of fertility disorders. Diagnoses were established by experts on Reproductive Medicine, and all decisions for invasive procedures and the choice of ART and specific technique to employ (IVF or ICSI) were based on clinical criteria.

The proposed research work was assessed by the Bioethics Unit, Biolaw and Economy of Health of the Department of Social Sciences and Health—Faculty of Medicine, University of Porto, which in turn issued a positive opinion to this study

as it was approved without any ethical constraints since the principles and established protocol were always respected.

Procedures were performed after patient informed consent, and under no circumstances, CC harvesting and study compromised the ordinary course of ART procedures.

Including criteria were male factor, previous tubal ligation, and infertility of unknown cause. Women with endometriosis, suspected past pelvic infection, polycystic ovary syndrome (PCOS), and other identifiable anovulatory conditions were excluded. Women's age was assigned to two groups: younger (<35 years) and older (≥35 years), assuming that oocyte quality significantly decreases from that age onwards. The demographic and baseline characteristics of these women and their treatments are presented in Table 1.

Ovarian stimulation protocols

A flexible antagonist protocol with recombinant, urinary, or both types of gonadotropin was employed. Gonadotropin administration started on day 2 of the menstrual cycle, and the GnRH antagonist was administered upon ultrasound identification of one follicle ≥14 mm diameter or more than 1 follicle exceeding 12 mm. When three follicles reached ≥17 mm in diameter and women had completed approximately 9 days of ovarian stimulation, 10,000 I.U. of human chorionic gonadotropin (HCG) was administered. Dosage was adjusted according to age and ovarian response. Ultrasound-guided transvaginal follicular puncture was performed in the next 34–36 h for follicle aspiration and oocyte-CC retrieval.

Collection of cumulus cells

After oocyte pickup under stereomicroscope observation, the cumulus-oocyte complexes (COCs) were washed in Sperm Preparation Medium (Origio, Denmark) and classified morphologically, employing previously described criteria [33, 34]. Each complex was analyzed individually.

CCs from COCs exhibiting apparently mature, fully expanded, and well defined *corona radiata* [34] were mechanically removed and placed in an anonymized sterile Eppendorf tube (Eppendorf International, Germany). They were washed with 1 mL of sterile DPBS 1× and centrifuged at 235×*g* for 6 min at 4 °C. Upon removal of the supernatant, a part of the sample was put in a cryotube and frozen in liquid nitrogen until processing for qPCR. Another part was digested with hyaluronidase (SynVitro® Hyadase; Origio, Denmark) for 30 s under continuous pipetting. Cells in suspension were then washed with 1 mL of sterile DPBS 1× and centrifuged as above. After supernatant removal, CCs were smeared on clean poly-L-lysine coated slides for immunocytochemistry (ICC) analysis.

Table 1 Characterization of the studied groups

	Younger women (<35 years old)	Older women (≥35 years old)
Number of patients (*n*)	36	20
Age (years)	31.7 ± 2.3	38.7 ± 3.4
Total FSH (IU) ministered during ovarian stimulation	1426.2 ± 661.5	2082.5 ± 1149.3
Total FSH days	8 ± 2.3	9 ± 1.2
Number of retrieved oocytes	5 ± 2.9	4 ± 2.1

Data are the mean ± SD of the two groups of women in which CCs (n_{total} = 56) were analyzed by qPCR

Extraction of RNA and cDNA production

In previously frozen CC samples, total RNA was extracted by RNeasy® Plus Mini Kit (Qiagen, Canada) according to the manufacturer's instructions, which included genomic DNA prior to removal through a gDNA eliminator column provided. RNA quantification was performed by spectrophotometric reading (NanoDrop ND-1000 ™ software 13.3.0, NanoDrop Technologies, USA) at 260 nm, and the degree of purity was estimated based on the ratio of absorbances at 260 and 280 nm, with reference values from 1.9 to 2.1. RNA was stored at −80 °C until subsequent tests were performed. Complementary DNA (cDNA) was produced from a 50 ng RNA sample through the qScript™ cDNA Synthesis Kit (Quanta BioSciences, USA), following the manufacturer's recommendations. As internal control, a similar reaction, but lacking reverse transcriptase, was made in parallel. A total of 56 RNA samples were successfully converted to 56 cDNA, labeled and stored at −20 °C until further analysis.

Real-time quantitative PCR

The designed sequences of each primer pair (Table 2) were analyzed using the software Beacon Designer™ (PREMIER Biosoft International, USA) to assess their applicability in qPCR.

Table 2 Human receptor gene sequences of forward (F) and reverse (R) cDNA primers and amplicon size

Target gene	Primer sequence (5′ → 3′)	Amplicon size (bp)
BMPR2	F: TCTTTGCCCTCCTGATTCTTG R: CACATAGCCGTTCTTGATTCTG	136
ALK6/BMPR1B	F: CATTCCTCATCAAAGAAGATC R: TCCTCTGTGGTGAAGAACAC	459
ALK5/TGFBR1	F: AAAACTTGCTCTGTCCACGGC R: TGTCCTGGGAAAGAAGCGTTC	826
ALK4/ACVR1B	F: TGTGATCAGAAGCTGCGTCC R: GGCATACCAACACTCTCGCA	96
GAPDH	F: GGTGAAGGTCGGAGTCAACG R: CAAAGTTGTCATGGATGACC	497

Other conditions for qPCR were optimized, including the program to be used, summarized in Table 3.

All qPCR reactions were performed in duplicate, including a negative control with all the PCR components except first-strand cDNA, in a final volume of 20 μL using 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad, USA). *GAPDH* was chosen as housekeeping/reference gene.

Upon qPCR run, Ct values for each amplification reaction were determined using the Bio-Rad IQ5 Optical System Software (Bio-Rad, USA); to estimate the absolute value of expression of the gene of interest, we employed the delta Ct method (ΔCt), with the following formula: $2^{(Ct(reference\ gene)-CT(target\ gene))}$. This approach uses the value of expression of a reference gene in place of a mass unit, in order to standardize the analysis. The choice of this method was mainly due to the low amount of sample.

Immunocytochemistry

CC smears on poly-L-lysine-coated slides were incubated with the membrane glycoprotein marker concanavalin A (Con-A), when applicable, and fixed by brief immersion in formaldehyde in PBS 0.1 M, pH 7.4. Cells were then permeabilized with 1% Triton X-100, blocked with 10% normal goat serum (NGS) in PBS 0.1 M, pH 7.4 (for detection of anti-hBMPR1B and anti-hTGF beta R1), and 5% bovine serum albumin (BSA) in PBS 0.1 M, pH 7.4 (for detection of anti-BMPR2). Incubation with primary antibodies, goat anti-hBMPR2 (1:20; AF811, R&D Systems®, USA), rabbit anti-hTGF beta RI (1:50; V-22, Santa Cruz Biotechnology, Inc., USA), and mouse anti-h/mBMPR1B (1:20; MAB505, R&D Systems®, USA) was performed overnight. Cells were thoroughly washed and then incubated with fluorescent secondary probes diluted in PBS 0.1% Tween 20 (anti-hBMPR2 reaction) or in blockage solution (anti-hBMPR1B and anti-hTGF beta RI reactions) for 1 h at room temperature, followed by binding induction with 488 streptavidin or appropriate Alexa fluorochrome (Alexa Fluor®, Invitrogen, UK). Cells were washed again in PBS-$Tween_{20}$ and briefly exposed to DAPI, no longer than 2 min. For the negative controls, the ICC procedure was the same as that mentioned above, without adding the primary antibodies. Slides were immediately examined with the ApoTome fluorescence microscope (Zeiss, Germany), and the images were captured with the digital imaging acquisition equipment and analyzed by the software AxioVision LE (Zeiss, Germany).

Table 3 Programs used in qPCR

	GAPDH					
	ALK4/ALK6/BMPR2			*ALK5*		
Cycle phase	T (°C)	Time (s)		T (°C)	Time (s)	
Denaturation	94	600		94	600	
	94	45	40 cycles	94	45	40 cycles
Annealing	58	30		61	60	
Extension	72	60		72	60	

Statistical analysis

Assuming to work with independent samples, without a normal distribution, the Mann-Whitney non-parametric test was used to compare the median values of transcript expression levels, between the two groups of samples. Data analysis was conducted with IBM SPSS Statistics 20.0 (IBM Corporation, USA); $p < 0.05$ was considered as statistically significant. To check whether our results were due to a lack of statistical power, post hoc power analyses were conducted using G*Power 3.0.10 (Franz Faul, Universität Kiel, Germany) with power $(1 - \beta)$ set at 0.80 and $\alpha = 0.05$, one-tailed.

Results

TGFB receptor mRNA expression levels

Results of receptor mRNA expression levels in the studied groups are depicted in Fig. 1.

Both *BMPR2* and *ALK6* messenger RNA (mRNA) was significantly decreased in samples from older women compared to younger women, with significance levels of $p = 0.002$ and $p = 0.013$, respectively. Post hoc power analysis detected differences between the two independent groups ($p < 0.05$), 0.92 for *BMPR2* and 0.84 for *ALK6*. As for *ALK4*, a significantly decreased mRNA was quantified in older women ($p = 0.022$), however, with weaker power (0.49). Regarding *ALK5* mRNA, it registered an increase in samples from older women without statistical significance ($p = 0.067$). In order to have a difference to be detected (95% chance) as significant at the 5% level ($\alpha = 0.05$), for *ALK4* and *ALK5*, a total of 74 and 397,260 samples, respectively, would be required. Due to the low amount of starting material in the CC samples, TGFB receptor mRNA expression detection may have conditioned the deviations noted.

ICC detection of TGFB receptors in dissociated CCs

In unstained smears, hyaluronidase-dissociated CCs exhibited irregular shape and variable size and were surrounded by rough deposits, likely due to dried albumin and salts. Simple staining was rendered difficult. In contrast, hyaluronidase-dissociated cells subsequently washed in PBS allowed staining improvement and the ICC study. Cells appeared more frequently as single cells, sometimes as pairs and occasionally

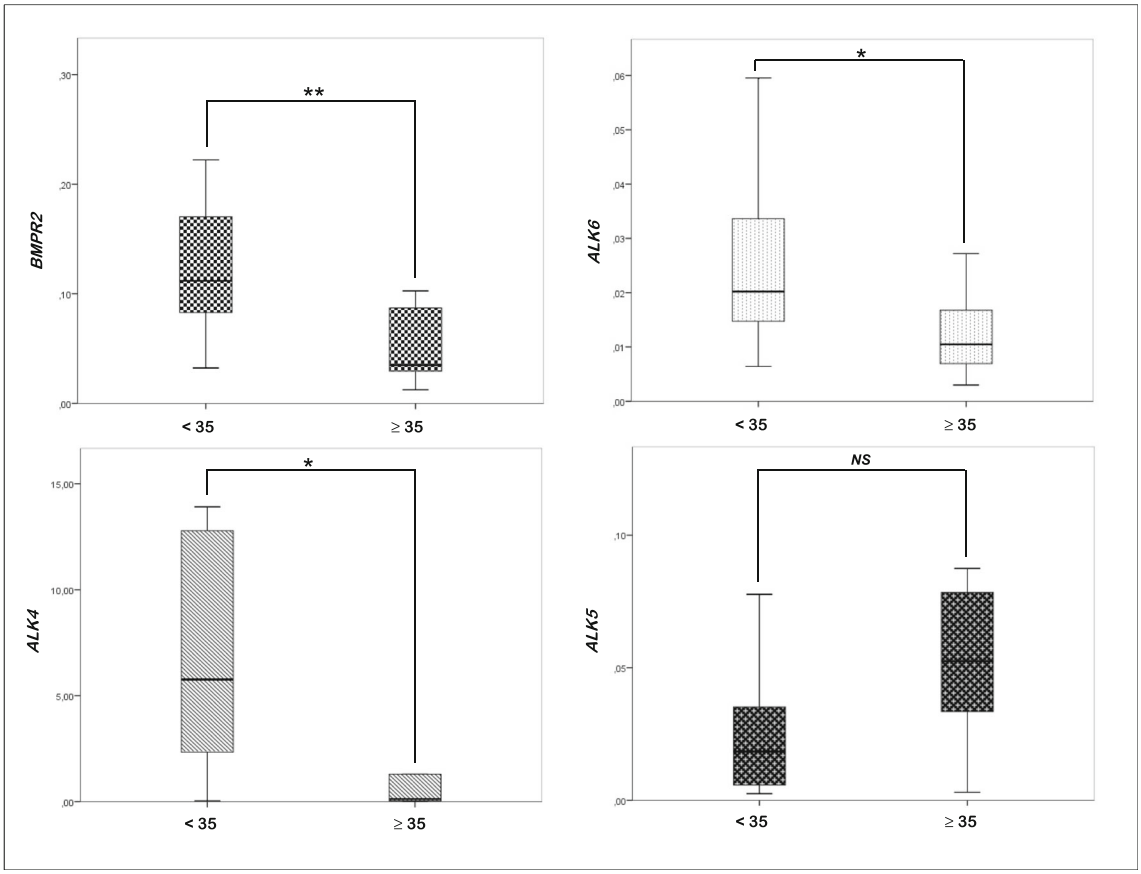

Fig. 1 TGFB receptor transcript (ΔCt) expression according to age group (<35 and ≥35 years). For each receptor, a *box-and-whisker* plot is presented, illustrating the median, 1st and 3rd quartiles, and minimum and maximum values (*lower* and *upper bars*). Differences are expressed as $^{**}p < 0.01$, $^{*}p < 0.05$, or NS (non-significant)

in larger groups, exhibiting a round shape and a conspicuous nucleus (Fig. 2).

The ICC study evidenced the preferential cell surface localization of type 1 receptors (as ALK5 and ALK6) although ALK6 showed considerable labeling intensity in the cytoplasm (Fig. 2a, b). Both type I receptors co-localized with BMPR2 as depicted in Fig. 2c, d. Age did not result in altered receptor localization (data not shown).

Discussion

The reciprocal and continuous paracrine communication at the cumulus cell-oocyte complex interaction [35, 36] has been emphasized as necessary for successful follicular growth, cumulus cell differentiation, ovulation, and fecundity (see [12] for a comprehensive review).

GDF9 and BMP15 are important oocyte communication signals that are expressed early in primordial follicles and attain stronger intensity from the primary/secondary follicle stage onwards. They were identified in a variety of mammalian species, including humans and other essentially mono-ovulatory mammals as sheep [37], goat [38], and bovine [39]. In humans, GDF9 and BMP15 expression was observed mainly in the oocytes at various ages as the fetal period, premenarche, and adulthood [40–43]. Upon controlled ovarian stimulation (COS), as is the condition of the present investigation, oocyte GDF9 expression increases until ovulation, when higher levels of expression are attained [44, 45]; in contrast, BMP15 is expressed later in the cycle, when primary follicles are well established [41], and the labeling intensity is smaller compared to GDF9 [45].

OSFs have important effects on the cells surrounding the human oocyte. In *ex vivo* studies, GDF9 promoted primordial follicle growth, differentiation, and viability [46], in agreement with the G*df9*$^{-/-}$ mice' lack of folliculogenesis progress [7]. In contrast, *Bmp15* inactivation in mice did not result in important developmental or structural changes, even upon stimulation, indicating that BMP15 effects on folliculogenesis are minor and established later [11].

However, mouse model information may not extend to other mammalian species as mono-ovulatory mammals, where absent functional BMP15 resembles mouse GDF9 inactivation. In fact, women with *BMP15* mutations exhibit premature ovarian failure [47, 48], similarly to some homozygous *BMP15* sheep mutants, whose folliculogenesis is

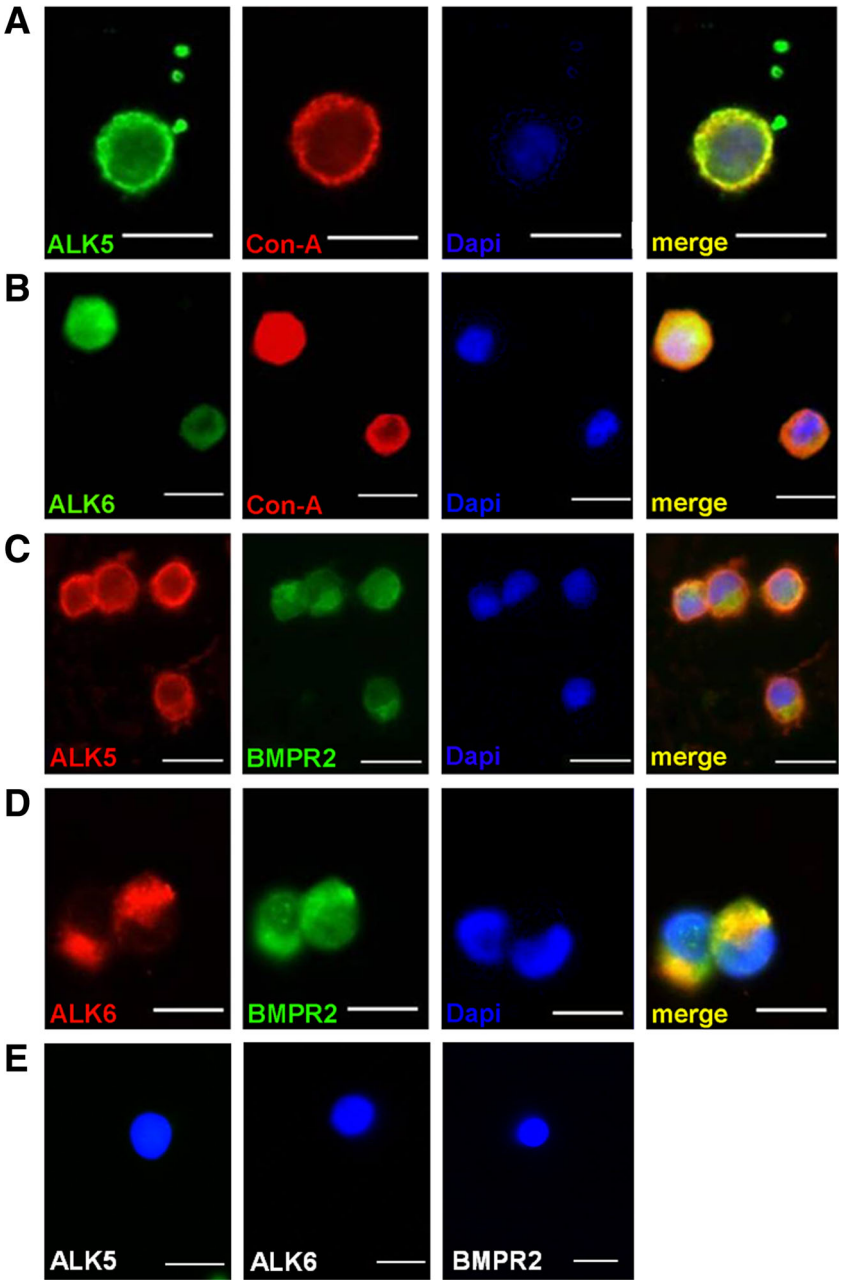

Fig. 2 Representative immunocytochemical labeling of TGFB receptors in human *cumulus oophorus* cells. **a** ALK5 (*green*) and the membrane marker Con-A (*red*) are detected and co-localize at the periphery of the cells (*yellow*). **b** ALK6 (*green*) and Con-A (*red*) are detected and co-localize (*yellow*) at the periphery of the cells too; note that ALK6 also exhibits a considerable intracellular labeling. **c** ALK5 and BMPR2 detection in human CCs; ALK5 (*red*) and BMPR2 (*green*) co-localize especially on cell periphery (*yellow*). **d** ALK6 (*red*) and BMPR2 (*green*) were detected and co-localize predominantly in the cytoplasm (*yellow*). **e** Negative controls, without the corresponding primary antibodies. **a**–**e** Nuclei were counterstained with DAPI (*blue*). Bars = 10 μm

blocked [49]. In addition, oocyte BMP15 expression in mice remains low until close to ovulation [50], whereas in human [41] and sheep [37] oocytes, the expression is already evident at the primary follicle stage. This point adds further to specific effects of BMP15 in mono-ovulatory mammals, compared to non-mono-ovulatory mammals, and emphasizes an intervention in follicle growth and differentiation, despite the knowledge gap on its modulatory role in humans [51].

In the present investigation, we addressed GDF9 and BMP15 receptors in the human granulosa-derived CCs, where they have been localized [6, 10, 14], including upon COS [21, 24]. These members of the TGFB receptor superfamily are synthesized in the rough endoplasmic reticulum, transported to the Golgi complex as proproteins, and then routed to the cell membrane [52]. The cytoplasmic and cell surface immunocytochemical labeling of CCs here described generally correspond to those different cell locations. However, receptor mRNA expression was different when young and aged women's cells were compared. In fact, there was reduction in mRNA amount of all three BMPR2, ALK4, and ALK6 receptors, accompanying the reduced reproductive fitness that occurs in aged women. Such joint reduction in expression adds importance to previous observations favoring ALK4 as a major type 1 component of the receptor complex that responds strongly to the GDF9:BMP15 heterodimer, by activating SMADs in vivo and in vitro [14, 15, 53]. In this point, it

emphasizes the value of the GDF9 and BMP15 interaction in COC maturation.

In contrast, to the three receptors, the slight increase in ALK5 indicates that it has a different, or secondary, involvement, eventually non-reproductive, in human cumulus cells. In fact, SMAD 2/3 cascade activation upon stimulation with GDF9/BMP15 heterodimers is still evidenced in *Alk5* cKO mice [13].

Due to scarcity of the data on human OSF receptor regulation, the interpretation of the changes here reported benefits from observations in other mono-ovulatory species such as some sheep strains that carry mutations on the BMP15 coding sequence of regulatory elements and *ALK6* itself [54]. In the Woodland variety of sheep, there is a putative X chromosome mutation, $FecX2^{W}$, that modulates *BMP15* expression. When compared to wild-type subjects, heterozygous carriers show reduced expression of oocyte BMP15, unchanged GDF9 expression, and reduced expression of BMPR2 and ALK6 receptors on granulosa cells from antral follicles [55]. It is thus conceivable that the age-related reduced level of expression of BMPR2/ALK6/ALK4 receptors that we observed in human CCs relates to reduced secretion of one or both OSFs and their reduced heterodimerization. As in the same variety of mutants the granulosa cell ALK5 receptor was slightly increased, the findings in Woodland sheep parallel those we found in aged women.

The resemblance of receptor expression variations compels to an additional observation. It is known that homozygous Woodland ewes are usually sterile, exhibit streak ovaries, and evidence folliculogenesis block at early stages, whereas their heterozygote counterparts have higher ovulatory rates and are more prolific [55]. In women, mutations in BMP15 and GDF9 also result in premature ovarian failure and streak ovaries [47, 48, 51, 56, 57], elevated serum FSH [56] and, occasionally, dizygotic twining [58]. Interestingly, FSH elevation was noticed in Booroola sheep variety, which has a mutation in the ALK6 receptor [59]; moreover, enhanced follicular sensitivity to FSH, in the setting of reduced bioactive BMP15 level, was the proposed mechanism [54] for the prolific heterozygous Woodland sheep. In fact, these ewes exhibited a larger number of antral follicles, although the size of follicles and oocytes was smaller [55], indicating that from the original cohort, more oocytes had been recruited to ovulate. It is noteworthy that the slight elevation of blood FSH observed later in women's reproductive life, resulting in the occasional ovulation of two smaller follicles, was the proposed mechanism for the recognized enhanced incidence of dizygotic twining observed at that age [60]. In those healthy older women, reduction of BMP15 and GDF9 secretion was also suggested as the plausible cause [61].

In the clinical setting, when older and younger women are compared regarding infertility causes, an increase in tubal obstruction and infertility of unknown cause is noticed [62]. While the first condition associates to the effects of external causes, as infections or endometriosis, the second condition is likely to result from intrinsic, age-related, functional disturbances at the COC. In the current study, we found a decrement in CC receptors, notably the type 1 ALK4 and ALK6 receptors that assemble for GDF9/BMP15 heterodimer interaction. One might speculate that age-related reduced secretion of OSFs is a fundamental event that hinders heterodimerization, prevents CCs activation, and leads to COC development disturbances and reduced fertility.

This study also shows that CC assessment is able to provide relevant information on COC functioning. Similarly to other views [23, 63], we are convinced that they are a mirror of oocyte quality and their study will help to fine tune the diagnosis and ameliorate the prognosis for fertility disorders.

Acknowledgement This study had financial support of Merck Serono Grant for Fertility Innovation.

Compliance with ethical standards

Ethical approval All procedures performed, involving human participants, were in accordance with the ethical standards of the institutional (please refer to the "Materials and methods" section for more information) and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in this study.

References

1. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. Endocr Rev. 1996;17(2):121–55.
2. Yoo SW, Savchev S, Sergott L, Rezai T, Lopez MF, Von Wald T, Eaton JL, Reindollar R, Usheva A. A large network of interconnected signaling pathways in human ovarian follicles is supported by the gene expression activity of the granulosa cells. Reprod Sci. 2011;18(5):476–84.
3. Erickson GF, Shimasaki S. The role of the oocyte in folliculogenesis. Trends Endocrinol Metab. 2000;11:193.
4. Makabe S, Naguro T, Stallone T. Oocyte–follicle cell interactions during ovarian follicle development, as seen by high resolution scanning and transmission electron microscopy in humans. Microsc Res Tech. 2006;69:436–49.
5. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science. 2002;296(5576):2178–80.
6. Juengel JL, McNatty KP. The role of proteins of the transforming growth factor-b superfamily in the intraovarian regulation of follicular development. Hum Reprod Update. 2005;11(2):144–16.
7. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature. 1996;383:531–5.
8. Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. Dev Biol. 1998;204:373–84.
9. Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM.

Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol Endocrinol. 2001;15:854–66.
10. Moore RK, Otsuka F, Shimasaki S. Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. J Biol Chem. 2003;278:304–10.
11. Mazerbourg S, Hsueh AJ. Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands. Hum Reprod Update. 2006;12(4):373–83.
12. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update. 2008;14(2):159–77.
13. Li Q, Agno JE, Edson MA, Nagaraja AK, Nagashima T, Matzuk MM. Transforming growth factor β receptor type 1 is essential for female reproductive tract integrity and function. PLoS Genet. 2011;7(10):e1002320.
14. Peng J, Li Q, Wigglesworth K, Rangarajan A, Kattamuri C, Peterson RT, Eppig JJ, Thompson TB, Matzuk MM. Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. Proc Natl Acad Sci U S A. 2013 Feb 19;110(8):E776–85.
15. Mottershead DG, et al. Cumulin, an oocyte-secreted heterodimer of the transforming growth factor-β family, is a potent activator of granulosa cells and improves oocyte quality. J Biol Chem. 2015;290(39):24007–20.
16. Russell DL, et al. Bidirectional communication between cumulus cells and the oocyte: old hands and new players? Theriogenology. 2016;86(1):62–8.
17. Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction. 2010;139(4):685–95.
18. Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: role of cumulus cells. Biol Reprod. 2000;63:805–10.
19. Matos L, Stevenson D, Gomes F, Silva-Carvalho JL, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. Mol Hum Reprod. 2009;15(7):411–9.
20. Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev. 2002;61(3):414–24.
21. Assou S, Anahory T, Pantesco V, Le Carrour T, Pellestor F, Klein B, Reyftmann L, Dechaud H, De Vos J, Samir H. The human cumulus–oocyte complex gene-expression profile. Hum Reprod. 2006;21:1705–19.
22. Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Rème T, Dechaud H, De Vos J, Hamamah S. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. Mol Hum Reprod. 2008;14(12):711–9.
23. Huang Z, Wells D. The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. Mol Hum Reprod. 2010;16(10):715–25.
24. Li Y, Li R-Q, Song-Bang O, Zhang N-F, Ren L, Wei L-N, Zhang Q-X, Yang D-Z. Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. Reprod Biol Endocrinol. 2014;12:81.
25. Alviggi C, Humaidan P, Howles CM, Tredway D, Hillier SG. Biological versus chronological ovarian age: implications for assisted reproductive technology. Reprod Biol Endocrinology. 2009;7:101.
26. Hansen KR, Knowlton NS, Thyer AC, Charleston JS, Soules MR, Klein NA. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. Hum Reprod. 2008;23:699–708.
27. Menken J, Trussell J, Larsen U. Age and infertility. Science. 1986;233:1389–94.
28. Schwartz D, Mayaux MJ. Female fecundity as a function of age: results of artificial insemination in 2193 nulliparous women with azoospermic husbands. Federation CECOS. N Engl J Med. 1982;306(7):404–6.
29. Kupka MS, Ferraretti AP, de Mouzon J, Erb K, D'Hooghe T, Castilla JA, Calhaz-Jorge C, De Geyter C, Goossens V. European IVF-Monitoring Consortium, for the European Society of Human Reproduction and Embryology. Assisted reproductive technology in Europe, 2010: results generated from European registers by ESHRE†. Hum Reprod. 2014;29(10):2099–113.
30. Centers for Disease Control and Prevention. American Society for Reproductive Medicine, Society for Assisted Reproductive Technology. In: Assisted reproductive technology national summary report. Atlanta (GA): US Dept of Health and Human Services; 2014. p. 2016.
31. Assisted reproductive technology in Australia and New Zealand 2010 [Internet]. Camberra; ACT: Australian Institute of Health and Welfare. http://www.aihw.gov.au/WorkArea/DownloadAsset.aspx?id=10737423255. Accessed 1 December 2015.
32. Broekmans FJ, Knauff EA, teVelde ER, Macklon NS, Fauser BC. Female reproductive ageing: current knowledge and future trends. Trends Endocrinol Metab. 2007;18(2):58–65.
33. Rattanachaiyanont M, Leader A, Léveillé MC. Lack of correlation between oocyte-corona-cumulus complex morphology and nuclear maturity of oocytes collected in stimulated cycles for intracytoplasmic sperm injection. Fertil Steril. 1999;71(5):937–40.
34. Ebner T, Moser M, Shebl O, Sommergruber M, Yaman C, Tews G. Blood clots in the cumulus-oocyte complex predict poor oocyte quality and post-fertilization development. Reprod BioMed Online. 2008;16(6):801–7.
35. Albertini DF, Combelles CM, Benecchi EH, Mary CJ. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction. 2001;121:647–53.
36. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. Reproduction. 2001;122(6):829–38.
37. Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, et al. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet. 2000;25:279–83.
38. Silva JRV, van den Hurk R, Van Tol HTA, Rolen BAJ, Figueiredo JR. Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and BMP receptors in the ovaries of goats. Mol Reprod Devel. 2005;70:11–9.
39. Bodensteiner KJ, Clay CM, Moeller CL, Sawyer HR. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. Biol Reprod. 1999;60(2):381–6.
40. Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L, Ritvos O. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab. 1999;84:2744–275.
41. Teixeira Filho FL, Baracat EC, Lee TH, Suh CS, Matsui M, Chang RJ, Shimasaki S, Erickson GF. Aberrant expression of growth differentiation factor-9 in oocytes of women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2002;87:1337–44.
42. Margulis S, Abir R, Felz C, Nitke S, Krissi H, Fisch B. Bone morphogenetic protein 15 expression in human ovaries from fetuses, girls, and women. Fertil Steril. 2009;92(5):1666–73.

43. Sun RZ, Lei L, Cheng L, Jin ZF, Zu SJ, Shan ZY, Wang ZD, Zhang JX, Liu ZH. Expression of GDF-9, BMP-15 and their receptors in mammalian ovary follicles. J Mol Histol. 2010;41(6):325–32.
44. Sidis Y, Fujiwara T, Leykin L, Isaacson K, Toth T, Schneyer AL. Characterization of inhibin/activin subunit, activin receptor, and follistatin messenger ribonucleic acid in human and mouse oocytes: evidence for activin's paracrine signaling from granulosa cells to oocytes. Biol Reprod. 1998;59(4):807–12.
45. Wei LN, Liang XY, Fang C, Zhang MF. Abnormal expression of growth differentiation factor 9 and bone morphogenetic protein 15 in stimulated oocytes during maturation from women with polycystic ovary syndrome. Fertil Steril. 2011;96(2):464–8.
46. Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJW, Hovatta O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. J Clin Endocrinol Metab. 2002;87:316–21.
47. Di Pasquale E, Beck-Peccoz P, Persani L. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet. 2004;75:106–11.
48. Di Pasquale E, Rossetti R, Marozzi A, Bodega B, Borgato S, Cavallo L, Einaudi S, Radetti G, Russo G, Sacco M, Wasniewska M, Cole T, Beck-Peccoz P, Nelson LM, Persani L. Identification of new variants of human BMP15 gene in a large cohort of women with premature ovarian failure. J Clin Endocrinol Metab. 2006;91(5):1976–9.
49. Otsuka F, McTavish KJ, Shimasaki S. Integral role of GDF-9 and BMP-15 in ovarian function. Mol Reprod Dev. 2011;78(1):9–21.
50. Guéripel X, Brun V, Gougeon A. Oocyte bone morphogenetic protein 15, but not growth differentiation factor 9, is increased during gonadotropin-induced follicular development in the immature mouse and is associated with cumulus oophorus expansion. Biol Reprod. 2006;75(6):836–43.
51. Persani L, Rossetti R, Di Pasquale E, Cacciatore C, Fabre S. The fundamental role of bone morphogenetic protein 15 in ovarian function and its involvement in female fertility disorders. Hum Reprod Update. 2014;20(6):869–83.
52. Wells RG, Yankelev H, Lin HY, Lodish HF. Biosynthesis of the type I and type II TGF-beta receptors. Implications for complex formation. J Biol Chem. 1997;272(17):11444–51.
53. Mazerbourg S, Klein C, Roh J, Kaivo-Oja N, Mottershead DG, Korchynskyi O, Ritvos O, Hsueh AJ. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. Mol Endocrinol. 2004;18(3):653–65.
54. Moore RK, Shimasaki S. Molecular biology and physiological role of the oocyte factor, BMP-15. Mol Cell Endocrinol. 2005;234(1–2):67–73.
55. Feary ES, Juengel JL, Smith P, French MC, O'Connell AR, Lawrence SB, Galloway SM, Davis GH, McNatty KP. Patterns of expression of messenger RNAs encoding GDF9, BMP15, TGFBR1, BMPR1B, and BMPR2 during follicular development and characterization of ovarian follicular populations in ewes carrying the Woodlands FecX2W mutation. Biol Reprod. 2007;77(6):990–8.
56. Dixit H, Rao LK, Padmalatha VV, Kanakavalli M, Deenadayal M, Gupta N, Chakrabarty B, Singh L. Missense mutations in the BMP15 gene are associated with ovarian failure. Hum Genet. 2006;119:408–15.
57. Laissue P, Christin-Maitre S, Touraine P, Kuttenn F, Ritvos O, Aittomaki K, Bourcigaux N, Jacquesson L, Bouchard P, Frydman R, Dewailly D, Reyss AC, Jeffery L, Bachelot A, Massin N, Fellous M, Veitia RA. Mutations and sequence variants in GDF9 and BMP15 in patients with premature ovarian failure. Eur J Endocrinol. 2006;154(5):739–44.
58. Zhao SY, Qiao J, Chen YJ, Liu P, Li J, Yan J. Expression of growth differentiation factor-9 and bone morphogenetic protein-15 in oocytes and cumulus granulosa cells of patients with polycystic ovary syndrome. Fertil Steril. 2010;94(1):261–7.
59. Xia Y, O'Shea T, Murison R, McFarlane JR. Concentrations of progesterone, follistatin, and follicle-stimulating hormone in peripheral plasma across the estrous cycle and pregnancy in merino ewes that are homozygous or noncarriers of the Booroola Gene1. Biol Reprod. 2003;69:1079–84.
60. Lambalk CB, De Koning CH, Braat DD. The endocrinology of dizygotic twinning in the human. Mol Cell Endocrinol. 1998;145(1–2):97–102.
61. Inagaki K, Shimasaki S. Impaired production of BMP-15 and GDF-9 mature proteins derived from proproteins WITH mutations in the proregion. Mol Cell Endocrinol. 2010;328(1–2):1–7.
62. Maheshwari A, Hamilton M, Bhattacharya S. Effect of female age on the diagnostic categories of infertility. Hum Reprod. 2008;23:538–42.
63. Li Y, Li RQ, Ou SB, Zhang NF, Ren L, Wei LN, Zhang QX, Yang DZ. Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. Reprod Biol Endocrinol. 2014;12:81.