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MicroRNAs: new candidates for the regulation of the human cumulus–oocyte complex

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STUDY QUESTION: What is the expression pattern of microRNAs (miRNAs) in human cumulus–oocyte complexes (COCs)?

SUMMARY ANSWER: Several miRNAs are enriched in cumulus cells (CCs) or oocytes, and are predicted to target genes involved in biological functions of the COC.

WHAT IS KNOWN ALREADY: The transcriptional profiles of human MII oocytes and the surrounding CCs are known. However, very limited data are available about post-transcriptional regulators, such as miRNAs. This is the first study focussing on the identification and quantification of small RNAs, including miRNAs, in human oocytes and CCs using a deep-sequencing approach.

STUDY DESIGN, SIZE, DURATION: MII oocytes and CCs were collected from women who underwent IVF.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Using the Illumina/deep-sequencing technology, we analyzed the small RNAome of pooled MII oocytes ($n = 24$) and CC samples ($n = 20$). The mRNA targets of CC and MII oocyte miRNAs were identified using *in silico* prediction algorithms. Using oligonucleotide microarrays, genome-wide gene expression was studied in oocytes (10 pools of 19 ± 3 oocytes/each) and 10 individual CC samples. TaqMan miRNA assays were used to confirm the sequencing results in independent pools of MII oocytes (3 pools of 8 ± 3 oocytes/each) and CC samples (3 pools of 7 ± 3 CCs/each). The functional role of one miRNA, *MIR23a*, was assessed in primary cultures of human CCs.

MAIN RESULTS AND THE ROLE OF CHANCE: Deep sequencing of small RNAs yielded more than 1 million raw reads. By mapping reads with a single location to the human genome, known miRNAs that were abundant in MII oocytes (*MIR184*, *MIR100* and *MIR10A*) or CCs (*MIR29a*, *MIR30d*, *MIR21*, *MIR93*, *MIR320a*, *MIR125a* and the *LET7* family) were identified. Predicted target genes of the oocyte miRNAs were associated with the regulation of transcription and cell cycle, whereas genes targeted by CC miRNAs were involved in extracellular matrix and apoptosis. Comparison of the predicted miRNA target genes and mRNA microarray data resulted in a list of 224 target genes that were differentially expressed in MII oocytes and CCs, including *PTGS2*, *CTGF* and *BMPR1B* that are important for cumulus–oocyte communication. Functional analysis using primary CC cultures revealed that *BCL2* and *CYP19A1* mRNA levels were decreased upon *MIR23a* overexpression.

LIMITATIONS, REASONS FOR CAUTION: Only known miRNAs were investigated in the present study on COCs. Moreover, the source of the material is MII oocytes that failed to fertilize.

WIDER IMPLICATIONS OF THE FINDINGS: The present findings suggest that miRNA could play a role in the regulation of the oocyte and CC crosstalk.

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Introduction

The quality of oocytes obtained during IVF procedures varies considerably. Whilst most mature oocytes are amenable to fertilization, only half of those fertilized complete embryonic development and fewer implant. In the ovarian follicle, the maturing oocyte is nurtured and supported by cumulus cells (CCs), the surrounding somatic cells. CCs are highly specialized cells with trans-zonal cytoplasmic projections that form gap junctions at the oocyte surface (Albertini *et al.*, 2001) as part of the cumulus–oocyte complex (COC) (Cha and Chian, 1998; Goud *et al.*, 1998; Barrett and Albertini, 2010). Disruption or deregulation of the CC interactions with the oocyte can affect oocyte quality and consequently embryo development and pregnancy outcome. Much knowledge on human oocytes and CCs has been generated over recent years mainly owing to technological advances in gene expression analysis using microarray (Assou *et al.*, 2006; Gasca *et al.*, 2007; Assou *et al.*, 2009; Assou *et al.*, 2011), CGH array (Gutierrez-Mateo *et al.*, 2004; Fragouli *et al.*, 2010) and high-fidelity RNA amplification (Wood *et al.*, 2007). Such techniques have also allowed entire profiling of the transcriptional activity in single human oocytes (Grondahl *et al.*, 2010). We and others have identified several transcripts in human MII oocytes and the surrounding CCs that are crucial for oogenesis and folliculogenesis (Assou *et al.*, 2006; Kocabas *et al.*, 2006). However, the post-transcriptional regulation of oocyte and CC transcripts needs to be elucidated. This is particularly important also because the stability and translation of the maternal mRNAs, that are accumulated during oocyte maturation (Niakan *et al.*, 2012) and that drive human preimplantation development, are controlled by post-transcriptional regulatory mechanisms (Bettegowda and Smith, 2007).

Recently, it has been demonstrated that small (~19–25 nucleotides in length) endogenous non-coding transcripts, called microRNAs (miRNAs), execute key functions by silencing the expression of specific target genes in plant, animal and human genomes (Reinhart *et al.*, 2002; Lewis *et al.*, 2005; Nilsen, 2007; Krol *et al.*, 2010). In addition, miRNAs are involved in the regulation of many cellular processes, including cell proliferation, differentiation and apoptosis (Bartel, 2004). The miRNA repertoires are cell type specific and change markedly during development (Carthew and Sontheimer, 2009). Changes in miRNA expression profiles have been linked to pathologies, such as cancer (Ventura and Jacks, 2009). Moreover, miRNAs have been associated with infertility as shown in female mice in which Dicer, an essential factor in miRNA biogenesis, was genetically ablated (Murchison *et al.*, 2007; Nagaraja *et al.*, 2008). Futhermore, analysis of messenger RNA (mRNA) expression during mouse and bovine oogenesis shows that a large proportion of maternal genes are regulated by miRNAs (Tang *et al.*, 2007; Lingenfelter *et al.*, 2011). Thus, miRNA profiling might help us to better understand the regulation of transcripts involved in human reproduction.

The aim of the present study was (i) to identify and quantify small RNAs, including miRNAs, in human CCs and MII oocytes and (ii) to characterize the biological relationships between miRNAs and the mRNA expression profiles of MII oocytes and CCs.

Materials and Methods

Sample collection and processing

Human MII oocytes that failed to fertilize and CCs were collected from patients who underwent conventional IVF or ICSI. All patients signed informed consent forms. Moreover, the material used in the present study would have been discarded as all the MII oocytes used were IVF by-products.

Oocytes and CCs

MII oocytes that failed to fertilize were collected 24 h post-insemination as previously described (Assou *et al.*, 2006; Monzo *et al.*, 2012) and CCs were mechanically removed from MII oocytes before ICSI. MII oocytes were pooled for sequencing, microarray and validation by RT–qPCR. CCs were pooled for sequencing and RT–qPCR validation, whereas 10 individual CCs were used for microarray analysis (Supplementary data, Table SI). All samples were immediately transferred in 0.5 ml Eppendorf® tubes containing RLT lysis buffer (ref: 74004; Qiagen) and frozen at −80°C.

RNA extraction

The RNeasy Micro Kit (ref: 74004; Qiagen) was used to isolate both total and small RNAs from MII oocytes and CCs. Small RNA was extracted as described in the manufacturer's protocol for the RNeasy Micro Kit (ref: 74004; Qiagen) with the following modifications: the lysate/RLT was equilibrated at 37°C for a few minutes prior to RNA purification, and after addition of the carrier RNA and lysis (0.2 ng/μl), 1.5 volumes of 100% ethanol were added to the lysates instead of one volume of 70% ethanol. Total RNA (5 ng/μl for oocytes and 2 μg/μl for CCs) was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop ND-Thermo Fisher Scientific, Wilmington, DE, USA) and its integrity assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA, http://www.agilent.com).

Preparation of the small RNA cDNA libraries and sequencing

Small RNA cDNA libraries were prepared according to the Illumina's V1.0 protocol. The 5′ RNA adaptor (5′-GUUCAGAGUUCUACAGUCCGA CGAUC-3′) was ligated to the oocyte and CC small RNA pools (Supplementary data, Table SI) with 1 μl of T4 RNA ligase (10 U/μl) (ref: M0242L; NEB) in the presence of RNase Out (ref: 10777-019; Invitrogen) overnight at 25°C. The ligation reaction was stopped by addition of 2× formamide loading dye and size fractionated on a 15% TBE urea polyacrylamide gel. The 40–60 base pair fraction (RNA plus 5′ adaptor) was excised and the RNA was eluted by incubating the gel slice at 4°C overnight in 600 μl NaCl 0.3 M, precipitated and suspended in DEPC-treated water. The 3′ RNA adapter (5′-pUC GUAUGCCGUCUUCUGCUUGidT-3′; p, phosphate; idT, inverted deoxythymidine) was then ligated to the RNA at 25°C overnight with T4 RNA ligase (NEB) in the presence of RNase Out (Invitrogen). The RNA with the 5′ and 3′ adaptors was size fractionated on a 10% TBE urea polyacrylamide and the 60–100 base pair RNA fraction was extracted as described above. Superscript II reverse transcriptase (Invitrogen) was used to reverse transcribe the RNA using the Illumina small RNA RT-Primer (5′-CAAGCA GAAGACGGCATACGA-3′). The resulting cDNA was submitted to 15 amplification cycles using Hotstart Phusion DNA Polymerase (NEB) and the Illumina small RNA primer set (5′-CAAGCAGAAGACGGCAT ACGA-3′; 5′-AATGATACGGCGACCACCGA-3′). After purification on a 12% TBE urea polyacrylamide gel, the PCR products were eluted in buffer (5:1, 7.5 M ammonium acetate) at 4°C overnight. The resulting gel slurries were submitted to Spin-X filters (Corning) to purify the PCR products before ethanol precipitation and pellet suspension in water. The DNA was quantified using an Agilent DNA 1000 chip and diluted to 10 nM for sequencing using an Illumina 1G sequencer.

Complementary RNA preparation and microarray processing

Total RNA samples from 10 pools of MII oocytes (2.7 ± 1.4 ng/μl per pool) (Supplementary data, Table SI) were subjected to two rounds of linear amplification according to the manufacturer's 'double amplification' protocol (two-Cycle cDNA Synthesis Kit; Invitrogen). Labeled fragmented cRNA was hybridized to HG-U133 plus 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA, USA) as described in Assou *et al.* (2006, 2009; Monzo *et al.* 2012). Total RNA (50 ng) from 10 individual CCs was used to prepare cRNA using the Affymetrix 3′ IVT express protocol (ref.901229) as described in (Ouandaogo *et al.*, 2011). After fragmentation, the labeled anti-sense aRNA (15 μg) was hybridized to HG-U133 plus 2.0 GeneChip arrays (Affymetrix™). Each CC sample was processed individually on a microarray chip.

Data processing and gene expression profile analysis

After image processing using the Affymetrix Microarray Suite 5.0, the .CEL files were analyzed using the Affymetrix Expression Console™ software and normalized with the MAS5.0 algorithm by scaling each array to a target value of 100 using the global scaling method to obtain an intensity value signal for each probe set. Gene annotation was performed using NetAffx (http://www.affymetrix.com; March 2009). Genes with significant differential expression profiles between MII oocytes and CC samples were identified using the significance analysis of microarray (SAM) algorithm (http://www-stat.stanford.edu/~tibs/SAM/), which utilizes a Wilcoxon test statistic and sample-lable permutation to evaluate statistical significance between sample groups. SAM provides mean fold change values (FC > 2) and a false discovery rate (FDR $< 5\%$) confidence percentage based on data permutation ($n = 300$). Hierarchical clustering was carried out with CLUSTER and TREEVIEW software (Eisen *et al.*, 1998).

Small RNA annotation and deep-sequencing data analysis pipeline

First, short sequences of 19–22 nucleotides (nt) in length were independently analyzed as described in Philippe *et al.* (2009). Briefly, reads were mapped with the CRAC software and reads with a single location were annotated with a double-step process according to the ENSEMBL Genome Browser (Ensembl API version 66, http://www.ensembl.org/index.html): the distribution of reads relative to protein coding genes (exonic, intronic or intergenic part) and the distribution in non-coding regions (Fig. 1A). Then, all the annotated non-coding transcripts were compiled to specify their frequency and distribution. Finally, the GeneGo MetaCore pathway analysis software (St. Joseph, MI), which provides predicted validated targets for known miRNAs, was used for miRNA target prediction.

Taqman miRNA assays

Complementary DNA was synthesized from total RNA from pooled MII oocytes or CCs (Supplementary data, Table SI) using the TaqMan miRNA-specific primers, *LET7b*, *MIR21*, *MIR30d*, *MIR184* and *MIR10A* (ref: #4427975, Life Technologies), according to the TaqMan MicroRNA RT protocol (Applied Biosystems). For reverse transcription, 5 μl (10 ng) of RNA sample, 0.15 μl (100 mM) dNTPs, 1 μl of 50 U μl^{-1} MultiScribe reverse transcriptase, 1.5 μl 10× RT buffer, 0.19 μl of 20 U μl^{-1} RNase inhibitor and 3 μL of 50 nM stem-loop RT primer (all from the TaqMan MicroRNA Reverse Transcription Kit; Applied Biosystems) were used. Reaction mixtures (15 μl) were incubated first at 16°C for 30 min and then at 42°C for 30 min, inactivated at 85°C for 5 min and then stored at 4°C. Quantitative PCR was performed using a Roche LightCycler 480 apparatus. The 10 μl PCR reaction mixtures included 4 μl of RT product, 4.5 μl 2× TaqMan (AmpErase UNG) Universal PCR Master Mix and 0.5 μl of primer and probe mix from the TaqMan MicroRNA Assay kit (ref: 4324018; Applied Biosystems). Reaction mixtures were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. *RN U6-1* was used as reference gene for normalization of the miRNA expression levels. This endogenous gene control showed a stable expression pattern between CCs and oocyte samples. The relative expression levels of target miRNAs were determined by using the equation $2^{-\Delta CT}$, in which ΔC_T were calculated as follows:

$$\Delta C_T = C_{T\,\text{miRNA of interest}} - C_{T\,\text{RN U6-1}}$$

CC culture and miRNA transfection

The fresh CCs were mechanically separated from the oocyte by using two needles. One needle was placed on the CC layer to keep the oocyte in place. The other needle was used to quickly cut off as much as possible of the cell layer, without damaging the oocyte. The CC clumps were transferred into a dish coated with 10 $\mu g/cm^2$ type I–III human collagen (in alpha-MEM medium) and cultured in serum-free medium (SPE-IV/EBM). At confluence, cells were washed by PBS and detached with TrypLE™ Select (3436D; Life Technologies) treatment for 5 min at 37°C. They were then seeded onto new culture dishes treated by human collagen I–III for expansion. The following experiments were performed on a primary culture of CCs obtained from one patient at passage 3 (P3). These cells were cultured in 100 mm culture dishes with an estimated plating density of 2.5×10^5 cells/well. When cells reach 50–60% confluence after 2 days in culture, they were transfected with 5 μg of wild-type *MIR23a* locus or *MIRD23* (mutated *MIR23a* locus that expresses only *MIR24* and *MIR27a*) cloned into the MIE retroviral vector using jetPEI® (www.polyplus-transfection.com) (Rathore *et al.*, 2012). The miR-23 constructs were tagged with GFP in order to assess the transfection efficiency, which ranges between 70 and 80%. Total RNA was extracted 48 h after transfection to perform quantitative RT–PCR analysis. The transfection experiments were repeated three times on P3 CC cultures and the qRT–PCR experiments were performed in triplicate. We verified that the P3 cells used in the above experiment retained the properties of CCs at the first passage (P1) by RT–qPCR analysis of three genes known to be linked to CC function, namely *AREG*, *STAR* and *PTX3*. It was reported in granulosa cell (GC) primary cultures that the cells retained their properties up to four passages (Brůcková *et al.*, 2008).

Quantitative PCR for mRNA

Total RNA derived from the primary CC cultures was reverse transcribed in a final volume of 20 μl with the SuperScript® First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed in 384-well plates (Sorenson BioScience, Inc.) on a Lightcycler® 480 Real-Time PCR System (Roche Diagnostics) using a reaction mix (final volume of 10 μl) that contained 2 μl cDNA, 5 μl SybrGreen (Roche Diagnostics) and 0.5 μM forward and reverse primers (*BAX*, forward primer 5′-CCAGCTGCCTTGGACTGT-3′ and reverse primer 5′-ACCC CCTCAAGACCACTCTT-3′; *BCL2*, forward primer 5′-GGCTGATATT CTGCAACACTG-3′ and reverse primer 5′-GGCAATGTGACTTTTT CCAA-3′; *CYP19A1*, forward primer 5′-TGCAAAGCACCCTAATGTTG-3′ and reverse primer 5′-TTTGTCCCCTTTTTCACTGG-3′) and the following conditions: incubation at 95°C for 10 min, then 40 cycles of 10 s at 95°C, 20 s at 63°C and 25 s at 72°C. At the end, a melting curve from 95 to 63°C was performed to control primer specificity. The geometric mean of the *GAPDH* showed stable expression pattern across the treatment groups that were compared. Therefore, *GAPDH* was used as endogenous control.

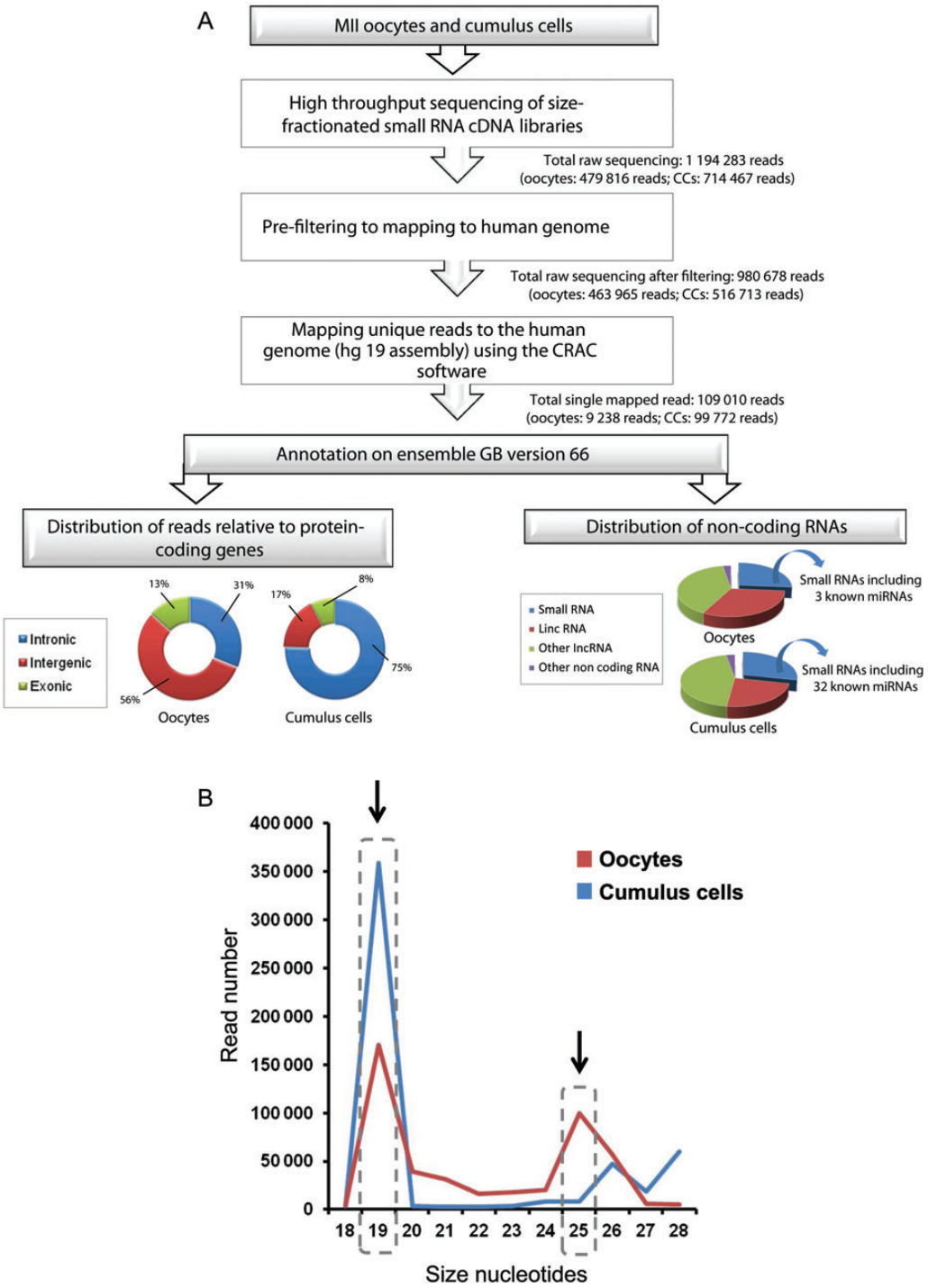

Figure 1 Computational pipeline for the analysis of the deep-sequencing data. (**A**) Small RNA cDNA libraries from human MII oocytes, and CCs were subjected to deep sequencing. Raw sequence reads were filtered, mapped to the reference human genome using the CRAC software and annotated according to the ENSEMBL Genome Browser (Ensembl API version 66). The location (intergenic, intronic or exonic) of reads that perfectly mapped to a single location in the human genome was determined. The distribution of the non-coding RNA classes, including small RNA, large intergenic non-coding RNAs (lincRNAs), other large non-coding RNAs (lncRNAs) and other small RNAs in the MII oocyte or CC genomes is given as a percentage of all mapped tags. The small RNA category (blue) contains known miRNAs. (**B**) Size distribution of the sequence reads. The two histograms show the length distribution of small RNAs in the MII oocyte and CC libraries.

Statistical analysis

The quantitative PCR results were expressed as the mean ± standard error of mean. One-way ANOVA was used for multiple comparisons, using SPSS for Windows (SPSS 16.0, SPSS Inc., Chicago, USA) and *t*-test was used for comparison between two groups; $P < 0.05$ was considered as significant.

Results

Deep sequencing of the small RNAome of human MII oocytes and CCs

Deep sequencing of the two small RNA cDNA libraries from human MII oocytes and CCs produced over 1 million separated reads. The pipeline approach used to analyze the sequences and identify known miRNAs is summarized in Fig. 1A. Most reads had an intronic (75% of CC and 31% of MII oocyte reads) or intergenic (56% of MII oocyte reads) location (Fig. 1A). In both libraries, reads showed a size distribution peak at 19 nt (Fig. 1B) that corresponded to miRNAs and represented 22% of the reads in the CC library. Reads of the MII oocyte library had also a second size distribution peak at around 25–27 nt that should correspond to Piwi-interacting RNA (pi-RNA)-like sequences (26–31 nt in size) (Fig. 1B).

miRNAs expression in human MII oocytes and CCs

Only three known miRNAs were identified in the MII oocyte library, whereas the number of known miRNAs increased to 32 in the CC library (Table I). The most abundant miRNAs in CCs were *LET7b* (51 reads), *LET7c* (31 reads) and *MIR21* (28 reads). In MII oocytes, the most abundant miRNAs were *MIR184* (1988 reads) and *MIR10A* (555 reads). To validate the sequencing data, the relative expression levels of five randomly selected miRNAs was assessed in independent pools of mature MII oocytes and CCs (Supplementary data, Table SI) by RT-quantitative PCR. The results were in accordance with the sequencing data (Fig. 2).

Identification of miRNA targets and their function

Using the GenGo Metacore software, we found that 30 mRNAs, predicted and experimentally validated by other laboratories to have roles in transcription regulation and cell cycle, were targeted by the three miRNAs identified in the MII oocyte library (*MIR184*, *MIR100* and *MIR10A*) (Supplementary data, Table SII). For instance, *SMARCA5* (*SWI/SNF*-related matrix associated actin-dependent regulator of chromatin, subfamily a member 5) was shown to be a target of *MIR100* (Bhushan and Kandpal, 2011); interestingly *SMARCA5* may be implicated in oocyte reprogramming (Assou *et al.*, 2009). *NCOR2* (nuclear receptor co-repressor 2), which mediates the transcriptional repression activity of nuclear receptors, was a target of *MIR184* (Wu *et al.*, 2011). Finally *HOXA1*, a homeobox gene whose mRNA is abundant in oocytes was targeted by *MIR10A* (Lund, 2010); it is noteworthy that *HOXA1* is essential for the regulation of oocyte-specific gene expression in the mouse (Huntriss *et al.*, 2006). The 32 miRNAs enriched in CCs (Supplementary data, Table SIII) targeted 538 mRNAs experimentally validated mRNAs by other laboratories to be involved in several biological functions, including cell assembly and organization, development, cell death and survival. The CC miRNAs with the highest number of predicted mRNA targets were *MIR21* ($n = 115$), *MIR29a* ($n = 54$) and *MIR23* ($n = 30$). Moreover, more than 12% of the predicted mRNA targets were regulated by more than one miRNA. For instance, *HMGA2* (high mobility group AT-hook 2) was targeted by six CC miRNAs (*LET7b/7d/7e/7g/7i* and *MIR30a*), *PTEN* (phosphatase and tensin homolog) by five (*MIR93*, *MIR29a*, *MIR23*, *MIR21* and *MIR132*), *LIN28* by five (*MIR30a*, *MIR30d*, *MIR30e*, *MIR125a* and *LET7b*), *TGFBR3* (TGF-beta receptor type III) by four (*MIR21*, *MIR93*, *MIR23* and *LET7c*) and *ESR1* (estrogen receptor 1) also by four (*MIR93*, *MIR146b*, *LET7i* and *LET7b*) (Supplementary data, Table SIII). As a preliminary experiment to assess the effect of *MIR23a* on a predicted target, we analyzed the impact of its forced expression on *BCL2* mRNA level in primary cultures of human CCs isolated from mature COCs. *BCL2* mRNA level decreased upon *MIR23a* overepression as did the *CYP19A1* mRNA. In contrast, BAX mRNA that is not predicted as a target did not display a decrease but rather a significant increase (Fig. 3). It is worth mentioning that *BAX* is a pro-apoptotic gene, whereas *BCL2* is anti-apoptotic. Further experiments are required to propose a mechanism to account for these results.

Genes that are differentially expressed in oocyte and CCs are predicted targets of oocyte and CC miRNAs

To explore the biological relationships between miRNAs and the mRNA expression profiles of MII oocytes and CCs, microarray analyses were performed using 10 pools of MII oocytes or 10 individual CCs (Supplementary data, Table SI). Using SAM (with a fold change ≥2 and FDR < 1%), we identified a total of 10 169 genes that were differentially expressed in the two groups. Overall, 4207 genes were specifically up-regulated in MII oocytes and 5962 genes were up-regulated in individual CCs (Supplementary data, Tables SIV and SV). Comparison of these differentially expressed mRNAs with the predicted miRNA target genes resulted in a list of 224 genes (Supplementary data, Table SVI) that included genes that were up-regulated in MII oocytes and significantly down-regulated in CCs, such as *CDC25A* (cell division cycle 25 homolog A), a key regulator of oocyte meiosis (fold: 53; FDR = 0; target of *MIR21*, *MIR424* and *LET7b*), and genes associated with chromatin remodeling, such as DNA methyltransferase *DNMT3B* (fold: 58; FDR = 0; a target of *miR-29a*), *DNMT1* (fold: 36; FDR = 0; a target of *MIR21*), *DNMT3A* (fold: 6; FDR = 0; a target of *MIR29a*) and *SMARCA5* (fold: 5; FDR = 0; a target of *MIR100*). The expression of some CC-specific genes known to be regulated by the oocyte-secreted paracrine factor *GDF9* (Growth differentiation factor 9), such as prostaglandin-endoperoxide synthase 2 *PTGS2* (fold: 93; FDR = 0) and the connective tissue growth factor *CTGF* (fold: 38; FDR = 0), were predicted targets of *MIR542* and *MIR21*, respectively, and bone morphogenetic protein receptor *BMPR1B* (fold: 18; FDR = 0) was a predicted target of *MIR21* (Fig. 4A). Additionally, *MIR21* was predicted to target many transcription factors up-regulated in CCs, such as nuclear factor *NFIB* (fold: 49; FDR = 0), myocyte enhancer factor *MEF2C* (fold: 33; FDR = 0) and retinoid X receptor alpha *RXRA* (fold: 4; FDR = 0) and *MIR29a* was predicted to target several extracellular matrix (ECM) genes up-regulated in CCs, such as collagen *COL4A1* (fold: 52; FDR = 0), *COL3A1* (fold: 6; FDR = 0), *COL1A1* (fold: 24; FDR = 0) and *COL1A2* (fold: 9; FDR = 0). Moreover, many pro-apoptotic genes that were up-regulated in CCs, including

Table I Putative miRNAs identified in human CCs and MII oocytes

Micro RNAs	Ensembl ID	Occ.	Sequence	Chr.	Location
CCs					
hsa-LET7b	ENSG00000207875	51	TGAGGTAGTAGGTTGTGTG	22	46 509 570
hsa-LET7c	ENSG00000199030	31	TGAGGTAGTAGGTTGTATG	21	17 912 157
hsa-MIR21	ENSG00000199004	28	TAGCTTATCAGACTGATGT	17	57 918 633
hsa-MIR182	ENSG00000207990	7	CAATGGTAGAACTCACACT	7	129 410 286
hsa-MIR30d	ENSG00000199153	5	TGTAAACATCCCCGACTGG	8	135 817 164
hsa-MIR99b	ENSG00000207550	4	CACCCGTAGAACCGACCTT	19	52 195 870
hsa-MIR320a	ENSG00000208037	4	GCTGGGTTGAGAGGGCGAA	8	22 102 486
hsa-MIR132	ENSG00000207724	4	TAACAGTCTACAGCCATGG	17	1 953 225
hsa-MIR191	ENSG00000207605	3	CAACGGAATCCCAAAAGCA	3	49 058 108
hsa-MIR146b	ENSG00000202569	3	TGAGAACTGAATTCCATAG	10	104 196 276
hsa-MIR93	ENSG00000207757	2	CAAAGTGCTGTTCGTGCAG	7	99 691 441
hsa-MIR744	ENSG00000211589	2	TGCGGGGCTAGGGCTAACA	17	11 985 225
hsa-MIR508	ENSG00000207589	2	TGATTGTAGCCTTTTGGAG	X	146 318 466
hsa-MIR30a	ENSG00000207827	2	TGTAAACATCCTCGACTGG	6	72 113 300
hsa-MIR23	ENSG00000207563	2	ATCACATTGCCAGGGATTA	9	97 847 546
hsa-MIR140	ENSG00000208017	2	ACCACAGGGTAGAACCACG	16	69 967 045
hsa-LET7i	ENSG00000199179	2	TGAGGTAGTAGTTTGTGCT	12	62 997 470
hsa-LET7g	ENSG00000199150	1	TGAGGTAGTAGTTTGTACA	3	52 302 354
hsa-LET7e	ENSG00000198972	1	TGAGGTAGGAGGTTGTATA	19	52 196 045
hsa-LET7d	ENSG00000199133	1	AGAGGTAGTAGGTTGCATA	9	96 941 122
hsa-MIR542	ENSG00000207784	1	TGTGACAGATTGATAACTG	X	133 675 396
hsa-MIR425	ENSG00000199032	1	TGACACGATCACTCCCGTT	3	49 057 633
hsa-MIR424	ENSG00000199097	1	CAGCAGCAATTCATGTTTT	X	133 680 712
hsa-MIR379	ENSG00000199088	1	TGGTAGACTATGGAACGTA	14	101 488 407
hsa-MIR30e	ENSG00000198974	1	TGTAAACATCCTTGACTGG	1	41 220 042
hsa-MIR29a	ENSG00000198981	1	TAGCACCATCTGAAATCGG	7	130 561 509
hsa-MIR25	ENSG00000207547	1	CATTGCACTTGTCTCGGTC	7	99 691 196
hsa-MIR210	ENSG00000199038	1	CTGTGCGTGTGACAGCGGC	11	568 114
hsa-MIR202	ENSG00000199089	1	GAGGTATAGGGCATGGGAA	10	135 061 041
hsa-MIR197	ENSG00000207709	1	CACCACCTTCTCCACCCAG	1	110 141 563
hsa-MIR183	ENSG00000207691	1	TATGGCACTGGTAGAATTC	7	129 414 809
hsa-MIR125a	ENSG00000208008	1	TCCCTGAGACCCTTTAACC	19	52 196 520
MII oocytes					
hsa-MIR184	ENSG00000207695	1988	TGGACGGAGAACTGATAAG	15	79 502 181
hsa-MIR10A	ENSG00000207777	555	TACCCTGTAGATCCGAATTTGT	17	46 657 266
hsa-MIR100	ENSG00000207994	38	AACCCGTAGATCCGAACTT	11	122 022 985

Occ, occurrence; Chr, chromosome.

BCL2-associated X protein *BAX* (fold: 14; FDR = 0), caspase *CASP7* (fold: 6; FDR = 0) and *CASP3* (fold: 2; FDR = 0), were predicted targets of the *LET7* family (*LET7b*/7d/7g/7e), *MIR30d* and *MIR29a*. Anti-apoptotic genes that were up-regulated in CCs, such as myeloid cell leukemia sequence *MCL1* (fold: 23; FDR = 0) and *BCL2* (fold: 4; FDR = 0), were predicted to be targeted by *MIR29a*, *MIR125a*, *MIR21* and *MIR30e*, whereas *BIRC5/Survivin* (fold: 7; FDR = 0), which was up-regulated in MII oocytes, was targeted by both *MIR542* and *MIR320a*. To visually assess the differentially expressed predicted miRNA target genes in CC and oocyte samples, we performed a supervised hierarchical clustering analysis (Fig. 4B).

Discussion

In this work we investigated the miRNA content of human MII oocytes and CCs. Analysis of the sequencing data from the two small RNA cDNA libraries indicates that overall the miRNA composition of the two cell types is different. Notably, the number of sequences

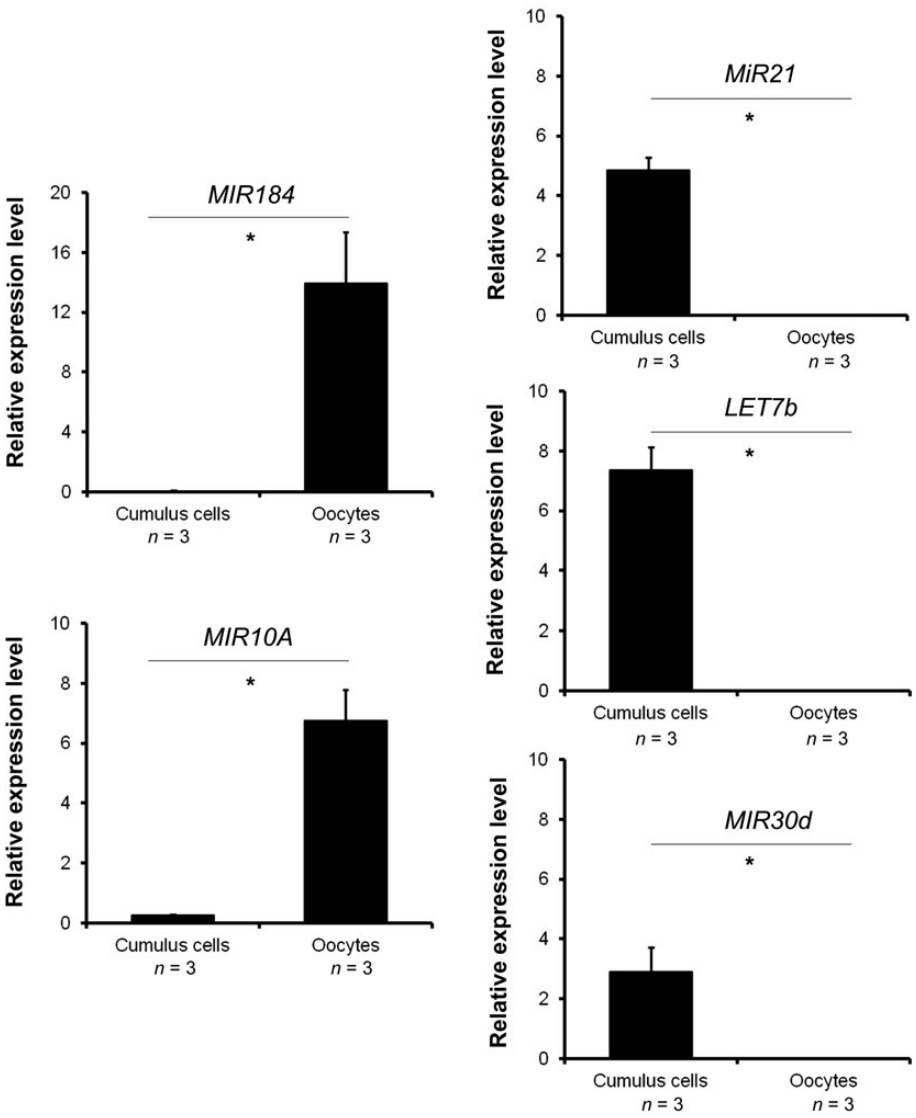

Figure 2 Relative expression levels of *MIR21*, *MIR30d*, *LET7b*, *MIR184* and *MIR10A* in MII oocytes and cumulus cells (CCs) by qRT–PCR. The transcript levels were calculated relative to the expression of RN *U6-1*. The mean ± SEM for each sample is presented in the bar graphs. Asterisk = $P < 0.01$.

corresponding to known miRNAs was higher in the CC library than in the mature MII oocyte library. This is consistent with the hypothesis that in germ cells, the number of expressed miRNAs is stage specific and decreases during development while the number of piRNAs increases (Malone *et al.*, 2009; Faunes *et al.*, 2012). Accordingly, the two size distribution peaks for the small RNA sequences of the MII oocyte library suggest that the transition of a major small RNA class from miRNA to piRNA might play a crucial role in the human oocyte–cumulus crosstalk, in line with previous findings (Girard *et al.*, 2006; Ohnishi *et al.*, 2010; Suh *et al.*, 2010; Tam *et al.*, 2008; Yang *et al.*, 2012a). Moreover, comparison of the miRNA expression profiling data and the list of target mRNAs that were differentially expressed in MII oocytes and CCs indicated that many genes that are up-regulated in MII oocytes are potential targets of CC miRNAs, thus suggesting that the oocyte–CC crosstalk might be mediated also via miRNAs. In addition, miRNAs have been reported to show a dynamic change during oocyte maturation in the mouse (Tang *et al.*, 2007), bovine (Tesfaye *et al.*, 2009; Mondou *et al.*, 2012) and human (Xu *et al.*, 2011).

Expression profiling of miRNA by deep sequencing indicated that *MIR100*, *MIR184* and *MIR10A* are specifically expressed in human MII oocytes. These miRNAs appear to be involved in the regulation of gene transcription, cell cycle and oocyte reprogramming. Interestingly, the comparison of our data on human oocytes with those reported for miRNA profiles in oocytes from other species (mouse, bovine and human) indicates that *MIR10* and *MIR100* are restricted to human and bovine oocytes (Tang *et al.*, 2007; Abdel El Naby *et al.*, 2013). It is worth noting that these miRNAs of interest are not affected by using MII oocytes that have failed to fertilize as source of material.

In human CCs, the *LET7* miRNA family is the most abundant miRNA cluster and *let-7b* is the most abundant individual miRNA. This supports previous miRNA expression profile studies that identified the *LET7* miRNA family as abundantly expressed in mouse and bovine ovaries

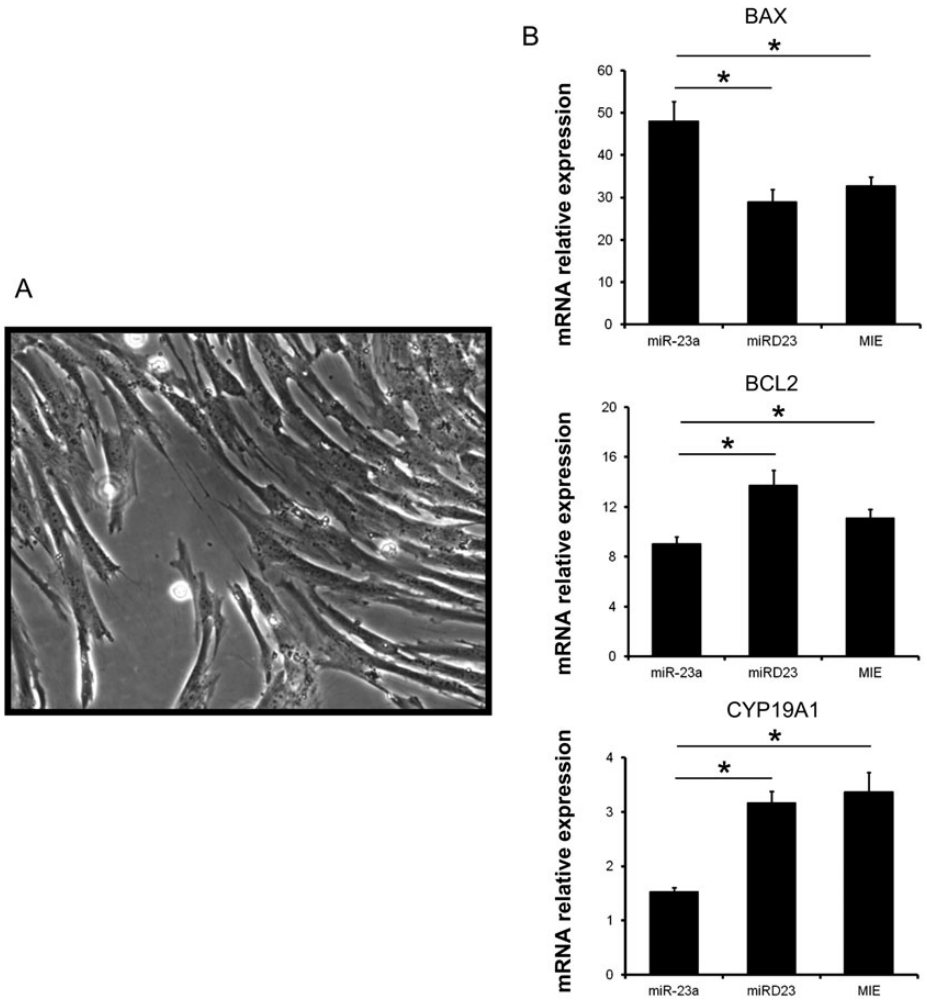

Figure 3 Biological effects of *MIR23a* in cultured CCs. (**A**) Phase contrast micrographs of primary CC cultures used as a model to test the functionality of *miR-23a*. (**B**) Primary CCs (P3) were transfected with the wild-type *MIR23a* locus, *MIRD23* (locus without *MIR23a*) or the MIE retroviral vector alone. 48 h after transfection, the expression of *BAX, BCL2* and *CYP19A1* was checked by qRT–PCR. Asterisk, $P \leq 0.05$. The transfection experiments were repeated three times and the qRT–PCR experiments were performed in triplicate.

(Reid *et al.*, 2008; Wyman *et al.*, 2009; Miles *et al.*, 2012) and in developing human ovary (Childs *et al.*, 2012). Additionaly, the comparison between our study on human CCs and previously published study on bovine CCs (Abdel El Naby *et al.*, 2013) reveals that *LET7b*, *LET7c LET7g MIR210* and *MIR125a* are expressed in both.

Globally, miRNAs that are abundant in CCs appear to be associated with the regulation of ECM and apoptosis. ECM formation through the production of ECM components by CCs is an important process that determines oocyte maturation and fertilization (Russell and Salustri, 2006; Dunning *et al.*, 2012). Analysis of the microarray data from individual CCs revealed that collagen genes (*COL4A1*, *COL4A5*, *COL3A1*, *COLA2*, *COL1A1*) are up-regulated in CCs compared with MII oocytes and are predicted to be *MIR29a* targets. Among the various biological functions of this miRNA, there is also the regulation of the expression of ECM components in different organs, including heart, lung, kidney and liver (van Rooij *et al.*, 2008; Jiang *et al.*, 2010; Cushing *et al.*, 2011). It is clear that the biological functions of *MIR29a* are complex, but the direct participation of this miRNA in regulating the expression of ECM components in human CCs needs further investigation.

CC apoptosis may compromise the oocyte developmental competence (Lee *et al.*, 2001) and elevated CC apoptosis has been associated with oocyte maturation delay and poor pregnancy outcome (Lee *et al.*, 2001; Host *et al.*, 2002). Here we show that the anti-apoptotic *BCL2*, *MCL1* and the pro-apoptotic *BAX*, *CASP3* and *CASP7* are up-regulated in CCs and are predicted targets of several CC miRNAs, suggesting a role for miRNAs in the regulation of apoptosis in COCs. Our findings also indicate that some miRNAs can target genes with opposite functions. For instance, *MIR29a* appeared to target both anti-apoptotic (*BCL2* and *MCL1*) and pro-apoptotic (*CASP7*) genes, suggesting that it may play roles in pathological conditions that might lead to ovarian failure. A recent study showed that *MIR23a* induces apoptosis in human GC (Yang *et al.*, 2012b). Similarly, we observed that after transient transfection of the whole *MIR23a* locus in primary cultures of CCs isolated from mature COCs, the mRNA expression of the anti-apoptotic

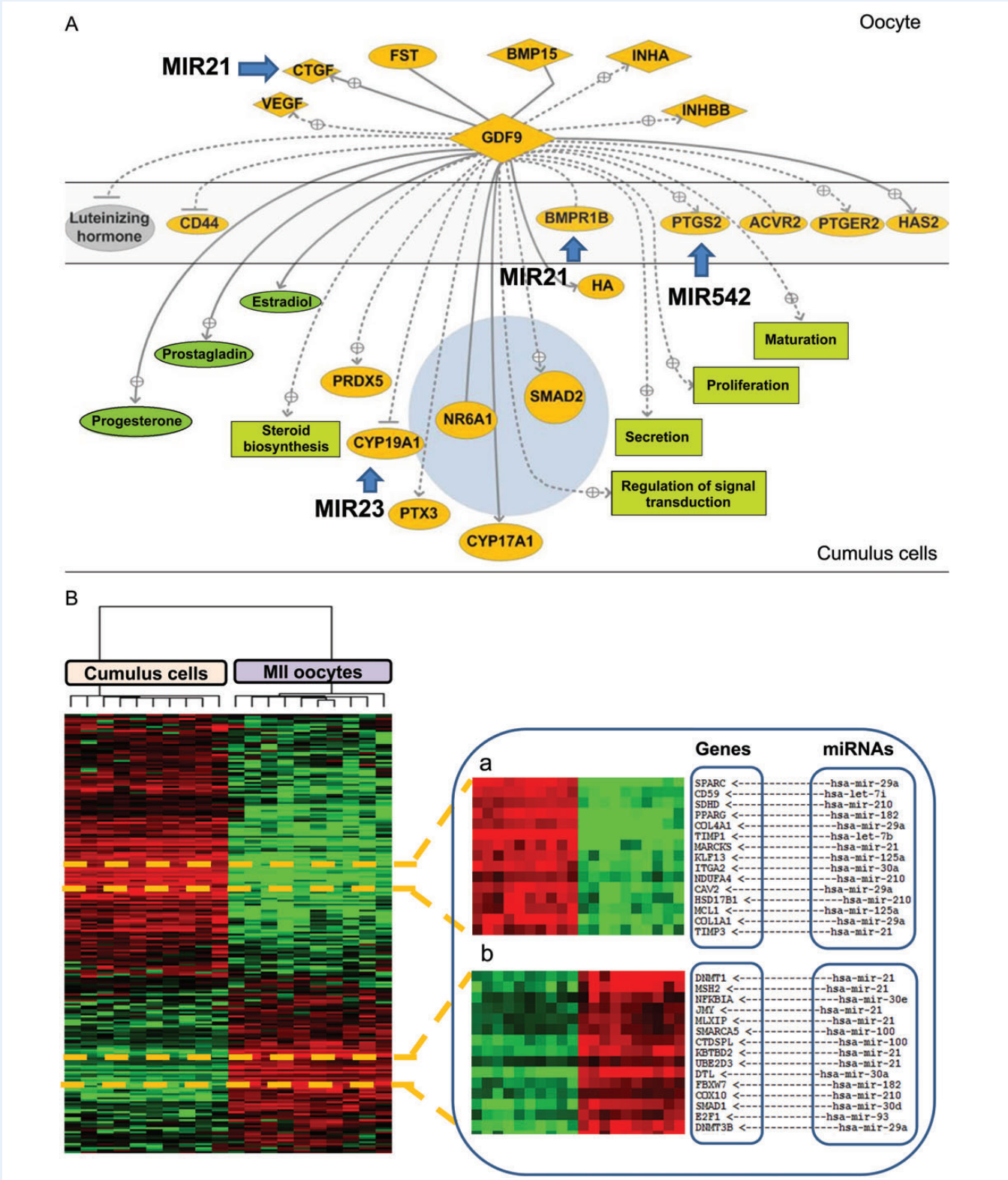

Figure 4 (**A**) Schematic representation of the components of the *GDF9* signaling pathway and targeting CC miRNAs. Some CC-specific genes known to be regulated by the oocyte-secreted paracrine factor (*GDF9*) (Elvin *et al.*, 1999), such as prostaglandin endoperoxide synthase 2 [*PTGS2* or cyclooxygenase-2 (*COX-2*)] and connective tissue growth factor (*CTGF*), are predicted targets of miRNAs that were identified in the CC small RNA cDNA library (*MIR542* and *MIR21*, respectively) (Adam *et al.*, 2012; Moore *et al.*, 2012). (**B**) Heat map representation of the 224 genes that are differentially expressed in MII oocytes and CCs and that are potential targets of the miRNAs identified by deep sequencing. (a) Cluster of targeted genes that are up-regulated in CCs but not MII oocytes. (b) Cluster of targeted genes that are highly expressed in MII oocytes but not CCs. Over-expression (red) and under-expression (green). Rows, genes; columns, profiled samples. The detailed list of the target genes and miRNAs is in Supplementary data, Table VI.

BCL2 was reduced and that of the pro-apoptotic *BAX* was increased in comparison with CC cultures transfected with the *MIRD23* construct or with control vector (MIE). Additionally, *MIR21*, which was one of the most abundant miRNAs in the CC library, has a critical role in maintaining the survival of GC in periovulatory follicles in response to luteinizing hormones and acts as an anti-apoptotic factor in cultured murine GC (Fiedler *et al.*, 2008; Carletti *et al.*, 2010; Hennebold, 2010). All these results suggest that both *MIR21* and *MIR23* play an important role in controlling transcripts that are involved in the ovulatory follicle apoptosis. Understanding the mechanisms through which the *MIR21*, *MIR23* and *MIR29a* affect CC apoptosis may help to explain their potential role in the pathogenesis of ovarian failure (Yang *et al.*, 2012b).

Finally, our study shows that several DNA methyltransferases (*DNMT*s) are up-regulated in MII oocytes are targeted by *MIR29a* and *MIR21* (Fabbri *et al.*, 2007; Zhang *et al.*, 2011), two miRNAs which were identified in the CC miRNA library. This suggests a CCs–oocyte miRNA trafficking possibly via gap junctions and points to novel functions for miRNAs in the COC crosstalk. Moreover, alteration of the *MIR21* or *MIR29a*-dependent regulation of *DNMT* expression could be one of the key molecular events leading to abnormal DNA methylation in oocytes and could be associated with a decrease in reproductive potential (Yue *et al.*, 2012).

Conclusion

This study provides the first characterization of the miRNA profile in human CCs and MII oocytes using a deep-sequencing approach combined with genome-wide gene expression arrays. The present findings suggest that miRNAs could play a role in the regulation of oocyte and CC cross-talk.

Supplementary data

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

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Authors' roles

S.A. conceived, designed and performed the experiments, analyzed and interpreted the data and wrote the paper; T.A. and D.P. performed the experiments; D.H., NP., C.H.L., D.P., T.C. and H.D. contributed to the data analysis; O.A. contributed to data interpretation, paper redaction and manuscript revision. S.H. conceived the study, analyzed and interpreted the data, wrote the paper and gave final approval.

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Conflict of interest

The authors of the study have no conflict of interest to report.

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