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Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF

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BACKGROUND: A biochemical marker for embryo development would increase the chance of a successful pregnancy with IVF by optimizing oocyte and embryo selection, and allow fewer embryos to be transferred. In this study, we correlated cumulus granulosa cell gene expression of hyaluronic acid synthase 2 (*HAS2*), cyclooxygenase 2 (*COX2*; *PTGS2*) and gremlin (*GREM1*) with subsequent embryo development in search of a parameter for embryo selection. METHODS: Cumulus cell gene expression was determined prospectively on eight consecutive patients undergoing IVF with ICSI. Immediately following oocyte retrieval, the cumulus was stripped from the oocyte, and cumulus gene expression for *PTGS2*, *HAS2* and *GREM1* was assessed using a one-step real-time quantitative RT–PCR assay. Oocyte quality, fertilization and embryo morphology were correlated to relative gene expression. RESULTS: Gene expression data were available on cumulus cells from 108 oocytes that developed into 70 embryos (64.8% fertilization rate). Cumulus *PTGS2*, *HAS2* and *GREM1* expression was higher from oocytes that developed into higher quality embryos (grades 3, 4 and 5) compared with lower quality embryos (grades 1 and 2) ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively). *HAS2* and *GREM1* expression was also higher from the cumulus surrounding oocytes that gave rise to higher grade embryos ($P < 0.001$). The expression of *PTGS2* and *HAS2* was 6-fold higher, and that of *GREM1* was 15-fold higher in cumulus yielding higher grade embryos versus lower grade embryos. CONCLUSION: *PTGS2*, *HAS2* and *GREM1* gene expression correlates to morphological and physiological characteristics and provides a novel approach to predict human embryo development. Ultimately, with better predictors of follicular and embryonic health, higher quality embryos can be selected and transferred, reducing higher order pregnancy rates.

Key words: embryo selection/growth differentiation factor 9/oocyte-regulated genes/reproductive success

Introduction

After IVF, the primary criterion for embryo selection is the morphological appearance of the embryos based on a combination of cleavage rates and fragmentation of cells (Van Royen *et al.*, 2001). The morphological appearance of the oocyte and embryo, however, does not accurately predict the health of the embryo. Seventy to eighty percent of morphologically abnormal embryos have abnormal chromosomes, but up to 40% of embryos with normal morphology have abnormal chromosomes (Munné *et al.*, 1993, 1994). Thus, the only option to maintain high pregnancy rates is to transfer 3–4 early stage embryos or 2–3 blastocysts. However, this results in frequent high order multiple gestations associated with increased fetal morbidity and mortality (Keith and Oleszczuk, 2002; Blickstein and Keith, 2003). Therefore, any biochemical markers for fertilization potential and subsequent embryo development would increase the chance of a successful pregnancy by optimizing oocyte and embryo selection, allowing fewer embryos to be transferred.

Pre-ovulatory follicles contain subpopulations of granulosa cells, including mural and cumulus cells. Mural granulosa cells line the follicle wall, are in close proximity to the theca cells and express genes important for follicular rupture. Cumulus cells, which remain apposed to the oocyte during follicular development and ovulation, are likely mediators of oocyte development and fertilization, and are in turn regulated by oocyte factors (Matzuk *et al.*, 2002). In the peri-ovulatory period, the cumulus cells expand to form a radiating layer in response to the mid-cycle surge of luteinizing hormone or exogenous HCG. This cumulus expansion denotes a grade III oocyte, the optimal stage for fertilization (Veek, 1999). During expansion, cumulus cells secrete a hyaluronic

acid-rich matrix that binds the oocyte and cumulus together, facilitates follicular extrusion and oviductal fimbrial capture, and allows sperm penetration and fertilization (Salustri *et al.*, 1996). Cumulus expansion is imperative for normal oocyte development *in vivo*, and oocytes not associated with cumulus expansion have limited potential for implantation (Veek, 1999). Expansion of cumulus is regulated by growth differentiation factor 9 (GDF9), an oocyte-secreted member of the transforming growth factor-β (TGF-β) superfamily (Elvin *et al.*, 1999a; Chang *et al.*, 2002; Vanderhyden *et al.*, 2003).

GDF9 has been shown to induce several genes in cumulus granulosa cells, including those for hyaluronic acid synthase 2 (*HAS2*), cyclooxygenase 2 (*COX2*; *PTGS2*), *GREM1* and steroidogenic acute regulator protein (*StAR*), and to repress those for luteinizing hormone receptor (*LHR*) and urokinase plasminogen activator (*uPA*), which play critical roles in follicular development and cumulus expansion (Elvin *et al.*, 1999a; Pangas *et al.*, 2004). Thus, regulated expression of downstream GDF9 targets in the cumulus cells may reflect GDF9 activity, and could ultimately predict oocyte health.

Alterations in cumulus expansion may occur as women age and may be responsible for their reproductive disadvantage, either as a direct cause or as a reflection of a decline in the functional and structural qualities of the oocyte (Navot *et al.*, 1991). The biochemical assessment of follicles, including the cumulus and oocytes, may generate additional information necessary for an understanding of successful and unsuccessful fertilization, both *in vivo* and *in vitro*. The expression of genes (e.g. *PTGS2*, *HAS2* and *GREM1*) expressed in the cumulus during expansion, and discarded at the time of IVF, may give a direct assessment of the fertility potential of an individual oocyte without compromising the oocyte integrity. Thus, by determining the 'optimal' embryos that are produced and transferred, we may be able to replace fewer embryos and still maintain an acceptable pregnancy rate.

Methods and materials

Study population

Baylor College of Medicine Institutional Review Board approval was obtained for this study (IRB # H-10863). The study population included eight consecutive patients undergoing IVF with ICSI at the Baylor Assisted Reproductive Technology programme. Patient age, diagnosis, size and number of follicles generated, estradiol level at time of HCG trigger, number of oocytes retrieved, medication used for stimulation and number of prior IVF cycles were recorded for all patients. Exclusion criteria were recent cigarette use and body mass index above 25 kg/m^2.

All patients underwent a standard institutional stimulation protocol of ovarian downregulation with GnRH agonist (Lupron®, TAP Pharmaceuticals, Lake Forest, IL) followed by controlled ovarian stimulation exclusively with HMG (Pergonal®, Serono, Rockland, MA). Follicular monitoring was performed via ovarian ultrasonography and serum estradiol measurements.

Average patient age was 30 years (range 22–36) and diagnoses included male factor only ($n = 3$), unexplained ($n = 1$), male factor and endometriosis ($n = 1$), male factor and tubal disease ($n = 1$) and known genetic disorder ($n = 2$). The two couples with known genetic disorders underwent preimplantation genetic diagnosis (PGD); a known female carrier of Hunter's disease and a known male balanced translocation carrier involving chromosomes 5 and 9. Average estradiol prior to HCG administration was 2319 pg/ml (range 600–4942). Oocyte retrieval was performed 36 h following HCG administration. A total of 108 oocytes (average = 13.6 oocytes per patient, range 6–25) were obtained from the 134 scanned follicles >10 mm in maximal diameter.

Semen analyses were obtained on all male partners. Mean semen concentration was 20×10^6/ml (range $1–51 \times 10^6$/ml), mean motility was 33.5% (range 3–75) and mean normal morphology (by Kruger's strict criteria) was 2.3% (range 0–7).

Pregnancy outcomes were recorded on all eight patients. One patient had no unaffected embryos available for transfer following PGD. Three patients have ongoing pregnancies, for a 37.5% ongoing pregnancy rate per initiated cycle.

Specimens

Immediately following ooctye retrieval, cumulus cells were stripped from the oocyte with a micropipette and frozen at −80°C within 10 min of oocyte aspiration. Oocytes were maintained in individual microdroplets of culture medium during cumulus recovery, insemination and culture.

ICSI

ICSI was routinely performed 5 h after aspiration using straight needles (Humagen, Charlottesville, VA) in sperm wash medium with HEPES buffer (Irvine Scientific, Santa Ana, CA) on a heated stage (Narishige micromanipulator McHenry, IL).

RNA isolation

RNA from the cumulus cells was extracted using a commercial RNA isolation kit (Picopure RNA Isolation Kit, Arcturus, Mountain View, CA, catalogue no. KIT0204). Total RNA was eluted in a volume of 25 μl of elution buffer and stored at −80°C.

Real-time PCR

RNA expression was assessed using a one-step real-time quantitative RT–PCR assay to measure *PTGS2*, *HAS2* and *GREM1* mRNA and 18S rRNA transcripts. The procedure was performed for all mRNA expression using the ABI Prism 7700 Sequence Detector (PE Applied Biosystems) and the proprietary Taq Man® assay. Primers for *PTGS2*, *GREM1* and *HAS2* were intron-spanning and obtained through Applied Biosystems [Assays on Demand®: Hs00153133_m1 (*PTGS2*), Hs00171951_m1 (*GREM1*) and Hs00193435_m1 (*HAS2*)]. 18S rRNA was used as a normalizer to correct for the amount of RNA in each sample (Applied Biosystems PDAR with MGB probe; catalogue no. 4319413E). RT–PCR was performed in a reaction volume of 20 μl containing 10 μl of One Step PCR Master Mix (Applied Biosystems catalogue no. 4309169), 0.5 μl of reverse transcriptase, 1 μl of primer, 6.5 μl of RNase-free water (Ambion®, Austin, TX) and 2 μl of extracted RNA. Each sample was analysed in duplicate, and multiple water blanks were included with the analysis.

The thermal profile used for the analysis was as follows: reverse transcription was performed at 48°C for 30 min. Following a 10 min denaturation at 95°C, 40 cycles of PCR were performed at 95°C for 15 s, and 60°C for 1 min.

Amplification data were collected and analysed with the ABI Prism 7700 Sequence Detector software SDS1.2. (PE Applied Biosystems). Relative gene expression was calculated via the Delta-delta C (T) method as described by Livak and Schmittgen (2001).

Replicate RT–PCR analysis indicated that the coefficients of variation of *PTGS2*, *GREM1* and *HAS2* mRNA relative to 18S rRNA expression were 3.1, 2.8, 3.3 and 1.4%, respectively.

Outcomes

Morphological characteristics of the cumulus and embryos were recorded. The cumulus mass for each cumulus–oocyte complex was assessed as compacted, expanded or hyperexpanded, and the percentage of dark versus light cells was recorded. Based on these characteristics, the oocytes were classified as either mature or immature. Fertilization was assessed the morning after fertilization and was defined by the presence of two pronuclei. Embryo morphology (blastomere number, shape, size, fragmentation and ooplasm characteristics) was recorded at 72 h. Embryos were graded according to a standard institutional grading scale as described (Wolf, 1984).

Statistical analysis

Statistical analysis was performed using Spearman's rank correlation test and Mann–Whitney rank sum test (SigmaStat 2.0 software). Logistic regression analysis and receiver operating characteristic (ROC) curves were constructed to determine the predictive value of clinical outcomes using SPSS® (SPSS Inc., Chicago, IL). Data points determined to be outliers (<5% of data points) were excluded from graphic representation, but they were, however, included in statistical analysis.

Results

Gene expression data were available on cumulus cells from all 108 oocytes. A total of 103 oocytes had expression data for all four genes of interest (*PTGS2*, *HAS2*, *GREM1* and 18S rRNA), yielding 412 data points. Five out of five total oocytes from one patient did not reveal any expression data for *PTGS2* or *HAS2*; however, *GREM1* and 18S rRNA assays yielded amplification results (10 data points). All five cumulus–oocyte complexes were expanded, and one of the five oocytes failed to fertilize. The subsequent embryo development of those four samples was variable; two were high grade and two were low grade.

Cumulus cell *PTGS2* expression is 6-fold higher (0.5 versus 3.2, respectively; $P < 0.05$), *GREM1* expression is 15-fold higher and *HAS2* expression is 6-fold higher (1.0 versus 15 for *GREM1* and 1.1 versus 6.5 for *HAS2*; $P < 0.001$ for both) in cumulus cells yielding healthier embryos (grades 3, 4 and 5) versus poor quality embryos (grades 1 and 2) (Figures 1–3).

After statistically significant correlations of gene expression to embryo quality were found, ROC curves were constructed to determine the predictive value of *PTGS2*, *HAS* and *GREM1* for oocyte maturity, fertilization and embryo development. Although all three genes were found to be statistically significant predictors of clinical outcomes ($P < 0.05$) (Figures 4–6), the genes were not found to be statistically significantly different from one another.

Logistic regression was then performed to characterize further the candidate genes as predictors of ooctye maturity, fertilization and embryo grade. By logistic regression, *HAS2* expression is predictive of oocyte maturity ($P < 0.05$), and both *PTGS2* and *GREM1* are predictors of fertilization

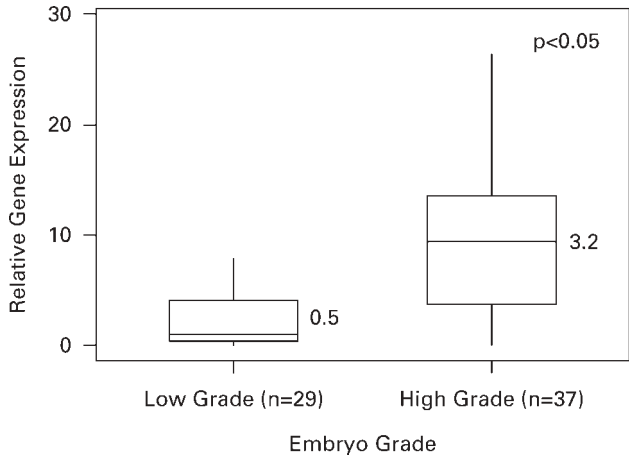

Figure 1. Relative expression of cyclooxygenase (*PTGS2*) in low grade versus high grade embryos. The horizontal line represents the median expression value, and the box encompasses 50% of data points (1st quartile to 3rd quartile). The upper adjacent value and lower adjacent value (vertical lines) represent maximum and minimum data points.

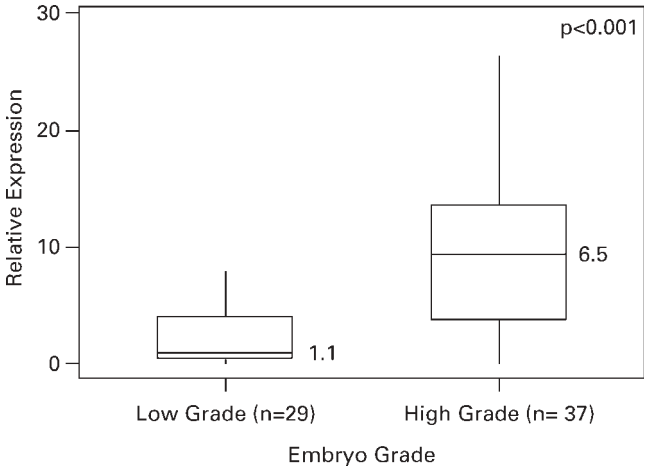

Figure 2. Relative expression of hyaluronic acid synthase 2 (*HAS2*) in low grade versus high grade embryos.

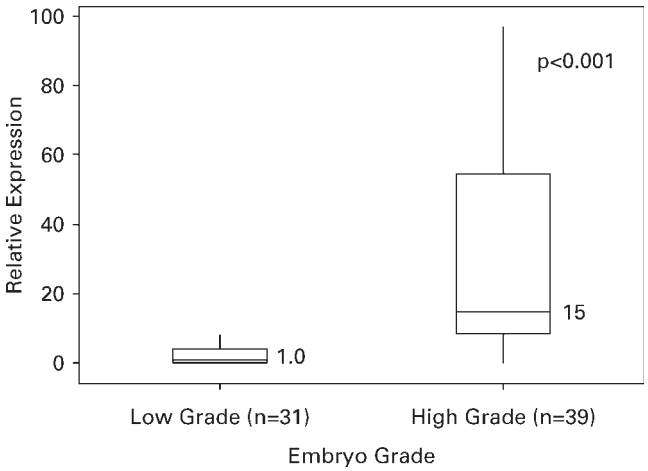

Figure 3. Relative expression of *GREM1* in low grade versus high grade embryos.

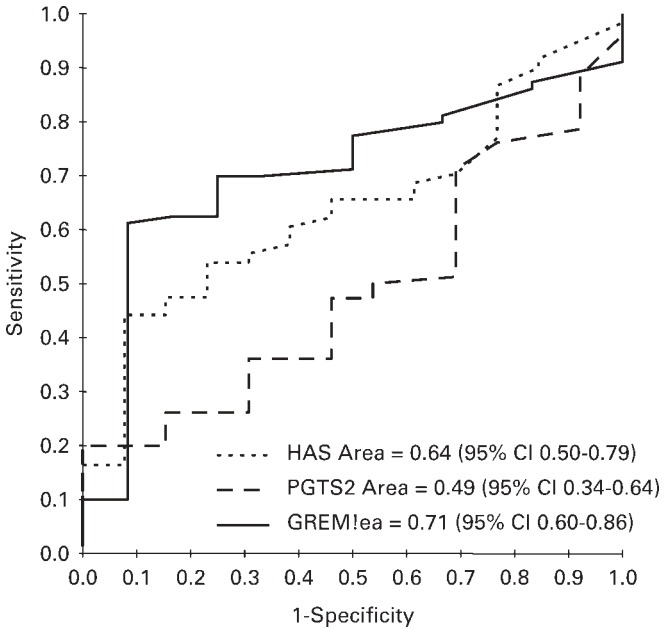

Figure 4. Receiver operating characteristics (ROC) curves for *PTGS2*, *HAS2* and *GREM1* expression as predictors of oocyte maturity. The ROC is a graphic representation of the false positives and false negatives for a particular diagnostic test (false positive on the *x*-axis; 1−false negative on the *y*-axis). The larger the area under the curve, the better the diagnostic test.

($P < 0.05$). *PTGS2* and *GREM1* are predictive of embryo development ($P < 0.05$).

The best single predictor for oocyte maturity, fertilization and embryo quality is *GREM1* expression, with areas under the curve of 0.71, 0.76 and 0.81, respectively (Figures 4–6). A 5.2-fold increased relative expression of *GREM1* yields a sensitivity and specificity for oocyte maturity of 63 and 93%, for fertilization 72 and 78%, and for embryo quality 83 and 81% (Table 1). Combining *PTGS2* and *GREM1* expressions to improve predictive efficiency increases the predictive value for embryo development only slightly (area under the curve of 0.82 versus 0.81), a difference which is not statistically significant.

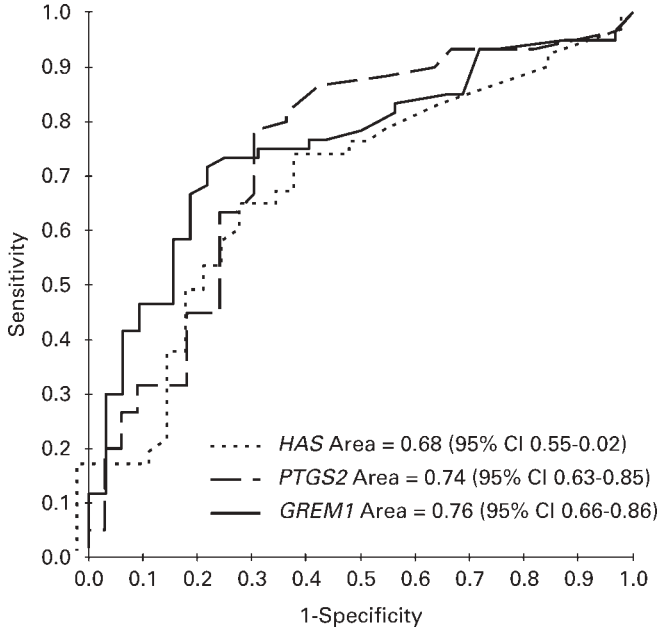

Figure 5. Receiver operating characteristics (ROC) curves for *PTGS2*, *HAS2* and *GREM1* expression as predictors of oocyte fertilization.

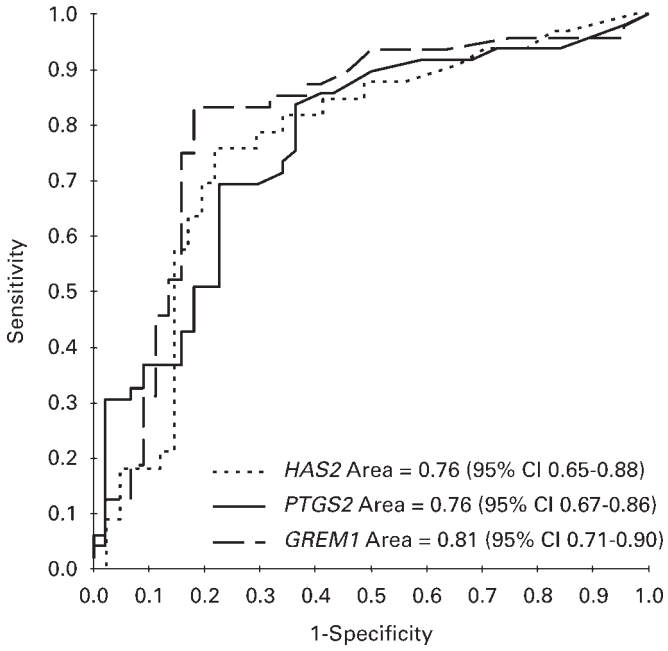

Figure 6. Receiver operating characteristics (ROC) curves for *PTGS2*, *HAS2* and *GREM1* expression as predictors of embryo quality.

Additional genes were analysed including pentraxin 3 (*PTX3*) (Applied Biosystems #Hs00357041_m1) and tumour necrosis factor-α-induced protein 6 (*TNFAIP6*) (Applied Biosystems #Hs00200178_m1). *PTX3* gene expression was not detected in the cumulus granulosa cells, and *TNFAIP6* expression did not differ as a function of embryo quality (data not shown).

Discussion

The developmental potential of human embryos is reflected in cumulus cell gene expression. Specifically, we demonstrate that expression of three genes downstream of *GDF9* activity (*HAS2*, *PTGS2* and *GREM1*) correlates with embryo development. Cumulus cells from oocytes that develop into higher quality embryos (grades 3, 4 and 5) have 6- to 15-fold increased median expression of these *GDF9* targets, and may be of clinical use in human assisted reproduction.

GDF9, a TGF-β superfamily member, is required for normal folliculogenesis in female mice and regulates human folliculogenesis (Dong *et al.*, 1996; Aaltonen *et al.*, 1999; Elvin *et al.*, 1999a, b). Elvin *et al.* demonstrated that GDF9 protein is expressed in all mouse oocytes beginning at the 3a follicle stage including antral follicles. *GDF9* gene mutation leads to infertility due to a block at the type 3b (primary) follicle stage, absence of thecal layer formation and defects in oocyte

Table I. Specificity and sensitivity of *GREM1*, *PTGS2* and *HAS2* expression for embryo development

	Relative expression[a]	Specificity	Sensitivity
Cyclooxygenase 2	1.5	77%	69%
Hyaluronic acid synthase 2	4.1	75%	78%
Gremlin	5.2	83%	81%

[a] Maximum efficiency as determined by ROC curves.

meiotic competence (Dong *et al.*, 1996; Carabatsos *et al.*, 1998; Aaltonen *et al.*, 1999; Elvin *et al.*, 1999b; Yan *et al.*, 2001). Additional data have shown that *GDF9* is also required for later stage development (Elvin *et al.*,1999a; Yan *et al.*, 2001).

The oocyte itself plays an active role in cumulus expansion (for review, see Eppig, 2001; Matzuk *et al.*, 2002), inducing expansion by secretion of GDF9. GDF9 induces *HAS2* in cumulus cells (Elvin *et al.*, 1999a) that stimulates the production of hyaluronic acid and results in cumulus expansion (Buccione *et al.*, 1990). GDF9 also facilitates cumulus expansion via the induction of cyclooxygenase 2 (*PTGS2*) and the resultant prostaglandin E_2 (PGE2) production (Elvin *et al.*, 2000b), both of which are involved in cumulus expansion in cattle (Calder *et al.*, 2001). Mice lacking functional *PTGS2* have defects in ovulation, fertilization, decidualization and implantation (Lim *et al.*, 1997). Systemic treatment with PGE2 restores the ability of *PTGS2* knockout mice to ovulate (Davis *et al.*, 1999). Calder *et al.* (2001) examined the expression of *PTGS2* and prostaglandin receptor mRNA in bovine cumulus–oocyte complexes, and found that expression of *PTGS2* and *EP2* mRNA is dependent on time of maturation and oocyte quality. Complexes in which the oocyte is microsurgically removed and cultured with recombinant GDF9 can be induced to undergo cumulus expansion (Elvin *et al.*, 1999a).

GREM1, recently described as another downstream gene upregulated by GDF9, selectively inhibits bone morphogenic protein (*BMP*) signalling and not *GDF9* signalling (Pangas *et al.*, 2004). It is hypothesized that the differential signalling inhibition may facilitate the luteinization of mural granulosa cells, while allowing cumulus cell expansion (Pangas *et al.*, 2004). If this hypothesis is correct, continued expression of *GREM1* in cumulus granulosa cells should represent a mature oocyte and may predict improved embryo development. The role of *GREM1* in human reproduction clearly merits further investigation.

Interestingly, despite the known role of *PTGS2* in cumulus expansion,*PTGS2* had a lower ROC curve for oocyte maturity than *GREM1* or *HAS2*. The ROC curves for *PTGS2* increased for fertilization and embryo development, although *GREM1* was the best predictor for all three clinical parameters. *HAS2* has an established role with cumulus expansion (Buccione *et al.*, 1990), and *GREM1* may function in cumulus expansion via the luteinization of mural granulosa cells (Pangas *et al.*, 2004). Further elucidation of the role of *GREM1* in cumulus expansion is required, as it may prove to be a better predictor of cumulus expansion than *PTGS2* expression.

In this report, we describe the gene expression of *PTGS2*, *HAS2* and *GREM1*, which function under the influence of GDF9. Expression profiling of other GDF9-induced genes in periovulatory granulosa cells, such as *TNFAIP6*, *EP2 PGE2* receptor and *StAR* (Elvin *et al.*, 2000a; Varani *et al.*, 2002; Fulop *et al.*, 2003) may also prove to be predictive of embryo quality. Oocyte-secreted factors other than GDF9 may also mediate cumulus physiology. Bone morphogenic protein 15 (BMP15), which is largely uncharacterized in humans, has been shown to be essential for normal follicular development in sheep (Galloway *et al.*, 2000; Juengel *et al.*, 2002; Hanrahan *et al.*, 2004). In contrast to results in mice, sheep with inactivating mutations of the *BMP15* gene are infertile, with primary stage arrest of follicular development. Expression of downstream *BMP15* targets may further define gene expression profiles in high grade embryos and be of additional clinical use.

A mutation in *BMP15* has been described recently in two sisters with premature ovarian failure (Di Pasquale *et al.*, 2004), indicating that the action of *BMP15* is required for normal human folliculogenesis. Dysregulation of downstream targets of *BMP15* or *GDF9* may result in abnormal embryo development. Patients with markedly abnormal *PTGS2*, *HAS2* or *GREM1* expression may have an underlying defect in *GDF9* or other oocyte-secreted mediators of cumulus physiology.

Ultimately, a finite set of cumulus genes may function as an assay for clinical utilization, creating an expression profile useful for embryo selection. In this study, the use of a single gene, *GREM1*, could accurately predict all three outcomes: maturity, fertilization potential and embryo quality. No additional predictive value was gained by including *PTGS2* or *HAS2* in the analysis. Cumulus cells are an ideal surrogate for the developmental potential of the oocyte as they are typically discarded tissues during IVF and represent a non-invasive means to identify embryo quality. Thus, cumulus expression profiling may be a parameter, other than morphology, by which to facilitate embryo selection.

The expression of these candidate genes (*PTGS2*, *HAS2* and *GREM1*) can be correlated to morphological and physiological characteristics and may provide a novel approach to predict human embryo development. Ultimately, with better predictors of follicular and embryonic health, we can better select the embryos for transfer and reduce higher order pregnancy rates.

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