—Original Article—

Mouse oocytes suppress miR-322-5p expression in ovarian granulosa cells

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Abstract. This study tested the hypothesis that oocyte-derived paracrine factors (ODPFs) regulate miRNA expression in mouse granulosa cells. Expression of mmu-miR-322-5p (miR-322) was higher in mural granulosa cells (MGCs) than in cumulus cells of the Graafian follicles. The expression levels of miR-322 decreased when cumulus cells or MGCs were co-cultured with oocytes denuded of their cumulus cells. Inhibition of SMAD2/3 signaling by SB431542 increased miR-322 expression by cumulus-oocyte complexes (COCs). Moreover, the cumulus cells but not the MGCs in $Bmp15^{-/-}/Gdf9^{+/-}$ (double-mutant) mice exhibited higher miR-322 expression than those of wild-type mice. Taken together, these results show that ODPFs suppress the expression of miR-322 in cumulus cells. Gene ontology analysis of putative miR-322 targets whose expression was detected in MGCs with RNA-sequencing suggested that multiple biological processes are affected by miR-322 in MGCs. These results demonstrate that ODPFs regulate miRNA expression in granulosa cells and that this regulation may participate in the differential control of cumulus cell versus MGC functions. Therefore, the ODPF-mediated regulation of cumulus cells takes place at both transcriptional and post-transcriptional levels.
Key words: Cumulus cells, miRNA, Ovarian follicles

Granulosa cells of the Graafian follicles are classified into two distinct cell types: cumulus cells and mural granulosa cells (MGCs). Cumulus cells are located in close proximity to the oocyte and support oocyte development, whereas MGCs line the inner wall of the follicle and are involved in the endocrine function of ovaries. Normal development and function of both cell types are important for the development of follicles as well as the formation of functional oocytes.

MicroRNAs (miRNAs), small non-coding RNAs of 19–25 nucleotides, are important regulators of cell differentiation and function [1]. They regulate gene expression post-transcriptionally, mainly by repressing the translation of target transcripts. The expression profiles of ovarian miRNAs have been reported for many mammalian species, including humans [2], mice [3], horses [4], and pigs [5]. Moreover, several miRNAs have been identified as regulators of granulosa cell development and function [6]. For example, miR-224, miR-383, and miR-320 have been shown to mediate the effects of

transforming growth factor, beta 1 (TGFB1) on estrogen production by mouse granulosa cells [7–9]. Similarly, miR-378 has been shown to regulate estrogen production by granulosa cells in pigs by directly targeting the expression of CYP19A1 (also known as aromatase). In addition, female mice harboring a hypomorphic mutation of the *Dicer* gene, which encodes an enzyme required for miRNA production, are infertile due to an insufficiency of the corpus luteum [10]. Conditional deletion of *Dicer* in granulosa cells results in an increase in atretic follicles and a reduced ovulation rate [11, 12]. Therefore, as in other cell-types, miRNAs play important roles in regulating the development and function of granulosa cells [6, 13, 14].

Mammalian oocytes, by producing paracrine factors (ODPFs), play key roles in regulating the development and function of granulosa cells [15–20]. ODPFs include members of the transforming growth factor beta (TGF-β) superfamily, such as growth differentiation factor 9 (GDF9) and bone morphogenetic proteins 6 and 15 (BMP6 and 15); and members of the fibroblast growth factor (FGF) family, such as FGF8. Animals deficient in genes encoding ODPFs exhibit phenotypes with impaired fertility. For example, $Gdf9^{-/-}$ female mice are infertile due to the arrest of folliculogenesis at the primary follicle stage and have a high incidence of ovarian cysts [21], and female mice deficient in *Bmp15* and/or *Bmp6* are subfertile with defects in ovulation and lower developmental competence of oocytes [22, 23]. Interestingly, double-mutant (hereafter designated DM) $Bmp15^{-/-}/Gdf9^{+/-}$ female mice show an even more severe reduction in fertility,

which is at least partly attributable to the impaired development of granulosa cells [22, 24, 25]. This synergistic interaction between BMP15 and GDF9 seems to be species dependent [26, 27], and recent studies have suggested the involvement of a BMP15/GDF9 heterodimer in this interaction [28, 29]. The actions of these TGF-β superfamily proteins in granulosa cells are mediated by SMAD signaling [30–32].

Many mRNAs are differentially expressed between cumulus cells and MGCs. Cumulus cells express higher levels of transcripts encoding enzymes or transporters involved in metabolic pathways, such as glycolysis, cholesterol biosynthesis, and amino acid uptake. The higher expression of these transcripts by cumulus cells requires stimulation by ODPFs [24, 33–35]. By contrast, while MGCs express high levels of *Lhcgr* mRNA, which encodes a receptor of luteinization hormone, *Lhcgr* expression in cumulus cells is suppressed by ODPFs and is thus barely detectable [36]. Therefore, oocytes regulate the levels of mRNA expression in associated granulosa cells, and this ability of oocytes appears to be crucial for the oocyte-mediated heterogeneity in granulosa cell development and function.

Considering the important role of miRNAs in regulating cellular differentiation and function in numerous cell types including ovarian cells, we tested the hypothesis that ODPFs could regulate spatial differences in cumulus cell and MGC function, at least in part by controlling miRNA expression. If so, this would suggest a mechanism by which ODPFs could affect the differential functions of cumulus cells and MGCs at both the transcriptional and post-transcriptional levels.

Materials and Methods

Mice

Experiments were conducted using B6D2F1 female mice purchased from Sankyo Labo Service Corporation (Tokyo, Japan), or produced and raised in the research colonies of investigators at The University of Tokyo. Some experiments employed 3-week-old female DM mice [22] and wild-type littermates with a B6/129/DBA2 mixed genetic background that were produced in the research colonies of the authors. Mutant mice were originally produced and generously gifted by Dr Martin M. Matzuk of the Baylor College of Medicine. All animal protocols were approved by the Animal Care and Use Committees at the University of Tokyo.

Isolation and culture of cumulus-oocyte complexes (COCs), cumulus cells, MGCs, and denuded oocytes

Minimum Essential Medium alpha (MEMα) with 75 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, 0.23 mM pyruvate, 3 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA), and 10 μM of the phosphodiesterase inhibitor, milrinone (Sigma-Aldrich) was used as the basic culture medium. Milrinone was added to the medium to maintain oocytes at the germinal vesicle stage because the ability of oocytes to regulate surrounding granulosa cells significantly changes before and after the resumption of meiotic maturation. Milrinone was added to all cultures for experimental balance. In some experiments, 10 μM of the inhibitor of SMAD2/3 activation, SB431542 (Wako Pure Chemical, Osaka, Japan), was added to the basic culture medium. All cultures were maintained at 37ºC in 5% O_2, 5% CO_2, and 90% N_2.

COCs and MGCs were isolated from female mice injected with equine chorionic gonadotropin (eCG) 42–46 h prior to each experiment, as reported previously [34]. To collect fresh samples of cumulus cells, cumulus cells and oocytes were separated by repeatedly pipetting with a fine-bore pipette. For experiments requiring the culture of cumulus cells, oocytectomized (OOX) cumulus cells were produced by microsurgically removing oocytes from COCs, as reported previously [37]. For culture experiments of MGCs, MGCs were resuspended in the basic culture medium supplemented with 5% fetal bovine serum, and 2×10^4 viable cells were transferred to each well of a 96-well plate (IWAKI). After an overnight culture in medium containing FBS to attach the cells to the bottom of the wells, the medium was changed to the serum-free basic culture medium. For oocyte co-culture experiments, oocytes were collected from early- to middle-stage antral follicles of 3-week-old female mice that were not treated with eCG, and added to the medium at a density of 2 oocytes per μl. Co-culture experiments were conducted for 24 h.

Isolation of total RNA, reverse transcription, and quantitative PCR for miRNAs

Total RNA was extracted using a miRCURY RNA Isolation Kit (Exiqon, Vedbaek, Denmark) and reverse transcribed using a Universal cDNA Synthesis kit or Universal cDNA Synthesis kit II (Exiqon) according to manufacturer protocols. Real-time PCR analyses were performed using Power SYBR Green PCR master mix (Applied Biosystems) and ABI Step One real-time PCR systems (Applied Biosystems) according to the manufacturer protocols. Expression levels of miRNA were normalized to the levels of U6 snRNA (*Rnu6*) using the $2^{-\Delta\Delta Ct}$ method [38]. The primers used for real-time RT PCR analysis for miRNA detection (LNA primers) were purchased from Exiqon.

Isolation of total RNA, reverse transcription, and quantitative PCR for mRNAs

Real-time PCR was conducted as previously reported [39]. Briefly, total RNA was extracted from cumulus cells or MGCs using a ReliaPrep RNA Cell Miniprep System (PROMEGA, Tokyo, Japan) and reverse transcribed using a ReverTra Ace qPCR Master Mix with a gDNA Remover kit (TOYOBO, Osaka, Japan) according to manufacturer protocols. Real-time PCR analyses were performed using the THUNDERBIRD qPCR Mix (TOYOBO) and an ABI StepOne real-time PCR system (Applied Biosystems) according to manufacturer protocols. Expression levels of mRNA were normalized to the levels of the housekeeping gene, ribosomal L19 (*Rpl19*) by the $2^{-\Delta\Delta Ct}$ method [37]. The PCR primers used to amplify *Ccnd2* were 5′-GCGTGCAGAAGGACATCCA-3′ and 5′-CACTTTTGTTCCTCACAGACCT-3′. The PCR primers used to amplify *Rpl19* have been reported previously [40].

RNA-seq analysis

Libraries for the RNA-seq were prepared from total RNA extracted from the MGCs of eCG-primed mice according to the manufacturer protocol (the TruSeq RNA sample preparation v2 low sample (LS) protocol; Illumina). The qualities of the libraries were measured using a Bioanalyzer (Agilent), and the concentrations were determined using a KAPA Library Quantification Kit (KAPA Biosystems). Ten

libraries, including two MGC libraries, were labeled by Illumina 1500 standard indexes and sequenced together using an Illumina HiSeq system (50-bp single-end reads). The sequenced reads were mapped to the mouse genome (mm9 assembly) using TopHat2 [41]. Read counts on genes and gene expression indexes normalized to reads per kilobase of exons per million reads (RPKM) were calculated using our in-house program. Expression changes were estimated using the DESeq package [42]. Raw data used in this study were deposited in the GEO database GSE80326.

miR-322 target transcripts and Gene ontology (GO) analysis

Putative target transcripts of miRNAs were predicted using DIANA (http://diana.imis.athena-innovation.gr/) [43, 44], Target Scan (http://www.targetscan.org/) [45], and miRDB (http://mirdb.org/miRDB/) [46]. The transcripts predicted to be miR-322 targets by at least one of the three websites were defined as the miR-322 targets.

The GO analysis for the miR-322 target transcripts was conducted using VLAD (http://proto.informatics.jax.org/prototypes/vlad/).

Immunoblotting

Immunoblot analysis was conducted as previously reported [47]. The antibodies used were anti-Cyclin D2 monoclonal antibody (DCS-5, Medical & Biological Laboratories, Nagoya, Japan) and anti-β-actin polyclonal antibody (GTX109639, GeneTex, Irvine, CA, USA). The protein-bound antibodies were visualized using peroxidase-conjugated goat anti-rabbit IgG (AP132P, Millipore) or goat anti-mouse IgG + IgM (115-035-044, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) as the secondary antibodies, and ImmunoStar LD Western blotting detection reagent (Wako Pure Chemicals, Osaka, Japan) according to manufacturer protocols. The images were scanned using a C-DiGit Blot Scanner and Image Studio for C-DiGit (LI-COR, Nebraska, USA).

Statistical analyses

Student's t test was used for pair-wise comparisons. P values of less than 0.05 were considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

Results

The miRNA mmu-miR-322-5p (hereafter designated miR-322) was differentially expressed between cumulus cells and MGCs

Oocyte-regulated transcripts are known to be differentially expressed in cumulus cells and MGCs due to differences in the distances of these cell-types from oocytes [48]. Therefore, we first searched for miRNAs that were differentially expressed between cumulus cells and MGCs using an miRNA-microarray. We identified mmu-miR-322-5p (miR-322), which is a processed product of the *Mir322* gene, as one of the miRNAs most differentially expressed between cumulus cells and MGCs (data not shown). Quantitative PCR confirmed that MGCs expressed levels of miR-322 about 10-fold higher than those expressed in cumulus cells (Fig. 1). In addition to U6 snRNA (*Rnu6*), 5S RNA (*Rn5s*) was also used as an internal control and the results were essentially the same (data not shown).
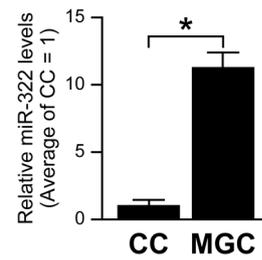
Fig. 1. Cumulus cells (CC) and mural granulosa cells (MGCs) differentially express miR-322. Expression levels of miR-322 in CC and MGCs were compared using qPCR (n = 3). * P < 0.05.
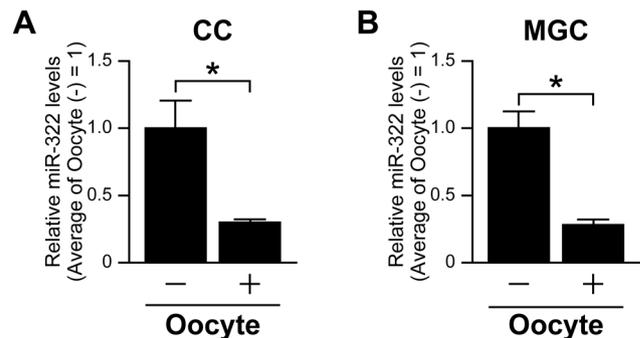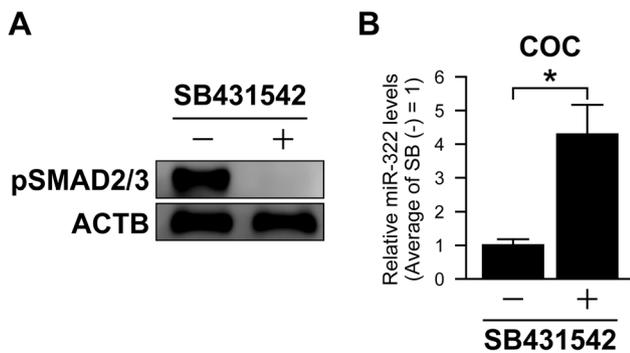

Fig. 2. Oocytes suppress miR-322 expression in granulosa cells. Oocytectomized cumulus cells (CC) (A) or MGCs (B) were co-cultured with oocytes (2 oocytes/μl) for 24 h and the expression levels of miR-322 were examined using qPCR (n = 4). * P < 0.05.

Oocytes suppressed miR-322 expression in both cumulus cells and MGCs in vitro

To test the possibility that the differential expression of miR-322 between cumulus cells and MGCs was due to regulation by ODPFs, cumulus cells and MGCs were each co-cultured with oocytes to examine the effect on their expression of miR-322 (Fig. 2). OOX cumulus cells or MGCs were cultured with or without denuded fully-grown oocytes (2 oocytes/μl) and the expression levels of miR-322 were assessed. As shown in Fig. 2A and B, both cumulus cells and MGCs co-cultured with oocytes exhibited significantly lower levels of miR-322 expression compared to those cultured without oocytes.

SMAD2/3 signaling is required for the suppression of miR-322 in cumulus cells by oocytes

To identify ODPFs that suppress miR-322, OOX cumulus cells were treated with SB431542, an inhibitor of SMAD2/3 signaling that mediates the GDF9 signal, and the expression levels of miR-322 were determined using qPCR. In addition, phosphorylated SMAD2/3 (pSMAD2/3) was detected using immunoblotting. As shown in Fig. 3A, pSMAD2/3 was barely detectable in SB431542-treated COCs, whereas a strong pSMAD2/3 signal was detected in COCs that were not treated with SB431542. Moreover, the levels of miR-322

A

SB431542

    −   +

pSMAD2/3

ACTB

B

Fig. 3. SMAD2/3 signaling is required for the suppression of miR-322 expression in cumulus-oocyte complexes (COCs). COCs were treated with SB431542 for 24 h. The phosphorylation status of SMAD2/3 (A; n = 3) and the expression of miR-322 (B; n = 4) were determined in COCs. * P < 0.05.

A

B

Fig. 4. Cumulus cells, but not MGCs, from $Bmp15^{-/-}/Gdf9^{+/-}$ mice express higher levels of miR-322 than wild-type mice. Expression levels of miR-322 were compared between cumulus cells (A) or MGCs (B) of wild-type (WT) and $Bmp15^{-/-}/Gdf9^{+/-}$ (DM) mice (n = 3). * P < 0.05.

in SB431542-treated COCs were significantly increased compared with those in COCs that were not treated with the inhibitor (Fig. 3B).
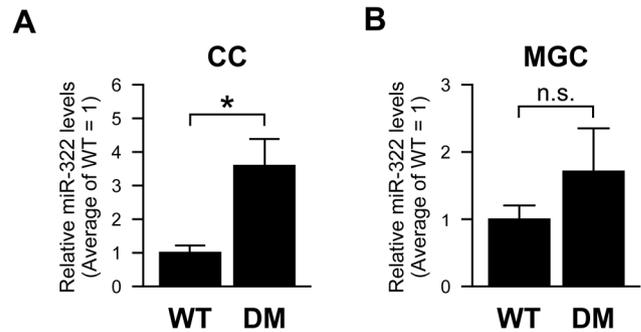
Cumulus cells from DM ($Bmp15^{-/-}/Gdf9^{+/-}$) mice expressed significantly higher levels of miR-322 than those from wild-type mice

The expression levels of miR-322 were compared in cumulus cells and MGCs from DM and wild-type mice. Homozygous mutant $Gdf9^{-/-}$ mice were not used because follicular development in $Gdf9^{-/-}$ mice is blocked at the primary follicle stage when cumulus cells have not yet developed [21]. As shown in Fig. 4A, cumulus cells from DM mice exhibited significantly higher levels of miR-322 than those from wild-type mice. On the other hand, miR-322 expression levels in MGCs from wild-type and DM mice were not significantly different (Fig. 4B). Therefore, oocyte-derived BMP15/GDF9 signals are required for suppressing miR-322 expression by cumulus cells in vivo. Moreover, the suppressive effect of oocytes on miR-322 expression may not reach MGCs, which are located further away from oocytes than are cumulus cells.

Identification of putative target transcripts of miR-322 in MGCs

The above results indicate that oocytes suppress miR-322 expression in cumulus cells, and, as a consequence of suppression by ODPFs, MGCs express higher levels of miR-322 than do cumulus cells. Therefore, we next assessed the potential target transcripts of miR-322 in MGCs. The 1687 transcripts that were predicted to be putative target transcripts of miR-322 were identified using the public databases, DIANA [43, 44], Target Scan [45], and miRDB [46]. Transcripts predicted to be miR-322 targets by at least one of the three databases were regarded as miR-322 targets in this study.

To focus on the miR-322 targets that are indeed expressed in MGCs, the transcriptome of MGCs was sequenced to identify mRNAs expressed in MGCs. Based on the criterion of an RPKM value greater than 10, 6080 transcripts were defined as being relatively highly expressed in MGCs. Among these MGC-expressed transcripts, 651 transcripts were predicted to be likely miR-322 targets (Fig. 5 and
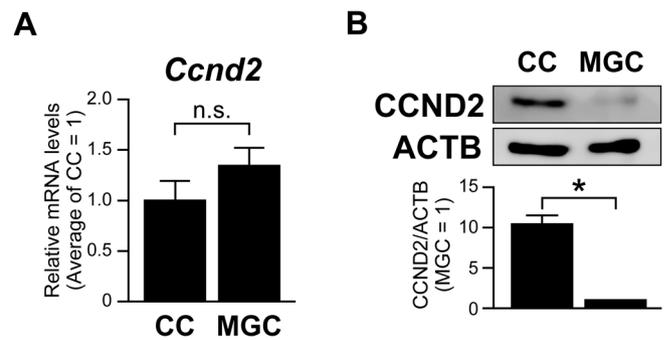
Supplementary Table S1: online only).

Since miRNA mainly suppresses gene function by repressing the translation of proteins, we compared both the protein and mRNA expression of cyclin D2 (CCND2), one of the MGC-expressed putative miR-322 targets (Table S1), in cumulus cells and MGCs (Fig. 6). While expression levels of the *Ccnd2* transcript were not significantly different between cumulus cells and MGCs, CCND2 protein levels were significantly lower in MGCs, which express higher levels of miR-322 than do cumulus cells. These results suggest that regulation of CCND2 protein expression is translational rather than transcriptional, and it is possible that the differential expression of miR-322 in cumulus cells and MGCs is involved in this translational control of CCND2.

Putative roles of miR-322 in MGCs

To further resolve the potential functions of miR-322 targets, we conducted a GO analysis of the putative miR-322 target transcripts expressed in MGCs. The complete list of biological processes (GO terms) significantly associated with the 651 transcripts is shown in Supplementary Table S2 (online only). The top three biological processes representing these transcripts were "cellular protein metabolic process" (GO:0044267, $Q = 1.23 \times 10^{-19}$), "cellular macromolecule metabolic process" (GO:0044260, $Q = 6.14 \times 10^{-18}$), and "cellular protein modification process" (GO:0006464, $Q = 3.25 \times 10^{-17}$) (Supplementary Table S2). The specific function of gene products that contribute to these GO terms are not clear because these GO terms are associated with a large number of transcripts (more than 1800 transcripts), and therefore we decided to focus on more specific GO terms that include fewer transcripts (less than 300 transcripts). The list of the top 10 GO terms (< 300 transcripts annotated) is shown in Table 1. The MGC-expressed miR-322 target transcripts were enriched in biological processes such as "regulation of translation" (GO:0006417), "regulation of cellular amide metabolic process" (GO:0034248), and "endoplasmic reticulum organization" (GO:0007029) (Table 1). Moreover, the targets were also enriched in the biological processes related to cell proliferation, including "positive regulation of cell cycle" (GO:0045787) and "positive regulation of mitotic cell cycle" (GO:0045931), and regulation of

Transcripts detected in MGCs (6080)

Putative miR-322 tragets (1687)

5429   651   1036
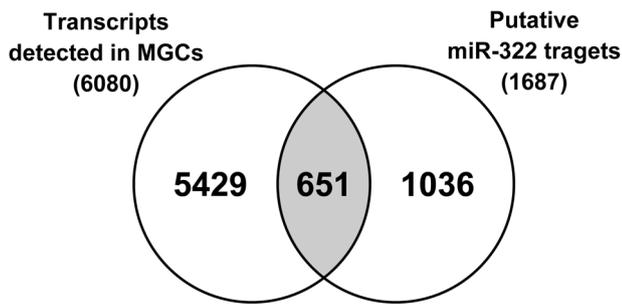
Fig. 5. Venn diagram illustrating the number of individual transcripts found to be highly expressed in MGCs by RNA-seq analysis, and those predicted to be putative miR-322 targets by public databases.

A

Ccnd2

n.s.

Relative mRNA levels (Average of CC = 1)

2.0
1.5
1.0
0.5
0

CC   MGC

B

CC   MGC

CCND2
ACTB

CCND2/ACTB (MGC = 1)

15
10
5
0

*

Fig. 6. CCND2 protein is differentially expressed between cumulus cells and MGCs. Expression levels of *Ccnd2* transcript (A) and CCND2 protein (B) were compared in cumulus cells (CC) and MGCs (n = 3). * P < 0.05; n.s., not significant.

Table 1. List of the top 10 biological processes (GO terms associated with less than 300 transcripts) significantly affected by miR-322 in MGCs

GOID	GO Term	Q value
GO:0006417	regulation of translation	3.13E-06
GO:0034248	regulation of cellular amide metabolic process	5.10E-06
GO:0007029	endoplasmic reticulum organization	2.77E-05
GO:0045787	positive regulation of cell cycle	3.62E-04
GO:0045931	positive regulation of mitotic cell cycle	5.37E-04
GO:0097191	extrinsic apoptotic signaling pathway	1.29E-03
GO:1903362	regulation of cellular protein catabolic process	2.87E-03
GO:0010498	proteasomal protein catabolic process	3.14E-03
GO:0042177	negative regulation of protein catabolic process	3.85E-03
GO:0051301	cell division	4.10E-03

apoptotic pathways including "extrinsic apoptotic signaling pathway" (GO:0097191). These processes are known to be augmented in cumulus cells compared with MGCs [49–51], and hence the suppression of miRNAs that target these transcripts in cumulus cells is a reasonable expectation.

Discussion

Mammalian oocytes play critical roles in the regulation of granulosa cell differentiation and function by secreting ODPFs. In addition, miRNAs are now well recognized as regulators of function in a variety of cell types, including granulosa cells. Nevertheless, whether oocytes influence the regulation of miRNA expression in granulosa cells has not yet been determined. Here, we showed that miR-322 is differentially expressed in cumulus cells and MGCs, and that miR-322 expression in granulosa cells is suppressed by co-culturing with oocytes. Moreover, cumulus cells from DM mice exhibited higher levels of miR-322 expression than those from control wild-type mice. Therefore, the present results indicate that ODPFs suppress miR-322 expression in cumulus cells *in situ*, and provide evidence for a mechanism that may contribute to differences in the function of cumulus cells and MGCs.

miR-322 and its human ortholog miR-424 are well known to negatively regulate cell proliferation by targeting the expression of cell cycle regulators such as cyclin D1 (CCND1) and CDC25A [52–55]. Granulosa cells do not require cyclin D1 for their proliferation, but instead use cyclin D2 (CCND2) as a D-type cyclin [56], which is also predicted to be a target of miR-322. The present results showed that CCND2 protein levels were significantly lower in MGCs compared with that in cumulus cells, while *Ccnd2* transcript levels were not significantly different. Therefore, it is possible that some miRNAs are involved in the control of CCND2 protein expression. In addition, miR-322 regulates apoptosis in rat intestinal epithelial cells by regulating TGF-β/SMAD signaling [57]. It is well known that mouse oocytes promote proliferation and suppress the apoptosis of granulosa cells [51, 58]. Although further studies are needed, it is possible that miR-322 may mediate, at least to some extent, the effects of oocytes on the proliferation and apoptosis of granulosa cells.

In a previous study, we identified a set of mRNAs that were enriched in cumulus cells, and whose expression in these cells was promoted by ODPFs [59]. Interestingly, many biological processes (GO terms) that were shown to be affected by MGC-expressed miR-322 in the present study were also relevant to the cumulus cell-enriched mRNAs identified in the previous study as being

promoted by ODPFs [59]. In fact, 7 out of the top 10 GO terms (< 300 transcripts annotated) associated with MGC-expressed miR-322 targets (Table 1) were significantly associated with ODPF-promoted cumulus cell transcripts. Notably, not many of the actual transcripts (less than 15%) were common to the two sets. Therefore, in addition to directly promoting the expression of some transcripts associated with certain biological processes, such as "positive regulation of mitotic cell cycle" (GO:0045787)[59], mouse oocytes appear to suppress the expression of miR-322, which targets other transcripts associated with the same biological processes (Table 1). Therefore, it is likely that an additional layer of mechanisms involving miRNAs contributes to oocyte regulation of the development and function of granulosa cells, in addition to the well-known oocyte regulation of steady-state levels of mRNAs in granulosa cells that has been actively studied using transcriptomic analyses. Studies are underway to seek novel miRNAs regulated by oocytes. Clarifying the physiological consequences of this regulation will broaden our understanding of the mechanisms regulating both granulosa cells and ovarian development.

Acknowledgements

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