Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy

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Abstract

The close relationship between cumulus cell function and oocyte developmental competence indicates that analysis of cumulus gene expression is a potential non-invasive method to aid embryo selection and IVF outcome. Cumulus was isolated from 674 oocytes from 75 women undergoing ICSI and gene expression analysed by quantitative RT-PCR. Cumulus expression of cyclooxygenase 2 (*PTGS2*) was higher with mature oocytes, whereas brain-derived neurotrophic factor (*BDNF*) was lower when fertilisation was normal. Expression levels of gremlin (*GREM1*) and *BDNF* were weak positive and negative predictors of embryo quality respectively. Ranking of *GREM1* expression within cohorts of oocytes showed that oocytes associated with the highest *GREM1* expression were more likely to be transferred or cryopreserved than discarded (49 vs 33%, $P < 0.02$), although the clinical pregnancy rate was not significantly different. This study demonstrates both the feasibility and difficulties of this method of analysis in the largest such group studied thus far. Novel relationships between *BDNF* expression and fertilisation were identified, and the potential value of *GREM1* expression as a marker of embryo quality supports the further assessment of *GREM1* analysis in the context of embryo selection.

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Introduction

Improvements in all aspects of assisted conception have led to steady rises in pregnancy rates, but the final criteria by which embryos are selected for transfer remain based on observation of their morphology. Aspects of embryo morphology, which have been identified as of prognostic importance, include the speed of cell division (number of blastomeres at any given stage of development), regularity of cell division and degree of fragmentation, based on data from single embryo transfers (Giorgetti *et al.* 1995, Ziebe *et al.* 1997, Van Royen *et al.* 1999). While embryo morphology has been correlated with implantation potential, its accuracy has proved limited. Alternative non-invasive approaches to this problem have led to the development of methods for the assessment of early embryo metabolism (Brison *et al.* 2004) and analysis of the function of the cumulus cells surrounding the oocyte (McKenzie *et al.* 2004, Cillo *et al.* 2007, Feuerstein *et al.* 2007) including by the use of microarrays (Assou *et al.* 2008) as well as analysis of

factors in follicular fluid (Fanchin *et al.* 2007). Potentially, these methods will also add to understanding of the basic biology of the human cumulus–oocyte complex (COC) and early embryo by allowing exploration of, for example, gene function derived from animal knockout experiments (Li *et al.* 2008).

During follicular development, the granulosa cells differentiate into two distinct phenotypes, the mural population lining the follicular antrum and the cumulus population enclosing the oocyte. The former is essential for oestrogen production and follicular rupture, while the latter is closely associated with oocyte development. Cumulus cell function is in part regulated by oocyte-derived factors and, in turn, contributes to oocyte maturation and subsequent developmental potential (Eppig 2001). In response to the ovulatory LH surge, mediated partly by epidermal growth factor-like ligands released by mural granulosa, cumulus cells show dramatic changes in morphology and function (Hernandez-Gonzalez *et al.* 2006, Russell & Robker 2007). They become expanded and produce a hyaluronan-rich extracellular matrix, which continues

to enclose the oocyte following follicular rupture and through fertilisation and early embryo development before implantation. A number of key cumulus genes have been identified including hyaluronin synthase 2 (*HAS2*), prostaglandin synthase 2 (*PTGS2*, also known as *COX2*), tumour necrosis factor- α -induced protein 6 (*TNFAIP6*, also known as *TSG6*) and pentraxin 3 (*PTX3*): gene deletion models of several of these have revealed effects on fertility through a range of mechanisms (Russell & Robker 2007).

The interaction between oocyte and cumulus is in substantial part mediated by growth differentiation factor 9 (GDF9) and bone morphogenetic proteins (BMPs), particularly BMP15, members of the transforming growth factor β family. The importance of these factors was originally identified in mice (Dong *et al.* 1996, Elvin *et al.* 1999, Yan *et al.* 2001, Dragovic *et al.* 2005) and their critical roles in the regulation of ovulation have been confirmed in sheep (Galloway *et al.* 2000, Hanrahan *et al.* 2004) and man (Montgomery *et al.* 2004, Di Pasquale *et al.* 2004). GDF9 regulates expression of several cumulus cell genes involved in expansion, including *HAS2*, *PTGS2* and *GREM1*, encoding the BMP-antagonist gremlin (Elvin *et al.* 1999, Pangas *et al.* 2004, Hussein *et al.* 2005). Gremlin has been proposed to allow differential regulation of cumulus function by oocyte-derived GDF9 and some BMPs, as it selectively prevents signalling from the latter but not the former. Other key cumulus genes include *TNFAIP6* and *PTX3*, which are involved in stabilisation of the cumulus complex during fertilisation and oviductal transport (Varani *et al.* 2002, Fulop *et al.* 2003). Expression of some of these genes in cumulus has been associated with the development of higher quality embryos (McKenzie *et al.* 2004, Zhang *et al.* 2005, Cillo *et al.* 2007). Novel factors acting during COC maturation may also have a major impact on developmental competence. One example is the demonstration that members of the neurotrophin family of growth factors, specifically brain-derived neurotrophic factor (BDNF), are expressed by human cumulus (Seifer *et al.* 2002a, Feng *et al.* 2003), and improve oocyte maturation and subsequent embryo development in both mouse and a large mammalian species, the cow (Seifer *et al.* 2002b, Kawamura *et al.* 2005, Martins da Silva *et al.* 2005).

Cumulus function may therefore both reflect and determine oocyte function, and subsequent embryo developmental potential. Understanding of these processes may be of value in optimising COC maturation *in vitro* for assisted conception as well as being of importance physiologically. The aim of this study was therefore to analyse cumulus gene expression from a large population of unselected oocytes from couples undergoing ICSI in relation to oocyte maturity, fertilisation, embryo development, implantation and subsequent pregnancy outcome.

Results

A total of 674 cumulus samples were obtained from the 75 women. Successful mRNA extraction and RT-PCR were achieved in all samples (based on *GAPDH* expression). Expression of all six genes (*HAS2*, *BDNF*, *GREM1*, *PTGS2*, *TNFAIP6* and *PTX3*) was identified in cumulus cells. However, the proportion of samples from which quantitative data could be obtained varied from 84% of samples for *BDNF* to 45% for *GREM1*.

The majority of COCs was of grade 2 'fully expanded' cumulus (51% of samples), with only a few grade 0 (5%). Analysis of gene expression comparing grades 0 and 2 showed only *HAS2* was more highly expressed in mature cumulus, although this did not reach statistical significance, but *BDNF* (0.78-fold, $P=0.001$), *TNFAIP6* (0.36-fold, $P=0.003$) and *PTX3* (0.47-fold, $P<0.001$) were all expressed at significantly lower levels in expanded cumulus (Fig. 1). Expression of all genes was similar in grades 1 and 2.

Oocyte maturity (metaphase II (MII) versus immature) was associated with 4.7-fold-increased *PTGS2* expression (relative expression 4.1×10^{-4} vs 0.86×10^{-4} , $P=0.001$), but differences were not observed with other genes. Analysis by fertilisation revealed differences in expression of *BDNF* but not expression of other genes analysed, with higher expression in cumulus from oocytes that showed failed fertilisation compared with normal fertilisation (0.045 ± 0.006 vs 0.033 ± 0.005 ; $n=71$ and 375 respectively, $P=0.001$), whereas *BDNF* expression was similar in normal versus abnormal fertilisation (abnormal: 0.036 ± 0.008 , Fig. 2A). Receiver-operating characteristic (ROC) analysis of *BDNF* and failed versus normal fertilisation gave an area under the curve of 0.39 (95% confidence interval (CI) 0.33–0.45, $P=0.001$, Fig. 2B). Analysis of early embryo cleavage

Figure 1 Relationship between cumulus expansion and gene expression. Open columns, grade 0 expansion; filled columns, grade 2 expansion. Mean $\pm$ S.E.M., $n=34$ immature, $n=345$ mature. Expression of *BDNF*, *TNFAIP6* and *PTX3* was significantly lower in grade 2 than grade 0 cumulus (all $P<0.01$). Data are expressed relative to *GAPDH*: to allow comparison on the same scale, data have been multiplied by factors of 10 for *PTX3*, 100 for *HAS2* and *BDNF*, 10 000 for *PTGS2* and *GREM1* and 100 000 for *TNFAIP6*.

Figure 2 (A) *BDNF* expression in cumulus from oocytes that showed normal or abnormal fertilisation or failed to fertilise. Mean $\pm$ S.E.M., $n=375$, 38 and 71 respectively, $*P=0.001$ versus normal fertilisation. (B) Receiver-operating characteristics for *BDNF* expression as a predictor of normal versus failed fertilisation. Area under the curve of 0.39, $P=0.001$.

also indicated a relationship with *PTGS2* expression, but not with other genes: *PTGS2* expression was slightly lower (0.78-fold, $P=0.04$) where early cleavage was observed at 25 h post-insemination.

Consistent with these findings, expression of *PTGS2* was higher and that of *BDNF* was lower in cumulus where the oocyte resulted in an embryo ($P<0.001$ for *PTGS2*; $P=0.015$ for *BDNF*) and also when graded by graduated embryo score (GES) using a cut-off of either 85 or 95 (85: *PTGS2* $P=0.001$, *BDNF* $P=0.005$), although differences between groups were small (1.6-fold for *PTGS2* and 0.78-fold for *BDNF* using GES 85 as discriminator). *GREM1* expression was also higher in cumulus from oocytes that resulted in high-quality embryos (2.4-fold: 2.7 ± 0.9 vs 1.1 ± 0.2 using GES 85, $P=0.09$), but this did not reach statistical significance. There were no significant direct correlations between GES and expression of any of the genes analysed. Because of the apparent positive relationship between embryo quality with *PTGS2* and *GREM1* and negative relationship with *BDNF*, ROC analysis was performed using data from embryos graded according to a GES cut-off of 85 (Fig. 3). This demonstrated significant positive predictive ability of *GREM1* expression (area under the curve 0.61, 95% CI 0.53–0.70, $P=0.012$) and significant

Figure 3 Receiver-operating characteristics for *GREM1* and *BDNF* expression as predictors of embryo grade. Area under the curve for *GREM1* 0.61 (solid line, 95% CI 0.53–0.70; $P=0.012$) and for *BDNF* 0.40 (dotted line, 95% CI 0.32–0.49; $P=0.03$).

negative predictive ability of *BDNF* (area under the curve 0.40, 95% CI 0.32–0.49, $P=0.03$), but not *PTGS2* (area under the curve 0.59, ns).

Cumulus gene expression was also investigated according to the establishment of pregnancy. Overall, 142 embryos were transferred, resulting in positive pregnancy tests in 43 out of 74 women, of whom 20 had singleton clinical pregnancies and 12 twin clinical pregnancies. This allowed classification into embryos that definitely did not result in pregnancy, those that definitely did (i.e. twin pregnancy following two embryo transfer or singleton pregnancy in the six cases where only one embryo was transferred, the latter resulting in three pregnancies) and those that might have resulted in pregnancy (i.e. singleton pregnancy following two embryo transfer). Expression of *GREM1* was 5.1-fold higher in embryos that definitely resulted in pregnancy compared with those that definitely did not, but this did not reach statistical significance (Fig. 4). Expression of other genes was similar between these three groups.

Figure 4 *GREM1* expression by the establishment of pregnancy. The differences between groups were not statistically significant.

As embryos are chosen for transfer only from within the cohort available to an individual woman, analysis of *GREM1* expression following ranking within a cohort was also performed. This allowed comparison of the 70 samples with the highest *GREM1* expression within each woman's cohort of COCs with the remaining population. These 70 oocytes resulted in 49 embryos compared with 604 other oocytes, which resulted in 385 embryos (70 vs 64%, ns). Out of these 49 embryos, 23 were transferred (47%) compared with 120 transferred embryos from 385 oocytes (31%, $P=0.04$). Thus, embryos from COCs with the highest *GREM1* expression had an increased probability of being selected for transfer. Top quality embryos may also be selected for cryopreservation: 34 out of the 49 high *GREM1* embryos were either transferred or cryopreserved compared with 204/385 (70 vs 53%; $P=0.03$); thus, overall, 49% of the oocytes with the highest *GREM1* expression resulted in embryos that were transferred or cryopreserved compared with 33% of the remainder ($P=0.02$). Analysis of the fate of the top four embryos in each cohort ranked by *GREM1* expression (Fig. 5A) shows the clear relationship between *GREM1* expression and embryo selection for transfer,

Figure 5 Embryo outcome by *GREM1* ranking. (A) The proportion of embryos selected for transfer (black columns), discard (grey columns) or cryopreservation (white columns) grouped by ranking of *GREM1* expression, with rank 1 having the highest *GREM1* expression. (B) GES score by *GREM1* ranking. Data are mean $\pm$ S.E.M. GES score for each group of embryos with *GREM1* ranking 1–4. (C) Percentages of embryos with GES ≥ 85 by *GREM1* ranking. $N=49$, 32, 29 and 23 for *GREM1* ranking 1–4 respectively.

Table 1 Summary of main findings.

Analysis	Genes implicated
Cumulus expansion	<i>BDNF</i> , <i>TNFAIP6</i> and <i>PTX3</i> lower in expanded cumulus
Oocyte maturity	<i>PTGS2</i> increased with mature oocytes
Fertilisation	<i>BDNF</i> lower with normal fertilisation
Embryo formation	<i>PTGS2</i> higher and <i>BDNF</i> lower
Embryo quality	<i>GREM1</i> -positive predictor and <i>BDNF</i> -negative predictor
Embryo selection for transfer	<i>GREM1</i> higher

cryopreservation or discard. There were significant differences in the outcomes by *GREM1* ranking at both high and low expression ($P=0.005$ for rank 1 and $P<0.0001$ for rank 4). These differences were marked for selection for transfer or discard, but with little differences between embryos selected for cryopreservation; thus, 47% of rank 1 embryos were transferred and 34% discarded whereas for rank 4, 14% were transferred and 64% discarded. Average GES for the high *GREM1* embryos did not, however, differ from the remaining population (76.6 ± 4.2 vs 70.5 ± 1.5 , ns); and while there were trends for higher *GREM1* ranking to be associated with higher GES score (Fig. 5B) and higher proportions of embryos with high GES scores (Fig. 5C), these also did not reach statistical significance. Double-embryo transfers were performed in almost all women in this study preventing clear analysis of embryo outcome. The implantation rate of high *GREM1* embryos appeared slightly but not significantly higher than the remaining population: 11 women achieved a clinical pregnancy after receiving one of the 23 embryos with the highest *GREM1* expression (47.8%) versus 21 out of 51 women (41.2%) who did not. A summary of the major findings is provided in Table 1.

Discussion

The ability to select embryos with the highest developmental competence is central to optimising success rates in assisted conception. Given the essential nature of the interaction between cumulus cells and the maturing oocyte and that many aspects of cumulus function are regulated by the oocyte (Eppig 2001, Matzuk *et al.* 2002), the analysis of cumulus cells appears a logical potential approach to the non-invasive assessment of oocyte developmental competence (Li *et al.* 2008). Cumulus cells are normally discarded during ICSI, thus can readily be obtained with the subsequent outcome of the oocyte recorded individually. This allows tracking right through to pregnancy outcome, although in the present study, the use of two embryos transfer precluded clear correlation with implantation and pregnancy.

The present data confirm the feasibility of this approach to the analysis of several genes from each cumulus sample from women recruited non-selectively. Other groups have previously reported relationships between cumulus gene

expression and embryo quality (McKenzie *et al.* 2004, Zhang *et al.* 2005, Cillo *et al.* 2007, Feuerstein *et al.* 2007), although in none was gene expression related to the establishment of pregnancy. In the present study, we included the genes identified by McKenzie *et al.* (2004) in an enlarged set including *PTX3* that they were unable to detect, but which was subsequently identified as a potential maker of embryo development in one study (Zhang *et al.* 2005) but not another (Cillo *et al.* 2007). *TNFAIP6* was also included as animal data have demonstrated that it is essential for female fertility (Fulop *et al.* 2003), as was *BDNF* that is expressed by human cumulus (Seifer *et al.* 2002a) and has been associated with oocyte maturation and subsequent embryo development in both mouse and a large mammalian species, the cow (Seifer *et al.* 2002b, Kawamura *et al.* 2005, Martins da Silva *et al.* 2005).

HAS2 is essential for cumulus expansion as its product, hyaluronic acid, is the main component of the matrix, which forms at cumulus expansion in response to the ovulatory LH surge. *HAS2* expression is also regulated by oocyte-derived GDF9. While *HAS2* expression was higher in cumulus graded as 'mature', this did not reach statistical significance: this may reflect the relative infrequency of immature cumulus. Expression of *BDNF*, *TNFAIP6* and *PTX3* was lower in mature than immature cumulus. *BDNF* expression is regulated by LH in the human (Feng *et al.* 2003) and that of *TNFAIP6* by prostaglandins (Yoshioka *et al.* 2000), as is *HAS2* (Eppig 1981). *PTX3* acts to stabilise the expanded cumulus, and is regulated by GDF9. Fertilisation *in vivo* is very low in *PTX3* knockout mice, whereas *in vitro* it was normal (Varani *et al.* 2002). It is unclear why *BDNF*, *TNFAIP6* and *PTX3* expression should be lower in mature cumulus, but it may reflect the time point at which the cumulus was recovered as expression of some cumulus genes can be transient (Yoshino *et al.* 2006).

All fertilisation in the present study was by ICSI, minimising variability due to sperm function. *BDNF* expression was higher in cumulus from oocytes that failed to fertilise, whereas expression of other genes showed no relationships with fertilisation. This relationship with abnormal fertilisation may reflect intrinsic cumulus function or the interaction with the oocyte. As discussed above, *BDNF* production in cumulus is known to be stimulated by LH/hCG, but its regulation by oocyte factors is unknown. *BDNF* has been reported to increase oocyte maturation to MII and developmental potential after parthenogenetic activation in mouse, cow and pig (Seifer *et al.* 2002a, Kawamura *et al.* 2005, Martins da Silva *et al.* 2005, Lee *et al.* 2007), but data in the human suggest a negative effect on oocyte developmental competence (Anderson *et al.* 2009). Further experimental data are required to elaborate the nature of the interaction between *BDNF* and fertilisation, but the present data support the conclusion that *BDNF* may have an important role at this critical point.

These data did not confirm the previously reported difference in cumulus expression of *HAS2* according to embryo grade (McKenzie *et al.* 2004), but relationships between *PTGS2*, *GREM1* and embryo development were confirmed (McKenzie *et al.* 2004, Cillo *et al.* 2007) and a novel relationship between *BDNF* and embryo grade was identified. In contrast to *PTGS2* and *GREM1*, the relationship between *BDNF* and embryo grade was negative. Thus, as with fertilisation, higher *BDNF* expression was associated with poorer developmental potential. None of these three genes, however, showed such clear relationships as previously described (McKenzie *et al.* 2004). This may reflect the larger size of the study, with 75 women instead of 10 increasing the interindividual variability. Additionally, data were not obtained on all genes from all cumulus preparations. This has been previously recognised (McKenzie *et al.* 2004), although its extent is unclear in other studies (Feuerstein *et al.* 2007). Methodological developments were required for *PTGS2* as initially it was undetectable in many samples, and undetectable readings were also relatively common for *GREM1*. Similarly, *PTX3* was not detectable in any samples in one study (McKenzie *et al.* 2004), although was readily detectable here.

Cumulus expression of both *Ptgs2* and *Grem1* are regulated by oocyte-derived GDF9 in the mouse (Elvin *et al.* 2000, Pangas *et al.* 2004), providing a molecular mechanism whereby cumulus gene expression can reflect oocyte function. *PTGS2* is essential for ovulation, but is also required for fertilisation (Lim *et al.* 1997). *GREM1* is one of the family of secreted proteins originally isolated from *Xenopus* that act as BMP antagonists (Hsu *et al.* 1998). In mammalian cumulus, it acts as a selective inhibitor of signalling by BMPs but not GDF9, and has been proposed as a component of the pathway that prevents premature cumulus luteinisation (Pangas *et al.* 2004). The present data also suggested a relationship between *GREM1* expression and establishment of pregnancy, although the fivefold higher expression associated with definite establishment of clinical pregnancy did not reach statistical significance. This study was not designed primarily to investigate such relationships as most women had two embryos transferred; thus, the number of embryos included in this analysis is limited. Furthermore, it is perhaps unlikely that *GREM1* expression would, on its own, closely reflect pregnancy potential.

A further analysis was performed to reflect more closely the clinical situation, as only embryos derived from an individual woman's oocytes are available for transfer to her. Thus, ranking by *GREM1* within a cohort may provide useful information more relevant to clinical practice. When embryos within a woman's cohort were ranked by *GREM1* expression, strong relationships between *GREM1* ranking and selection for transfer or discard were identified for both high and low ranks. However, there appeared to be little or no relationship between cryopreservation and *GREM1* ranking. This is

Table 2 Graduated embryo score assessment of cleavage stage embryos on day 2 or 3 of development.

Evaluation	Hours after insemination	Characteristic	Score
(A) Day 2			
1	40–42	Cleavage	
		Regular and symmetrical	35
		Slightly irregular	25
2	40–42	Very irregular	0
		Fragmentation	
		Absent	35
3	40–42	<20%	25
		>20%	0
		Cell number and grade ^a	
		4/1, 4/2	30
		3/1, 3/2, 2/2, 2/1, 5/2	20
		6/2, 7/2, 4/3, 3/3, 2/3, +MNB	0
Total score			100
(B) Day 3			
1	40–42	Cleavage	
		Regular and symmetrical	35
		Slightly irregular	25
2	40–42	Very irregular	0
		Fragmentation	
		Absent	35
3	66–68	<20%	30
		>20%	0
		Cell number and grade ^a	
		7/1, 7/2, 8/1, 8/2	30
		9/1, 9/2, 10/1, 10/2, 6/2 compact	20
		7/3, 8/3, 9/3, 10/3, 4/2, 2/2, 2/1, 4/3, 5/2, 4/2, 5/3, 6/3, any +MNB, any >10 cells	0
Total score			100

^aGrade 1, symmetrical blastomeres and absent fragmentation; grade 2, slightly uneven blastomeres and <20% fragmentation; grade 3, uneven blastomeres and >20% fragmentation. MNB, multinucleate blastomere.

likely to reflect the wider range of embryo quality acceptable for cryopreservation, whereas, within a woman's cohort, those embryos of the highest quality are generally chosen for transfer and those of the poorest quality for discard; thus, they are more clearly separated.

As analysis of *GREM1* expression was independent of clinical decision making, these results suggest that *GREM1* expression may reflect similar aspects of embryo development to those identified by conventional embryological assessment. This was not, however, reflected in analysis of GES score, although there were trends between *GREM1* expression and both GES score and the proportion of embryos with a high GES score. This indicates that there are aspects of embryo development, which are used by embryologists for selection that may reflect or underlie *GREM1* expression but are not readily categorised in scoring systems.

In conclusion, these data confirm the feasibility of large-scale analysis of cumulus gene expression from individual COCs that can then be followed through

to the establishment of pregnancy. Developments of this and other non-invasive techniques are emerging (Hillier 2008), and will require larger-scale validations such as that described here to assess their true value. The functional roles of the several gene products investigated here in the regulation of oocyte developmental competence merit further study. This study confirms previous reports of a positive relationship between *GREM1* and embryo development, and supports further investigation of the function of *GREM1* in human cumulus and as a marker of embryo quality.

Materials and Methods

Subjects

This study was approved by Lothian Research Ethics Committee (REC 06/S1104/50). Women undergoing superovulation for ICSI for male factor infertility were approached and informed, written consent given prior to oocyte collection. All women undergoing oocyte collection for ICSI were regarded as eligible for recruitment. A total of 75 women were recruited, average age 35.2 (25.5–44.6 years). Controlled ovarian stimulation was achieved using a standard long protocol of GnRH-agonist downregulation followed by gonadotrophin administration in most cases or an antagonist protocol ($n=4$), in which FSH was administered from cycle day 2 to 3 with addition of GnRH antagonist from day 6. All patients were administered hCG (10 000 IU Pregnyl, Organon or 0.25 mg Ovitrelle, Serono) 34–36 h before transvaginal oocyte collection under ultrasound guidance.

In the majority of patients, two embryos were selected for transfer on either day 2 or 3 post-insemination. Only one embryo was available for transfer in six patients. Embryos were cultured to the blastocyst stage in four patients; transfer in this case was carried out on day 5.

Cumulus preparation

COCs were collected into and incubated in equilibrated (37 °C, 6% CO₂ in air) fertilisation medium (Cook Medical, Limerick, Ireland) for 3 h prior to cumulus removal. COCs were exposed to SynVibro Cumulase (Medicult, Jyllinge, Denmark) for 2 min and then transferred to individual wells of a four-well dish (Nunc, Fisher Scientific, Loughborough, UK) containing 0.5 ml equilibrated fertilisation medium. Following a 5-min incubation period, cumulus cells were removed from the oocyte using a fine bore pipette. Stripped oocytes were rinsed in fresh fertilisation medium and then moved to an individual wash drop prior to injection. The cumulus cell samples were then collected into individual microcentrifuge tubes, subjected to brief centrifugation and the supernatant removed to leave a pellet of cumulus cells. About 100 µl cell lysis RLT buffer (Qiagen) containing 0.143 mol/l β-mercaptoethanol was added to each sample, which was immediately transferred to ice before storage at –80 °C until analysis.

Table 3 Primers used for quantitative RT-PCR.

Gene	GenBank Acc. No.	Forward primer	Reverse primer	Annealing Temp.	Product Size
<i>HAS2</i>	NM_005328	GCCTCATCTGTGGAGATGGT	ATGCACTGAACACACCCAAA	54°C	182bp
<i>PTGS2 (COX2)</i>	NM_000963	TTCAAATGAGATTGTGGAAAAAT	AGATCATCTCTGCCTGAGTATCTT	54°C	304bp
<i>TNFAIP6 (TSG-6)</i>	NM_007115	ATATGGCTTGAACGAGCAGC	TGGCCGCTTCAAATTCACA	54°C	107bp
<i>GREMLIN</i>	NM_013372	TGCTGGAGTCCAGCCAAGA	GCACCACTCTCGCTTCAGGTA	54°C	65bp
<i>BDNF</i>	NM_001709	AACAATAAGGACGCAGACTT	TGCAGTCTTTTGTCTGCCG	54°C	222bp
<i>Hs_PTX3_1_SG</i>	NM_002852	Quantitect Primer Assay		54°C	140bp
<i>GAPDH</i>	NM_002046	GACATCAAGAAGGTGGTGAAGC	GTCCACCACCTGTTGCTGTAG	54°C	212bp

Assessment of oocytes and embryos

Cumulus morphology score

Just prior to removal of cumulus cells, COCs were scored individually for maturity by observation with an inverted microscope at magnification $\times 200$. The scoring system was based on that originally proposed by Veeck (1988), whereby the cumulus and corona layers were ranked according to degree of expansion and spacing between individual cells (grade 0, least expansion; grade 2, most expansion).

Oocyte maturity

The maturity of individual denuded oocytes was evaluated immediately following removal of cumulus cells. Individual oocytes were categorised as prophase I (presence of a germinal vesicle (GV)); metaphase I (absence of both GV and polar body); MII (absence of GV and the presence of polar body); empty or fractured zona pellucida (no viable oocyte within zona pellucida).

Fertilisation assessment

Oocytes were assessed for fertilisation 16–18 h post-insemination using an inverted stereo microscope and categorised as follows: 2PN (presence of two pronuclei, normally fertilised); 1PN (presence of less than two pronuclei, abnormally fertilised); 3PN or >3PN (presence of more than two pronuclei, abnormally fertilised); OPN (pronucleus not visible, unfertilised); degenerated.

Subsequent embryo score

Cleavage and morphology of normally fertilised embryos were assessed at 24 h intervals following fertilisation check. Embryos were given an individualised GES to reflect their quality on day 2 or day 3 (depending on day of transfer) according to the characteristics outlined in Table 2, slightly modified from Fisch *et al.* (2001). Embryos with GES scores of 85 and above would be considered good quality.

RNA extraction and quantitative PCR

Total RNA was extracted from frozen samples of cumulus cells obtained from individual oocytes using the RNeasy micro kit (Qiagen), with an on-column DNase digestion step to remove residual genomic DNA contamination. Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer. RNA from each sample was used to generate cDNA using the

Expand Reverse Transcriptase kit (Roche Diagnostics). Total RNA was incubated with 50 pmol oligo (dT)₁₆ primer for 10 min at 65 °C and then placed on ice. A reaction mix comprising buffer, 10 mmol/l dithiothreitol, 1 mmol/l each dNTP, 40 IU RNasin RNase inhibitor (Promega) and 50 IU RT+ was added to each tube in a total volume of 20 μ l. Equivalent reactions with RT omitted provided RT controls. All tubes were incubated at 42 °C for 1 h.

Real-time RT-PCR was used to quantify the mRNA transcript levels of *HAS2*, *PTGS2*, *BDNF*, *GREM1*, *TNFAIP6* and *PTX3*, with *GAPDH* mRNA transcripts as endogenous references. Real-time PCR amplification reactions were carried out using an ABI 7900 HT Fast Real-Time PCR System apparatus (Applied Biosystems, Warrington, UK). Reverse-transcribed RNA samples were diluted in water, and 1 μ l of diluted first-strand cDNA was added to a final volume of 10 μ l containing 0.5 μ mol/l each of forward and reverse primers (Table 3) in SYBR Green master mix (Invitrogen). Initial analyses demonstrated very low expression of *PTGS2*, at the limit of quantification. Expression of *PTGS2* and *GAPDH* was therefore also quantified using the Lightcycler (Roche Diagnostics Ltd) using the QuantiTect SYBR Green PCR kit (Qiagen) as above, which allowed ready detection and quantification of *PTGS2*.

All samples were run in duplicate in 96-well plates including quality control standards for each gene. A melting curve analysis was recorded at the end of the amplification to evaluate the absence of contaminants or primer dimers. Standard curves were determined for each gene using fourfold serial dilutions of cDNA from pooled human cumulus cells and the $\Delta\Delta C_t$ method was used to calculate relative concentration of each transcript, which was expressed relative to *GAPDH*.

Statistical analysis

Gene expression data were not normally distributed, thus were analysed using non-parametric testing. Analysis was performed using Kruskal–Wallis or Mann–Whitney tests as appropriate, Spearman's rank correlation test and by construction of ROC curves (SPSS v13, SPSS Inc., Chicago, IL, USA). Proportions were compared using Fisher's exact test.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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