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ORIGINAL ARTICLES

Transcriptional characteristics of different sized follicles in relation to embryo transferability: potential role of hepatocyte growth factor signalling

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STUDY HYPOTHESIS: We hypothesized that a better discrimination between follicles containing oocytes with high developmental competence and those containing oocytes with low competence, based on a combination of a follicle's size and transcriptomic signature, will provide a reliable method to predict embryonic outcome of IVF.

STUDY FINDING: This study provides new insights on the impact of follicular size on oocyte quality as measured by embryonic development and demonstrates that medium follicles yield a better percentage of transferable embryos.

WHAT IS KNOWN ALREADY: Although it is generally accepted that large ovarian follicles contain better eggs, other studies report that a better follicular size subdivision and a better characterization are needed.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: Individual follicles ($n = 136$), from a total of 33 women undergoing IVF, were aspirated and categorized on the basis of their follicular liquid volume (small, medium or large) and the embryonic outcome of the enclosed oocyte: poor or good development. Comprehensive gene expression analysis between cells from the different sized follicles was performed using microarrays and quantitative RT–PCR to find molecular markers associated with follicular maturity and oocyte developmental competence.

MAIN RESULTS AND THE ROLE OF CHANCE: The analysis of embryonic outcome in relation to follicular size indicates that the medium-sized follicles category yielded more transferable embryos (35%) compared with the largest follicles (30%) (NS). Gene expression analysis revealed expression markers with significant ($P < 0.05$) discrimination between the poor development groups for all three follicle sizes, and good development medium-size follicles, including up-regulation of thrombomodulin, transforming growth factor, beta receptor II and chondrolecti, and those associated with hyaluronan synthesis, coagulation and hepatocyte growth factor signalling.

LIMITATIONS, REASONS FOR CAUTION: These analyses were performed in a single cohort of patients coming from a single clinic and the biomarkers generated will require validation in different geographical and biological contexts to ensure their global applicability.

WIDER IMPLICATIONS OF THE FINDINGS: Medium-size follicles seem to be the optimal size for a positive embryonic outcome and are associated with competence markers that may help in understanding the ideal differentiation status during late folliculogenesis.

LARGE SCALE DATA: The data discussed in this publication have been deposited in The National Center for Biotechnology Information Gene Expression Omnibus database and are accessible through GEO Series accession number GSE52851.

STUDY FUNDING AND COMPETING INTEREST(S): This study was supported by Canadian Institutes of Health Research (CIHR) and Natural Sciences and Engineering Research Council of Canada (NSERC) to M.A.S. There are no competing interests to declare.

Key words: follicular cells / granulosa cells / follicular size / oocyte competence / gene expression / hepatocyte growth factor

Introduction

In order to improve the success rate of assisted reproductive technologies (ART), the analysis of the reasons for failure is a key component. Nearly 64% of ART cycles using fresh own oocytes fail to produce a pregnancy (CDC report USA national survey http://www.cdc.gov/art/pdf/2013-report/art_2013_national_summary_report.pdf#page=21), raising medical, scientific, public health, economic and social issues for patients and society. The most important research question is why some oocytes have higher developmental competence potential than others for a given cycle and patient, or across patients.

In the past years, much evidence has demonstrated a relationship between oocyte developmental acquisition and the surrounding somatic cell environment acquired through folliculogenesis (Sugiura and Eppig, 2005; Li and Albertini, 2013; Dumesic *et al.*, 2015). Moreover, efforts have been made to link follicle size to developmental competence using different evaluation criteria (Nataprawira *et al.*, 1992; Wittmaack *et al.*, 1994; Arnot *et al.*, 1995; Dubey *et al.*, 1995; Miller *et al.*, 1996; Ectors *et al.*, 1997; Bergh *et al.*, 1998; Salha *et al.*, 1998; Teissier *et al.*, 2000; Trounson *et al.*, 2001; Triwitayakorn *et al.*, 2003; Rosen *et al.*, 2008; Farhi *et al.*, 2010; Lee *et al.*, 2010; Mehri *et al.*, 2014). Most studies have compared two populations of follicle sizes (variable threshold) and reached the conclusion that larger follicles are superior in terms of developmental competence (a list of relevant publications is summarized in Supplementary Table SI). Using the likelihood of cleavage as a criterion for evaluating developmental competence, Ectors and collaborators (Ectors *et al.*, 1997), defined follicles in the 16–23 mm diameter size range as superior to the <16 mm and >23 mm categories. Based on the early cleavage timing, Lee and co-authors (Lee *et al.*, 2010), determined that follicles with a volume of 3–5 ml (18–21 mm follicles) were superior to follicles >5 ml (>21 mm). More recently, Farhi *et al.* (2010) showed that when the size of the largest follicle in a patient is 18–22 mm, the likelihood of pregnancy is greater than when it is 26 mm (Farhi *et al.*, 2010).

In a previous study, we have found molecular markers of oocyte competence associated with individual oocytes collected from individual follicles (Hamel *et al.*, 2008, 2010a, b). Since a correlation between follicular physiological status and embryo outcome has been demonstrated, we want to explore if a classification of these follicles based on their size and embryonic outcome could explain the reasons for success or failure. Therefore, this study has two main goals. The first one is to determine whether or not the sizes of human follicles are associated with oocyte competence for embryonic development. The second one is to attempt to understand the impact of follicular status by associating a differential gene expression list to each follicular size category. This study thus allowed identification of markers of follicular maturity and embryo transferability and highlighted a new physiological pathway, namely hepatocyte growth factor (HGF) signalling, unknown until now in follicular physiology.

Materials and Methods

This work utilized the same database and samples as used previously (Hamel *et al.*, 2008, 2010a, b), and the institutional review board approval was obtained for those studies. The specific tissues and procedures for this analysis are outlined below as well as all the information related to sample preparation.

Follicular cells recovery

Follicular cells (FC) composed principally of granulosa cells but containing contaminating cumulus cells from individual follicles ($n = 136$) were collected from 33 consenting patients who were undergoing IVF treatment at the Ottawa Fertility Centre (Ottawa, ON, Canada). Samples were collected irrespective of hormonal treatment (FSH or hMG) or age. Patients with polycystic ovary syndrome, severe male factor and ovarian failure were excluded from the study.

FCs were collected from individual follicular aspiration and the cumulus-oocyte complexes were used for IVF/ICSI procedures, as previously described (Hamel *et al.*, 2008, 2010a, b). Only follicles containing a mature oocyte were kept for the analysis. Cells collected from the aspirated follicular fluid are mainly granulosa cells. Ovulation was triggered when the 2 largest follicles reached 17 mm (measured by ultrasound) in a given cycle and oocyte retrieval was carried out 34 h later. Patient follicular population repartition was normal, with a mean of 5.7 ± 2.3 (SEM) follicles >15 mm diameter and 12.1 ± 6.3 (SEM) follicles <15 mm diameter at trigger. Although no particular treatment was used to remove contaminating blood cells, the fact that individual aspiration was used has minimized the contaminant and we believe that the contamination would be similar across follicular sizes and therefore masked by the contrast analysis. Data from individual follicles, including follicular fluid volume, oocyte maturity, embryonic development and treatment outcomes, were collected. Depending on the aspirated follicular volume, the samples were categorized on the basis of size (small (S): 0.5–1.5 ml, medium (M): 2–3 ml or large (L): >3 ml) and embryo outcome: poor development/blocked (−), or transferable (transferred or cryopreserved) embryos. A positive embryo development (+) refers to a transferable embryo, at Day 3 (67%) or Day 5 (33%) and these embryos have been cryopreserved for potential transfer later.

From the dataset associated with the 33 patients, a subset of samples with specific follicular size and enclosed oocyte competence outcome were selected for gene expression analysis with microarrays and quantitative RT–PCR (qRT–PCT) (Supplementary Table SII).

Experimental design

For the microarray and qRT–PCR analyses, the four categories (S−, M−, M+ and L−) each containing four different individual samples were included and three comparisons were performed in triplicate. The medium-sized follicles associated with transferable embryos (M+) were compared with all follicle size categories correlated with poor or blocked embryonic development (S−, M− and L−). Schematic overview of the general experimental design for microarrays and qRT–PCR is provided in Supplementary Fig. S1. Considering the limited individual tissues, the same samples utilized for the microarray experiment have also been used before amplification for the qRT–PCR assays.

RNA extraction and amplification

Total RNA was extracted using the RNeasy mini kit (Qiagen, Mississauga, Canada) following the manufacturer's protocol and including a DNAse digestion with the RNase-free DNase Set (Qiagen), directly on the extraction column. Total RNA integrity and concentration were evaluated on a 2100-Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6000 Pico Kit (Agilent Technologies) and the RNA integrity number threshold for usable samples was set as >7. To generate enough material for hybridization, the samples were amplified using the RiboAmp HS RNA amplification kit (Life Technologies Inc., Burlington, ON, Canada). After two amplification rounds of 6 h each, the amplified RNA (aRNA) output was quantified using the NanoDrop ND-1000 system (NanoDrop Technologies, Wilmington, DE, USA).

Sample labelling and microarray hybridization

For each sample, 2 μg of aRNA were labelled using the ULS Fluorescent Labelling Kit for Agilent arrays (with Cy3 and Cy5) (Kreatech Diagnostics, Amsterdam, Netherlands). The labelled product was then purified with the PicoPure RNA Isolation Kit but without DNase treatment. Labelling efficiency was measured using the Nano-Drop ND-1000 (NanoDrop Technologies). Samples were hybridized on the human gene expression 4 × 44K v2 microarray (Agilent).

Overall, 18 hybridizations were performed, corresponding to M+ versus S−, M+ versus M− and M+ versus L− with three biological replicates samples for each group and including a technical dye-swap replicate. A total of 825 ng of each labelled sample (Cy3 and Cy5) was incubated in a solution containing 2× blocking agent and 5× fragmentation buffer in a volume of 55 μl at 60°C for 15 min and put on ice immediately afterwards. 2X HI-RPM hybridization buffer (Agilent) was added (55 μl of 2× GEx) for a total volume of 110 μl. The hybridization mix was added onto the array and hybridization was performed at 65°C for 17 h using an Agilent Hybridization chamber in a rotating oven. Slides were then washed with gene expression wash buffer 1 (containing 0.005% Triton X-102) for 3 min at room temperature and then transferred to gene expression wash buffer 2 (also containing 0.005% Triton X-102) for 3 min at 42°C. Final washes at room temperature with acetonitrile (for 10 s at room temperature) and with drying and stabilization solution (for 30 s at room temperature) were performed before air-drying slides. The slides were then scanned using the Tecan PowerScanner microarray scanner (Tecan Group Ltd, Männerdorf, Switzerland) and features were extracted using ArrayPro 6.4 (Media Cybernetics, Bethesda, MD, USA).

Microarray data analysis

Microarray data were processed with Flexarray 1.6.1 (Efron and Tibshirani, 2002) using a background correction (simple subtraction method) performed on raw intensities, Loess within-array normalization, and between-array Quantile normalization based on intensities. To improve the power and gain a more stable inference with our small number of arrays, the Limma package in Bioconductor was used in FlexArray. Limma uses linear models to analyse the microarray data and an empirical Bayes method to assess differential expression by moderating the standard errors of the estimated log-fold changes (Smyth, 2004). Differences between treatments were considered significant when the Limma *P*-value was less than 0.05. The data discussed in this publication have been deposited in The National Center for Biotechnology Information Gene Expression Omnibus database and are accessible through GEO Series accession number GSE52851.

Ingenuity pathway analysis

Data were also analysed through the use of Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com). IPA is able to construct networks showing the known potential direct or indirect interactions between molecules, and to overlay our microarray data on it.

Complementary DNA (cDNA) preparation and qRT–PCR

Total RNA from 12 individual follicles (10 ng) (three biological replicates of M+, S−, M− and L−) was reverse transcribed using a qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) with oligo $dT_{(20)}$ primers following the manufacturer's recommendations. Based on the *P*-values and fold changes observed in the microarray analysis, in combination with a known gene function, eight genes were selected for validation by qPCR. The primers used in qRT–PCR are listed in Supplementary Table SII and were designed using the IDT PrimerQuest tool (http://www.idtdna.com/Scitools/Applications/Primerquest/, accessed May 2012). To confirm the specificity of each pair of primers, electrophoresis on a standard 1.2% agarose gel was performed for each amplified fragment. The PCR product was then purified using the QIAquick Gel Extraction kit (Qiagen), quantified using the NanoDrop ND-1000, and then sequenced. The products were used to create the standard curve for the quantification experiment, with dilutions ranging from 2×10^{-4} to 2×10^{-8} ng μl^{-1}. qRT–PCR was performed on a LightCycler 480 (Roche Diagnostics, Laval, QC, Canada) using SYBR incorporation. Each reaction mix (in a final volume of 20 μl) contained 2 μl (0.5 ng) of the cDNA product, 0.25 mmol L^{-1} of each primer and 1× SYBR mix (LightCycler 480 SYBR Green I Master, Roche Diagnostics). The PCR conditions used for all genes were as follows: denaturing cycle for 10 min at 95°C, 50 PCR cycles (denaturing, 95°C for 1 s; annealing, for 5 s; extension, 72°C for 5 s), a melting curve (94°C for 5 s, 72°C for 30 s and a step cycle starting at 72°C up to 94°C at 0.2°C/s), and a final cooling step at 40°C. cDNA quantification was performed using LightCycler 480 Software Version 1.5 (Roche Diagnostics) by comparison with the standard curve. PCR specificity was confirmed by melting-curve analysis.

Statistical analysis of qRT–PCR results

Analysis of gene expression stability over the different follicles was performed using the GeNorm VBA applet software as described by Vandesompele *et al.* (2002). The most stable reference genes were identified by the stepwise exclusion of the least stable gene and recalculating the M values. Following GeNorm analysis, beta actin (ACTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were the most stable genes among 4 tested (ACTB, GAPDH, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), cyclophilin A (PPIA)), with M values of 0.545 (i.e. <1.5, as recommended by the software developer). Evaluation of mRNA differences was performed using Mann–Whitney test for the comparisons of two conditions with GraphPad Prism Version 5.0 (GraphPad Software Inc., CA, USA). Differences were considered statistically significant at the 95% confidence level ($P < 0.05$). Data are presented as mean ± SEM.

Results

Follicle volume analysis and its association with embryonic outcome

The complete dataset, including 136 follicles from 33 patients, was analysed and follicles were grouped into three categories based on volume. Small (S) correspond to follicles with a volume between 0.5 and 1.5 ml, medium (M) follicles have a volume of 2–3 and large (L) have >3 ml volume. Also, the follicles were also classified in two categories based on the oocyte developmental competence from them: (i) poor development (blocked) and (ii) transferred/cryopreserved. The relative mean frequency of each embryonic developmental category per follicular size is illustrated in Fig. 1. The 'no development' group was not used since it can be indicative of an IVF or sperm issue instead of considering the level of oocyte competence. Additional information regarding data and relative proportions of each follicular size groups per development category is shown in Table I.

The largest proportion of transferable embryos is associated with medium-size follicles (2–3 ml of follicular fluid, Fig. 1) and also associated with the smallest proportion of blocked embryos. Consequently three times more follicles were available for this category. Consistent with previous studies (see references in Supplementary Table SI), the smallest follicles are correlated with the smallest proportion of transferable

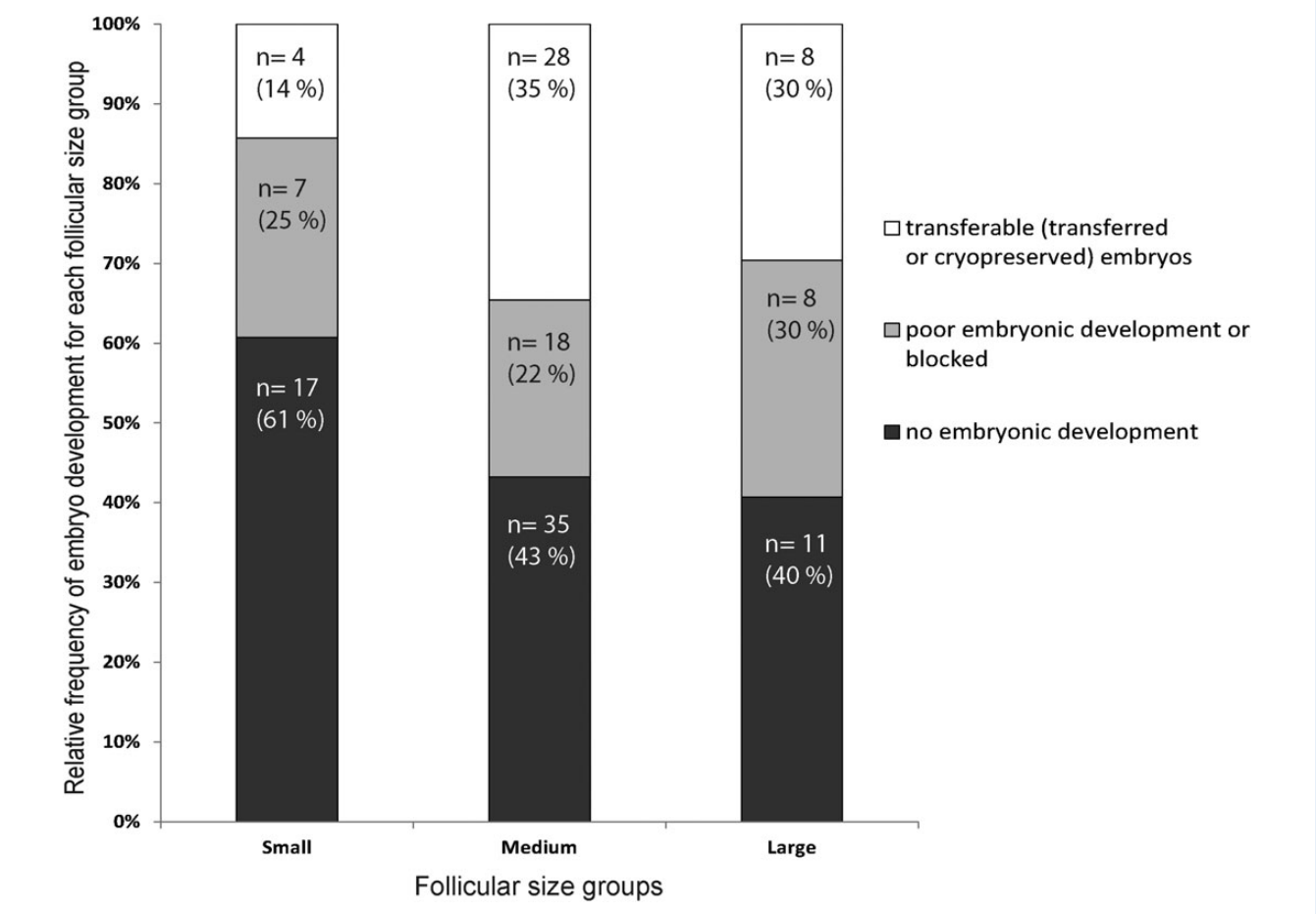

Figure 1 Individual human follicle and embryo development dataset analysis. Relative mean frequency of embryo development category per follicular size group. The number of embryos in each developmental category from each follicular size range and their percentage (%) of the total is indicated.

Table I Information regarding human follicular cells and embryonic developmental stage used in microarrays experiments.

	Small follicle Poor or blocked embryonic development	Medium follicle Poor or blocked embryonic development	Large follicle Poor or blocked embryonic development	Medium follicle Transferable (transferred or cryopreserved) embryos
Follicular fluid	0.5–1.5 ml	2–3 ml	>3 ml	2–3 ml
Equivalent diameter based on fluid volume	9.8–14.2 mm	15.6–17.9 mm	>18 mm	15.6–17.9 mm
Embryonic developmental stages associated with enclosed oocytes	Zygote to up to 10 cells	Zygote to up to 10 cells	Zygote to up to 10 cells	Blastocyst stage at Day 5 or 6

embryos. Conversely, the poor development (blocked) embryos are more likely to come from large follicles (>3 ml) than medium-sized follicles (2–3 ml) but the majority of them are from the smallest size category (0.5–1.5 ml). Using two-way analysis of variance, the follicular volume has a significant effect on embryo development (P-value <0.0001) and the correlation (r) of follicle size and embryonic development is also significant (P-value = 0.0086) showing that the embryo development is influenced by follicular size.

Based on our analyses, the best oocyte developmental competence was associated with the medium-size category. Using regression analysis, it was found that within the small and medium categories, a bigger follicle volume is associated with higher occurrence of a transferable embryo (P-value = 0.009, $R^2 = 0.99$, data not shown). However, this is not the case for the large follicle group (P-value = 0.163). Although our data set was small (136 follicles), each follicle was tracked individually, thus providing much higher resolution of the outcome.

Gene expression analysis

The microarray experiment was performed using the medium volume follicles associated with transferred or cryopreserved embryos groups

(M+) as the group of interest since they have been shown to have a higher proportion of transferable embryos. The M+ follicles were compared to the poor or blocked embryonic development follicular groups of small, medium and large follicular volumes (S−, M− and L−). The experimental microarray design is illustrated in Supplementary Fig. S1. Principal component analysis (PCA), which groups the categories or samples based on their gene expression, shows that the small negative (S−), medium positive (M+), and medium negative (M−) follicles genes have a more homogenous expression pattern, and much less dispersed, compared to the large negative population (Fig. 2A). PCA also indicates that biological replicates tightly co-segregate, which adds value to our gene analysis by showing homogeneity within categories.

A total of 1817 targets/probes were detected to be significantly changed between M+/S−, M+/M− and M+/L− follicular groups (Fig. 2B). Details regarding gene ID and fold-change (>1.5) of the differentially expressed genes (DEG) among the three comparisons can be found in Supplementary Tables SIII–SV.

Quantitative RT–PCR validation

Validation of the microarray results was performed using qRT–PCR (Fig. 3). Candidate selection for qRT–PCR validation was based on different expression between the M+ and the M− groups transforming growth factor, beta receptor II (TGFBR2), thrombomodulin (THBD) and chondrolectin (CHODL) transcripts) and also compared across S− and M+ follicles (THBD). Moreover, Supplementary Fig. S2 illustrates the gene expression comparison of the qRT–PCR validation and the microarray results for the four different follicular groups (S−, M−, L− and M+) and shows a similar profile for both methods.

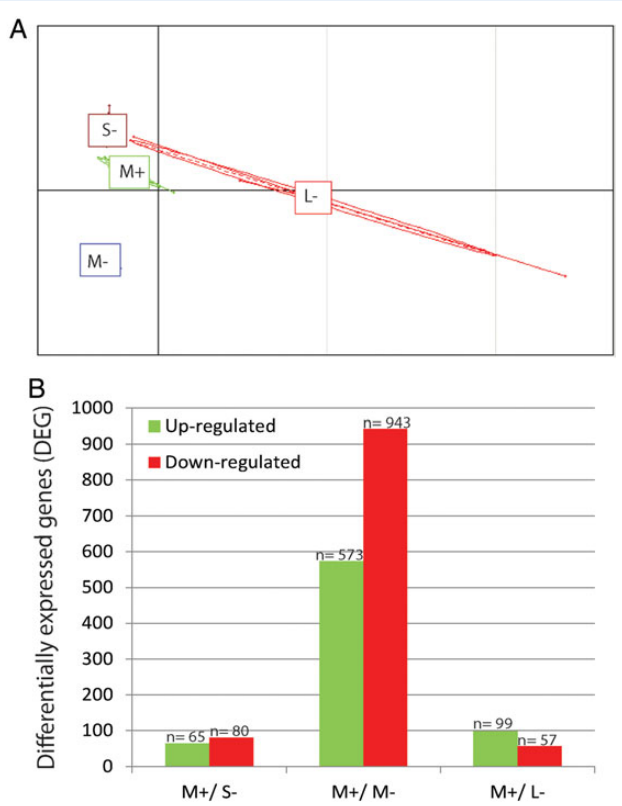

Figure 2 Characterization of the three different sizes of follicle by microarrays. (**A**) Principal component analysis of follicle group comparisons using biological triplicate microarray data. (**B**) Changes in gene expression profile among the different follicular sizes. Unigenes up-regulated (green) and down-regulated (red) between the different microarrays. M+, medium follicles associated with transferable (transferred or cryopreserved) embryos; S− small, M− medium and L− large follicles linked to a poor or blocked embryo development.

Ingenuity pathway analysis

Among the tools available in Ingenuity software, it is possible to select extracellular proteins as a way to explore the possible secretion or interaction of molecules between granulosa or cumulus cells. The extracellular molecules represented, respectively, 9.6, 6.4, and 5.3% of the DEG (*P*-value <0.05 and 1.5-fold change) in the comparisons M+/S−, M+/M− and M+/L− (Supplementary Tables SIII–SV). There were six molecules in common between the M+/S− and M+/M− groups: epidermal growth factor containing fibulin-like extracellular matrix protein 1 (EFEMP1), lectin, galactoside-binding, soluble, 12 (LGALS12), lectin, galactoside-binding, soluble, 3 (LGALS3), nidogen 1 (NID1), PLBD1 (phospholipase B domain containing 1), vitronectin (VTN). Three molecules are shared between M+/M− and M+/L− groups: (apolipoprotein C-I (APOC1), proprotein convertase subtilisin/kexin type 9 (PCSK9), metallopeptidase inhibitor 1 (TIMP1)) (Supplementary Tables SIV and SV).

Ingenuity software has a function to identify upstream regulators by looking for pathways and genes that can have increased activity based on expression levels of their downstream targets. Among the different upstream regulators associated with oocyte competence, the HGF pathway has been identified (Fig. 4). Indeed, many DEG in M+ follicles seem to be driven by the upstream regulator HGF. HGF is also up-regulated when M+ and M− groups are compared (Supplementary Table SIV). The majority of DEG (Z score of 2.8, where 2 is considered to be a very significant threshold) under this influence is also activated in M+/M− (Supplementary Table SIV). However, we note with interest that HGF is upstream of TGFBR2, THBD, toll-like receptor 2 (TLR2), alpha-2-macroglobulin (A2M), PDE8B and annexin A3 (ANXA3), which are all up-regulated in M+/M− (Fig. 4) and hence associated with developmental competence.

Discussion

To our knowledge, this is the first study where FC samples from individually punctured follicles are compared in relation to follicular volume and embryonic outcome. Indeed, no gene analysis has ever been done in conjunction with such precise knowledge of follicle volume and the developmental competence of the enclosed oocyte.

The present study included an analysis of a dataset of 136 follicles to correlate follicular volume to embryonic development outcomes. The results show that the medium follicles yielded more transferable embryos compared with the largest and the smallest ones. These results are consistent with studies by Ectors (Ectors *et al.*, 1997) and Lee (Lee *et al.*, 2010), who linked medium-sized follicles to a superior cleavage rate compared with the largest follicles. Our study goes further by examining embryo transferability, a more advanced criterion of embryo development evaluation.

The first conclusion emerging from the volume-outcome analysis is that oocyte quality seems to be associated with specific follicular

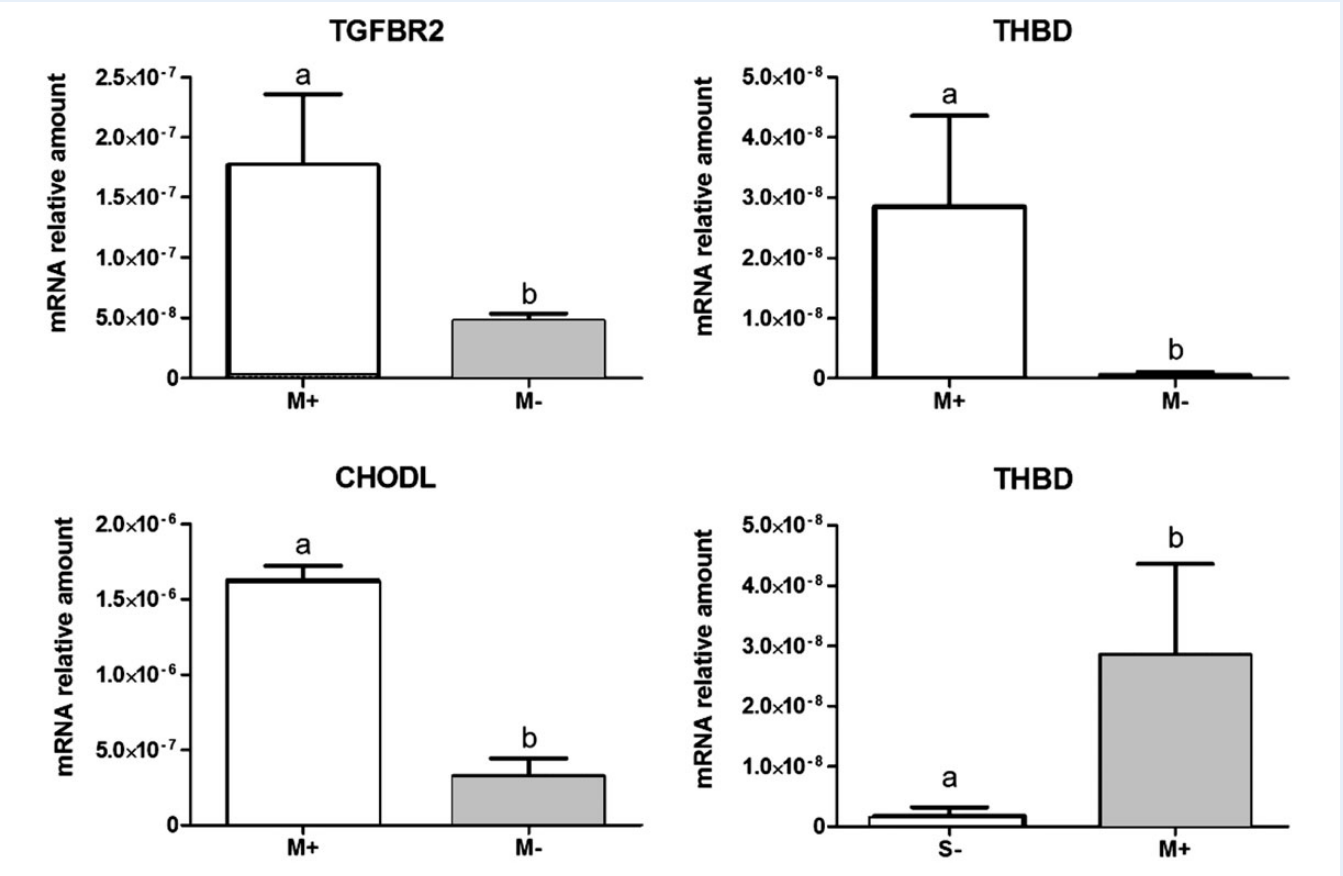

Figure 3 Validation by quantitative RT–PCR of the potential markers of follicles associated with transferable embryos. M+: medium-sized follicles associated with developmental competence (transferable embryos); M−: medium-sized follicles associated with poor or blocked embryonic development; S−: small follicles associated with poor development or blocked embryonic development; TGFBR2: transforming growth factor receptor 2; CHODL: chondrolectin; THBD: thrombomodulin. Different superscripts represent significant differences (Mann–Whitney test, $P = 0.05$).

conditions, since follicles that are too small or too large contain oocytes that are less likely to become transferable embryos. However, follicle size does not explain oocyte quality completely, since both competent and incompetent oocytes are observed in all categories. This intra-size diversity supports our hypothesis of the existence of a potential relationship between granulosa cell gene expression and the developmental competence of the enclosed oocyte. Based on PCA, the volume-outcome groupings have reduced overlap and M+ follicles (the best quality) distinguish themselves from M− follicles and relatively well from S− but with more difficulty from L−, which corroborates the results obtained using qRT–PCR. Gene expression analysis using the microarray revealed particularities of each of the four follicle categories, which may hold the key to explaining the variations in oocyte potential.

Distinctive gene expression patterns associated with small incompetent follicles (S−) were related principally to follicle growth, for example, small follicles overexpress angiotensin I converting enzyme 2 (ACE2) (fc: 2.7) (Goncalves *et al.*, 2012), glutathione S-transferase theta 3 (GSTA3) (fc: 2.3), and fascin homologue 1 (FSCN1) (fc: 2.3) (Kostopoulou *et al.*, 2008). It is noted with interest that secreted phosphoprotein 1 (SPP1), a gene associated with antral follicle growth in bovine (Skinner *et al.*, 2008; Hayashi *et al.*, 2010), is down-regulated (fc: −3.9) in S− follicles and therefore could be related to low follicular competence (Supplementary Table SIII).

Among the differentially overexpressed genes in the largest incompetent follicles uromodulin (UMODL1) (fc: 37.3) is particularly interesting. Umod1l is regulated by gonadotrophins. Mice carrying extra copies of functional Umod1l were generated by bacterial artificial chromosome transgenesis showing reduced or diminished fertility (Wang *et al.*, 2012). Among the multilayered pre-antral follicles, elevated apoptosis was observed in both the oocytes and surrounding granulosa cells (Wang *et al.*, 2012). Genes associated with cellular proliferation, such as filamin A interacting protein 1-like (FILIP1L) (fc: 17,3) (Burton *et al.*, 2011), Y box binding protein 1 (YBX1) (fc: 2.1) (Raffetseder *et al.*, 2009) and TGFBR2 (fc: 1.9) (Kingsley, 1994), and with apoptosis, for example NOD-like receptors (NLR) family, pyrin domain containing 12 (NLRP12) (fc: 5.1) (Wang *et al.*, 2002) and B-cell lymphoma 2 (BCL2)-like 13 (BCL2L13) (fc: 1.9) an apoptotic facilitator (Kataoka *et al.*, 2001), have been up-regulated (Supplementary Table SV).

In the M+/M− DEG list (Supplementary Table SIV), and also identified as a major upstream regulator, HGF has been shown to act positively on the reduction of apoptosis in granulosa cells (Uzumcu *et al.*, 2006). It corroborates with the down-regulation in M+ follicles of molecules associated with hypoxia, like spectrin alpha non-erythrocytic

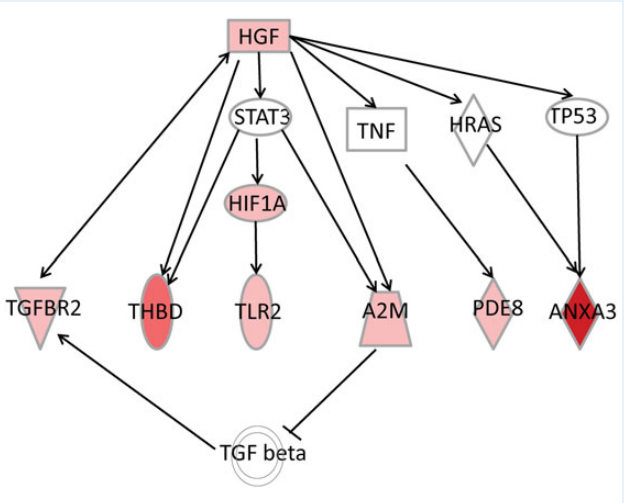

Figure 4 Ingenuity pathway analysis revealed hepatocyte growth factor (HGF) as an upstream regulator. Various downstream targets found among over-expressed genes in medium follicular cells associated with developmental competence (M+) compared to medium-sized follicles associated with poor or blocked embryonic development (M−) are also shown. Gene symbol colour (red to pink) indicates degrees of expression. Full arrow line indicates 'acts on' and arrow dotted line: 'acts on indirectly'. TLR2: toll-like receptor 2; ANXA3: annexin-3; A2M: alpha 2 macroglobulin; PDE8B: phosphodiesterase 8B; HIF1A: hypoxia induced factor 1A; TNF: tumour necrosis factor; STAT3: signal transducer and activator of transcription 3; HRAS: v-Ha-ras Harvey rat sarcoma viral oncogene homologue; TP53: tumour protein P53; TGF beta: transforming growth factor beta.

1 (SPTAN1) (fc: 9.4) (Weigand *et al.*, 2012), oxidative stress with dual oxidase 2 (DUOX2) (fc: 3.4) (Ameziane-El-Hassani *et al.*, 2005) and apoptosis, epithelial membrane protein 3 (EMP3) (fc: 3.1) (Taylor and Suter, 1996). HGF acts positively on angiogenesis, which is consistent with ANXA3 downstream over-expression (Park *et al.*, 2005). Among the genes associated with M+ and downstream HGF, TLR2 (fc: 1.7) is of potential interest from a physiological perspective. Toll-like receptors are well known to be associated with antigen detection but also have endogenous ligands (Zhang and Schluesener, 2006). TLR2 and TLR4 recognize extracellular matrix (ECM) degradation products, such as fragments of hyaluronan and heparin sulphate, a function that represents a key element in our study (Supplementary Fig. S3). Supplementary Figure S3 illustrates how the medium follicles associated with oocyte competence prepare for ovulation by programming the right context for blood coagulation, an important feature to prevent intra-abdominal bleeding and for corpus luteum formation. Also TLR2 acts positively on the production of cytokines interleukin 6 and tumour necrosis factor-alpha, which promote tissue regeneration. This positive feedback constitutes a system in which post-inflammatory wound defence and repair processes are promoted. TLR2 expression might be associated with follicles that have responded adequately to stimuli associated with ECM degradation, and hence with an appropriate inflammatory response.

With respect to inflammation, TGFBR2 (also downstream from HGF) is over-expressed in M+ compared with M− (fc: 1.8) and down-regulated in L− (fc: 1.9) (Supplementary Tables SIV and SV). The tissue repair process involving the TLR2 gene also involves hyaluronic acid and is positively regulated by members of the TGF-beta family. Some of these are produced by ovarian follicles (anti-Mullerian hormone, bone morphogenetic protein 2 (BMP2), BMP4, BMP6) and others are secreted by oocytes, for example BMP6, BMP15 and growth differentiation factors (GDFs) (Pangas, 2007). TGFBR1 acts positively on hyaluronic acid via its action on hyaluronan synthase, the enzyme necessary for hyaluronic acid synthesis. Hyaluronic acid is incorporated into the plasma membrane or into the ECM (Weigel *et al.*, 1997), acting on specific receptors such as CD44 and hyaluronan-mediated motility receptor. Hyaluronic acid is involved in ECM regulation, cellular motility, and has a positive impact on cellular proliferation, preparing the luteal cells for differentiation (Stern, 2004).

THBD, downstream from HGF, is overexpressed in follicles above a certain size and associated with developmental competence. THBD forms a complex with thrombin, which produces activated protein C by cleavage of the inactive form. Activated protein C binds to endothelial protein C receptor, which stimulates Prader–Willi/ Angelman gene region 1 (PAR1) and PAR4 receptors and G protein signalling (Riewald *et al.*, 2003; Griffin *et al.*, 2006). This system is activated in granulosa cells from pre-ovulatory follicles following the LH surge (Cheng *et al.*, 2012). Furthermore, PAR1 and PAR4 act negatively on cyclic AMP production and inhibit progesterone production (Cheng *et al.*, 2012). THBD could therefore regulate the luteinization process in M+ follicles and could be representative of an optimal pre-luteinization state 34 h hCG as in this case.

Finally, expression of another molecule downstream of HGF, the enzyme PDE8B which inactivates cyclic AMP, would have a transitory expression associated with the oocyte's developmental competence in terms of capacity to reach the morulae/blastocyst stage. The PDE8B transcript has been detected in human ovaries (Hayashi *et al.*, 1998), and found under-expressed in granulosa cells in patients with diminished ovarian reserve (Skiadas *et al.*, 2012). It appears that PDE8B could also modulate the level of luteinization in concert with THBD.

Also the role of THBD in the coagulation cascade might be important (Fig. 5). It is clear that the preparation of the follicles towards ovulation requires the appropriate anti-coagulant factors to be present and ready to be activated to promote optimal blood clotting for corpus luteum formation.

HGF downstream signalling seems to play a key role in follicle developmental competence and acts at various levels, including apoptosis reduction, ECM organization and inflammatory processes, luteinization and angiogenesis. The potential cross signalling of HGF also involves the theca cells, as HGF increased expression of the SCF (Stem cell factor c-kit) gene in granulosa cells, and SCF reciprocally increased expression of the HGF gene in theca cells (Ito *et al.*, 2001). Several molecules associated with negative regulation of the coagulation canonic pathway, notably tissue factor pathway inhibitor (TFPI) (fc: 1.6)), THBD (fc: 3.7), alpha 2 macroglobulin (A2M) (fc: 1.6), ANXA3 (fc: 36.0) and ANXA4 (fc: 1.6), are up-regulated in M+/M− follicles (Fig. 4). The anticoagulation process could facilitate the ovulation process of medium-sized follicles associated with competent oocytes. This study has allowed us to identify several potential new indicators (THBD, TLR2, PDE8B, UMODL1L, TGFBR2 and ANXA3) associated with embryo transferability. The markers identified herein may be useful for identifying the best follicles but may also serve to help assess whether or not a given cycle has created permissive conditions for oocyte competence.

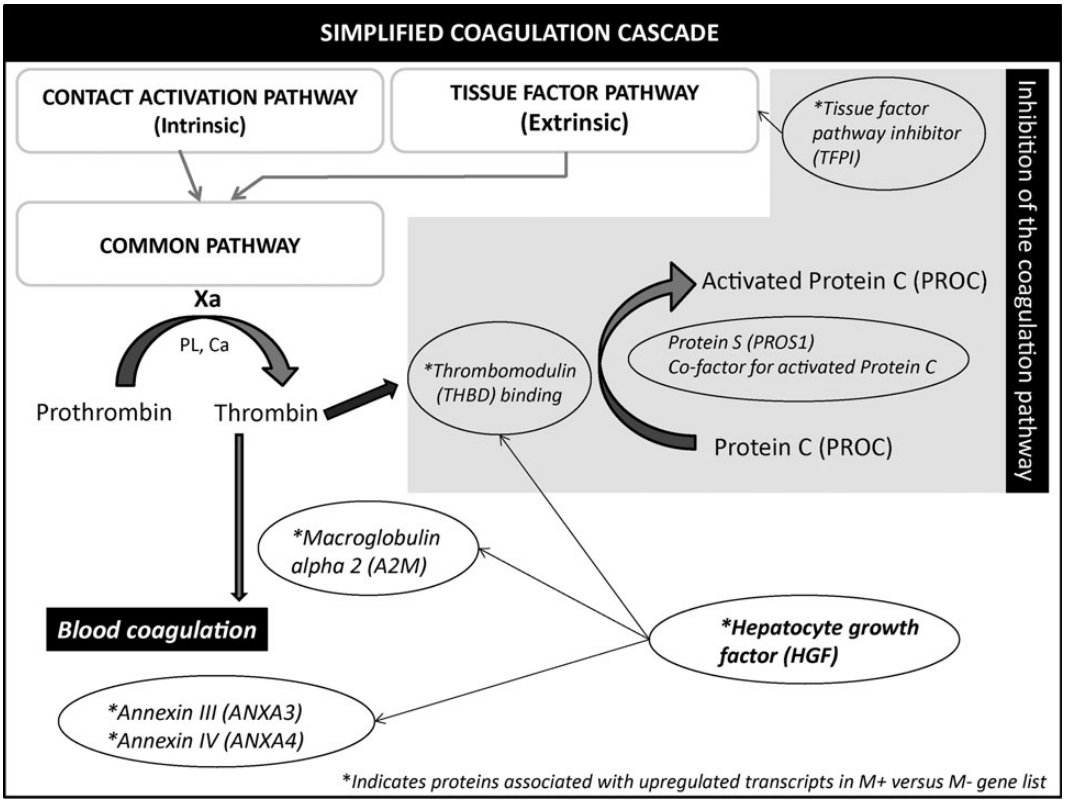

Figure 5 Schematic illustration of the coagulation pathway and its inhibitor systems. Three pathways are implicated in the classical blood coagulation: (i) intrinsic pathway (ii) extrinsic and (iii) the common pathway. The majority of the molecules identified in this study is in the common pathway. * represents transcripts over-expressed in medium follicular cells associated with developmental competence (M+/ M−). The grey box contains proteins associated with anticoagulation. Xa, factor Xa; PL, phospholipids; Ca, calcium.

Conclusions

Based on three gene expression comparisons between follicles of different volumes, this study brings important new information on the impact of follicular size on oocyte quality and the potential explanation(s) for such differences. Several biomarkers have been identified with positive and negative associations with the outcome. Moreover, a key modulator of important downstream targets, HGF, has been identified and singled out for further characterization.

Supplementary data

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

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Authors' roles

A.L.N. participated in the conception and design of the study, analysed the complete dataset, processed all the samples (RNA extraction, microarrays experiment and real-time PCR) and analysed, performed the statistical analysis, interpreted the data, and drafted the manuscript. M.C.L. participated in the conception and design of the study, participated in the coordination of the sample collection and the dataset acquisition, and revised the manuscript. A.L. participated in the conception and design of the study, participated in the coordination of the sample collection and the dataset acquisition, and revised the manuscript. M.A.S. participated in the conception and design of the study, the analysis and the interpretation of the data, and improved the manuscript. All the authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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