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Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis

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Besides the established selection criteria based on embryo morphology and blastomere number, new parameters for embryo viability are needed to improve the clinical outcome of IVF and more particular of elective single-embryo transfer. Genome-wide gene expression in cumulus cells was studied, since these cells surround the oocyte inside the follicle and therefore possibly reflect oocyte developmental potential. Early cleavage (EC) was chosen as a parameter for embryo viability. Gene expression in cumulus cells from eight oocytes resulting in an EC embryo (EC-CC; $n = 8$) and from eight oocytes resulting in a non-EC (NEC) embryo (NEC-CC; $n = 8$) was analysed using microarrays ($n = 16$). A total of 611 genes were differentially expressed ($P < 0.01$), mainly involved in cell cycle, angiogenesis, apoptosis, epidermal growth factor, fibroblast growth factor and platelet-derived growth factor signalling, general vesicle transport and chemokine and cytokine signalling. Of the 25 selected differentially expressed genes analysed by quantitative real-time PCR 15 (60%) genes could be validated in the original samples. Of these 8 (53%) could also be validated in 24 (12-EC-CC and 12 NEC-CC) extra independent samples. The most differentially expressed genes among these were *CCND2*, *CXCR4*, *GPX3*, *CTNND1 DHCR7*, *DVL3*, *HSPB1* and *TRIM28*, which probably point to hypoxic conditions or a delayed oocyte maturation in NEC-CC samples. This opens up perspectives for new molecular embryo or oocyte selection parameters which might also be useful in countries where the selection has to be made at the oocyte stage before fertilization instead of at the embryonic stage.

Keywords: assisted reproductive technology; early cleavage; gene expression; cumulus cells; microarray

Introduction

The only way to prevent a dizygotic twin pregnancy in IVF, which is regarded as one of the most serious complications, is single-embryo transfer (SET). As most patients have more than one embryo available for transfer, selecting the most viable one is of pivotal importance. Most clinics rely for embryo selection on the non-invasive examination of developmental and morphological aspects of the embryos. In every stage of oocyte and embryonic development, characteristics have been defined which appear to be prognostic indicators of successful pregnancy. Among these are zona pellucida thickness and cytoplasmic granularity of the oocyte, size of pronuclei and alignment of nuclear polar bodies in the zygote, early cleavage (EC) in the cleavage stage embryo and number and size of blastomeres, fragmentation and multinucleation in the 4–8-cell stage embryo [see Borini *et al*. (2005) for a review and Gerris (2005) for a more extensive list of references]. Especially, EC appears to be a good parameter for embryo viability as it is highly correlated with the blastocyst formation rate (Fenwick *et al*., 2002) and the implantation and pregnancy rate (Shoukir *et al*., 1997; Sakkas *et al*., 1998; Lundin *et al*., 2001), not only in double but also in SETs (Salumets *et al*., 2003; Van Montfoort *et al*., 2004). For embryos in the intermediate syngamy state, the pregnancy rate was in between that of EC and non-early cleavage (NEC) embryos (Wharf *et al*., 2004).

As the developmental potential of an embryo cannot be fully determined by characteristics visible by microscopy alone, several other markers are studied (Pearson, 2006). For instance, several investigators focused on the influence of the follicular micro-environment on subsequent embryonic development. The follicular fluid LH and growth hormone levels at the time of oocyte retrieval were higher in embryos with good morphology (Mendoza *et al*., 2002). Furthermore, the concentrations of hormones (17β-estradiol, LH, growth hormone, prolactin, leptin), growth factors (insulin-like growth factor-I), cytokines (interleukin-1) and proteinases (matrix-metalloproteinase-9) in follicular fluid differ according to the probability of pregnancy (Mendoza *et al*., 2002; Anifandis *et al*., 2005; Hammadeh *et al*., 2005; Lee *et al*., 2005). Also vascularization of the follicles has been examined as a potential marker for the developmental potential of an embryo. The peri-follicular blood flow characteristics are related to oocyte oxygenation (Van Blerkom *et al*., 1997) and can differ between the follicles in one ovary. Nargund *et al*. (1996) found, by Doppler imaging of the follicular blood flow, that oocytes from poorly vascularized follicles developed in morphologically inferior embryos as compared to those from well-vascularized

follicles. Several other studies confirmed a positive relationship between perifollicular vascularization and pregnancy (Chui *et al*., 1997; Van Blerkom *et al*., 1997; Borini *et al*., 2004). Pregnancies were only achieved with embryos from oocytes which had vascularity detected in >50% of their follicular circumference and live births only from oocytes with >75% follicular vascularity (Chui *et al*., 1997). Gaulden *et al*. (1992) suggested that hypoxic intracellular conditions might result in a diminished level of oxidative metabolism in the oocyte and a lower intracellular pH. The latter in turn could lead to meiotic spindle instabilities and chromosomal abnormalities. Indeed,Chui *et al*. (1997) and Van Blerkom *et al*. (1997) reported a significantly higher incidence of aneuploidy and spindle defects in oocytes derived from follicles with poor vascularization as compared to those from well-vascularized follicles. In addition, ATP content of the oocyte and dissolved oxygen content of the follicle fluid are related to oocyte/embryo development (Van Blerkom *et al*., 1995,1997).

As the oocyte is in dialogue with the surrounding cumulus cells via paracrine and gap-junctional signalling (Sutton *et al*., 2003), we hypothesized that differences in intra-follicular processes which are responsible for oocyte and embryonic development and subsequently implantation are reflected in the gene expression pattern of cumulus cells. The bi-directional communication between the oocyte and the cumulus cells is necessary for oocyte development as oocytes fail to grow in the absence of (a connection with) cumulus cells (Ackert *et al*., 2001; Matzuk *et al*., 2002). Zhang *et al*. (2005) reported that the expression of several genes in cumulus cells, particularly pentraxin 3 (*PTX3*), was indicative of oocyte and embryo quality. In addition, the expression of cyclooxygenase 2 (*COX2*), gremlin (*GREM*) and hyaluronic acid synthase 2 (*HAS2*) is also positively correlated with embryo quality (McKenzie *et al*., 2004). In turn, oocyte factors like growth and differentiation factor-9 (GDF-9) are necessary for cumulus expansion (Sutton *et al*., 2003).

The aim of this study was to analyse the genome-wide expression of genes in cumulus cells as indicators of embryo viability. By analysing gene expression in cumulus cells, the understanding of the regulation of oogenesis and embryonic development might be improved. This information might lead to new molecular non-invasive embryo selection parameters reflected in cumulus cells that can be used in addition to the existing morphological parameters or might result in an oocyte selection tool for those who are obliged to select a limited number of oocytes for fertilization (Ludwig *et al*., 2000).

Materials and Methods

Patients and human cumulus cell collection

Patients visiting the IVF clinic of the academic hospital Maastricht underwent an IVF or ICSI treatment as described previously (Van Montfoort *et al*., 2006). For the study, which was approved by the local Ethics Committee, in consenting patients, immediately following ultrasound-guided cumulus–oocyte complex (COC) retrieval, a proportion of the cumulus cells surrounding a single oocyte were removed using a sharp needle, lysed in 100 μl Trizol reagent (Invitrogen, Carlsbad, USA) supplemented with 1% (v/v) 2-mercapto-ethanol (Merck, Darmstadt, Germany), snap-frozen in liquid nitrogen and stored at −80°C (cumulus cells from one oocyte per vial). The oocytes were cultured and fertilized individually in 5 μl droplets covered by mineral oil. Between 23–26 h post-injection or 25–28 h post-insemination EC status of embryos was assessed. A 2 h time difference is necessary to compensate for the time difference in early development between IVF- and ICSI-derived embryos (Van Montfoort *et al*., 2004). Subsequently, on Day 2 of development, the embryos were examined for morphology, number of blastomeres and the presence or absence of multinucleated blastomeres (MNBs) (Van Montfoort *et al*., 2005).

Experimental design

EC was chosen as a marker for embryo viability. Gene expression in cumulus cells from eight oocytes resulting in an EC embryo (EC-CC; $n = 8$) and from eight oocytes resulting in a non-EC embryo (NEC-CC; $n = 8$) derived from six patients were analysed using microarrays ($n = 16$). To exclude a differential gene expression due to differences in patient characteristics, samples were paired. From four patients both an EC-CC and a NEC-CC sample were used. From two additional patients two EC-CC as well as two NEC-CC samples were used. The microarray results were validated by quantitative RT–PCR (qRT–PCR) on the original samples analysed by microarray as well as on 24 new samples.

The cumulus cell samples (for microarray and RT–PCR) from EC and NEC embryos were derived from normally fertilized (2PN) oocytes, which developed into embryos with comparable characteristics on Day 2, i.e. 4-cell with good morphology and no MNBs present.

RNA isolation

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions with some adaptations for the small quantity of RNA. RNA was precipitated with isopropyl alcohol for 2 h and the RNA pellet was washed three times with 75% ethanol. To be able to track the small RNA pellet, 5 μg glycogen (Ambion, Woodward, USA) was added to the sample before RNA precipitation. Total RNA was resuspended in 20 μl RNase-free water and stored at −80°C. For all RNA samples quantity and purity were determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) and RNA integrity was determined using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, USA).

Two cycle amplification and microarray hybridization

Fifty nanogram total RNA was amplified using the two-cycle cDNA synthesis kit (Affymetrix, Santa Clara, USA) in combination with the MEGAscript T7 *in vitro* transcription system (Ambion). Biotin labelled target complementary RNA was fractionated and hybridized to Human Genome U133A Plus 2.0 Arrays (Affymetrix). Each array contained >54 000 oligonucleotide probe-sets corresponding to 38 500 characterized human genes.

Microarray analysis

To identify probe sets which were differentially expressed between eight EC-CC and eight NEC-CC samples, a three step process was applied. First, Affymetrix GeneChip Operating Software (GCOS, version 1.4) was used to analyse image data. For each transcript represented on the array by a probe set, the expression algorithm computed the detection call (present, absent or marginal), the detection *P*-value, and the signal which is an average intensity value for each probe set. This resulted in a table with 54 675 probe sets. Second, for each probe set the 16 detection calls were used to determine whether the probe set was reliably detected or not and should or should not be selected for further analysis (McClintick and Edenberg, 2006). To this end, for every group of eight arrays (the early and the non-early) the number of present calls was counted (a number ranging from 0 to 8). If six or more calls were present, the probe set was denoted present. If the probe set was in at least one of the two groups denoted present, it was selected for further analysis. Finally, the over- or underexpression of the remaining probe sets in one of the two groups was analysed using the class comparison method in BRB ArrayTools software package applying a univariate test composed of a paired *t*-test with random variance model. This was developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html). Hierarchical clustering of samples was also performed using BRB ArrayTools. Samples were clustered by comparing their expression profiles.

The genes showing significant differential expression between both groups ($P < 0.01$) were classified into functional groups using the Panther classification system (http://www.pantherdb.org) (Thomas *et al*., 2003). The gene expression data analysis tool (Thomas *et al*., 2006) was used to determine which biological process or pathway was significantly overexpressed in one of the two groups. This program uses binomial statistics with Bonferroni correction to analyse whether the proportion of genes from a certain biological process or pathway present in a gene list (i.e. the list of differentially expressed

genes from an array study) is significantly different from the proportion of genes in that process or pathway in the whole human genome ($P < 0.05$).

Quantitative real-time PCR

For the qRT–PCR, TaqMan low density arrays (TLDAs) (Applied Biosystems, Foster City, USA) were used. Each 2 μl well of the TLDA contains user-defined primers and probes selected from an online catalogue (http://myscience.appliedbiosystems.com) for a single gene. One well contains primers and probes for 18S rRNA, a mandatory endogenous control from the manufacturer.

cDNA was prepared from 100 ηg total RNA per sample using the High Capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. To each cDNA sample (20 μl), 80 μl nuclease-free water and 100 μl 2× TaqMan Universal PCR Master Mix (Applied Biosystems) was added. This mixture was then equally divided over two sample-loading ports of the TLDA, each connected to one set of all the genes of interest. The arrays were centrifuged twice (1′, 331 *g*) to equally distribute the sample over the wells. Subsequently, the card was sealed to prevent an exchange between wells. qRT–PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system with the following thermal cycler conditions: 2 min at 50°C and 10 min at 94.5°C, followed by 40 cycles of 30 s at 97°C and 1 min at 59.7°C.

qRT–PCR analysis

The RQ manager 1.2 software was used to generate Ct values corrected for variances in fluorescent signal strength by using a passive reference dye. The geNorm program (Vandesompele *et al*., 2002) was used to determine the most stably expressed housekeeping genes. Briefly, the average pair-wise variation of a housekeeping gene with all other housekeeping genes was calculated. Stepwise exclusion of the gene with the highest variation resulted in a combination of two housekeeping genes that have the most stable expression. The geometrical mean of the Ct values of these two genes was used as a normalization factor which was substracted from the Ct values of the genes of interest to obtain normalized Ct values (ΔCt). Subsequently, the mean ΔCt of the NEC-CC samples was substracted from the mean ΔCt of the EC-CC samples generating a ΔΔCt. This ΔΔCt was recalculated into a relative expression quantity ($2^{-\Delta\Delta Ct}$) of the gene of interest in EC-CC as compared with NEC-CC samples (Livak and Schmittgen, 2001).

Results

Microarray analysis

For the gene expression analysis, 8 EC-CC and 8 NEC-CC samples (from 6 patients) have been analysed using 16 microarrays. The raw microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE9526. From the 54 675 probe sets on the array, 18 480 had a present call. Most of these probe sets showed similar expression between the EC-CC and NEC-CC group, except for 737 probe sets that were differentially expressed ($P < 0.01$). For 59 of these probe sets, the corresponding gene is not yet known. Of the 678 remaining probe sets, which correspond to 611 different genes, 162 (24%) were up-regulated and 516 (76%) were down-regulated in EC-CC compared with NEC-CC. Clustering analysis of the arrays based on the 500 most significantly differentially expressed genes perfectly clustered the EC-CC samples and the NEC-CC samples (Fig. 1). The two EC-CC samples derived from the same patient (sample 1-EC a and b and sample 6-EC a and b) and the NEC-CC samples from the same patient (sample 1-NEC a and b and sample 6-NEC a and b) were highly correlated.

Of the 611 genes that are differentially expressed between EC and NEC ($P < 0.01$), 426 could be categorized into one or more of the biological processes listed in Table I. Of these processes, protein modification, nucleic acid, lipid, fatty acid and steroid metabolism, apoptosis, general vesicle transport, cell cycle, cell structure and

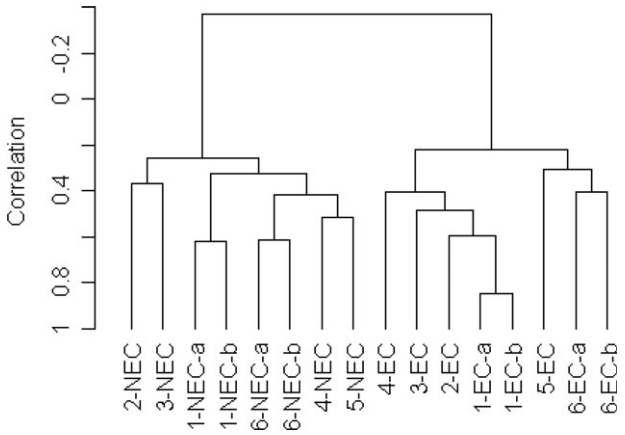

Figure 1: Cluster dendrogram of an hierarchical clustering of eight samples from cumulus cells from oocytes resulting in an early cleavage embryo (EC-CC) and eight samples from cumulus cells from oocytes resulting in a non-early cleavage embryo (NEC-CC). The analysis is run on a selected subset of genes, i.e. the 500 most significantly differentially expressed genes in EC-CC ($n = 8$) as compared to NEC-CC ($n = 8$) according to the Affymetrix GCOS and BRB ArrayTools software. From patients 1 and 6, two EC-CC and two NEC-CC samples were used, denoted as a and b.

motility, chromatin packaging and remodelling, transport and other signalling are significantly overrepresented ($P < 0.05$, as compared to the whole human genome) according to the Panther gene expression tool. In Table II, the significantly differentially expressed genes are shown, categorized into their most prominent role. To economize space, only the genes ($n = 95$) with $P < 0.001$ are shown. Information on all of the 426 differentially expressed genes (with $P < 0.01$) is provided in the Supplementary Data, Table S1.

Furthermore, the significantly ($P < 0.05$) overrepresented pathways in which the genes differentially expressed between EC and NEC are involved in are Ras, chemokine and cytokine signalling, epidermal, fibroblast and platelet-derived growth factor (EGF, FGF and PDGF) signalling and angiogenesis. In Table III, the genes classified to these pathways are shown.

qRT–PCR

From six out of eight NEC-CC samples and from all EC-CC samples sufficient RNA remained for a validation of the microarray results by qRT–PCR. Furthermore, an additional 12 EC-CC and 12 NEC-CC samples were analysed to validate the results in independent samples. qRT–PCR was performed on genes selected either because of their highly significant differential expression in both study groups or because of their involvement in a biological relevant pathway or cellular process ($n = 25$). Furthermore, the most stably expressed housekeeping genes from the microarray ($n = 4$, *EIF4G2*, *PARK7*, *SRP14* and *RHOA*) and *18S* (a mandatory control by the manufacturer) were included in the qRT–PCR analysis. *SRP14* and *RHOA* were the two most stably expressed housekeeping genes, i.e. the genes with the lowest variation in expression levels. The expression values of the other 25 genes were normalized against the geometrical mean of these two genes.

In the original samples, which were also analysed by microarray, for 22 out of the 25 selected genes (88%) the differential expression between EC-CC and NEC-CC could be confirmed by qRT–PCR (Fig. 2). When fold changes between 0.9 and 1.1 were excluded 15 of the 25 (60%) genes could be confirmed. Of these, the differential expression of 10 genes (67%) could be confirmed in the independent samples of which 8 remained after exclusion of the fold changes between 0.9 and 1.1 (Fig. 3). These genes were cyclin D2

Table I. Number of significantly differentially expressed transcripts ($P < 0.01$) up or down-regulated in EC-CC as compared to NEC-CC, categorized per biological process (% per category).

	Up-regulated (%)		Down-regulated (%)	
Antioxidation and free radical removal	0	(0)	3	(100)
Apoptosis*	6	(19)	26	(81)
Extracellular matrix, cell communication and cell adhesion	16	(29)	39	(71)
Cell cycle*	16	(33)	33	(67)
DNA metabolism, repair and replication	4	36)	7	(64)
Cell motility and structure*	8	(16)	41	(84)
Chromatin packaging and remodelling*	2	(15)	11	(85)
Defense	12	(26)	34	(74)
Growth factor	5	(83)	1	(17)
Amino acid metabolism and transport	1	(7)	14	(93)
Carbohydrate metabolism	5	(19)	21	(81)
Lipid, fatty acid and steroid transport and metabolism*	7	(21)	26	(79)
Phospholipid metabolism	1	(14)	6	(86)
Oxidative phosphorylation	0	(0)	3	(100)
Protein biosynthese	5	(25)	15	(75)
Protein modification*	17	(26)	49	(74)
Proteolysis	5	(15)	28	(85)
Calcium mediated signalling	3	(30)	7	(70)
Cytokine and chemokine mediated signalling	5	(50)	5	(50)
G-protein mediated signalling	3	(16)	16	(84)
Other signalling*	22	(27)	60	(73)
Stress response	03	(23)	10	(77)
Transcription factor	12	(21)	44	(79)
mRNA transcription and posttranslational modification	14	(19)	58	(81)
Nucleoside, nucleotide and nucleic acid metabolism*	23	(21)	89	(79)
Purine metabolism	3	(30)	7	(70)
RNA processing	0	(0)	3	(100)
Cation transport	6	(33)	12	(67)
Mitochondrial transport	1	(17)	5	(83)
General vesicle transport*	5	(23)	17	(77)
Transporter*	15	(21)	56	(79)
Other or unknown function	53	(27)	146	(73)

*Biological processes significantly overrepresented as compared to the whole human genome.

(*CCND2*), catenin delta-1 (*CTNND1*), CXC chemokine receptor 4 (*CXCR4*), 7-dehydrocholesterol reductase (*DHCR7*), dishevelled dsh homolog 3 (*DVL3*), glutathione peroxidase 3 (*GPX3*), heatshock 27 kDa protein 1 (*HSPB1*) and tripartite motif-containing 28 (*TRIM28*).

Discussion

To improve the clinical outcome of elective single-embryo transfer (eSET), the embryo selection needs to be optimized. Besides the established selection criteria based on embryo morphology and blastomere number, new selection parameters should be developed. Information about the oocyte and its development might be a valuable contribution to the existing selection criteria. As cumulus cells surround the oocyte inside the follicle, a microarray analysis was performed on these cells. Both cumulus cells from oocytes developing into an EC-CC as well as from oocytes developing into a NEC-CC embryo were compared. Our analysis revealed that 18 480 genes were expressed in cumulus cells, 611 of which showed significant differential expression between EC-CC and NEC-CC. A cluster analysis could perfectly separate the EC-CC and NEC-CC samples, indicating that differences in embryonic implantation potential can already be detected as early as folliculogenesis. These differences were not manifested in blastomere number and morphology of the embryo as these were similar in both groups.

The differences in gene expression could not be due to differences in age, ovarian stimulation or other patient characteristics as from each patient one EC-CC and one NEC-CC ($n = 4$ patients) sample or two EC-CC and two NEC-CC samples ($n = 2$ patients) were used. By pairing the samples from each patient, the differential gene expression due to different patient characteristics could be ruled out. Furthermore, while other studies analyzing gene expression in human cumulus cells pooled the cumulus cells from several oocytes (Zhang *et al*., 2005; Assou *et al*., 2006), in this study each sample consisted of the cumulus cells from one oocyte. This prevented loss of information.

Of the 611 differentialy expressed genes 24% was overexpressed in EC-CC, whereas 76% was overexpressed in NEC-CC. The most abundant functions or pathways these genes were involved in were EGF, FGF and PDGF signalling as well as chemokine and cytokine signalling, lipid, fatty acid and steroid metabolism, cell cycle, apoptosis and angiogenesis. Twenty-five genes were selected for validation by quantitative real-time PCR. The gene expression profile found by microarray analysis could be validated for 15 of the 25 (60%) selected genes. In literature, a 84–88% concordance between microarray and quantitative real-time PCR has been described (Rajeevan *et al*., 2001a; Dallas *et al*., 2005). Microarray results can be influenced by labelling

Table II. Genes differentially expressed ($P < 0.001$) in EC-CC versus NEC-CC categorized into biological process.

Gene ID	Gene description	Probe set	Fold[a]
Antioxidation and free radical removal			
GPX3	Glutathione peroxidase 3 (plasma)	201348_at	0.5
PRDX2	Peroxiredoxin 2	39 729_at	0.7
Apoptosis			
CLU	Clusterin (complement lysis inhibitor, SP-40,40, sulphated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	208791_at 208792_s_at	0.6 0.6
Extracellular matrix, cell communication and cell adhesion			
CSPG2	Chondroitin sulfate proteoglycan 2 (versican)	211571_s_at	0.6
CTNND1	Catenin (cadherin-associated protein), delta 1	211240_x_at	0.7
GPC4	Glypican 4	204983_s_at	0.6
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1553678_a_at	0.7
Cell cycle			
76P	Gamma tubulin ring complex protein (76p gene)	213266_at	0.8
APRIN	Androgen-induced proliferation inhibitor	229704_at	1.3
CCND2	Cyclin D2	200953_s_at	0.6
PRC1	Protein regulator of cytokinesis 1	218009_s_at	0.6
DNA metabolism, repair and replication			
DKFZP564I0422	THAP domain containing, apoptosis associated protein 2	212202_s_at	0.7
Cell motility and structure			
CFL1	Cofilin 1 (non-muscle)	1555730_a_at	0.6
MSN	Moesin	200600_at	0.7
PFN1	Profilin 1	200634_at	0.6
WASL	Wiskott–Aldrich syndrome-like	205809_s_at	1.6
Chromatin packaging and remodelling			
ARID1A	AT rich interactive domain 1A (SWI- like)	210649_s_at	0.7
Defense			
IFITM1	Interferon induced transmembrane protein 1 (9–27)	214022_s_at	0.7
ILF2	Interleukin enhancer binding factor 2, 45 kDa	200052_s_at	0.7
Amino acid metabolism and transport			
AKAP13	A kinase (PRKA) anchor protein 13	237018_at	0.8
GATM	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	216733_s_at	0.6
Carbohydrate metabolism			
C17orf25	Chromosome 17 open reading frame 25	209092_s_at	0.8
PGD	Phosphogluconate dehydrogenase	201118_at	0.7
UGP2	UDP-glucose pyrophosphorylase 2	231698_at	1.6
Lipid, fatty acid and steroid transport and metabolism			
ACAD8	Acyl-coenzyme A dehydrogenase family, member 8	221669_s_at	0.7
DHCR7	7-dehydrocholesterol reductase	201791_s_at	0.8
ELOVL5	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	1567219_at	1.5
PLD3	Phospholipase D family, member 3	201050_at	0.7
Protein biosynthese			
METAP2	Methionyl aminopeptidase 2	209861_s_at	0.7
RPL14	Ribosomal protein L14	219138_at	1.5
RPS3	Ribosomal protein S3	208692_at	0.8
Protein modification			
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	208810_at	0.7
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-*N*-acetylglucosamine:polypeptide-*N*-acetylglucosaminyl transferase)	229787_s_at	1.4
RYK	RYK receptor-like tyrosine kinase	216976_s_at	0.7
SUMO2	SMT3 suppressor of mif two 3 homolog 2 (yeast)	208739_x_at	0.7
Proteolysis			
CST3	Cystatin C (amyloid angiopathy and cerebral haemorrhage)	201360_at	0.6
HTRA1	HtrA serine peptidase 1	201185_at	0.7
USP11	Ubiquitin specific peptidase 11	208723_at	0.7
XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	208453_s_at	0.7
Cytokine and chemokine mediated signalling			
CXCR4	Chemokine (C-X-C motif) receptor 4	209201_x_at 211919_s_at	0.5 0.5

Continued

Table II. *Continued*

Gene ID	Gene description	Probe set	Fold[a]
G-protein mediated signalling			
APLP2	Amyloid beta (A4) precursor-like protein 2	208703_s_at	0.6
		208248_x_at	0.7
HRB	HIV-1 Rev-binding protein	213926_s_at	1.7
Other signalling			
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	229010_at	1.6
LMBR1	Limb region 1 homolog (mouse)	224410_s_at	0.7
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	211536_x_at	0.8
RAB5B	RAB5B, member RAS oncogene family	201276_at	0.7
Stress response			
HSPB1	Heat shock 27 kDa protein 1	201841_s_at	0.6
Transcription factor			
NFIB	Nuclear factor I/B	209289_at	1.4
PQBP1	Polyglutamine-binding protein 1	214527_s_at	0.7
TRIM28	Tripartite motif-containing 28	200990_at	0.6
TSC22D3	TSC22 domain family, member 3	208763_s_at	0.8
WWTR1	WW domain containing transcription regulator 1	202132_at	1.5
mRNA transcription and posttranslational modification			
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	212107_s_at	1.5
SNRP70	Small nuclear ribonucleoprotein 70 kDa polypeptide (RNP antigen)	201221_s_at	0.7
SNRPA	Small nuclear ribonucleoprotein polypeptide A	201770_at	0.8
Nucleoside, nucleotide and nucleic acid metabolism			
AK1	Adenylate kinase 1	202587_s_at	0.7
BAT1	HLA-B associated transcript 1	200041_s_at	0.7
CSNK2A1	Casein kinase 2, alpha 1 polypeptide	229212_at	1.3
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	212515_s_at	0.7
RANBP9	RAN-binding protein 9	242143_at	0.7
Purine metabolism			
DKC1	Dyskeratosis congenita 1, dyskerin	201478_s_at	0.7
		201479_at	0.7
GUK1	Guanylate kinase 1	213621_s_at	1.7
LARP1	La ribonucleoprotein domain family, member 1	212193_s_at	0.6
PRPS2	Phosphoribosyl pyrophosphate synthetase 2	230352_at	1.5
Cation transport			
SLC39A9	Solute carrier family 39 (zinc transporter), member 9	217859_s_at	1.3
General vesicle transport			
ARF6	ADP-ribosylation factor 6	214182_at	1.6
DCTN1	Dynactin 1 (p150, glued homolog, *Drosophila*)	201082_s_at	0.7
RAB6IP2	RAB6 interacting protein 2	1563947_a_at	1.5
TRAPPC1	Trafficking protein particle complex 1	225294_s_at	0.8
Transporter			
FOLR1	FOLAte receptor 1 (adult);FOLR1	211074_at	2.2
Unknown function			
AG1	AG1 protein	201104_x_at	0.7
C18orf10	CHromosome 18 open reading frame 10	212055_at	0.7
C20orf45	Chromosome 20 open reading frame 45	229835_s_at	1.4
C21orf34	Chromosome 21 open reading frame 34	239999_at	0.5
C3orf10	Chromosome 3 open reading frame 10	224023_s_at	1.6
CKLFSF3	Chemokine-like factor superfamily 3	1555705_a_at	0.7
CKS1B	CDC28 protein kinase regulatory subunit 1B	201897_s_at	0.8
CRTAP	Cartilage associated protein	1554464_a_at	0.7
DJ328E19.C1.1	Hypothetical protein DJ328E19.C1.1	212854_x_at	0.8
EHBP1	EH-domain-binding protein 1	212650_at	1.4
ILF3	Interleukin enhancer binding factor 3, 90kDa	208931_s_at	0.7
KIAA0256	KIAA0256 gene product	212451_at	1.4
KIAA0652	KIAA0652	203364_s_at	0.7
LY6E	Lymphocyte antigen 6 complex, locus E	202145_at	0.7
MEIS4	Meis1, myeloid ecotropic viral integration site 1 homolog 4 (mouse)	214077_x_at	1.3
MGEA5	MEningioma expressed antigen 5 (hyaluronidase)	223494_at	0.6
NAG8	Nasopharyngeal carcinoma associated gene protein-8	210109_at	0.7
PMF1	Polyamine-modulated factor 1	202337_at	0.7
RIG	Regulated in glioma	221127_s_at	1.5
RTN3	Reticulon 3	219549_s_at	0.8
SEL1L	Sel-1 suppressor of lin-12-like (C. elegans)	230265_at	1.5
SPTAN1	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	208611_s_at	0.7
THOC3	THO complex 3	224623_at	0.7
UST	Uronyl-2-sulfotransferase	205139_s_at	0.7
WBP11	WW-domain-binding protein 11	217821_s_at	0.8

[a] EC-CC : NEC-CC expression ratio.

Table III. Genes differentially expressed ($P < 0.01$) in EC-CC versus NEC-CC per significantly overrepresented ($P < 0.05$) pathway.

Gene ID	Gene description	Probe set	Fold[a]
Ras pathway			
AKT1	v-akt murine thymoma viral oncogene homolog 1	207163_s_at	0.8
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	201895_at	0.8
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	201466_s_at	1.3
MAP2K2	Mitogen-activated protein kinase kinase 2	202424_at	0.8
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	211536_x_at	0.8
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	211537_x_at	0.8
RAF1	v-raf-1 murine leukaemia viral oncogene homolog 1	201244_s_at	0.8
RHOA	ras homolog gene family. member A	1555814_a_at	0.8
SEC5L1	SEC5-like 1 (S. cerevisiae)	226270_at	0.7
SOS2	Son of sevenless homolog 2 (*Drosophila*)	233369_at	0.7
STAT1	Signal transducer and activator of transcription 1	200887_s_at	0.8
STK4	Serine/threonine kinase 4	211085_s_at	1.3
Inflammation mediated by chemokine and cytokine signalling			
AKT1	v-akt murine thymoma viral oncogene homolog 1	207163_s_at	0.8
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	201895_at	0.8
C15orf24	Chromosome 15 open reading frame 24	217898_at	0.8
CAMK1	Calcium/calmodulin-dependent protein kinase I;CAMK1	204392_at	0.7
CXCR4	Chemokine (C-X-C motif) receptor 4	209201_x_at	0.5
CXCR4	Chemokine (C-X-C motif) receptor 4	211919_s_at	0.5
CXCR4	Chemokine (C-X-C motif) receptor 4	217028_at	0.7
GNAQ	Guanine nucleotide-binding protein (G protein). q polypeptide	224862_at	0.7
IFNAR2	Interferon (alpha, beta and omega) receptor 2	204786_s_at	1.3
ITGB1	Integrin, beta 1	1553678_a_at	0.7
ITPR1	Inositol 1.4.5-triphosphate receptor. type 1	231329_at	0.7
ITPR2	Inositol 1.4.5-triphosphate receptor. type 2	240458_at	0.7
JAK1	Janus kinase 1	1552610_a_at	0.7
JAK1	Janus kinase 1	201648_at	0.8
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	201466_s_at	1.3
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	211537_x_at	0.8
MPP1	Membrane protein. palmitoylated 1. 55kDa	202974_at	0.7
NFATC3	Nuclear factor of activated T-cells. cytoplasmic. calcineurin-dependent 3	210555_s_at	0.7
RAF1	v-raf-1 murine leukaemia viral oncogene homolog 1	201244_s_at	0.8
RHOA	ras homolog gene family. member A	1555814_a_at	0.8
SOS2	Son of sevenless homolog 2 (*Drosophila*)	233369_at	0.7
STAT1	Signal transducer and activator of transcription 1	200887_s_at	0.8
STK4	Serine/hreonine kinase 4	211085_s_at	1.3
EGF receptor signalling pathway			
AKT1	v-akt murine thymoma viral oncogene homolog 1	207163_s_at	0.8
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	201895_at	0.8
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	229010_at	1.6
MAP2K2	Mitogen-activated protein kinase kinase 2	202424_at	0.8
MRPL38	Mitochondrial ribosomal protein L38	225103_at	0.7
PKD2	Polycystic kidney disease 2	203688_at	0.8
PPP2CA	Protein phosphatase 2 (formerly 2A). catalytic subunit. alpha isoform	208652_at	0.7
PPP6C	Protein phosphatase 6. catalytic subunit	206174_s_at	0.8
RAF1	v-raf-1 murine leukaemia viral oncogene homolog 1	201244_s_at	0.8
SOS2	Son of sevenless homolog 2 (*Drosophila*)	233369_at	0.7
STAT1	Signal transducer and activator of transcription 1. 91kDa	200887_s_at	0.8
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. eta polypeptide	201020_at	0.7
FGF signalling pathway			
AKT1	v-akt murine thymoma viral oncogene homolog 1	207163_s_at	0.8
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	201895_at	0.8
FGFR2	Fibroblast growth factor receptor 2	208229_at	1.3
MAP2K2	Mitogen-activated protein kinase kinase 2	202424_at	0.8
MRPL38	Mitochondrial ribosomal protein L38	225103_at	0.7
PPP2CA	Protein phosphatase 2 (formerly 2A). catalytic subunit. alpha isoform	208652_at	0.7
PPP2R1A	Protein phosphatase 2 (formerly 2A). regulatory subunit A (PR 65). alpha isoform	200695_at	0.7
PPP6C	Protein phosphatase 6. catalytic subunit	206174_s_at	0.8
RAF1	v-raf-1 murine leukaemia viral oncogene homolog 1	201244_s_at	0.8
SOS2	Son of sevenless homolog 2 (*Drosophila*)	233369_at	0.7
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein. eta polypeptide	201020_at	0.7
PDGF signalling pathway			
AKT1	v-akt murine thymoma viral oncogene homolog 1	207163_s_at	0.8
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	201895_at	0.8
ARHGAP10	Rho GTPase activating protein 10	239567_at	0.7

Continued

Table III. *Continued*

Gene ID	Gene description	Probe set	Fold[a]
ITPR1	Inositol 1.4.5-triphosphate receptor. type 1	231329_at	0.7
ITPR2	Inositol 1.4.5-triphosphate receptor. type 2	240458_at	0.7
JAK1	Janus kinase 1	1552610_a_at	0.7
JAK1	Janus kinase 1	201648_at	0.8
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	201466_s_at	1.3
MAP2K2	Mitogen-activated protein kinase kinase 2	202424_at	0.8
PDGFA	Platelet-derived growth factor alpha polypeptide;	205463_s_at	1.7
PDGFA	Platelet-derived growth factor alpha polypeptide;	229830_at	1.4
PDGFC	Platelet-derived growth factor C	222719_s_at	0.7
RAF1	v-raf-1 murine leukaemia viral oncogene homolog 1	201244_s_at	0.8
RHOA	ras homolog gene family. member A	1555814_a_at	0.8
SOS2	Son of sevenless homolog 2 (i)	233369_at	0.7
STAT1	Signal transducer and activator of transcription 1	200887_s_at	0.8
Angiogenesis			
AKT1	v-akt murine thymoma viral oncogene homolog 1	207163_s_at	0.8
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog	201895_at	0.8
DVL3	Dishevelled. dsh homolog 3 (*Drosophila*)	201908_at	0.8
FGFR2	Fibroblast growth factor receptor 2	208229_at	1.3
JAK1	Janus kinase 1	1552610_a_at	0.7
JAK1	Janus kinase 1	201648_at	0.8
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	201466_s_at	1.3
PDGFA	Platelet-derived growth factor alpha polypeptide;	205463_s_at	1.7
PDGFA	Platelet-derived growth factor alpha polypeptide;	229830_at	1.4
PDGFC	Platelet-derived growth factor C	222719_s_at	0.7
PKD2	Polycystic kidney disease 2	203688_at	0.8
RAF1	v-raf-1 murine leukaemia viral oncogene homolog 1	201244_s_at	0.8
RHOA	ras homolog gene family. member A	1555814_a_at	0.8
SOS2	Son of sevenless homolog 2 (*Drosophila*)	233369_at	0.7
STAT1	Signal transducer and activator of transcription 1	200887_s_at	0.8
STK4	Serine/threonine kinase 4	211085_s_at	1.3
WNT5A	Wingless-type MMTV integration site family. member 5A	213425_at	0.6

[a] EC-CC : NEC-CC expression ratio.

and hybridization efficiency, whereas quantitative PCR is dependent on the efficiency of the enzymes and primers. Especially, small fold changes between study groups are sensitive to these variations, which explains the lower concordance in our study (Rajeevan *et al*., 2001b). The differential expression of 8 of the 15 genes (53%) could be verified in extra independent samples. This indicates that gene expression validation in independent samples is very important to control for genes not consistently over- or underexpressed in the tested conditions and that with microarray analysis alone some genes can show differential expression between the examined conditions by chance. The validated genes are *CCND2, CTNND1, CXCR4, DHCR7, DVL3, GPX3, HSPB1* and *TRIM28*.

CCND2 is an important cell cycle regulator and plays an essential role in cumulus cell proliferation as a null mutation in *Ccnd2* in mice impairs cumulus cell proliferation and leads to small follicles unable to ovulate (Sicinski *et al*., 1996). Cumulus cell proliferation

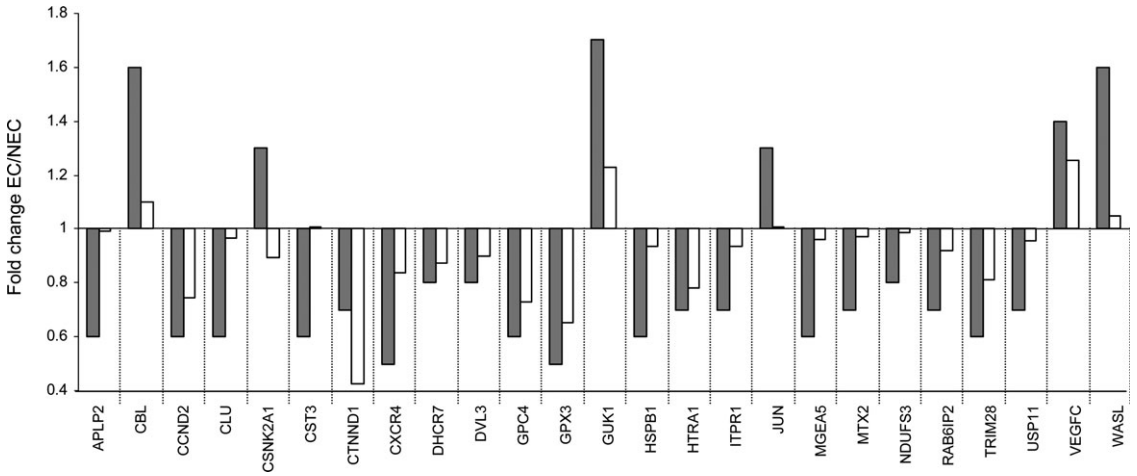

Figure 2: Relative gene expression of 25 genes expressed in cumulus cells from oocytes resulting in an early cleavage embryo (EC-CC) as compared to cumulus cells from oocytes resulting in an non-early cleavage embryo (NEC-CC). The relative gene expression in EC-CC and NEC-CC samples derived from 6 patients was measured using two different platforms. The gray bars show the relative gene expression as measured with Affymetrix microarrays and the BRB ArrayTools software (EC-CC: $n = 8$; NEC-CC: $n = 8$). The white bars show the relative gene expression measured with qRT–PCR using TaqMan low density arrays and analysed with the delta Ct method (EC-CC: $n = 8$; NEC-CC: $n = 6$). For microarray analysis and qRT–PCR the same samples were used.

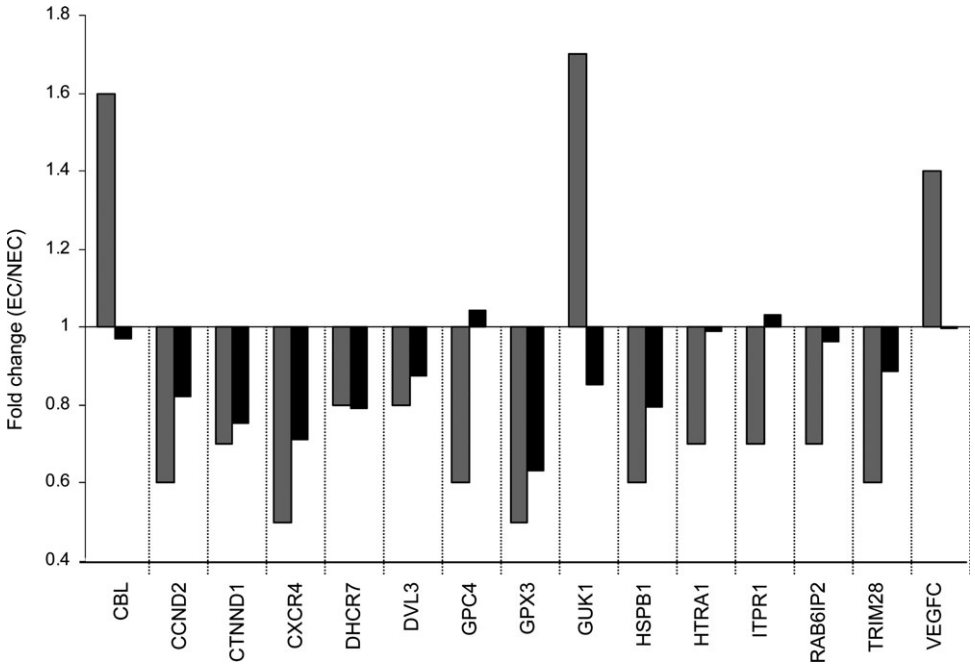

Figure 3: Relative gene expression of 15 genes expressed in cumulus cells from oocytes resulting in an early cleavage embryo (EC-CC) as compared to cumulus cells from oocytes resulting in an non-early cleavage embryo (NEC-CC). The relative gene expression was measured using two different platforms. The gray bars show the relative gene expression as measured with Affymetrix microarrays and the BRB ArrayTools software (EC-CC: $n = 8$; NEC-CC: $n = 8$). The black bars show the relative gene expression measured with qRT–PCR using TaqMan low density arrays and analysed with the delta Ct method (EC-CC: $n = 12$; NEC-CC: $n = 12$). For microarray analysis and qRT–PCR different independent samples were used.

is regulated by factors secreted by fully grown oocytes (Eppig, 2001). At least in bovine oocytes, this cumulus cell promoting activity decreases upon meiotic maturation (Lanuza *et al*., 1998). The increased proliferation in the NEC-CC group as compared to the EC-CC group therefore suggests that the enclosed oocyte in the first group was less mature at the time of oocyte retrieval. This delayed maturation might also explain the increased *CTNND1* expression in NEC-CC. δ-Catenin forms, together with E-cadherin, a major cell–cell adhesion protein complex (Keirsebilck *et al*., 1998). This might result in an enhanced cumulus cell adhesion as compared to EC-CC, and thus a delayed cumulus expansion, which was not visible by microscopy at the time of cumulus cell collection. The cumulus cell membrane receptor CD44 (Schoenfelder and Einspanier, 2003), which attaches the extracellular matrix to the cumulus cells (Yokoo *et al*., 2002) was significantly underexpressed in NEC-CC, also confirming a disturbed cumulus cell expansion. Cumulus expansion is necessary for a proper oocyte and embryo development (Zhang *et al*., 1995) and is induced by the oocyte factors BMP-15 and GDF-9 (Sutton *et al*., 2003). In our study, no indication was found for underexpression of *BMP-15* or *GDF-9*, as the expression of the target genes *PTGS2, HAS2, GREM1* and *PTX3* (McKenzie *et al*., 2004; Zhang *et al*., 2005) was not different between the two groups. There might be an oocyte factor other than BMP-15 or GDF-9 that induces cumulus expansion.

An increase in cumulus cell apoptosis has also been associated with immaturity of oocytes and with an impaired fertilization (Host *et al*., 2002) and pregnancy rate (Lee *et al*., 2001). In our study, of the 10 differentially expressed genes ($P < 0.01$) whose most prominent function is a role in apoptosis, 8 point to an increase in apoptosis in the NEC-CC samples (6 pro-apoptotic genes were overexpressed and 2 anti-apoptotic genes were underexpressed as compared to EC-CC). It is not exactly known how these apoptotic signals exert their negative effect on oocyte and embryo development. The apoptotic signals can easily be transferred from the cumulus cells to the oocyte through the gap junctions. After the LH peak, at the time the oocyte reaches metaphase I, these junctions close (Sutton *et al*., 2003). The timing of gap junction closure might be important for oocyte and embryo development. A delayed response to LH also emerges from the increased expression of both CCND2 (Muniz *et al*., 2006) and CTNND1 (Sasson *et al*., 2004), whose expression is inhibited by LH and the increased expression of CD44, which is induced by LH (Schoenfelder and Einspanier, 2003).

TRIM28 (also known as KAP-1 or TIF1beta) is a universal co-repressor of gene transcription that acts via interaction with KRAB domains in Kruppel-type zinc-finger proteins. It also has a role in DNA repair. Upon DNA damage, KAP1 is phosphorylated through which the damaged DNA can decondensate (Ziv *et al*., 2006). The increased expression of TRIM28 in NEC-CC might thus lead to a reduced response to DNA damage or to transcriptional suppression of several genes.

The chemokine receptor *CXCR4* was up-regulated in NEC-CC samples. The expression of *Cxcr4* in cumulus cells was confirmed by Hernandez *et al*. (2006) who localized the protein at the cell surface. The role of this receptor and its ligand CXCL12 in oocyte development is however still undefined. *CXCR4* in endothelial and cancer cells is expressed via hypoxia inducible factor 1α (HIF1α)-mediated transcription, which in turn is activated under hypoxic conditions via the phosphatidylinositol 3-kinase (PI 3-kinase) pathway (Schioppa *et al*., 2003; Staller *et al*., 2003; Phillips *et al*., 2005). EGF can also induce PI 3-kinase signalling and up-regulate *CXCR4* transcription via HIF-1α (Phillips *et al*., 2005). HIF-1α is a regulator of oxygen homeostasis by functioning as a transcription factor for genes involved in angiogenesis, erythropoiesis, glycolysis and cell proliferation and survival (Semenza, 2002). How *CXCR4* can relieve hypoxic stress in cumulus cells needs further investigation.

Another gene which might indicate a hypoxic environment in NEC-CC samples is *GPX3*. Hypoxia is a strong transcriptional regulator of this gene through its HIF-1 binding sites. It is the only glutathione

peroxidase in which these binding sites have been detected (Bierl *et al*., 2004). Hypoxia leads to the formation of reactive oxygen species (ROS) which can cause lipid peroxidation, enzyme inactivation and cell damage, resulting in apoptosis (Buttke and Sandstrom, 1994) not only in cumulus cells, but also in the oocyte (Tatemoto *et al*., 2000). Both hypoxia (Van Blerkom *et al*., 1997) and a concentration of ROS above a certain level in follicular fluid have also been negatively associated with embryonic development, pregnancy outcome (Pasqualotto *et al*., 2004; Das *et al*., 2006) and a significantly higher incidence of aneuploidy and spindle defects in oocytes (Chui *et al*., 1997; Van Blerkom *et al*., 1997). Nowadays some clinics screen the embryos for aneuploidy by fluorescence in-situ hybridization on one or two blastomeres biopsied from the embryo (Munne *et al*., 2003). Although our data need further examination, it would be very promising if intrafollicular hypoxia and thus the enhanced chance for aneuploidy in the corresponding embryo can be analysed by using the cumulus cells instead of removing one or two blastomeres from the embryo.

A common response to hypoxia is the stimulation of angiogenesis. Recently, it has been established that Wnt signalling, besides in embryogenesis, also plays a role in angiogenesis (Masckauchan *et al*., 2006). *WNT5A*, which is up-regulated in NEC-CC (only analysed with microarray, but not included in the qRT–PCR assay) acts via non-canonical Wnt signalling to promote angiogenesis. It exerts its signal through *DVL3* phosphorylation (also up-regulated in NEC-CC) (Schulte *et al*., 2005).

In agreement with the existence of a stressful environment in NEC-CC is the increased expression of the stress-induced apoptosis inhibitor *HSPB1* (Arya *et al*., 2007). *HSPB1* can however also be expressed in the presence of estrogen instead of stress factors and act as a co-repressor of estrogen signalling by binding to the estrogen receptor beta (Al-Madhoun *et al*., 2007).

DHCR7 converts 7-dehydrocholesterol to cholesterol, which is a component necessary for progesterone and estrogen synthesis. A suppression of the cholesterol biosynthesis pathway in COC led to a decrease in progesterone production and subsequently to a decreased rate of germinal vesicle breakdown (Yamashita *et al*., 2003). It is however unknown how an increased cholesterol biosynthesis is related to a reduced embryonic development (as is the case in NEC samples), but it is consistent with the estrogen-related *HSPB1* expression and it fits with the theory of a delayed follicle maturation as estrogen and progesterone levels start to rise during follicle maturation.

The fact that EGF as well as PDGF and FGF signalling is overrepresented is mainly because the pathways share common components like *AKT1, ARAF, MAPK2K, RAF1* and *SOS2*. These factors were all more abundant in NEC-CC as compared to EC-CC. The EGF-like ligands epiregulin, amphiregulin and betacellulin, which have recently been discovered to be induced by LH (Conti *et al*., 2006) were not differentially expressed in this study. As these are the ligands that are mainly involved in oocyte maturation, this suggests that a disturbed EGF signalling might not be the cause of the decreased implantation potential in the NEC-CC group. The FGF signalling specific *FGFR2* was up-regulated in EC-CC as compared to NEC-CC and in the PDGF signalling *PDGFA* was down-regulated and *PDGFC* up-regulated, all known to be involved in angiogenesis. Chen *et al*. (2006) found in mice ovaries that FSH, partly via estradiol, modulated PDGF members, including e.g. *Pdgfa* and *Pdgfc*, in an opposite way, suggesting different functions for these two proteins. Angiogenesis is important for folliculogenesis as the concentration of VEGF in follicular fluid has been negatively correlated to IVF outcome and embryo development (Friedman *et al*., 1998; Barroso *et al*., 1999).

Although the ultimate goal was to find a new parameter predicting a pregnancy, in this study, EC, which has previously been shown to be a good marker for pregnancy, was used as an end-point. To correlate a differential gene expression directly to pregnancy, only cycles with SET should be included, as with double-embryo transfer it is not known which embryo implanted. SET, however, would make it impossible to perfectly match samples with a positive and samples with a negative pregnancy outcome for several patient characteristics which probably influence gene expression in cumulus cells. Furthermore, as pregnancy or implantation not only depends on embryonic factors, but also on e.g. endometrial receptivity, a considerable number of extra microarrays would have been needed in order to find significant intrafollicular differences in gene expression. As EC is a good parameter for pregnancy, independent of blastomere number and morphology (Salumets *et al*., 2003; Van Montfoort *et al*., 2004), this was chosen as a marker. Besides, there are indications that whether or not an embryo cleaves early is determined during oogenesis as the human embryonic genome is only activated between the four- and eight-cell stage (Braude *et al*., 1988; Eichenlaub-Ritter and Peschke, 2002). This means that the mature oocyte at ovulation must contain the proteins and mRNA necessary for fertilization and the early stages of embryonic development, including the first cleavage division. Several intrafollicular processes might influence the accumulation of these transcripts. Most investigators of EC concluded indeed after eliminating several explanations for EC like differences in oocyte maturity, that EC might be the result of an as yet unknown intrinsic oocyte factor (Shoukir *et al*., 1997; Sakkas *et al*., 1998; Lundin *et al*., 2001; Fenwick *et al*., 2002).

In conclusion, this study provides evidence that embryo viability is reflected in differential gene expression in the cumulus cells. The molecular discrimination of cumulus cells from different oocytes might lead to an improved embryo selection with improved eSET results or might serve as a tool for oocyte selection necessary in countries where not all oocytes are allowed to be fertilized. Although some genes point to hypoxic conditions as a negative regulator or to a delayed oocyte maturation, other processes or conditions might be disturbed as well. Probably, among different IVF patients, different processes can be disturbed leading to an impaired embryo development. To generate a group of genes predictive of embryo quality or even pregnancy, genes belonging to several processes that might be disturbed should be included.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org

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References

Ackert CL, Gittens JE, O'Brien MJ, Eppig JJ, Kidder GM. Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev Biol* 2001;**233**:258–270.

Al-Madhoun AS, Chen YX, Haidari L, Rayner K, Gerthoffer W, McBride H, O'Brien ER. The interaction and cellular localization of HSP27 and

ERbeta are modulated by 17beta-estradiol and HSP27 phosphorylation. *Mol Cell Endocrinol* 2007;**270**:33–42.

Anifandis G, Koutselini E, Louridas K, Liakopoulos V, Leivaditis K, Mantzavinos T, Sioutopoulou D, Vamvakopoulos N. Estradiol and leptin as conditional prognostic IVF markers. *Reproduction* 2005;**129**:531–534.

Arya R, Mallik M, Lakhotia SC. Heat shock genes—integrating cell survival and death. *J Biosci* 2007;**32**:595–610.

Assou S, Anahory T, Pantesco V, Le Carrour T, Pellestor F, Klein B, Reyftmann L, Dechaud H, De Vos J, Hamamah S. The human cumulus–oocyte complex gene-expression profile. *Hum Reprod* 2006;**21**:1705–1719.

Barroso G, Barrionuevo M, Rao P, Graham L, Danforth D, Huey S, Abuhamad A, Oehninger S. Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. *Fertil Steril* 1999;**72**:1024–1026.

Bierl C, Voetsch B, Jin RC, Handy DE, Loscalzo J. Determinants of human plasma glutathione peroxidase (GPx-3) expression. *J Biol Chem* 2004;**279**:26839–26845.

Borini A, Tallarini A, Maccolini A, Prato LD, Flamigni C. Perifollicular vascularity monitoring and scoring: a clinical tool for selecting the best oocyte. *Eur J Obstet Gynecol Reprod Biol* 2004;**115**(Suppl 1):S102–S105.

Borini A, Lagalla C, Cattoli M, Sereni E, Sciajno R, Flamigni C, Coticchio G. Predictive factors for embryo implantation potential. *Reprod Biomed Online* 2005;**10**:653–668.

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988;**332**:459–461.

Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. *Immunol Today* 1994;**15**:7–10.

Chen X, Aravindakshan J, Yang Y, Tiwari-Pandey R, Sairam MR. Aberrant expression of PDGF ligands and receptors in the tumor prone ovary of follitropin receptor knockout (FORKO) mouse. *Carcinogenesis* 2006;**27**:903–915.

Chui DK, Pugh ND, Walker SM, Gregory L, Shaw RW. Follicular vascularity—the predictive value of transvaginal power Doppler ultrasonography in an in-vitro fertilization programme: a preliminary study. *Hum Reprod* 1997;**12**:191–196.

Conti M, Hsieh M, Park JY, Su YQ. Role of the epidermal growth factor network in ovarian follicles. *Mol Endocrinol* 2006;**20**:715–723.

Dallas PB, Gottardo NG, Firth MJ, Beesley AH, Hoffmann K, Terry PA, Freitas JR, Boag JM, Cummings AJ, Kees UR. Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT–PCR—how well do they correlate? *BMC Genomics* 2005;**6**:59.

Das S, Chattopadhyay R, Ghosh S, Ghosh S, Goswami SK, Chakravarty BN, Chaudhury K. Reactive oxygen species level in follicular fluid–embryo quality marker in IVF? *Hum Reprod* 2006;**21**:2403–2407.

Eichenlaub-Ritter U, Peschke M. Expression in in-vivo and in-vitro growing and maturing oocytes: focus on regulation of expression at the translational level. *Hum Reprod Update* 2002;**8**:21–41.

Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001;**122**:829–838.

Fenwick J, Platteau P, Murdoch AP, Herbert M. Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. *Hum Reprod* 2002;**17**:407–412.

Friedman CI, Seifer DB, Kennard EA, Arbogast L, Alak B, Danforth DR. Elevated level of follicular fluid vascular endothelial growth factor is a marker of diminished pregnancy potential. *Fertil Steril* 1998;**70**:836–839.

Gaulden ME. Maternal age effect: the enigma of Down syndrome and other trisomic conditions. *Mutat Res* 1992;**296**:69–88.

Gerris JM. Single embryo transfer and IVF/ICSI outcome: a balanced appraisal. *Hum Reprod Update* 2005;**11**:105–121.

Hammadeh ME, Fischer-Hammadeh C, Amer AS, Rosenbaum P, Schmidt W. Relationship between cytokine concentration in serum and preovulatory follicular fluid and in vitro fertilization/intracytoplasmic sperm injection outcome. *Chem Immunol Allergy* 2005;**88**:80–97.

Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L, Richards JS. Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Mol Endocrinol* 2006;**20**:1300–1321.

Host E, Gabrielsen A, Lindenberg S, Smidt-Jensen S. Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. *Fertil Steril* 2002;**77**:511–515.

Keirsebilck A, Bonne S, Staes K, van Hengel J, Nollet F, Reynolds A, van Roy F. Molecular cloning of the human p120ctn catenin gene (CTNND1): expression of multiple alternatively spliced isoforms. *Genomics* 1998;**50**:129–146.

Lanuza GM, Fischman ML, Baranao JL. Growth promoting activity of oocytes on granulosa cells is decreased upon meiotic maturation. *Dev Biol* 1998;**197**:129–139.

Lee DM, Lee TK, Song HB, Kim CH. The expression of matrix metalloproteinase-9 in human follicular fluid is associated with in vitro fertilisation pregnancy. *Bjog* 2005;**112**:946–951.

Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. *J Assist Reprod Genet* 2001;**18**:490–498.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:402–408.

Ludwig M, Schopper B, Katalinic A, Sturm R, Al-Hasani S, Diedrich K. Experience with the elective transfer of two embryos under the conditions of the german embryo protection law: results of a retrospective data analysis of 2573 transfer cycles. *Hum Reprod* 2000;**15**:319–324.

Lundin K, Bergh C, Hardarson T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Hum Reprod* 2001;**16**:2652–2657.

Masckauchan TN, Agalliu D, Vorontchikhina M, Ahn A, Parmalee NL, Li CM, Khoo A, Tycko B, Brown AM, Kitajewski J. Wnt5a signaling induces proliferation and survival of endothelial cells in vitro and expression of MMP-1 and Tie-2. *Mol Biol Cell* 2006;**17**:5163–5172.

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 2002;**296**:2178–2180.

McClintick JN, Edenberg HJ. Effects of filtering by Present call on analysis of microarray experiments. *BMC Bioinformatics* 2006;**7**:49.

McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Hum Reprod* 2004;**19**:2869–2874.

Mendoza C, Ruiz-Requena E, Ortega E, Cremades N, Martinez F, Bernabeu R, Greco E, Tesarik J. Follicular fluid markers of oocyte developmental potential. *Hum Reprod* 2002;**17**:1017–1022.

Muniz LC, Yehia G, Memin E, Ratnakar PV, Molina CA. Transcriptional regulation of cyclin D2 by the PKA pathway and inducible cAMP early repressor in granulosa cells. *Biol Reprod* 2006;**75**:279–288.

Munne S, Sandalinas M, Escudero T, Velilla E, Walmsley R, Sadowy S, Cohen J, Sable D. Improved implantation after preimplantation genetic diagnosis of aneuploidy. *Reprod Biomed Online* 2003;**7**:91–97.

Nargund G, Doyle PE, Bourne TH, Parsons JH, Cheng WC, Campbell S, Collins WP. Ultrasound derived indices of follicular blood flow before HCG administration and the prediction of oocyte recovery and preimplantation embryo quality. *Hum Reprod* 1996;**11**:2512–2517.

Pasqualotto EB, Agarwal A, Sharma RK, Izzo VM, Pinotti JA, Joshi NJ, Rose BI. Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. *Fertil Steril* 2004;**81**:973–976.

Pearson H. Safer embryo tests could boost IVF pregnancy rates. *Nature* 2006;**444**:12–13.

Phillips RJ, Mestas J, Gharaee-Kermani M, Burdick MD, Sica A, Belperio JA, Keane MP, Strieter RM. Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem* 2005;**280**:22473–22481.

Rajeevan MS, Ranamukhaarachchi DG, Vernon SD, Unger ER. Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. *Methods* 2001a;**25**:443–451.

Rajeevan MS, Vernon SD, Taysavang N, Unger ER. Validation of array-based gene expression profiles by real-time (kinetic) RT–PCR. *J Mol Diagn* 2001b;**3**:26–31.

Sakkas D, Shoukir Y, Chardonnens D, Grace Bianchi P, Campana A. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. *Hum Reprod* 1998;**13**:182–187.

Salumets A, Hyden-Granskog C, Makinen S, Suikkari A, Tiitinen A, Tuuri T. Early cleavage predicts the viability of human embryos in elective single embryos transfer procedures. *Hum Reprod* 2003;**18**:821–825.

Sasson R, Rimon E, Dantes A, Cohen T, Shinder V, Land-Bracha A, Amsterdam A. Gonadotrophin-induced gene regulation in human granulosa cells obtained from IVF patients. Modulation of steroidogenic genes, cytoskeletal genes and genes coding for apoptotic signalling and protein kinases. *Mol Hum Reprod* 2004;**10**:299–311.

Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, Bernasconi S, Saccani S, Nebuloni M, Vago L *et al.* Regulation of the chemokine receptor CXCR4 by hypoxia. *J Exp Med* 2003;**198**:1391–1402.

Schoenfelder M, Einspanier R. Expression of hyaluronan synthases and corresponding hyaluronan receptors is differentially regulated during oocyte maturation in cattle. *Biol Reprod* 2003;**69**:269–277.

Schulte G, Bryja V, Rawal N, Castelo-Branco G, Sousa KM, Arenas E. Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation. *J Neurochem* 2005;**92**:1550–1553.

Semenza G. Signal transduction to hypoxia-inducible factor 1. *Biochem Pharmacol* 2002;**64**:993–998.

Shoukir Y, Campana A, Farley T, Sakkas D. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. *Hum Reprod* 1997;**12**:1531–1536.

Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD *et al.* Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 1996;**384**:470–474.

Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature* 2003;**425**:307–311.

Sutton ML, Gilchrist RB, Thompson JG. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 2003;**9**:35–48.

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**:805–810.

Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, Diemer K, Muruganujan A, Narechania A. PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* 2003;**13**:2129–2141.

Thomas PD, Kejariwal A, Guo N, Mi H, Campbell MJ, Muruganujan A, Lazareva-Ulitsky B. Applications for protein sequence-function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. *Nucleic Acids Res* 2006;**34**:W645–W650.

Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod* 1995;**10**:415–424.

Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Hum Reprod* 1997;**12**:1047–1055.

Van Montfoort AP, Dumoulin JC, Kester AD, Evers JL. Early cleavage is a valuable addition to existing embryo selection parameters: a study using single embryo transfers. *Hum Reprod* 2004;**19**:2103–2108.

Van Montfoort AP, Dumoulin JC, Land JA, Coonen E, Derhaag JG, Evers JL. Elective single embryo transfer (eSET) policy in the first three IVF/ICSI treatment cycles. *Hum Reprod* 2005;**20**:433–436.

Van Montfoort AP, Fiddelers AA, Janssen JM, Derhaag JG, Dirksen CD, Dunselman GA, Land JA, Geraedts JP, Evers JL, Dumoulin JC. In unselected patients, elective single embryo transfer prevents all multiples, but results in significantly lower pregnancy rates compared with double embryo transfer: a randomized controlled trial. *Hum Reprod* 2006;**21**:338–343.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT–PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3**: RESEARCH0034.

Wharf E, Dimitrakopoulos A, Khalaf Y, Pickering S. Early embryo development is an indicator of implantation potential. *Reprod Biomed Online* 2004;**8**:212–218.

Yamashita Y, Shimada M, Okazaki T, Maeda T, Terada T. Production of progesterone from de novo-synthesized cholesterol in cumulus cells and its physiological role during meiotic resumption of porcine oocytes. *Biol Reprod* 2003;**68**:1193–1198.

Yokoo M, Miyahayashi Y, Naganuma T, Kimura N, Sasada H, Sato E. Identification of hyaluronic acid-binding proteins and their expressions in porcine cumulus-oocyte complexes during in vitro maturation. *Biol Reprod* 2002;**67**:1165–1171.

Zhang L, Jiang S, Wozniak PJ, Yang X, Godke RA. Cumulus cell function during bovine oocyte maturation, fertilization, and embryo development in vitro. *Mol Reprod Dev* 1995;**40**:338–344.

Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. *Fertil Steril* 2005;**83**(Suppl 1):1169–1179.

Ziv Y, Bielopolski D, Galanty Y, Lukas C, Taya Y, Schultz DC, Lukas J, Bekker-Jensen S, Bartek J, Shiloh Y. Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. *Nat Cell Biol* 2006;**8**:870–876.

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