A miRNA-target network putatively involved in follicular atresia

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F. X. Donadeu and J. Ioannidis

The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK

*Corresponding author. Email: xavier.donadeu@roslin.ed.ac.uk

ABSTRACT

In a previous microarray study we identified a subset of miRNAs which expression was distinctly higher in atretic than healthy follicles of cattle. In the present study we investigated the involvement of those miRNAs in granulosa and theca cells during atresia. RT-qPCR confirmed that miR-21-5p/-3p, miR-150, miR-409a, miR-142-5p, miR-378, miR-222, miR-155 and miR-199a-5p were expressed at higher levels in atretic than healthy follicles (9-17 mm, classified based on steroidogenic capacity). All miRNAs except miR-21-3p and miR-378 were expressed at higher levels in theca than granulosa cells. The expression of 13 predicted miRNA targets was determined in follicular cells by RT-qPCR, revealing downregulation of *HIF1A, ETS1, JAG1, VEGFA* and *MSH2* in either or both cell types during atresia. Based on increases in miRNA levels simultaneous with decreases in target levels in follicular cells, several predicted miRNA-target interactions were confirmed that are putatively involved in follicular atresia, namely miR-199a-5p/miR-155-*HIF1A* in granulosa cells, miR-155/miR-222-*ETS1* in theca cells, miR-199a-5p-*JAG1* in theca cells, miR-199a-5p/miR-150/miR-378-*VEGFA* in granulosa and theca cells, and miR-155-*MSH2* in theca cells. These results offer novel insight on the involvement of miRNAs in follicle development by identifying a miRNA-target network that is putatively involved in follicle atresia.

Keywords: miRNAs, follicle, follicle atresia, bovine, granulosa, theca

1. Introduction

The overwhelming majority of follicles recruited from the primordial pool during a female's reproductive life will undergo atresia before they can reach the ovulatory stage. Atresia is an active process involving not only cell death but also resorption of follicular

tissue and its replacement by stromal and new follicular tissue; these processes involve infiltration by immune and other cells, very much resembling wound healing [1]. Among several key regulators of wound healing and tissue remodeling processes across body tissues are microRNAs (miRNAs) [2].

The involvement of miRNAs in different aspects of follicle development has been demonstrated in numerous studies [3,4]. Much of the existing evidence has been obtained using follicular cell cultures, mostly granulosa cells. Often reported effects of miRNAs include either the promotion or suppression of granulosa cell apoptosis [5-10]. Yet, in many cases, the site of expression, if any, of these miRNAs within follicles (i.e., granulosa and theca compartments) or whether their expression actually changes during follicle atresia, supporting their physiological role, has not been clarified. Moreover, although the posttranscriptional effects of miRNAs in tissues often involve targeting of a common gene simultaneously by several miRNAs and, at the same time, a single miRNA can simultaneously target multiple genes, previous functional studies in follicles have often used a one miRNA – one target approach thus providing limited information on the wider biological effects of miRNAs expressed simultaneously acting in co-ordination.

In a previous study we used microarray to profile miRNA expression across a wide range of antral follicle development stages in cattle [11], a species which follicular physiology closely resembles the human, particularly when compared to rodents; by comparing miRNA profiles between steroidogenic-active and steroidogenic-inactive follicles, we identified a subset of miRNAs that are putatively involved in the growth of healthy dominant follicles. In the present study we focused our attention on those miRNAs identified as upregulated during follicle atresia in our previous study. Specifically, we established and compared the

63 expression of miRNAs and their putative targets within the follicular granulosa and theca
64 compartments to gain insight into their involvement in follicle atresia.

65

66 **2. Materials & methods**

67 *2.1 Collection and processing of bovine tissues*

68 Follicles from ovaries of cycling beef cattle obtained at an abattoir were collected as part
69 of a separate study [11]. Individual follicles 9-17 mm in diameter were dissected out and the
70 follicular fluid aspirated and centrifuged at 800 *g* for 10 min. The resulting supernatant was
71 stored at –80 °C until further analyses and the cell pellet was combined with the follicular
72 wall free of surrounding stroma and snap-frozen in liquid nitrogen until RNA extraction.
73 Alternatively, after hemi-dissection, follicles were gently scraped with blunt-ended forceps to
74 collect granulosa and theca wall compartments. Theca walls were washed repeatedly to
75 remove any residual granulosa cells. Theca and granulosa cells from each individual follicle
76 were then separately snapped frozen in liquid nitrogen.

77 Intra-follicular concentrations of estradiol and progesterone were measured using
78 competitive double antibody radioimmunoassay kits (Siemens Healthcare Diagnostics Inc.,
79 USA) following the manufacturer's instructions. All assays were validated in our laboratory
80 by showing parallelism between serial sample dilutions and the provided assay standard
81 curve. Sensitivity of the assays was 0.56 ng / mL and 0.01 ng / mL, and the intra-assay CVs
82 were 6 % and 4.3 % for estradiol and progesterone, respectively.

83 *2.2 RT-qPCR*

84 Total RNA was extracted using the miRNeasy Mini kit (Qiagen, UK) and reverse-
85 transcribed using the miScript II RT kit (Qiagen), as described [11]. Messenger RNA levels
86 were quantified using the SensiFAST^{TM} SYBR Lo-ROX Kit (Bioline Reagents Ltd, UK) and
87 bovine-specific primers (Table 1). For miRNA quantification the miScript SYBR Green PCR
88 kit and miScript Primer Assays (Qiagen) were used. All PCRs were run on a MX3005P
89 QPCR system (Stratagene, CA, USA) using a standard curve to calculate copy numbers from
90 Cq values [11]. Messenger RNA data were normalized using *18S* values within each sample
91 and miRNA data were normalized using endogenous *RnU6-2*. Mean intra-assay CVs for
92 miRNA and mRNA qPCRs were 9.5% and 11.3%, respectively.

93 *2.3 miRNA target identification*

94 Identification of putative miRNA targets was done using miRTarBase release 6.0 and
95 TargetScan release 7.0 to select targets experimentally validated in human and/or rodents (by
96 reporter assay, western blot and/or RT-qPCR, as detailed in
97 http://mirtarbase.mbc.nctu.edu.tw/) and computationally predicted targets within the bovine
98 genome (http://www.targetscan.org), respectively. For convenience, each identified miRNA-
99 target interaction was classified as high-, medium- or low-confidence based on whether it was
100 present in both miRTarBase and TargetScan, miRTarBase only or TargetScan only.

101 *2.4 Statistical analyses*

102 ROUT outlier test was applied to data sets, and outlier values ($P < 0.01$) were excluded
103 from subsequent analyses. Gene expression data were assessed for normality using the
104 D'Agostino & Pearson normality test and were log-transformed prior to statistical analysis
105 where necessary. Two-way ANOVA followed by unpaired t-tests to identify differences in
106 gene expression between healthy and atretic follicles within each cell type were used.

Significance was considered at $P < 0.05$ whereas differences with P values < 0.1 were considered to approach significance. Nomenclature according to miRBase release 21 is used throughout the manuscript. All miRNAs referred to are bovine (bta-) except otherwise specified.

3. Results

3.1 miRNA expression analyses in follicular tissues

In a previous study in cattle [11], microarray analyses yielded a total of 11 unique bovine sequences which were expressed in greater abundance (> 1.5 fold) in atretic than in healthy pre-ovulatory-size follicles (Table 2). The status of the follicles analyzed in that study had been pre-determined on the basis of steroidogenic capacity and *LHGCR* expression in granulosa cells (Figure 1). Microarray results were validated by RT-qPCR in the present study, confirming the up-regulation of 9 miRNAs in atretic follicles (Table 2).

To gain insight on the involvement of these miRNAs in follicle development we quantified their relative expression in granulosa and theca cell compartments. This showed that all miRNAs except for miR-21-3p and miR-378 were expressed in greater abundance (between 2-fold and 25-fold) in theca than in granulosa cells (Cell type, $P < 0.05$; Figure 2). Moreover, an effect of Follicle Status ($P < 0.05$), owing to overall higher expression levels in atretic follicles, was detected for all miRNAs except miR-21-5p and miR-378; for miR-378, an interaction approached significance ($P = 0.07$), reflecting higher expression levels in granulosa but not theca cells from atretic follicles.

3.2 Identification and expression analyses of miRNA targets

129 To identify putative targets of the 9 miRNAs in cattle we used miRTarBase, a database
130 containing experimentally validated targets of human, mouse and rat miRNAs, and we
131 selected 36 genes that were simultaneous targets of ≥ 2 of those miRNAs (Table S1). To
132 increase confidence in our target selection, we then searched all 36 genes in TargetScan and
133 selected those, 16 in total, which bovine homologues were computationally predicted targets
134 of one or more of the corresponding bovine miRNA sequences (Table S1). Finally, we
135 assessed the validity of these predictions by analyzing by qPCR the expression in follicular
136 cells of 8 of the 16 genes identified (Table 3 and Figure 3; an additional two targets, *E2F2*
137 and *SIRT1*, were also selected but could not be detected in follicular cells by RT-qPCR). For
138 completeness, our qPCR analyses also included one gene (*MYD88*) which interactions with
139 two miRNAs were experimentally validated but not computationally predicted in bovine
140 (Table 3 and Figure 3), and 2 genes, *IGF1* and *PAPPA*, which were computationally
141 predicted bovine targets of 2 miRNAs (miR-222 and miR-378) and 3 miRNAs (miR-142-5p,
142 miR-150 and miR-378), respectively, but none of which were experimentally validated in any
143 species, i.e., they were present in TargetScan but not in miRTarBase (Table 3 and Figure 3).

144 As shown in Figure 3, out of the remaining miRNA targets, 8 were enriched (Cell type, P
145 < 0.05) in either granulosa cells (*HIF1A*, *IGF1R* and *PAPPA*) or theca cells (*ETS1*, *JAG1*,
146 *MSH2*, *IGF1* and *TIMP3A*), and 5 targets were differentially expressed according to follicle
147 status, in all cases involving a reduction in atretic follicle cells, as indicated by a significant
148 effect of Follicle Status or a Follicle Status x Cell Type interaction (*HIF1A, ETS1, JAG1,*
149 *VEGFA* and *MSH2*).

150 *3.3 Validation of miRNA-target interactions*

151 Comparing miRNA and mRNA expression profiles (Figures 2 and 3) allowed for the
152 testing, based on a negative association between the expression of a miRNA and that of its

predicted target(s) within a follicular cell type, of a total of 12 high-confidence miRNA-target interactions (i.e., obtained from both miRTarBase and TargetScan; indicated by dark grey in Table 3), 8 medium-confidence interactions (obtained from miRTarBase but not present in TargetScan; indicated by light grey in Table 3) and 5 low-confidence interactions (identified in TargetScan but not present in miRTarBase; indicated by an "X" in Table 3).

Out of the 12 predicted high–confidence interactions analyzed, 5 were confirmed by RT-qPCR, specifically involving miR-199a-5p and *HIF1A* in granulosa cells, miR-155/miR-222 and *ETS1* in theca cells, miR-199a-5p and *JAG1* in theca cells, and miR-199a-5p and *VEGFA* in both granulosa and theca cells (miRNAs indicated in bold in Figure 3A). For another 3 predicted high-confidence interactions, differences in mean miRNA and target levels did not reached significance ($P < 0.1$). These involved *JAG1* and miR-21-5p in theca cells, *MSH2* and miR-21-5p in theca cells and *IGF1R* and miR-378 in granulosa cells (Figure 3A,B). Four predicted high-confidence interactions involving *TIMP3* and *RECK1* were not confirmed as the levels of these transcripts did not change significantly according to Follicle status (Figure 3B).

Out of 8 predicted medium-confidence interactions analyzed (Table 3), 4 were confirmed involving miR-155 and *HIF1A* in granulosa cells, miR-150 and *VEGFA* in both granulosa and theca cells, miR-378 and *VEGFA* in granulosa cells, and miR-155 and *MSH2* in theca cells (Figure 3A). For another 2 medium-confidence interactions, involving miR-21-5p and *VEGFA* in both granulosa and theca cells, and *IGF1R* and miR-21-5p in granulosa cells (Figure 3A,B), differences in mean miRNA and target levels did not reached significance ($P < 0.1$), while the remaining 2 medium-confidence interactions were not confirmed as the levels of *MYD88* did not change with follicle status (Figure 3B).

Finally, none of the 5 low-confidence interactions (Table 3) tested were confirmed as transcript abundance of *IGF1* and *PAPPA* did not change according to Follicle status (Figure 3B).

4. Discussion

A limited number of studies in cattle [11,12] and pigs [8] have reported genome-wide miRNA expression profiles associated with follicle atresia. Five of the 9 miRNAs confirmed to be upregulated in atretic follicles in the present study (miR-21-5p, miR-21-3p, miR-222, miR-155, miR-199a-5p) were also found to be increased in subordinate relative to dominant follicles on Day 3 of the bovine estrous cycle using deep-sequencing rather than microarray [12]. Another miRNA, miR-378, was previously shown, together with miR-21-5p, to increase in expression in subordinate and anovulatory follicles in horses [13,14]. Taken together, these results are consistent with an involvement of these miRNAs in follicular atresia in the monovular ovary. Indeed, all 9 miRNAs identified in atretic follicles in this study can reportedly regulate cell survival and/or tissue turnover [15-21]. Specifically in the ovary, miR-21 promotes cell survival during luteinization [10] while at the same time is expressed at very high abundance in the regressing corpus luteum [22], suggesting a multifaceted, developmental stage-dependent involvement in follicle and corpus luteum function. A putative involvement of miR-378 in regulating luteal cell survival in bovine has been suggested but not proven [23]. However, in the pig, miR-378 targets aromatase and progesterone receptor in granulosa cells and regulates both ovarian estradiol production and oocyte maturation [24-26] Finally, another of the miRNAs investigated in our study, mir-222, may reportedly regulate steriodogenesis of granulosa cells [27].

Our miRNA-target pair analyses provides novel insight on the molecular regulation of follicular atresia by identifying specific miRNA networks putatively involved within different follicular compartments (summarized in Figure 4). To identify high-confidence bovine miRNA targets we selected genes that both 1) had already been experimentally validated for 2 or more miRNAs in different cellular contexts in humans and/or rodents (as bovine-specific information is not available) and 2) contained predicted miRNA target sites in the bovine homolog 3'UTR. In choosing this approach we took into consideration that 1) effective target downregulation often involves multiple miRNAs simultaneously binding the 3'UTR of a gene and 2) computational prediction of miRNA targets is relatively inaccurate in terms of both false targets being identified and true targets being missed. A similar proportion of predicted miRNA-target interactions classified as high-confidence (identified from both miRTarBase and TargetScan) and medium-confidence (identified from miRTarBase only) were confirmed by qPCR (5 out of 12 and 4 out 8, respectively), with another 3 and 2 interactions failing to be validated because miRNA and/or mRNA expression differences only approached significance ($P < 0.1$). These results highlight the notion that a significant number of true miRNA targets are normally missed using computational prediction approaches, and that the fact that a target has been experimentally validated in other species may provide the strongest rationale for target selection, particularly considering that many miRNAs are functionally conserved. It needs to be pointed out that failure to detect differences in predicted target levels by RT-qPCR (e.g., *MYD88* in this study) does never by itself provide conclusive evidence that the gene in question is not an actual target, as miRNAs may in general have greater effects on protein than transcript levels. Unfortunately, quantification of protein levels is not always possible in bovine due to the limited availability of species-specific antibodies.

223 Among the genes confirmed as miRNA targets in atretic follicles was *VEGFA*, which
224 expression was significantly downregulated in both granulosa and theca cells, putatively
225 through the effects of at least 3 different miRNAs. Within the follicle, VEGFA has strong
226 trophic effects not only in vascular cells but also in steroidogenic cells [28,29]. A key
227 transcriptional activator of VEGFA is HIF1A [30]. This is a gonadotropin-induced, master
228 regulator of cellular responses to hypoxia which expression in granulosa cells mediates
229 survival, steroidogenic and angiogenic responses within the follicle [30,31]. HIF1A is
230 reportedly expressed at higher levels in granulosa than theca within non-atretic follicles [32].
231 These observations are consistent with our finding of a simultaneous decrease in the
232 expression of *HIF1A* and *VEGFA* during bovine follicle atresia, particularly in granulosa
233 cells. Our results implicate a network of miRNAs, namely, miR-199a-5p, miR-155, miR-150
234 and miR-378, in the down-regulation of the HIF1A-VEGF effector system during atresia,
235 with one miRNA, miR-199a-5p, simultaneously targeting both genes, as reported in other cell
236 types [33].

237 Two miRNA targets identified in theca cells and which, albeit much less characterized,
238 may also be involved in angiogenesis, are the transcription factor, ETS1, and the NOTCH1
239 ligand, JAG1. ETS1 is a proto-oncogen highly expressed in immune and vascular cells. In the
240 ovary, it has been shown to be expressed in theca and granulosa cells and its expression to
241 dynamically change during the estrous cycle [34,35]. An involvement of ETS1 in regulating
242 RGS2 expression during ovulation has been shown [36]. Moreover, its role in promoting
243 angiogenesis or as a pro-apoptotic factor in different cell types [37] could account for the
244 changes in expression during follicle atresia. Likewise, apart from its reported role in
245 promoting early follicle development, little information is available on the role of JAG1 in
246 the ovary. An involvement of JAG1 in follicular angiogenesis is suggested by its expression

247 in endothelial and other vascular mural cells in mouse ovaries [38]. The reason for the
248 opposite trends in *JAG1* expression in granulosa and theca cells of healthy and atretic
249 follicles in our study is unknown, warranting further study of the functions of this gene in the
250 adult ovary.

251 *MSH2*, a gene involved in DNA mismatch repair, was confirmed as a target of miR-155 in
252 theca cells. This is consistent with the finding in cattle that atresia involves the
253 downregulation of cell cycle and DNA replication genes in theca cells rather than the
254 downregulation of apoptotic genes as occurs in granulosa cells [39]. Interestingly, a study
255 showed that another miRNA, miR-26a, targeted the cell cycle checkpoint kinase, *ATM*, in
256 porcine follicular cells during atresia, leading to increased DNA breaking and apoptosis, and
257 raising the possibility that miR-155 could exert a similar effect through targeting *MSH2* in
258 bovine theca cells.

259 Finally, because of the heterogeneous nature of follicular tissue, particular theca, it is not
260 possible to determine, from our data, the specific cell types involved in the identified
261 miRNA-target interactions. For example, although most of the miRNAs analyzed in this
262 study are known not to be cell-specific, miR-150 and miR-155 are highly expressed and
263 primarily regulate hematopoietic and vascular cells. Nonetheless, paracrine regulation of gene
264 expression by miRNAs has also been described whereby miRNAs are produced by one cell
265 type and then secreted to regulate gene expression of a different cell type within a tissue [40],
266 adding further complexity to the role of miRNA-target interactions during follicular atresia.

267 In summary, by establishing the expression patterns of miRNAs and their putative targets
268 in granulosa and theca cells of healthy and atretic follicles in cattle we have identified a
269 network of miRNAs including miR-199a-5p, miR-155, miR-222, miR-150 and miR-378
270 which we propose are involved in follicle atresia through combined targeting of genes

involved in cell survival, proliferation and differentiation; namely *HIF1A* and *VEGFA* in granulosa cells, and *MSH2, ETS1, JAG1* and *VEGFA* in theca cells. Although the identified miRNA-target interactions should be confirmed by gene targeting or other molecular approaches in future studies, our results will provide fertile ground for further hypothesis-testing towards a better understanding of the molecular mechanisms involved in follicle atresia.

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Figure legends

Figure 1. Mean (± SE) concentrations of Estradiol, Progesterone, and transcript levels of *CYP19A1* and *LHCGR* in bovine follicles (9-17 mm) classified as healthy (n = 26) or atretic (n = 15). Differences between group means are indicated by asterisks ($P \leq 0.05$). Data adapted from Sontakke *et al.* 2014

Figure 2. Relative miRNA levels (Mean ± SE; normalized to levels of *RnU6-2*) in granulosa and theca cells from bovine healthy (n = 9) and atretic (n = 6) follicles. Differences between group means within each Cell type are indicated by an asterisk ($P < 0.05$).

Figure 3. Relative mRNA levels (Mean ± SE; normalized to levels of *18S*) in granulosa and theca cells from bovine healthy (n = 9) and atretic (n = 6) follicles. Genes confirmed as miRNA targets by PCR are shown in A), other genes are shown in B). Putative targeting miRNAs are shown on top of the corresponding graph bars in A); all indicated miRNA-target interaction were obtained from miRTarBase (database of experimentally validated miRNA-target interactions in human and/or rodents), and interactions that were in addition

computationally predicted in cattle (obtained from TargetScan) are indicated by miRNAs in bold (e.g., miR-199a-5p-*HIF1A*). Differences between group means within each Cell type are indicated by an asterisk ($P < 0.05$).

Figure 4. Schematic summary of putative miRNA-target interactions in granulosa and theca cells during follicular atresia identified in this study.

Table 1. Primer sequences used in mRNA analyses

Gene	Sequence (5'-3') sense / antisense
18S	GCTGGCACCAGACTTG / GGGGAATCAGGGTTCG
CYP19A1	CGCAAAGCCTTAGAGGATGA / ACCATGGTGATGTACTTTCC
E2F2	TCGCTATGACACATCGCTGG / CGTCACGTAGGCCAGTCTCT
ETS1	CACAGTCTCTCCGGCAAAGT / GTGGATGATAGGCCGACTGG
HIF1A	CAGAAGAACTTTTGGGCCGC / TCCACCTCTTTTGGCAAGCA
IGF1	AGTGCTGCTTTTGTGATTTCTTGA / GCACACGAACTGGAGAGCAT
IGF1R	AAGCTGAGAAGCAGGCAGAG / CGGAGGTTGGAGATGACAGT
JAG1	GAGTGTGAGTGTTCTCCGGG / TTGGCCTCGCATTCATTTGC
LHCGR	GGACTCTAGCCCGTAGG / ACACATAACCACCATACCAAG
MSH2	TGGGCAGAAGTGTCCATTGT / CCCACGCTAATCCAAACCCA
MYD88	AAGTTGTGCGTGTCTG / GGAAATCACATTCCTTGCT
PAPPA	TTGCTGCGCTTCTACAGTGA / GCACAGTCACCCTGTAGGTC
RECK	GTGCTTCCTTCTCTTGTCTGGA / GGCTTGACAGTATTCTCGGC
SIRT1	GCTTACAGGGCCTATCCAGG / TATGGACCTATCCGAGGTCTTG
TIMP3	GGATTCACCAAGATGCCCCA / GAGCTGGTCCCACCTCTCTA
VEGFA	TGTAATGACGAAAGTCTGGAG / TCACCGCCTCGGCTTGTCACA

Table 2. Bovine miRNA sequences which expression was upregulated (> 1.5 fold) in Atretic relative to Healthy follicles*.

miRNA	Microarray		RT-qPCR	
	Fold Change	Adjusted P-value**	Fold Change	P-value
bta-miR-483/hsa-miR-483-3p	3.64	0.001	0.88	0.409
bta-miR-21-3p/hsa-miR-21-3p	3.09	0.002	3.38	0.021
bta-miR-150/hsa-miR-150-5p	2.54	0.001	2.92	0.001
bta-miR-21-5p/ hsa-miR-21-5p	2.39	0.001	4.90	0.001
bta-miR-409a/hsa-miR-409a-5p	2.36	0.000	1.85	0.001
bta-miR-744/hsa-miR-744-5p	2.36	0.002	0.73	0.057
bta-miR-142-5p/hsa-miR-142-5p	2.03	0.001	2.81	0.001
bta-miR-378/has-miR-378a-3p	1.91	0.001	1.51	0.017
bta-miR-222/hsa-miR-222-3p	1.84	0.000	1.92	0.001
bta-miR-155/hsa-miR-155-5p	1.66	0.019	5.66	0.001
bta-miR-199a-5p/hsa-miR-199a-5p	1.63	0.004	1.66	0.001

* Microarray analyses were performed in 6 healthy and 5 atretic follicles (12-17 mm in diameter) in a previous study (data adapted from Sontakke et al., 2014). Microarray data were validated by qPCR in the present study using 26 healthy and 15 atretic follicles (9-17 mm in diameter).

**FDR; Benjamini and Hochberg adjustment

Table 3. Candidate miRNA-target interactions that were analysed by qPCR (see Figure 3)

	miR-142-5p	miR-150	miR-155	miR-199a-5p	miR-21-5p	miR-222	miR-378
ETS1							
JAG1							
RECK							
TIMP3							
HIF1A							
IGF1R							
MSH2							
VEGFA							
MYD88							
IGF1						X	X
PAPPA	X	X					X

High-confidence miRNA-target interactions obtained from both miRTarBase and TargetScan (i.e., that both have been experimentally validated in human, rat and/or mouse and are computationally predicted in bovine) are shown in dark grey. Medium-confidence interactions obtained from miRTarBase only are shown in light grey. Low-confidence interactions obtained from TargetScan only are indicated by an "X".

Fig 1

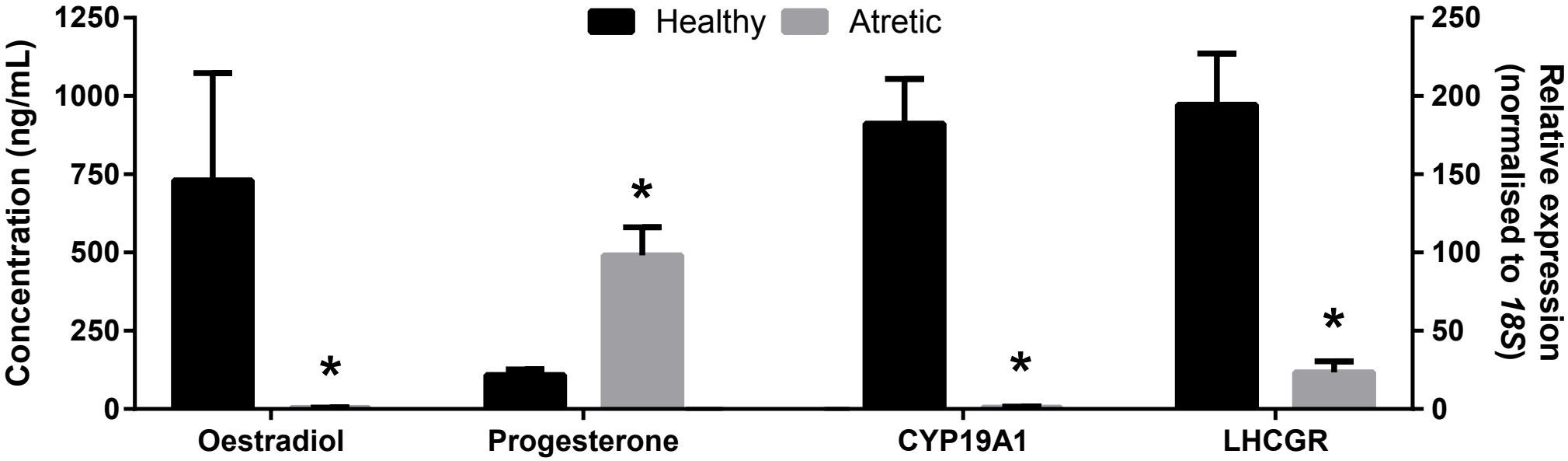

Healthy
Atretic
Concentration (ng/mL)
1250
1000
750
500
250
0
Relative expression (normalised to 18S)
250
200
150
100
50
0
*
*
*
*
Oestradiol
Progesterone
CYP19A1
LHCGR

Fig 2

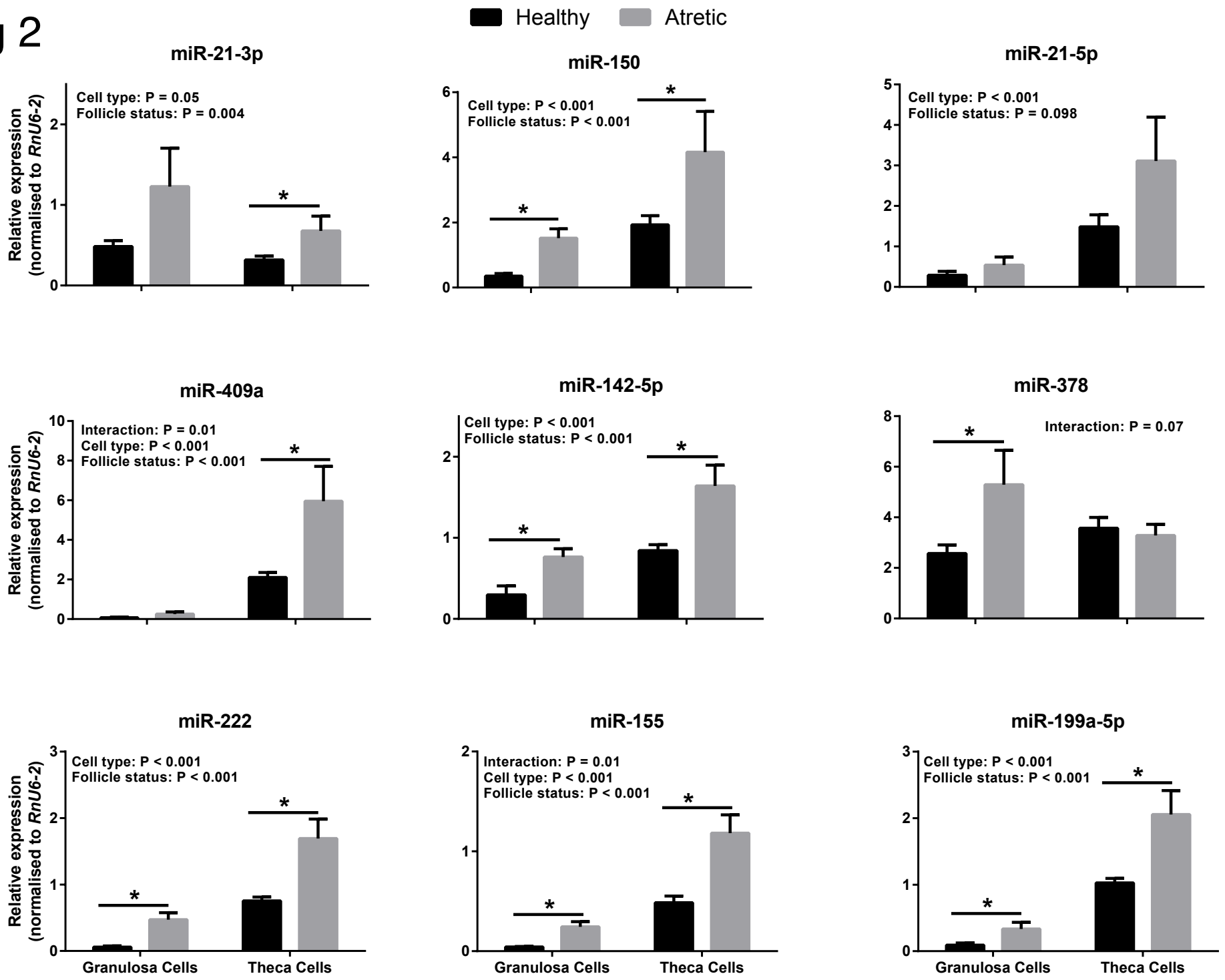

Healthy
Atretic
miR-21-3p
Cell type: P = 0.05
Follicle status: P = 0.004
Relative expression (normalised to RnU6-2)
miR-150
Cell type: P < 0.001
Follicle status: P < 0.001
miR-21-5p
Cell type: P < 0.001
Follicle status: P = 0.098
miR-409a
Interaction: P = 0.01
Cell type: P < 0.001
Follicle status: P < 0.001
miR-142-5p
Cell type: P < 0.001
Follicle status: P < 0.001
miR-378
Interaction: P = 0.07
miR-222
Cell type: P < 0.001
Follicle status: P < 0.001
miR-155
Interaction: P = 0.01
Cell type: P < 0.001
Follicle status: P < 0.001
miR-199a-5p
Cell type: P < 0.001
Follicle status: P < 0.001
Granulosa Cells
Theca Cells

Fig 3

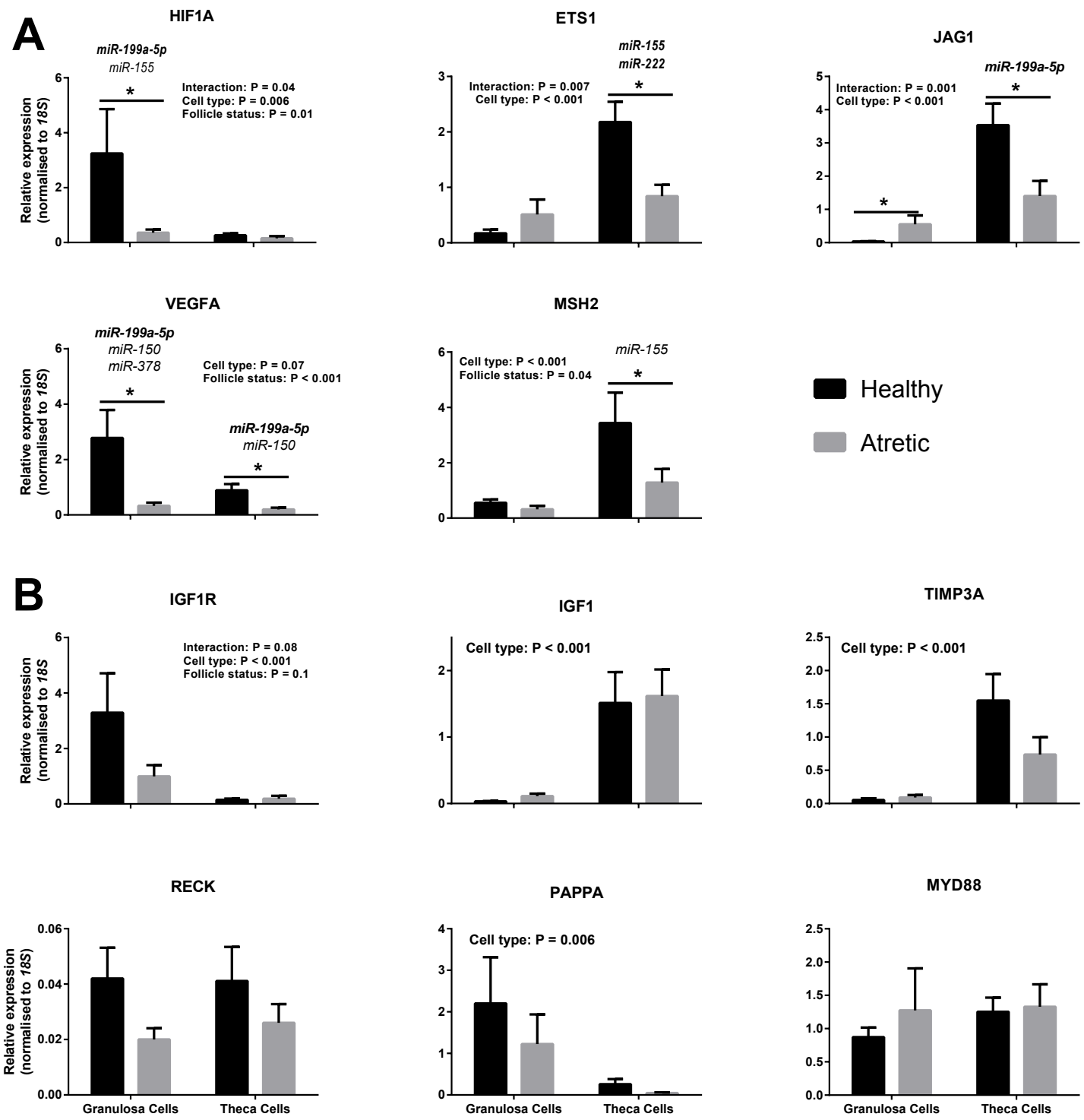
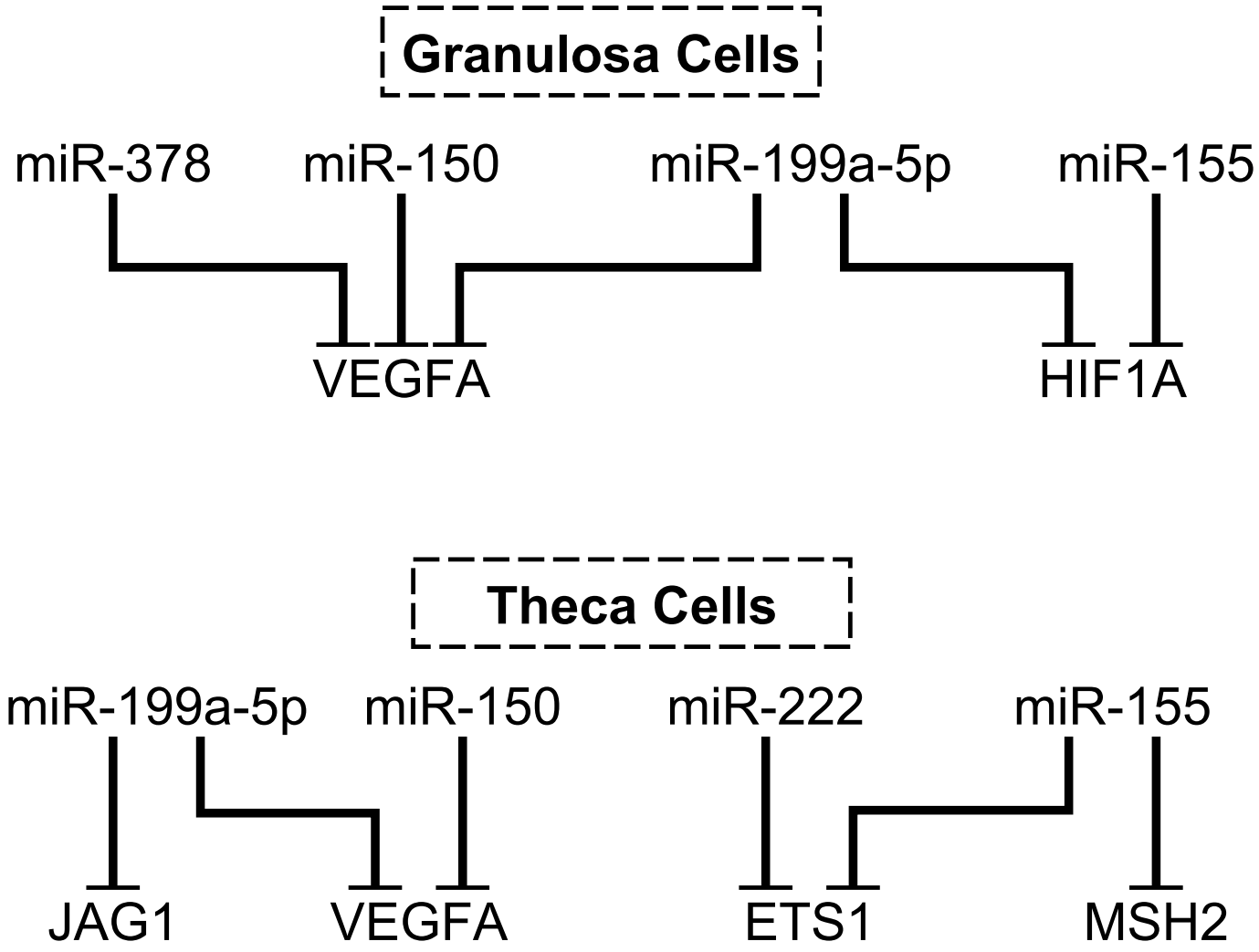

A
HIF1A
miR-199a-5p
miR-155
Interaction: P = 0.04
Cell type: P = 0.006
Follicle status: P = 0.01
Relative expression (normalised to 18S)
ETS1
miR-155
miR-222
Interaction: P = 0.007
Cell type: P < 0.001
JAG1
miR-199a-5p
Interaction: P = 0.001
Cell type: P < 0.001
VEGFA
miR-199a-5p
miR-150
miR-378
Cell type: P = 0.07
Follicle status: P < 0.001
miR-199a-5p
miR-150
MSH2
miR-155
Cell type: P < 0.001
Follicle status: P = 0.04
Healthy
Atretic
B
IGF1R
Interaction: P = 0.08
Cell type: P < 0.001
Follicle status: P = 0.1
IGF1
Cell type: P < 0.001
TIMP3A
Cell type: P < 0.001
RECK
PAPPA
Cell type: P = 0.006
MYD88
Granulosa Cells
Theca Cells

Fig 4


Granulosa Cells
miR-378
miR-150
miR-199a-5p
miR-155
VEGFA
HIF1A
Theca Cells
miR-199a-5p
miR-150
miR-222
miR-155
JAG1
VEGFA
ETS1
MSH2