Association between human oocyte developmental competence and expression levels of some cumulus genes

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Abstract

At present, oocyte selection is mainly based upon morphological criteria but it is generally acknowledged that its reliability requires further improvement. The aim of this study was to determine whether transcript levels in cumulus cells can provide a useful marker of oocyte developmental competence *in vitro*. A retrospective study was performed on cumulus cells isolated from 90 oocytes retrieved from 45 patients. Upon fertilization, 35 oocytes originated good-quality embryos and 36 developed into poor-quality embryos, whereas 19 failed to be fertilized. Semi-quantitative measurement of hyaluronic acid synthase 2 (HAS2), gremlin1 (GREM1), and pentraxin 3 (PTX3) mRNAs was performed and data for all genes were obtained from all the samples. Cumulus cells isolated from oocytes that originated high-quality embryos on day 3 of culture had HAS2 and GREM1 transcript levels higher than those detected in cells from oocytes that did not fertilize or developed into poor-quality embryos. No differences were observed in PTX3 levels. Results indicate that the measurement of HAS2 and GREM1 levels in cumulus cells would reliably complement the morphological evaluation providing a useful tool for selecting oocytes with greater chances to be fertilized and develop *in vitro*.

Reproduction (2007) **134** 645–650

Introduction

Embryo selection for transfer into the uterus is a crucial step in human-assisted reproduction in order to achieve an effective balance between reducing the risk of multiple gestation and maximizing the probability of pregnancy. To date, non-invasive embryo selection has been based mainly on morphological and developmental criteria performed during *in vitro* development (Scott 2003). Recent results have suggested that the evaluation of amino acid metabolism (Brison *et al.* 2004) or the incidence of apoptosis in cumulus cells (Lee *et al.* 2001) may provide additional predictive parameters. However, it is generally acknowledged that the reliability of current methods for predicting embryo viability requires further improvement which may arise by combining the evaluation of different parameters at successive time points. Moreover, in some countries, not all retrieved oocytes can be fertilized due to legal limitations (Ragni *et al.* 2005). In such situations, predicting embryo quality is even more challenging because the time when the predictive evaluation can be performed is limited to the interval between oocyte retrieval and fertilization. This has prompted the search for additional parameters that can

support morphological and metabolic evaluations of the oocyte in order to appropriately select those that have the greater chance of fertilization and development. In this respect, the analysis of cumulus cells is a good approach for providing such supplementary information. These cells, in fact, are closely connected to the oocyte through a gap junction network during follicular development and ovulation, forming a reciprocal functional interconnection (Gilchrist *et al.* 2004, Pangas & Matzuk 2005).

Cumulus expansion, in particular, is a critical process for normal oocyte development, ovulation, and fertilization (Elvin *et al.* 1999, Chang *et al.* 2002, Vanderhyden *et al.* 2003). Oocytes, whose maturation is not associated with cumulus expansion, have limited potential for implantation (Veeck 1999).

Growth differentiation factor 9 (GDF9), a member of the transforming growth factor- β superfamily, was the first oocyte-specific factor shown to cause cumulus expansion (Elvin *et al.* 1999). GDF9 functions as an oocyte-secreted paracrine factor that regulates several key granulosa cell enzymes involved in cumulus cell expansion and creates a microenvironment optimal for acquisition of oocyte developmental competence (Pangas & Matzuk 2005).

The expression levels of GDF9 downstream target genes in the cumulus cells may reflect GDF9 activity and could ultimately predict oocyte health (McKenzie *et al.* 2004). Indeed, recent data indicate that an association exists between the expression level of some GDF9 target genes and the grade of the resulting embryos (McKenzie *et al.* 2004, Zhang *et al.* 2005). However, these results were obtained with different methods and some inconsistencies between laboratories were observed.

Therefore, aim of the present work is to verify whether the expression level of GDF9 target genes in cumulus cells can be correlated with oocyte quality, which was evaluated as competence to be fertilized and to cleave *in vitro*. To this purpose, we examined the levels of hyaluronic acid synthase 2 (HAS2), pentraxin 3 (PTX3), and gremlin1 (GREM1), which previous work indicated as possible markers of developmental competence (McKenzie *et al.* 2004, Zhang *et al.* 2005) and critical for cumulus function.

Results

Cumulus cells isolated from 90 oocytes retrieved from 45 patients were analyzed. Upon fertilization, 35 oocytes originated good-quality embryos, 36 developed into poor-quality embryos, whereas 19 failed to be fertilized. The fertilization outcome of the 16 oocytes that did not satisfy all the morphological criteria of cytoplasmic appearance did not influence this distribution since seven originated good-quality embryos, seven poor-quality embryos, and two failed to be fertilized.

Semi quantitative RT-PCR analysis was performed in order to measure the relative abundance of the transcripts of interest in cumulus cells, and expression data for all four genes were obtained from all the samples.

No statistically significant difference of PTX3 mRNA levels was observed in cumulus cells isolated from oocytes that developed into high-quality embryos and those isolated from oocytes that developed into poor-quality embryos or those that failed to be fertilized (Fig. 1A). On the contrary, HAS2 mRNA expression was greater ($P < 0.05$) in cumulus cells isolated from oocytes that developed into high-quality embryos than that of cumulus cells isolated both from oocytes developing into poor-quality embryos, and from those that failed to be fertilized (Fig. 1B).

The relative abundance of GREM1 mRNA showed an expression pattern similar to that described for HAS2 and displayed higher levels ($P < 0.05$) of expression in cumulus cells isolated from oocytes that developed into high-quality embryos compared with cumulus cells isolated from oocytes that developed into low-grade embryos or isolated from oocytes that failed to be fertilized (Fig. 1C).

Finally, analysis of the transcript levels detected in cumulus cells isolated from the 16 oocytes that did not

Figure 1 Relative abundance of (A) PTX3, (B) HAS2 and (C) GREM1 mRNA expression in cumulus cells isolated from human oocytes that failed to be fertilized, originated low-grade embryos, or high-grade embryos. The horizontal line represents the median expression value and the box encompasses 50% of data points (first quartile to third quartile). Maximum and minimum data points are represented by the upper adjacent value and lower adjacent value (vertical lines). Outliers are present in which case the ends of the vertical line extend to a maximum of 1.5 times the inter-quartile range. These outliers are indicated by closed dots. Different superscript letter indicate a statistically significant difference ($P < 0.05$).

satisfy all the morphological criteria of cytoplasmic appearance was performed. The results demonstrated that HAS2 mRNA levels in cumulus cells isolated from the nine oocytes that either did not fertilize or developed into low-quality embryos were significantly lower compared to that detected in cells isolated from the seven oocytes that developed into good-quality embryos (median values of 0.159 vs 0.327 respectively, $P=0.04$). No statistically significant differences were observed in the mRNA levels of GREM1 and PTX3.

Discussion

The identification of objective markers that may be added to the morphological evaluation of an individual oocyte to improve the efficiency of predicting its developmental competence would increase the chances of a pregnancy, lowering the risk of multiple gestations.

The results of this study confirmed that the semi-quantitative analysis of selected genes expressed in the cumulus cells may reflect the quality of the enclosed oocyte, at least in terms of *in vitro* development.

The predictive role of gene expression in cumulus cells has been described before, with cyclooxygenase 2, GREM1, HAS2 (McKenzie *et al.* 2004), PTX3 (Zhang *et al.* 2005), and progesterone receptor (Hasegawa *et al.* 2005) being identified as useful markers.

With the exception of progesterone receptor, all other genes are downstream targets of GDF9 (Elvin *et al.* 1999, Varani *et al.* 2002, Pangas *et al.* 2004), confirming the hypothesis that the close morphological and functional interactions between oocytes and cumulus cells make the latter useful candidates as a source of non-invasive oocyte quality markers. In fact, the analysis of cumulus cells has some distinctive advantages compared with the direct evaluation of the oocyte. Metaphase II oocytes are delicate cells which may be, even if slightly, damaged by repeated morphological examinations. On the contrary, since cumulus cells can be separated from oocytes immediately after retrieval, their analysis does not add any further stress to the oocyte itself.

Our data confirmed a relationship between the amount of GREM1 and HAS2 transcripts in cumulus cells and the developmental competence of the respective oocytes, as previously described (McKenzie *et al.* 2004). The results have been obtained from an equivalent amount of cumulus–oocyte complexes (90 vs 103) but in our work a higher number of patients have been involved (45 vs 8), further reducing any bias related to a possible population effect.

Cumulus expansion is correlated with oocyte quality (Veeck 1999) and HAS2 controls the production of hyaluronan, which is one of the main components of the extracellular matrix secreted by cumulus cells during this process (Weigel *et al.* 1997). Since HAS2 is specifically expressed by cumulus cells in response to the combined effect of FSH and GDF9 (Dragovic *et al.* 2005) and the

latter is secreted by the oocyte, it is not surprising that this gene expression is associated with oocyte quality.

The relationship between oocyte quality and Grem1 expression is less clear. Its role as a downstream target of GDF9 has been described only recently in mice and it has been proposed that Grem1 modulates the crosstalk between GDF9 and bone morphogenetic protein (BMP) signaling that is necessary during follicle development because both ligands use components of the same signaling pathway (Pangas *et al.* 2004). In particular, Grem1 is an antagonist of BMPs, and recent data indicate its involvement in an intrafollicular BMP signaling pathway which contributes to the negative regulation of thecal androgen production (Glister *et al.* 2005). However, this pathway is unlikely to include SMAD4 because, in ovarian-specific SMAD4 knockout mice, GREM levels are not affected even if, in these animals, severe cumulus cell defects were observed both *in vivo* and *in vitro* (Pangas *et al.* 2006).

As for HAS2, a clear role in cumulus expansion has been described also for PTX3 (Varani *et al.* 2002, Salustri *et al.* 2004). This molecule co-localizes with hyaluronic acid throughout the cumulus matrix from the periphery to the zona pellucida. However, PTX3 is unable to bind directly to hyaluronan (HA); therefore, it is believed that PTX3 interacts with tumor necrosis factor α -induced protein 6 (TNFAIP6), which has the ability to specifically bind HA molecules. PTX3 actually forms large multimer complexes that interact with TNFAIP6, serving as an anchoring site for multiple HA molecules (Salustri *et al.* 2004). However, the relationship between PTX3 levels and oocyte competence is controversial. In previous studies, neither PTX3 transcript levels in cumulus cells (McKenzie *et al.* 2004) nor PTX3 protein concentration in follicular fluid (Paffoni *et al.* 2006) were related to oocyte quality. In partial contrast with these data, an extensive study of 98 cumulus–oocyte complexes from 16 patients indicated a statistical difference in PTX3 mRNA levels between cumulus cells derived from unfertilized oocytes and cells isolated from oocytes that developed to the eight-cell stage and led to the establishment of clinical pregnancies. However, no significant differences were observed between all the other groups, which included early cleavage embryos and 8-cell embryos that did not establish a pregnancy (Zhang *et al.* 2005). Our results confirmed that PTX3 transcript level does not show significant association with oocyte quality. It must be noted, however, that only the study by Zhang *et al.* (2005) took pregnancy as an end point, whereas in the other studies, end points were limited to *in vitro* development after 72 h from fertilization (McKenzie *et al.* 2004) or to oocyte morphology and fertilization (Paffoni *et al.* 2006). Therefore, it is possible that differences in PTX3 levels are related to the ability of the oocyte to develop to later stages of early embryonic development and, more importantly, to implant.

Our results add further evidence to the data available in the literature supporting the concept that cumulus cells are a promising source of reliable markers for predicting oocyte quality. Moreover, it is important to note that the oocytes evaluated in this study had been previously selected on the basis of morphological parameters, since, under Italian legislation, only three oocytes for each patient can be fertilized. Therefore, this study indicates that the determination of cumulus cell transcript levels provides useful information that adds to the morphological selection. The high developmental competence of 7 out of 16 oocytes that did not completely fulfill the morphological parameters, in fact, further indicated the limits of this kind of selection. On the contrary, the higher levels of HAS2 transcript present in the seven high-quality oocytes with sub-optimal morphology indicated that cumulus gene expression provides a further degree of evaluation independently of morphology itself.

In the future, in order to perform a prospective analysis of cumulus cells expression, it will be necessary to develop methods compatible with a timely selection of the oocytes destined for fertilization.

Materials and Methods

All chemicals were purchased from Sigma–Aldrich and Invitrogen unless otherwise indicated.

Patients and treatment

Forty-five patients with an average age of 36.5 (± 3.2) years were recruited for this study. Male factor was their main indication to assisted reproductive technologies. All patients provided informed consent prior to participation in this study.

Oocytes were obtained following controlled ovarian hyperstimulation using a long protocol with gonadotropin-releasing hormone analog, triptoreline (Decapeptyl; Ipsen S.p.A., Milan, Italy), or a protocol using gonadotropin-releasing hormone antagonist, cetrorelix (Cetrotide; Serono, Milan, Italy), depending on the clinical situation. In the first case, triptoreline (0.1 mg per day) was administered for at least 14 days for pituitary depression before gonadotropin stimulation. For the second type of stimulation protocol, cetrorelix (0.25 mg per day) was given by a daily injection as soon as one or more follicles ≥ 14 mm were seen on transvaginal ultrasound during ovarian stimulation protocol. In both cases, to achieve ovarian hyperstimulation, recombinant follicle-stimulating hormone (rFSH, Gonal-F 75; Serono) was initiated on cycle day 3 of the menstrual cycle onwards by once-daily injection at a dose determined by the physician, based on the patient's characteristics and history; follicular development was monitored via ovarian ultrasonography and serum estradiol-17 β assay. When at least three follicles with a mean diameter ≥ 18 mm were present, patients were instructed to administer 5000 IU of human chorionic gonadotropin (hCG, Gonasi; AMSA, Rome, Italy).

Transvaginal follicular aspiration for oocyte retrieval was performed 36 h post-hCG.

Oocytes evaluation and selection

In Italy, only three oocytes for each patient can be fertilized in the *in vitro* fertilization clinics, therefore only the cumulus cells isolated from oocytes subjected to fertilization were analyzed. As a consequence, all oocytes used in this study underwent a preliminary morphological examination in order to select the three best ones destined for fertilization. This allowed us to verify if the quantitative measurement of gene transcripts in cumulus cells could provide a further element of evaluation in addition to the morphological assessment.

Oocyte selection was performed using the following criteria: (1) presence of a single polar body in the perivitelline space and (2) clear cytoplasm, uniform texture, and fine granularity. Conversely, dark oocytes with granularity affecting the whole cytoplasm or the central portion of the cytoplasm, oocytes showing cytoplasmic inclusions, vacuoles, or refractile bodies were discarded. However, 16 of the 90 oocytes evaluated in this study were fertilized by ICSI even if they did not fulfill all the morphological criteria of cytoplasmic appearance. This was done when fewer than three oocytes with all the desired characteristics were retrieved from a single patient in order to reach the number of oocytes to be inseminated for each patient allowed by law. These oocytes were included in order to verify whether a suboptimal morphological evaluation would coincide with the results of cumulus cell transcript levels analysis.

Cumulus cells isolation

During ovum pick up, oocyte–cumulus complexes were immediately separated from follicular fluid, washed in flushing medium, and transferred to 1 ml IVF medium (G-FERT; Vitrolife Sweden AB, Kungsbäck, Sweden). Following a 2- to 3-h incubation at 37 °C in an atmosphere of 6% CO₂ in air, cumuli oophori were completely removed from all oocytes by pipetting them through 140 or 170 μ m internal diameter pipettes (Flexipet; Cook Medical Inc., Bloomington, IN, USA), after a brief exposure to 40 IU/ml hyaluronidase (Type IV; Sigma–Aldrich Srl) in Gamete Handling Medium (G-MOPS Vitrolife Sweden AB).

Cumulus cells were washed in PBS and then subjected to centrifugation at 1000 *g* for 10 min. The supernatant was removed and the pellet was resuspended in 500 μ l Trizol and stored in liquid nitrogen until used.

Embryo evaluation

After 1 h denudation, oocytes selected for fertilization were microinjected using a previously described ICSI procedure (Van Steirteghem *et al.* 1993) and then transferred to culture medium (G1; Vitrolife Sweden AB). Eighteen to twenty hours post microinjection, oocytes were checked for the presence of two pronuclei and the extrusion of the second polar body, as signs of normal fertilization. Zygotes were transferred to fresh G1 medium and cultured into single 40 μ l drops until day 3 when embryos were transferred to the patients. Embryos were scored on day 2 (42–44 h post insemination) and day 3 (66–68 h post insemination) according to the developmental

Table 1 Sequence of primers used for measuring cumulus cell gene expression with amplification conditions, number of cycles for exponential amplification, amplification product size, and accession number of the sequence used for primer design.

Genes	Primer sequences	Annealing temperature (°C)	Cycles for exponential amplification no.	Fragment size (bp)	NCBI accession no.
28S ribosomal RNA	5' primer: GTGTTGACGCGATGTGATTT 3' primer: GGGGCCTCCCACTTATTCTA	56	32	305	M11167
Pentraxin 3	5' primer: ATTCAGAGGAAGGGCTCACA 3' primer: TGCTCTCCGGTCTCTCTTA	57	32	241	NM_002852
Gremlin1	5' primer: GTCACACTCAACTGCCCTGA 3' primer: GCACACGAACACGCACAAG	57	33	251	NM_013372
Hyaluronan synthase 2	5' primer: ATCATCCAAAGCCTGTTTGC 3' primer: GGACCTTTTCGTGGAAGTT	56	36	301	NM_005328

stage and the morphological quality, as specified in the previously reported grading system by Veeck (1999). This classification is primarily based on equal-sized blastomeres, pattern of fragmentation, and cytoplasmic appearance; grade 1 represents perfect morphology and grade 5 represents the worst.

RNA isolation and reverse transcription

Cumulus cells were grouped into three classes depending on the fate of the oocytes from which they were isolated: (1) those that failed to be fertilized (unfertilized); (2) those that were fertilized but developed into grades 3–5 embryos (low quality); (3) those that developed into grades 1–2 embryos (high quality).

Total RNA from individual cumulus cell complexes was extracted using Trizol reagent according to the manufacturer's instructions. After quantification by spectrophotometer, RNA was incubated with DNase (1 U/μg RNA) and 10× DNase I Reaction Buffer (1 μl/μg RNA) for 15 min at room temperature, then 25 mM EDTA (1 μl/μg RNA) was added to the sample in order to inactivate the enzyme for 10 min at 65 °C. The amount of RNA extracted from each cumulus ranged from 0.3 to 0.7 μg.

DNase-treated RNA from each cumulus was precipitated with ethanol and then reverse transcribed into cDNA in a total volume of 10.5 μl reaction mixture containing 8.5 μl sterile water, 1 μl 10 mM dNTPs, and 1 μl random primers (250 ng/μl). RNA was denatured at 65 °C for 5 min, then 4 μl of 5× first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, and 15 mM MgCl₂), 1.5 μl MgCl₂ 50 mM, 2 μl of 0.1 M dithiothreitol, and 1 μl RNaseOUT recombinant ribonuclease inhibitor (40 U/ml) were added. Reverse transcription was performed with 200 U (1 μl) Superscript II reverse transcriptase for 1 h at 42 °C. Enzymes were inactivated at 70 °C for 15 min. Tubes without RNA or reverse transcriptase were prepared as negative controls during the RT reaction (data not shown).

Polymerase chain reaction

Primer sequence, amplification product size, and accession number of the sequence used for designing the primers for each gene are presented in Table 1. Ribosomal 28S subunit RNA was used as internal standard to normalize reactions for the amount of RNA in each sample.

The optimal cycle number at which the transcript was amplified exponentially was established from running a linear

cycle series and the value for each gene is reported in Table 1. PCR was carried out in an automated thermal cycler (iCycler, Bio-Rad), in a final volume of 20 μl, using the following conditions: 28S and HAS2: 1 min at 94 °C (denaturation), 1 min at 56 °C (annealing), and 1 min at 72 °C (extension); GREM1 and PTX3: 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C.

Detection and semi-quantification of RT-PCR products

The amplification products were subjected to electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris-acetate and 1 mM EDTA) containing 0.5 μg/ml ethidium bromide. After electrophoresis at 80 V for 45 min, the fragments were visualized on a 312 nm u.v. transilluminator. The image of each gel (Fig. 2) was recorded using a digital Kodak camera (DC290). The intensity of each band was assessed by densitometric analysis performed using the public domain NIH Image 1.63 program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The quantification procedure based on direct digitalization of the PCR product after separation on agarose gel provides a well-established and sensitive method to detect even small differences in amounts of mRNA from different biological samples (Grover *et al.* 2001, Ringhoffer *et al.* 2001). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each gene by the intensity of the 28S amplification product as previously

Figure 2 Representative gels of PCR amplification products for PTX3 (241 bp), HAS2 (301 bp), GREM1 (251 bp), and 28sRNA (305 bp). Numbers at the top of the panels indicate patient identification codes. The 50 bp ladder marker is on the left of each gel and the 350 bp band used to normalize each band of gene of interest is indicated by a small arrow.

described (Wrenzycki *et al.* 2002) and validated (Grover *et al.* 2001, Ringhoffer *et al.* 2001).

To account for gel–gel variations, each band was normalized against the 350 bp band of a 50 bp ladder marker (Invitrogen cat no. 10416-014; Stenman *et al.* 1999).

Statistical analysis

Statistical analysis was performed using Kruskal–Wallis ANOVA on rank (SigmaStat 3.1 software, Systat Software Inc., San Jose, CA, USA). Values were considered significantly different for $P \leq 0.05$.

Acknowledgements

This work was supported by Ministry of Health (decree 4 August 2004). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 20 April 2007

First decision 17 May 2007

Revised manuscript received 13 July 2007

Accepted 8 August 2007