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Functional studies of microRNA 17-92 cluster members in bovine granulosa cells and oocyte maturation

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Dedicated to my beloved wife and son

Functional studies of microRNA 17-92 cluster members in bovine granulosa cells and oocyte maturation

Dynamic transcript of genes expression are believed to occur during follicular development and oocyte maturation, which one way or the other is regulated by a post-transcriptional modifier, namely microRNA. In the previous work, among others, miR-17-92 cluster members were overexpressed in granulosa cell of subordinate follicle at day 19 of estrous cycle compared to the dominant ones. Thus, we hypothesized the potential involvement of miR-17-92 cluster members in follicular function at the late stage of the estrous cycle. Therefore, the aim of this thesis was to investigate the role of miR-17-92 cluster members in bovine granulosa cell and oocyte maturation. First, potential target gene of miR-17-92 cluster were predicted in silico followed by validation using luciferase assay. In order to investigate the role of miR-17-92 cluster member in granulosa cell function and oocyte maturation, we modulated the expression of those microRNAs in granulosa cells and cumulus-oocyte complexes (COCs) under in vitro condition. Target prediction and validation revealed that PTEN and BMPR2 are direct target genes of miR-17-92 cluster members. This result was confirmed by the alteration of PTEN and BMPR2 expression in granulosa cells transfected with miR-17-92 cluster members mimic and inhibitor. In this study, we observed that overexpression of miR-17-92 cluster increased proliferation and decreased differentiation rate of granulosa cells. On the other hand, inhibition of miR-17-92 cluster showed the opposite phenotypes. However, progesterone level in spent media of granulosa cells culture was not persistent with the cell differentiation rate. Further, cross-validation by target knockdown PTEN and BMPR2 genes simulated the results obtained from granulosa cells transfected miR-17-92 cluster member. In addition, the expression of one of miR-17-92 cluster members (miR-20a) in cumulus cells increased after in vitro maturation (IVM). Contrastively, it was decreased in oocytes after IVM. Moreover, the expression of miR-20a in cumulus cells and oocytes was affected by the presence or absence of their companion cells during culture. The expression of miR-20a in cumulus cells and oocytes from COCs cocultured with miR-20a mimic or inhibitor suggested that the transfection was restricted in the cumulus cells. In this study, miR-20a overexpression in COCs culture increased oocyte maturation rate and cumulus cell progesterone synthesis. On the other hand, inhibition of miR-20a did not affect the oocyte maturation rate, but decreased progesterone synthesis. In conclusion, the miR-17-92 cluster members involved in granulosa cell proliferation and differentiation, as well as oocyte maturation by targeting PTEN and BMPR2 genes.

Funktionelle Bedeutung des miRNA-17-92 Komplexes in bovinen Granulosazellen und in der bovinen Eizellreifung

Die Genexpression während der Follikelentwicklung und Eizellreifung ist räumlich/zeitlich dynamisch geregelt, unter anderem spielt die posttranskriptionelle Expressionsregulation durch miRNAs eine bedeutende Rolle. In vorangegangenen Arbeiten unserer Arbeitsgruppe wurde gezeigt, dass Mitglieder des miR-17-92 Komplexes in Granulosazellen subordinater Follikel, im Vergleich zu dominanten Follikeln, am Tag 19 des Östrus überexprimiert sind. Daraus ergab sich für diese Arbeit die Hypothese, dass der miR-17-92 Komplex im späteren Zyklus die follikuläre Dynamik beeinflusst. Daher war es das Ziel dieser Arbeit, die Rolle von miR-17-92 in bovinen Granulosazellen und während der Oozytenmaturation aufzuklären. Zunächst wurden potentielle Target-Gene des miR-17-92 Komplexes identifiziert, mittels Luziferase-Array validiert und die Expression der miRNAs in Granuloszellen und im Kumulusoozytenkomplex stimuliert und inhibiert. Die Zielgenidentifizierung und Validierung bestätigte PTEN und BMPR2 als direkte Zielgene des miR-17-92 Komplexes. Die Expression von PTEN und BMBR2 konnte mit miR-17-92 Agonisten und Inhibitoren moduliert werden. Die Überexpression von miR-17-92 erhöhte die Proliferation und verringerte die Differenzierungsrate der Granulosazellen. Die Inhibierung von miR-17-92 zeigte den gegenteiligen Phänotyp. Der Progesteronspiegel im Kulturmedium der Granulosazellen war nicht konsistent mit der Differenzierungsrate. Knockdown von PTEN und BMPR2 zeigten dieselben Ergebnisse wie die mit miR-17-92 transfizierten Granulosazellen. Die Expression von miR-20a in Kumuluszellen und Kumuluszelloozytenkomplexen, die mit miR-20a Agonisten oder Inhibitoren kokultiviert wurden, wiesen auf eine Expression in den Kumuluszellen hin. Die Expression von miR-20a in Kumuluszellen verbesserte die in vitro Maturationsrate und die Kumuluszell Progesteronsynthese; die Inhibierung von miR-20a reduzierte die Progesteronsynthese, hatte aber keinen Einfluss auf die Oozytenmaturationsrate. Es kann gefolgert werden, dass der miR-17-92 Komplex über die Zielgene PTEN und BMPR2 an der Oozytenmaturation und der Granulosaproliferation- und Differentiation regulierend beteiligt ist.

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List of abbreviations

ACVR2A	:	Activin receptor type-2A
ACVR2B	:	Activin receptor type-2B
AKT	:	Protein kinase B
ATM	:	Ataxia telangiectasia mutated
BDNF	:	Brain-derived neurotrophic factor
BMP	:	Bone morphogenetic protein
BMPR2	:	Bone morphogenetic protein receptor type II
CC	:	Cumulus cell
CCK-8	:	Cell-counting kit-8
CCND2	:	Cyclin D2
CDKN1A	:	Cyclin-dependent kinase inhibitor 1
CDNA	:	Complementary DNA
CO2	:	Carbon dioxide
COC	:	Cumulus-oocyte complex
CYCB2	:	Cytochrome C552
CYP11A1	:	Cholesterol side-chain cleavage enzyme
CYP19A1	:	Aromatase
DHCR24	:	24-dehydrocholesterol reductase
DMEM	:	Dulbecco’s modified Eagle's medium
E2F1-3	:	E2F transcription factor 1-3
EDTA	:	Ethylenediaminetetraacetic acid
EGFR	:	Epidermal growth factor receptor
ELISA	:	Enzyme-linked immunosorbent assay
FBS	:	Fetal bovine serum

FOXL2	:	Forkhead box protein L2
FOXO	:	Forkhead box protein O
GV	:	Germinal vesicle
GVBD	:	Germinal vesicle breakdown
HPGD	:	Hydroxyprostaglandin dehydrogenase 15-(NAD)
HSD3B2	:	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
INHBA	:	Inhibin beta A subunit
INHBB	:	Inhibin beta B subunit
IRAK1	:	Interleukin-1 receptor-associated kinase 1
IU	:	International unit
IVM	:	In vitro maturation
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
LH	:	Luteinizing hormone
LNA	:	Locked nucleic acid
MAP3KI	:	Mitogen-activated protein kinase kinase kinase 1
MAPK1	:	Mitogen-activated protein kinase 1
MII	:	Metaphase II
mRNA	:	Messenger RNA
MYC	:	V-myc avian myelocytomatosis viral oncogene homolog
NC	:	Negative control
NURR1	:	Nuclear receptor related 1 protein
OD	:	Optical density
Oo	:	Oocyte
P27	:	Cyclin-dependent kinase inhibitor 1B
PCNA	:	Proliferating cell nuclear antigen

PCR	:	Polymerase chain reaction
PGR	:	Progesterone receptor
PI3K	:	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLB	:	Passive lysis buffer
PTEN	:	Phosphatase and tensin homolog
PTGS2	:	Prostaglandin-endoperoxide synthase 2
PTX3	:	Pentraxin 3
qRT-PCR	:	Quantitative real-time PCR
RBMS1	:	RNA-binding motif, single-stranded-interacting protein 1
rRNA	:	Ribosomal RNA
SDS	:	Sodium dodecyl sulfate
SEM	:	Standard error of the mean
SFI	:	Splicing factor 1
siRNA	:	Small interfering RNA
SIRT1	:	Sirtuin 1
SMAD	:	Mothers against decapentaplegic
SMAD3	:	Mothers against decapentaplegic homolog 3
SMAD4	:	Mothers against decapentaplegic homolog 4
SMAD7	:	Mothers against decapentaplegic homolog 7
STAR	:	Steroidogenic acute regulatory protein
STAT3	:	Signal transducer and activator of transcription 3
TCM	:	Tissue culture media
TGFB1	:	Transforming growth factor beta 1
TRAF6	:	TNF receptor associated factor 6
UTR	:	Untranslated region
v	:	Volume

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1.1 Introduction

Follicular development is a complex process regulated by a vast number of intra- and extra-ovarian factors. Based on the developmental stage and their dependence on the gonadotropins, follicular development can be classified into three phases namely: primordial until preantral follicular phase (gonadotropin-independent phase), transition from preantral to early antral stage (gonadotropin-responsive), and gonadotropin-dependent follicular growth phase which includes follicle recruitment, selection and ovulation (Aerts and Bols 2010, McGee and Hsueh 2000). In bovine species, follicular development within one estrous cycle occurs in a wave-like pattern (Jaiswal et al. 2009). In every wave, several follicles will be recruited into the growth phase but one follicle will be selected to subsequently be a dominant (Ginther et al. 1989). Although some biological and regulatory mechanisms are known, several key elements of folliculogenesis remain unclear. Among several processes during follicular development in cattle, mechanisms that allowed selected follicle to grow into dominant follicle while the rest follicle regress (Evans and Fortune 1997), are not clearly understood. However, several studies have demonstrated the critical interaction between oocyte and surrounding somatic cells (theca and granulosa cells) (Cecconi et al. 2004, Gilchrist et al. 2004b, Orisaka et al. 2009) and within somatic cells as well (Parrott and Skinner 1998).

Granulosa cells are the most important follicular cells for supporting the follicular growth progression, oocyte developmental competence and ovulation (Voronina et al. 2007). At the late stage of the estrous cycle, follicles enter into non-exponential growth phase with slower increase in diameter (Ali et al. 2001, Manikkam and Rajamahendran 1997). This condition coincided with the declining of granulosa cell proliferation and progression while terminal differentiation of granulosa cell lead to granulosa lutein cells (Hirshfield 1991, Rao et al. 1978, Richards et al. 1986, Richards 1994). The granulosa cell proliferation and differentiation seem to be a critical cellular activity within the dominant follicle and is regulated by the balance between positive and negative regulators of cell cycle kinase cascades (Robker and Richards 1998a). For instance, deletion of the proliferation-related gene in mice granulosa cell, namely CCND2, resulted in decreased proliferation and a reduced number of granulosa cell. Similarly, the P27 gene deleted granulosa cell, a gene responsible for differentiation, exhibited impaired luteinization and reduced number of the granulosa lutein cell. These both phenomenons lead to incomplete folliculogenesis and ovulation failure (Robker and Richards 1998b).

The ovulation process is the result of a long and orchestrated process to release the competent oocyte which is fertilizable, followed by normal embryo development and eventually the birth of healthy offspring (Labrecque and Sirard 2014). During the oogenesis, the oocyte is arrested in the diplotene of the prophase stage of first meiosis cleavage within the ovarian follicle. Along with the follicle recruitment, the volume of the oocyte increases, undergoes replication and results in redistribution of cytoplasmic organelles (Picton et al. 1998). The resumption of the meiosis progression of the oocyte during folliculogenesis is influenced by the local microenvironment formed by companion somatic cells, namely, cumulus oophorus (Sanchez and Smitz 2012). The interaction between oocyte and cumulus cells is crucial for the development and functions of both cell types (Eppig 2001, Gilchrist et al. 2004a, Matzuk et al. 2002). For instance, the removal of cumulus cell before in vitro oocyte maturation inhibits oocyte developmental competence (Vozzi et al. 2001), and the similar result was obtained when the interaction of both cells was disrupted using gap junction inhibitors (Atef et al. 2005).

At the late stage of estrous cycle, granulosa cells from preovulatory follicle started to express the LH receptor (Bao et al. 1997). In response to the LH surge, the granulosa cell starts to differentiate into granulosa lutein cell, the expression of some genes related to progesterone synthesis was increased and followed by the increment of granulosa cell progesterone synthesis (Baufeld and Vanselow 2013, Chang et al. 2015, Havelock et al. 2004, Zhang et al. 2015). Apart from granulosa cells, six hours after LH surge, the germinal vesicle breakdown (GVBD) of the oocyte is started along with increased steroidogenesis-related genes in the cumulus cells, including HSD3B2, INHBA, PGR, HPGD and DHCR24 (Assidi et al. 2010, Dieleman et al. 1983, Sirard et al. 1989). Several studies proved that supplementation of LH and FSH within in vitro maturation (IVM) media induced oocyte maturation, cumulus cells expansion, synthesis of hyaluronic acid and progesterone production by cumulus cells (Ježová et al. 2001, Nagyova et al. 2011, Nagyova et al. 2012, Nagyová et al. 1999, Procházka et al. 1991). The progesterone synthesis during oocyte maturation process is essential for oocyte meiosis resumption and subsequent oocyte maturation processes (Aparicio et al. 2011, Choi et al. 2001, Ježová et al. 2001, Montano et al. 2009, Nagyova et al. 2011, Nagyova et al. 2012, Shimada et al. 2004c, van Tol et al. 1996, Yamashita et al. 2003). In addition, administration of progesterone during IVM is believed to promote oocyte maturation in various species in a dose-dependent manner (Jamnongjit et al. 2005, Siqueira et al. 2012, Yamashita et al. 2003). On the other hand, the inhibition of progesterone synthesis during oocyte maturation drastically decreased the percentage of mature oocyte (MII stage), ovulation rate and

subsequent embryonic development in mouse (Aparicio et al. 2011, Panigone et al. 2008, Siqueira et al. 2012, Sirotkin 1992), porcine (Kawashima et al. 2008, Shao et al. 2003, Shimada and Terada 2002, Shimada et al. 2004c) and bovine species (Aparicio et al. 2011, O'Shea et al. 2013, Roh et al. 1988, Shao et al. 2003, Shimada et al. 2004b, Shimada et al. 2004a, Wang et al. 2006).

Insights into the molecular mechanism regulating the follicular development, an orchestrated expression of genes are believed to occur in various follicular cells. Several studies have been conducted to investigate the differential expression of genes in granulosa cells derived from small, medium and large antral follicle in bovine (Douville and Sirard 2014, Hatzirodos et al. 2014b). The mRNA abundance in the different follicular size demonstrated numbers of genes and pathways associated with the regulation of follicular dominance (Girard et al. 2015, Hatzirodos et al. 2014a) and development of oocyte competence in the late stage of estrous cycle (Nivet et al. 2013). Apart from this, the dynamics of gene expression in oocyte and cumulus cells before and after maturation process indicates the spatiotemporal regulation of genes during oocyte maturation (Assidi et al. 2010, Fair et al. 2007, Regassa et al. 2011). We have shown in our previous work that the presence of oocyte and cumulus cells in the culture of oocyte-ectomized cumulus cells and denuded oocyte, respectively, altered the pattern of the differential expressed genes in both cells types (Regassa et al. 2011). It has been postulated that the differential expression of genes in one way or the other are believed under the regulation of post-transcriptional modifiers, namely microRNAs.

The role of microRNAs has been studied in the last decade to provide evidenced the role of microRNAs in the follicular cells function (Table 1). In our previous work, we demonstrated the dynamic change of the microRNAs global expression in granulosa cells derived from large and small follicles at day 3, 7 (Salilew-Wondim et al. 2014) and 19 (Gebremedhn et al. 2015) of the estrous cycle in bovine. Especially in the late stage of the estrous cycle (day 19), among several microRNAs, miR-17-5p, miR-19a, miR-20a and miR-92a, which are belong to miR-17-92 cluster, were found to be upregulated in the subordinate follicle compared to the dominant ones (Gebremedhn et al. 2015).

Table 1. List of microRNAs expressed in ovarian follicle cells.

Functions	MicroRNA	Target genes	Reference
Promote granulosa cells proliferation	Hsa-miR-93	CDKN1A	(Jiang et al. 2015)
	Mmu-miR-242	SMAD7	(Yao et al. 2010)

Suppress granulosa cell proliferation	Chi-miR-10b	BDNF	(Peng et al. 2016)
	Mmu-miR-181a	ACVR2A	(Zhang et al. 2013)
	Mmu-miR-145	ACVR1B	(Yan et al. 2012)
Induce granulosa cell apoptosis	Mmu-miR-125a-5p	STAT3	(Wang et al. 2016)
	Hsa-miR-146a	IRAK1	(Chen et al. 2015)
		TRAF6	
	Ssc-let-7g	TGFB1	(Zhou et al. 2015a)
		MAP3K1	(Cao et al. 2015)
	Ssc-miR-26b	SMAD4	(Liu et al. 2014a)
	Ssc-miR-34a	INHBB	(Tu et al. 2014)
	Ssc-miR-26b	ATM	(Lin et al. 2012)
Inhibit granulosa cell apoptosis	Mmu-miR-22	SIRT1	(Xiong et al. 2016)
	Ssc-miR-92	SMAD7	(Liu et al. 2014b)
Regulate 17β-estradiol synthesis of granulosa cells	Mmu-miR-764-3p	SF1	(Wang et al. 2015)
	Mmu-miR-132	NURR1	(Wu et al. 2015)
	Mmu-miR-133b	FOXL2	(Dai et al. 2013)
	Mmu-miR-383	RBMS1	(Yin et al. 2012)
	Ssc-miR-378	CYP19A1	(Xu et al. 2011a)
Regulate oocyte maturation	Ssc-miR-378	CYP19A1	(Pan et al. 2015)
Involve in the regulation of cumulus expansion	Mmu-miR-224	PTX3	(Yao et al. 2014)

MicroRNA 17-92 (miR-17-92) cluster is one of the best characterized polycistronic miRNAs, consist of six individual miRNAs, namely miR-17, miR-18a, miR-19a, miR-20, miR-19b and miR-92a (Kumar et al. 2013). Based on the sequence homology and seed region conservation, the six individual miRNA in the cluster belong to four different microRNA families, namely miR-17 (miR-17 and miR-20), miR-18, miR-19 (miR-19a and miR-19b) and miR-92 family (Tanzer and Stadler 2004). In addition, this microRNA cluster is transcribed from intergenic

region of chromosome 12 of the bovine genome and its expression is believed to be regulated by MYC (Kumar et al. 2013, O'Donnell et al. 2005), E2F1-3 (Sylvestre et al. 2007) and SMAD3 (Luo et al. 2014) transcription factor genes. Several studies have been conducted by modulating the expression of miR-17-92 cluster revealed that miR-17-92 cluster members play a role in cell differentiation, cell proliferation, self-renewal, cell apoptosis and motility of various cell types (Cohen et al. 2015, Dou et al. 2015, Li et al. 2014, Poitz et al. 2013, Qin et al. 2013, Wu et al. 2013, Wu et al. 2014, Xu et al. 2014). However, the functional role of this microRNA cluster in granulosa cell function and oocyte maturation is not yet known.

Taking all these information into account, we hypothesized that modulation of miR-17-92 cluster members play significant role in bovine granulosa cells to support the follicular development and oocyte growth. For this, two experiments were conducted to achieve the following objectives:

1. To investigate the role of miR-17-92 cluster in bovine granulosa cell function.
2. To investigate the role of this microRNA cluster in bovine oocyte maturation in vitro.

1.2 Materials and methods

To achieve the objectives of this study, several materials and methods were used. The details of materials and methods are described in the respective chapters of this thesis. The importance of the main methods and their description are briefly summarized here.

1.2.1 Bovine granulosa cell culture

Granulosa cells were isolated from healthy small follicle (3-5 mm of diameter) of bovine ovaries obtained from a local slaughterhouse. Cell concentration was determined using trypan blue exclusion method. A total 2.5 x 10^5 granulosa cells per well were seeded into 600 µl culture medium (DMEM/F12-HAM medium supplemented with 10% FBS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 2.5 µg/ml fungizone (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in CytoOne® 24-well plate (Starlab GmbH, Hamburg, Germany). Granulosa cell cultures were performed at 37 ºC in a humidified atmosphere with 5% (v/v) CO_2 in air.

1.2.2 Cumulus-oocyte complex (COC) collection and in vitro oocyte maturation

The COCs were isolated from healthy small follicles (2-8 mm of diameter) of bovine ovaries obtained from a local slaughterhouse. The good quality and morphologically uniform COCs were washed before set into culture to obtain matured oocytes or were directly frozen as immature COCs (germinal vesicle; GV). The COCs were cultured in groups of 50 in 400 µl of maturation media (modified parker medium supplemented with 12% estrus cow serum and 10 µg/ml Follitropin®) under mineral oil in five-well dishes. Maturation was performed for 22 h at 39 °C in a humidified atmosphere with 5% (v/v) CO_2 in air. The cumulus cells and oocytes from the immature and matured group of COCs were separated by gentle pipetting in TCM-199 supplemented with hyaluronidase (1 mg/ml). Matured oocyte (metaphase II stage; MII) was indicated by the presence of first polar body under an inverted microscope. The total numbers of recovered and matured oocytes after IVM were recorded. The maturation rate was calculated from the number of matured oocytes compared to the total number of recovered oocytes.

1.2.3 Cumulus cells and denuded oocytes culture

To investigate the effect of oocyte on cumulus cells microRNA expression and vice versa, cumulus cells and oocytes were cultured in the presence or absence of the others. For this, cumulus cells and oocytes from 100 collected COCs were separated and cultured independently in the maturation media at 39 °C in 5% (v/v) CO_2 incubator for 22 h. The cumulus cells (CCs-Oo) and denuded oocytes (Oo-CCs) were collected and stored at -80 °C until further analysis. The cumulus cells and oocytes from COCs culture were used as controls (CCs+Oo and Oo+CCs).

1.2.4 MicroRNA mimic and inhibitor transfection

To investigate the function of microRNA 17-92 cluster in bovine granulosa cell culture, 100 nM of individual or a pool of microRNA 17-92 cluster mimic or inhibitor (miR-17-5p, miR-19a, miR-20a, miR-92a; miRCURY LNA™; Exiqon, Vedbaek, Denmark) were transfected into subconfluent granulosa cells using Lipofectamine® 2000 transfection reagent (Life Technologies GmbH, Darmstadt, Germany). Cultured granulosa cells and spent media were collected 48 h post-transfection. To investigate the role of miR-20a during IVM, 50 nM of miR-20a mimic or inhibitor were transfected in COCs. Maturated COCs and spent media were collected 22 h post-transfection.

1.2.5 MicroRNA target prediction

Target gene prediction for members of the miR-17-92 cluster was performed by using miRWalk database (http://www.umm.uniheidelberg.de/apps/zmf/mirwalk/). The target genes were identified at least by four prediction tools and those with p-value<0.05 were selected for further analysis. Common target genes of all microRNA in the cluster were selected and binding site prediction was performed by using PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) with minimum 7-mer seed region. Pathway analysis was performed by KEGG pathways database (http://www.genome.jp/kegg/pathway.html) and microRNA-mRNA binding site prediction in bovine sequences was performed by using TargetScan (http://www.targetscan.org).

1.2.6 Plasmid construction and luciferase assay

Wild-type plasmid was constructed by ligating the 3´-UTR fragment into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega GmbH, Mannheim, Germany). Gene specific primers were used to amplify 3´-UTR region of PTEN and BMPR2 as listed in Table S1 (Chapter 2). Specific primers and 50 bp mutated-oligonucleotides were designed based on bovine PTEN (XM_613125) and BMPR2 (NM_001304285) mRNA sequences in GenBank. Afterwards, subconfluent cultured granulosa cells (70-80% of confluency) were co-transfected with 500 ng of plasmid harboring wild-type or mutated-sequences and 50 nM of the corresponding individual microRNA mimic or mimic negative control (mimic NC; Exiqon, Vedbaek, Denmark). Transfection was performed using Lipofectamine® 2000 (Life Technologies GmbH, Darmstadt, Germany) as transfection reagent. The cell lysate was extracted using 1x Passive Lysis Buffer (PLB; Promega GmbH, Mannheim, Germany) at 48 hours post transfection. Luciferase activity assay in cultured granulosa cells was performed using pmirGLO Dual Luciferase® Reporter Assay System (Promega GmbH, Mannheim, Germany) according to manufacturer's protocol. The absorbance of firefly and renilla luciferase activity was detected by Centro LB 960 Microplate Luminometer (Berthold Technologies GmbH).

1.2.7 Total RNAs isolation from granulosa and cumulus cells

Total RNAs from granulosa and cumulus cells were extracted using miRNeasy® mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. Before starting the total RNAs extraction, granulosa and cumulus cells were washed using 1x PBS to remove remaining culture media. At the end of the extraction protocol, trapped RNAs in the membrane of spin column were eluted using 30 µl RNase-free water. Quantity and quality of isolated RNAs were assessed using NanoDrop 8000 UV-Vis Spectrophotometers (Thermo Scientific, Wilmington, USA).

1.2.8 Total RNA isolation from denuded oocytes

In order to extract the total RNAs from denuded oocytes, a pool consisted of 50 denuded oocytes was washed using 0.9 ml 1 x PBS/0.1 ml 0.5 M EDTA followed by centrifugation at 3000 x g for 5 minutes. The supernatant was removed and the total RNAs was isolated from cell pellet using PicoPure® RNA isolation kit (Life Technologies GmbH, Darmstadt,

Germany). Total RNAs concentration and purity were determined using NanoDrop 8000 UV-Vis Spectrophotometers (Thermo Scientific, Wilmington, USA) for further analysis.

1.2.9 Candidate genes expression analysis

To investigate the expression of miR-17-92 cluster members target and candidate genes in granulosa and cumulus cells, the equal amount of total RNA was reverse transcribed using RevertAid first stand cDNA synthesis kit (Life Technologies GmbH, Darmstadt, Germany) with oligo (dT)18 primer. The primers used for gene expression analysis have been confirmed by sequencing analysis using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter GmbH, Krefeld, Germany). The quantitative PCR (qPCR) was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany) in Applied Biosystem® StepOnePlus™ (Applied biosystems, Foster City, CA, USA). The mRNAs expression data was analyzed using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schmittgen 2001) and the expression level of β-ACTIN was used for normalization.

1.2.10 MiR-20a expression analysis

The cDNA for miR-20a expression was constructed from an equal amount of total RNA using Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) following the manufacturer's instructions. MiR-20a expression was performed using ExiLENT SYBR® green master mix (Exiqon, Vedbaek, Denmark) in Applied Biosystem® StepOnePlus™ (Applied biosystems, Foster City, CA, USA). The relative expression of miR-20a was analyzed using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schmittgen 2001). The 5s rRNA expression was used as an internal control.

1.2.11 Western blot analysis

Total protein from granulosa and cumulus cells were isolated using 1x PLB (passive lysis buffer; Promega GmbH, Mannheim, Germany) and the protein concentration was determined using Coomassie Protein Assay Reagent (Life Technologies GmbH, Darmstadt, Germany). The same amount of protein was separated on 4-16% gradient SDS-polyacrylamide gel. Transfer protein from the gel into Immun-Blot® PVDF Membrane (Bio-Rad Laboratories

GmbH, Germany) was performed using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories GmbH, Germany). The membrane was blocked in Roti®-Block (Carl Roth GmbH, Germany) for 1 h at room temperature and then incubated overnight at 4 °C with an anti-BMPRII goat polyclonal antibody, anti-PTEN goat polyclonal antibody, anti-PCNA rabbit polyclonal antibody, anti-STAR rabbit polyclonal antibody or anti-β-ACTIN mouse monoclonal antibody (Santa Cruz Biotechnology Inc, Germany). The membrane was incubated for further 2 h at room temperature in donkey anti-goat, goat anti-rabbit or goat anti-mouse IgG-HRP (1:10000; Santa Cruz Biotechnology Inc, Germany). The protein bands were visualized using Clarity™ Western ECL Substrate (Bio-Rad Laboratories Inc, USA) and membrane image was captured using ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, Germany) and the ImageJ 1.48v software (http://imagej.nih.gov/ij) was used to analyze the signal intensities.

1.2.11 Determination of granulosa cell diameter

Granulosa cell diameter was measured as an indicator for cell differentiation using ImageJ 1.48v software (http://imagej.nih.gov/ij). The pictures of granulosa cells were captured from five different areas of counting chamber using 40 x magnifications. Cells with diameter ≥ 14 µm were considered as a differentiated cell while those with diameter ≤ 14 µm were considered as undifferentiated (Kuran et al. 1995).

1.2.12 Cell proliferation assay

To investigate the proliferation rate, a total 2 x 10^4 were cultured in 96-well plate and cultured in the culture medium (described in the previous section). MiR-17-92 cluster members mimic or inhibitor was transfected 48 h after seeding. Granulosa cell proliferation analysis was performed 48 h post-transfection using CCK-8 kit (Dojindo EU GmbH, München, Germany) according to manufacturer´s protocol. The proliferation rate was indicated by the absorbance at 450 nm wavelength using Synergy™ H1 Multi-Mode Reader (BioTek Germany, Bad Friedrichshall, Germany).

1.2.13 Progesterone assay

Progesterone level in spent media of granulosa cell culture and IVM were measured using progesterone ELISA kit (ENZO life sciences GmbH, Lörrach, Germany) according to the manufacturer´s instruction. Before analysis, the spent media was diluted using 1x PLB. The progesterone level was calculated from optical density at 405 nm wavelength using Synergy™ H1 Multi-Mode Reader (BioTek Germany, Bad Friedrichshall, Germany).

1.2.14 Targeted knockdown of PTEN and BMPR2 genes using small interfering RNA (siRNA)

Small interfering RNA (siRNA) targeting PTEN or BMPR2 gene was transfected into subconfluent cultured granulosa cell to cross-validate the results obtained from miR-17-92 cluster members transfection. Gene expression and other phenotype measurements, namely cell diameter, cell proliferation and progesterone levels analysis were performed 48 h post siRNA transfection.

1.2.15 Data analysis

All data are presented as mean ± standard error mean (SEM). The statistical significant difference between the groups was analyzed using t-test in GraphPad Prism® software version 5.02 (GraphPad Software, Inc., La Jolla, CA, USA). Mean differences at $p<0.05$ were considered as significant and indicated in the corresponding figure legend.

1.3 Results

The main results in this thesis are briefly described here. The detailed results can be found in the respective chapters in this thesis.

1.3.1 Target prediction and validation of miR-17-92 cluster member in granulosa cells

In this study, we first performed in silico analysis to predict potential genes targeted by miR-17-92 cluster members. Bioinformatic analysis by miRwalk revealed that 233 genes to be commonly targeted by the individual miRNA members of the cluster, namely miR-17-5p, miR-19a, miR-20a and miR-92a. Binding site prediction based on PITA identified 91 genes with a minimum 7-mer binding site. Pathway analysis of the top 26 genes showed that PI3K-AKT signaling pathway, FOXO signaling pathway, focal adhesion and hippo signaling pathway to be among the top 10 pathways. The PTEN and BMPR2 were selected as targets based on their conserved binding site between human and bovine, and their potential involvement in bovine granulosa cells function.

Validation of PTEN and BMPR2 as potential target genes of miR-17-92 cluster members was performed using luciferase assay. Here we showed that transfection of miR-17-92 cluster members mimic in granulosa cell harboring 3´-UTR of PTEN and BMPR2 genes reduced the luciferase activity compared to those granulosa cell transfected with microRNA mimic negative control. In addition, no difference was observed between relative luciferase activity in granulosa cells harboring the mutant-type constructed plasmid PTEN and BMPR2 3´-UTR transfected with miR-17-92 cluster members mimic compared to microRNA mimic control.

Further validation was performed by modulating the expression of individual or pooled miR-17-92 cluster members in cultured granulosa cells. In the present study, we found that overexpression of individual or pool of miR-17-92 cluster members in granulosa cells resulted in decreased mRNA expression of PTEN and BMPR2 genes. However, the expression of PTEN gene was upregulated in miR-17-5p and miR-19a inhibitor transfected granulosa cells while elevated BMPR2 gene expression was observed in granulosa cells transfected with miR-19a, miR-20a and pool of miR-17-92 cluster. These results from mRNA expression analysis were confirmed by the protein expression analysis of those target genes.

1.3.2 The effect of miRNA 17-92 cluster members modulation in granulosa cell proliferation and differentiation

We next examined the effect of overexpression and inhibition of miR-17-92 cluster in granulosa cell function. In this experiment, we showed that except miR-17-5p mimic, granulosa cells transfected with individual or a pool of miR-17-92 cluster members mimic increased cellular proliferation. On the other hand, proliferation rate in granulosa cells transfected with miR-92a and a pool of miR-17-92 cluster inhibitors was significantly reduced. These results were further confirmed by the expression of cell proliferation marker genes, namely CCND2 and PCNA. The overexpression of miR-17-5p and miR-19a in granulosa cells increased the expression of CCND2 gene, whereas the PCNA expression was increased in granulosa cells transfected with miR-20a and miR-92a mimic. Inhibition of miR-17-92 cluster members in granulosa cells resulted in decreased CCND2 gene expression while no difference was observed in the expression of PCNA gene. Nevertheless, PCNA protein analysis revealed an increasing trend in granulosa cells transfected with either miRNA-17-92 cluster mimic or inhibitor.

Granulosa cell differentiation as indicated by cell diameter revealed that except in miR-20a overexpression, granulosa cells transfected with miR-17-92 cluster mimic had a lower percentage of differentiated cells. No difference in cell differentiation rate was observed in granulosa cell transfected with miR-17-92 cluster members. In addition, progesterone level measured in spent media showed that miR-20a overexpressed granulosa cells resulted in increased progesterone synthesis. There was no effect of either miR-17-92 cluster members mimic or inhibitor in the mRNA expression of cell differentiation-related genes, namely CYP11A1 and STAR. However, the PCNA protein in granulosa cell transfected with miR-17-92 cluster members mimic and inhibitor showed decreasing and increasing trend, respectively.

1.3.3 Selective knockdown of PTEN and BMPR2 genes

Cross-validation of the results obtained from miR-17-92 cluster members mimic transfection was performed by selectively targeted knockdown of PTEN and BMPR2 in cultured granulosa cells using siRNA. The suppression of the PTEN gene resulted in increased proliferation rate while no difference was observed in BMPR2 suppression. The mRNA

expression of CCND2 and PCNA genes were relatively higher in PTEN and BMPR2 suppressed granulosa cells. However, the protein level of PCNA showed the opposite trend. Apart from cell proliferation, the percentage of differentiated granulosa cells was decreased in PTEN suppressed granulosa cells. The progesterone level detected in spent media indicated an increasing progesterone synthesis in granulosa cells transfected with PTEN and BMPR2 siRNA. These results were confirmed by the expression of CYP11A1 and STAR mRNA. However, we found inconsistencies between protein and mRNA expression of STAR gene.

1.3.4 Temporal expression of miR-20a in cumulus cells and oocytes

In this part of experiment, one of the most dominant members of the microRNA 17-92 cluster (miR-20a) was selected for further investigation during oocyte maturation. The transcript abundance showed that miR-20a expression in cumulus cells increased during in vitro maturation, while in the oocytes, the miR-20a expression is being suppressed after in vitro maturation. In order to know whether the expression of miR-20a in cumulus cells and oocytes is dependent on their communication, we have analyzed cumulus cells and oocytes cultures in the presence or absence of oocytes and cumulus cells, respectively. The expression of miR-20a in cumulus cells cultured with the presence of oocytes (CCs+Oo) was higher compared with those cumulus cells cultured without oocytes (CCs-Oo). On the other hand, miR-20a expression in oocytes cultured with cumulus cells (Oo+CCs) was lower compared to those oocytes cultured without their cumulus cells (Oo-CCs).

1.3.5 Modulation of miR-20a in cumulus oocyte complexes (COCs) during in vitro maturation

To investigate the role of miR-20a during in vitro maturation, we modulated the expression of this microRNA by coculturing COCs with miR-20a mimic, inhibitor or corresponding control. For feasibility study of miR-20a overexpression or inhibition, first we analyzed the expression of miR-20a in cumulus cells and oocytes after transfection. The results showed that the expression of miR-20a in cumulus cells was increased and decreased in COCs cocultured with miR-20a mimic and inhibitor, respectively. However, the expression of miR-20a in oocytes could not be detected either in miR-20a mimic or inhibitor cocultured group. In addition, we found that overexpression miR-20a leads to increased oocyte maturation rate while no difference in the maturation rate was observed in COCs cocultured with miR-20a

inhibitor compared to the control. This result was further confirmed by the analysis of oocyte maturation marker genes in cumulus cells and oocytes, namely INHBA, MAPK1, PTGS2, PTX3, EGFR and CYCB2. With the exception of PTGS2 and CYCB2 gene expression, overexpression of miR-20a increased the expression of oocyte maturation marker genes in cumulus cells and oocytes. On the other hand, inhibition of miR-20a suppressed the expression of those marker genes.

1.3.6 The effect of miR-20a in cumulus cell progesterone synthesis

Progesterone is one of the critical hormones to support oocyte maturation, produced and secreted by cumulus cells during IVM. In the present study, the cumulus cell progesterone synthesis, as measured in spent media, relatively increased and decreased in COCs cocultured with miR-20a mimic and inhibitor, respectively. The expression of CYP11A1 and STAR genes, which are involved in progesterone synthesis, increased in miR-20a overexpressed COCs. However, no difference in progesterone was observed between COCs transfected with miR-20a inhibitor compared to control.

1.3.7 MiR-20a regulates oocyte maturation and progesterone synthesis by targeting PTEN and BMPR2 in cumulus cells

To confirm whether miR-20a also regulates the expression of PTEN and BMPR2 genes during in vitro oocyte maturation, we next examined the expression of PTEN and BMPR2 in cumulus cells. The results showed that the expression of PTEN and BMPR2 genes were increased and decreased in cumulus cells obtained from COCs cocultured with miR-20a mimic and inhibitor, respectively.

Chapter 2 (Published in Cell and Tissue Research; doi:10.1007/s00441-016-2425-7)

MicroRNA 17-92 cluster regulates bovine granulosa cells proliferation and differentiation by targeting PTEN and BMPR2 genes

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Abstract

Granulosa cell proliferation and differentiation are the key developmental steps involved the formation of dominant follicle that could be eligible for ovulation. This process is in turn regulated by spatiotemporally emerging molecular events. Among others, microRNAs are one of the molecular signatures which are believed to regulate granulosa cells function by fine tuning the expression of genes. In previous study, we showed that miR-17-92 cluster was found to be differentially expressed in the granulosa cells from subordinate and dominant follicles at day 19 of the estrous cycle. However, the role of this miRNA cluster in bovine follicular cells function is not known. Therefore, in the present study, the role of miR-17-92 cluster in granulosa cell function was investigated using an in vitro model. Target prediction and luciferase assay analysis revealed that miR-17-92 cluster coordinately regulate PTEN and BMPR2 genes. Furthermore, overexpression of the miR-17-92 cluster using mimic promoted granulosa cell proliferation and reduced the proportion of differentiated cells. However, the cluster inhibitor resulted in decreased proliferation and increased differentiation in granulosa cells. This was further supported by the expression analysis of marker genes of proliferation and differentiation. Furthermore, the role of miR-17-92 cluster was cross-validated by selective knockdown of its target genes using siRNA technique. The suppression of PTEN and BMPR2 genes revealed similar phenotypic and molecular alterations as observed when the granulosa cells were transfected with miR-17-92 cluster mimic. In conclusion, the miR-17-92 cluster involved in granulosa cell proliferation and differentiation by coordinately targeting the PTEN and BMPR2 genes.

Keywords: microRNA 17-92 cluster, granulosa cells, proliferation and differentiation, PTEN, BMPR2

Introduction

Follicular development is the most important physiological process to ensure normal reproduction of mammalian species. Based on the developmental stage and their dependence on the gonadotropin, follicular development can be classified into three phases namely: primordial until preantral follicular stage (gonadotropin-independent phase), transition from preantral to early antral stage (gonadotropin-responsive), and gonadotropin-dependent follicular growth phase which includes follicle recruitment, selection and ovulation (Aerts and Bols 2010, McGee and Hsueh 2000). In bovine species, follicular development within one estrous cycle occurs in a wave-like pattern (Jaiswal et al. 2009). In every wave, several follicles will be recruited into the growth phase but one follicle will be selected to subsequently be a dominant follicle while the rest undergo atresia and regress (Ginther et al. 1989).

Among the follicular cells, granulosa cells are the key components of the follicular cells which are essential to support the progression of follicular growth and oocyte development (Voronina et al. 2007). Proliferation and terminal differentiation of granulosa cells are critical for ovulation and this phenomenon is regulated by the balance between positive and negative regulators of cell cycle kinase cascades (Robker and Richards 1998a). For instance, deletion of genes responsible for proliferation and differentiation, namely CCND2 and P27, respectively in mice resulted in a decrease of granulosa cells proliferation and impaired luteinization, which are leading to incomplete folliculogenesis and ovulation failure (Robker and Richards 1998b).

In order to have a better understanding about the molecular mechanisms associated with follicular development and granulosa cell function, several studies have been conducted to investigate the differential expression of genes in granulosa cells of different follicular size in bovine species (Douville and Sirard 2014, Hatzirodos et al. 2014b). More focus was laid on differentially expressed genes associated with follicular dominance (Girard et al. 2015, Hatzirodos et al. 2014a) and oocyte competence (Nivet et al. 2013). On the other hand, the expression of genes in bovine follicular cells are believed to be under the regulation of post transcriptional modifiers, namely microRNAs (Maalouf et al. 2015). On this regards, we have previously studied the global expression profile of microRNAs in bovine granulosa cells derived from dominant and subordinate follicle at day 3 and 7 (Salilew-Wondim et al. 2014), and 19 (Gebremedhn et al. 2015) of the estrous cycle. In the later study, among several clusters of microRNAs, the microRNA 17-92 cluster (miR-17-5p, miR-19a, miR-20a and

miR-92a) was found to be enriched in granulosa cells derived from subordinate follicle compared to the dominant ones. However, the role of this microRNA cluster in bovine granulosa cell function is not yet known. Interestingly, bioinformatic analysis of this microRNA cluster revealed that PTEN and BMPR2 genes to be the potential target genes of this microRNA cluster and they are known to play a vital role in bovine granulosa cells development in dominant follicle and small/subordinate follicle (Douville and Sirard 2014, Hatzirodos et al. 2014b). Therefore, this study has been designed to investigate the role of microRNA 17-92 cluster in bovine granulosa cell using an in vitro model.

Materials and Methods

Bovine granulosa cell culture

Bovine ovaries as sources of granulosa cells were collected from a local slaughterhouse and transported to the laboratory in warm 0.9% NaCl solution. Immediately after arrival, ovaries were washed three times using a new warm 0.9% NaCl solution. The granulosa cells were collected from healthy small follicle (3-5 mm of diameter) using a 20-gauge needle. The granulosa cells were transferred to a culture medium (DMEM/F12-HAM medium supplemented with 10% FBS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 2.5 µg/ml fungizone; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Cell viability and concentration were determined by using trypan blue exclusion method. Finally, a total of 2.5 x 10^5 cells per well were seeded into CytoOne® 24-well plate (Starlab GmbH, Hamburg, Germany) containing 600 µl culture medium at in 37 °C with 5% CO_2.

MicroRNA target prediction

Target genes of the miR-17-92 cluster members were predicted using miRWalk database (http://www.umm.uniheidelberg.de/apps/zmf/mirwalk/) and genes commonly predicted by at least four prediction tools with p-value<0.05 were selected as potential target gene for further analysis. Target genes were common for all members of miR-17-92 cluster were selected and the binding sites of those genes were further predicted using PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) and TargetScan (http://www.targetscan.org) with minimum 7-mer seed region. The canonical pathway enriched by the target genes of the miR-17-92 cluster were identified using KEGG pathways database (http://www.genome.jp/kegg/pathway.html).

Plasmid construction

Wild-type plasmid was constructed by ligating the 3´-UTR of PTEN or BMPR2 gene fragments into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega GmbH, Mannheim, Germany) while the mutant plasmid was constructed by ligating 50 bp mutated nucleotides of PTEN or BMPR2 gene. Gene specific primers (listed in Electronic Supplementary Material, Table S1) were used to amplify the 3´-UTR region of PTEN and BMPR2 genes. The gene specific primers and 50 bp mutated-oligonucleotides were designed on the bovine PTEN (XM_613125) and BMPR2 (NM_001304285) mRNA sequences.

Luciferase assay

Sub-confluent in vitro cultured granulosa cells (70-80% of confluency) were co-transfected with 500 ng of plasmid harboring wild type or mutant-sequences of the 3´-UTR of PTEN or BMPR2 and 50 nM of the corresponding individual miRCURY LNA™ microRNA mimics (Exiqon, Vedbaek, Denmark) or miRCURY LNA™ microRNA mimic negative control (mimic NC; Exiqon, Vedbaek, Denmark). Transfection was performed using Lipofectamine® 2000 transfection reagent (Life Technologies GmbH, Darmstadt, Germany). Afterward, the cell lysates were extracted using 1x Passive Lysis Buffer (PLB; Promega GmbH, Mannheim, Germany) 48 h post transfection. The luciferase activity assay in cultured granulosa cells was performed using pmirGLO Dual Luciferase® Reporter Assay System (Promega GmbH, Mannheim, Germany) according to manufacturer's protocol. Finally, the absorbance of firefly and renilla luciferase activity was detected by Centro LB 960 Microplate Luminometer (Berthold Technologies GmbH).

MicroRNA mimic and inhibitor transfection

To investigate the function of microRNA 17-92 cluster in bovine granulosa cells, sub-confluent cultured granulosa cells were transfected with 100 nM of individual microRNA (miR-17-5p, miR-19a, miR-20a, miR-92a) or pool of miR-17-92 cluster miRCURY LNA™ microRNA mimics or inhibitors (Exiqon, Vedbaek, Denmark). The granulosa cells transfected with 100 nM of mimic or inhibitor miRCURY LNA™ microRNA mimic or inhibitor negative controls (NC), respectively (Exiqon, Vedbaek, Denmark) were used as controls for appropriate treatments. The cultured granulosa cells and the spent media were then collected 48 h post transfection for further analysis.

Total RNA isolation and quantitative real-time PCR (qRT-PCR)

To confirm the expression of miRNA cluster target, and cell proliferation and differentiation marker genes in granulosa cells transfected with miRNA cluster mimic, inhibitor or NC, the cells were harvested 48 h post transfection and lysed using lysis buffer. Total RNA was then isolation in the cell lysates using miRNeasy® mini kit (Qiagen GmbH, Hilden, Germany) following manufacturer's protocol. After assessing the quality and concentration of the RNA samples, cDNA synthesis was performed using RevertAid first stand cDNA synthesis kit (Life Technologies GmbH, Darmstadt, Germany) with oligo $(dT)_{18}$ primer. The qRT-PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, München, Germany) in Applied Biosystem® StepOnePlus™ (Applied biosystems, Foster City, CA, USA). The mRNAs expression data was analyzed using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schmittgen 2001) and the expression level of β-ACTIN was used for normalization. Gene specific primers used for mRNA expression analysis are listed in Electronic Supplementary Material, Table S2.

Western blot analysis

Total protein from cultured granulosa cells was isolated 48 h post transfection using 1x PLB (Promega GmbH, Mannheim, Germany) and the protein concentration was determined using Coomassie Protein Assay Reagent (Life Technologies GmbH, Darmstadt, Germany). A total of 30 μg of protein was separated on 4-16% gradient SDS-polyacrilamide gel. Afterwards, the protein was transferred into Immun-Blot® PVDF Membrane (Bio-Rad Laboratories GmbH, München, Germany) using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad Laboratories GmbH, München, Germany). Following this, the membrane was incubated overnight with anti-PTEN goat polyclonal antibody (product no. sc-6818), anti-BMPRII goat polyclonal antibody (product no. sc-5682), anti-PCNA rabbit polyclonal antibody (product no. sc-7907), anti-STAR rabbit polyclonal antibody (product no. sc-25806) or anti-β-ACTIN mouse monoclonal antibody (product no. sc-47778; Santa Cruz Biotechnology Inc., Heidelberg, Germany). At the end of incubation period, the membrane was washed 6 times with 1x TBST (tris-buffered saline and tween 20) and incubated with donkey anti-goat, goat anti-rabbit or goat anti-mouse IgG-HRP secondary antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany). Detection of the protein signal was then performed using Clarity™ Western ECL Substrate (Bio-Rad Laboratories GmbH, München, Germany). Following this, the images

were developed using ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH, München, Germany).

Determination of granulosa cell diameter

Cultured granulosa cells were harvested using 0.25% trypsin-EDTA solution (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and the snapshots of trypsinized granulosa cells were captured using a camera from five different areas of counting chamber with 40x microscope magnification. Diameter of the granulosa cells was measured using ImageJ 1.48v software (http://imagej.nih.gov/ij). Granulosa cells with diameter ≥ 14 µm were considered as differentiated cell while those with diameter ≤ 14 µm were considered as undifferentiated (Kuran et al. 1995).

Cell proliferation assay

To perform the cell proliferation assay**,** 2 x 10^4 granulosa cells per well were seeded into 96-well plate and cultured in the growth medium (described in the previous section). Sub-confluent granulosa cells were then transfected with individual members or pooles of the members of the microRNA 17-92 cluster mimic, inhibitor and NCs. After 48 h culture, 10 µl of CCK-8 kit solution (Dojindo EU GmbH, München, Germany) was added into each well and the plate was incubated for another 2 h. The optical density (OD) associated with 450 nm wavelength was measured using Synergy™ H1 Multi-Mode Reader (BioTek Germany, Bad Friedrichshall, Germany).

Progesterone assay

The progesterone level was measured in cultured granulosa cells spent media 48 h post transfection. Prior to measuring progesterone level, the spent media was diluted in 1:25000 in phosphate buffer saline (PBS) and then the progesterone level was measured using progesterone ELISA kit (ENZO life sciences GmbH, Lörrach, Germany) according to the manufacture´s instruction and the 405 nm wavelength OD was detected using Synergy™ H1 Multi-Mode Reader (BioTek Germany, Bad Friedrichshall, Germany).

Targeted knockdown of PTEN and BMPR2 genes using small interfering RNA (siRNA)

Bovine specific Antisense LNA™ GapmeR (Exiqon, Vedbaek, Denmark) were used to inhibit the expression of PTEN and BMPR2 genes. For this, sub-confluent cultured granulosa cells were transfected with 50 nM of siRNA targeting PTEN or BMPR2. Cells transfected with 50 nM of siRNA negative control (siRNA NC) were used as controls. Gene expression and other phenotype measurements, namely cell diameter, cell proliferation and progesterone levels analysis were performed 48 h post siRNA transfection.

Data analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical significant difference between the groups was analyzed using t-test in GraphPad Prism® software version 5.02 (GraphPad Software, Inc., La Jolla, CA, USA). Mean differences at $p<0.05$ were considered as significantly different.

Results

The miRNA 17-92 cluster targets PTEN and BMPR2 genes

The bioinformatic analysis revealed that 233 genes to be commonly potentially targeted by the microRNA 17-92 cluster members among which 91 genes had a minimum 7-mer binding sites. Canonical pathway analysis showed that PI3K-AKT signaling pathway, FOXO signaling pathway, focal adhesion and hippo signaling pathway were among the top 10 pathways enriched by miR-17-92 cluster target genes. Among several bioinformatically predicted potential target genes, PTEN and BMPR2 were selected for further analysis based on their conserved binding site, and their potential role in bovine granulosa cells development. Therefore, to further investigate whether these two genes could be targeted by miR-17-92 cluster, luciferase assay system was performed using expression vector ligated with 3´-UTR of PTEN or BMPR2 genes. Luciferase activity in granulosa cells co-transfected with miR-17-5p, miR-19a, miR-20a or miR-92a mimic and plasmid vector harboring wild type PTEN or BMPR2 3´-UTR sequences were significantly reduced compared to those cells transfected with miRNA mimic control and plasmid vector harboring wild type PTEN or BMPR2 3´-UTR (Fig. 1).

Following the luciferase assay, we opted to look into whether manipulating the expression level of the miRNA 17-92 cluster could affect the expression levels of these target genes. For this, cultured granulosa cells were transfected with 100 nM of individual or pooled of mimic or inhibitor of miR-17-92 cluster members. Granulosa cells transfected with the negative control of inhibitor or mimic were used as controls. Subsequent gene expression analysis was performed 48 h post transfection showed that granulosa cells transfected with individual or pooled miRNA mimic exhibited a significant reduction in the relative abundance of PTEN and BMPR2 genes compared to cells transfected with controls (Fig. 2a). On the contrary, the expression levels of PTEN and BMPR2 genes were upregulated in cultured granulosa cells transfected with miR-17-92 cluster inhibitors (Fig. 2b). Furthermore, both qualitative and quantitative western blot analysis of the PTEN and BMPR2 protein levels showed that granulosa cells transfected with miR-17-92 cluster mimic exhibited lower PTEN and BMPR2 protein levels (Fig. 2c, d) indicating that miRNA 17-92 cluster targets both PTEN and BMPR2 genes.

MiRNA 17-92 cluster regulates bovine granulosa cell proliferation and differentiation by controlling the expression of PTEN and BMPR2 genes

Parallel analysis of cellular proliferation and differentiation post over or under expression of miR-17-92 cluster using mimic and inhibitor, respectively showed that except miR-17-5p mimic, granulosa cells transfected with individual or pool of miR-17-92 cluster members mimic significantly increased cellular proliferation activity (Fig. 3a) while, granulosa cells transfected with miR-92a inhibitor or pooled miR-17-92 cluster inhibitors significantly reduced cellular proliferation activity (Fig. 3b). This was further confirmed by the analysis of expression of cell proliferation marker genes, namely CCND2 and PCNA. Nevertheless, upregulation of CCDN2 gene expression was observed only in granulosa cells transfected with miR-17-5p and miR-19a mimic while PCNA gene expression level was increased only in granulosa cells transfected with miR-20a and miR-92a mimic (Fig. 3c). On the other hand, transfection of granulosa cells with individual or pooled inhibitors of miR-17-92 cluster resulted in a reduced expression of CCND2 gene (Fig. 3d) and no significant difference was observed in PCNA gene expression. However, the expression of PCNA protein revealed an increasing trend in granulosa cells transfected with either miRNA-17-92 cluster mimic or inhibitor (Fig. 3e, f).

Following cell proliferation, we also opted to understand whether miR-17-92 cluster could be involved in granulosa cells differentiation. For this, the proportion of differentiated cells was evaluated based on their size under inverted microscope. Those cells with a diameter of >14 μm were considered as differentiated while others with diameter of ≤ 14μm were categorized as undifferentiated ones. Based on that, except those transfected with miR-20a mimic, granulosa cells transfected with miR-17-92 cluster mimic had significantly lower differentiating cell compared to cells transfected with mimic NC (Fig. 4a). Moreover, except mimic of miR-20a, granulosa cells transfected with inhibitor or mimic of miRNA-17-92 cluster showed no significant difference in progesterone synthesis (Fig. 4c, d). The mRNA expression analysis of the cell differentiation marker genes, namely CYP11A1 and STAR genes, showed that except the transfection of miR-17-5p mimic, none of the microRNAs including the pooled microRNA mimic resulted in dysregulation of these marker genes (Fig. 4e, f). However, protein expression analysis in cultured granulosa cells showed that miRNA-17-92 cluster mimic transfection decreased the STAR protein (Fig. 4g) while miR-17-5p and miR-92a mimic transfection showed increasing trend (Fig. 4h).

Targeted knockdown of PTEN and BMPR2 genes in bovine granulosa cells using RNA interference increased cell proliferation and progesterone secretion

In order to validate the phenotypic variation observed in bovine granulosa cells after modulating the expression level of miRNA 17-92 cluster, we have performed an independent experiment to suppress the expression of the two target genes (PTEN and BMPR2) using siRNA technology (Fig. 5a-d). The transfection of granulosa cells with PTEN siRNA has effectively increased ($p<0.05$) cell proliferation while suppression of BMPR2 expression had no effect on proliferation of the cells (Fig. 5e). Similarly, relatively higher expressions of CCND2 and PCNA genes were observed following targeted suppression of PTEN and BMPR2 genes (Fig. 5h). Moreover, the percentage of differentiating cells was found to be decreased in granulosa cells transfected with PTEN siRNA (Figure 5f). On the other hand, suppression of PTEN and BMPR2 genes increased the expression of CYP11A1 and STAR genes ($p<0.01$; Fig. 5i) and the release of progesterone in spent media (Fig. 5g). However, the protein analysis of PCNA and STAR genes showed decreasing trend in granulosa cells transfected with both PTEN and BMPR2 siRNA (Fig. 5j).

Discussion

Orchestrated physiological processes are believed to occur in granulosa cells during follicular development. However, the molecular mechanisms that could trigger the selection of one follicle to develop into dominant follicle while the rest follicles are regressing in cattle are barely understood. It has been postulated that these processes are regulated by spatiotemporal expression of genes (Douville and Sirard 2014, Girard et al. 2015, Hatzirodos et al. 2014a, Hatzirodos et al. 2014b, Nivet et al. 2013) which are epigenetically regulated by miRNAs (Feng et al. 2015, Liu et al. 2016, Sontakke et al. 2014, Zhou et al. 2015b). In our previous work, among others, microRNA 17-92 cluster was upregulated in bovine granulosa cells derived from subordinate compared to the dominant follicle at day 19 of estrous cycles (Gebremedhn et al. 2015). Among these, microRNA 17-92 cluster was found to be increased in granulosa cells of subordinate follicles compared to preovulatory dominant follicle. This may hint that this miRNA cluster could have an important role in granulosa cell function during follicular development.

Based on the insilico target prediction results, miR-17-92 cluster are potentially targeting genes involved in several key developmentally related canonical pathways namely, PI3K/AKT signaling pathway, FOXO signaling, focal adhesion and hippo signaling pathways which are known to be the key pathways in follicular development and ovulation by targeting several key genes (Alam et al. 2004, Jagarlamudi et al. 2009, Klusza and Deng 2011, Xiang et al. 2015, Zhang et al. 2010). Moreover, among target genes enriched in those pathways, PTEN and BMPR2 were selected for wet lab target validation as those genes have conserved binding site (seed region) between human and bovine sequences, and their potential role in follicular development (Douville and Sirard 2014, Hatzirodos et al. 2014b). Accordingly, the luciferase assay validated that PTEN and BMPR2 genes to be direct target of miR-17-92 cluster. This was further confirmed by modulating the activity of this microRNA cluster in bovine granulosa cells using mimic and inhibitor under in vitro condition. Overexpression of microRNA 17-92 cluster resulted in reduced expression of both mRNA and protein level of PTEN and BMPR2 genes while inhibition resulted in elevated expression of both genes in cultured bovine granulosa cells. Therefore, miR-17-92 cluster could regulate the cell physiology by optimizing the expression level of PTEN and BMPR2.

The dysregulation of PTEN and BMPR2 gene expression has been associated with abnormalities in follicular development. For instance, the deletion of PTEN gene in mouse lead to a premature follicular growth and ovulation failure (Reddy et al. 2008) while targeted

disruption of PTEN in ovarian granulosa cells resulted in enhanced proliferation and repress structural luteolysis (Richards et al. 2012). Similarly, in the present study, suppression of PTEN and BMPR2 genes via overexpressing the microRNA 17-92 cluster increased granulosa cell proliferation and this was accompanied by increased expression of proliferation marker genes, namely CCND2 and PCNA. On the other hand, overexpressing the microRNA 17-92 cluster using miRNA mimic was found to decrease the proportion of differentiated granulosa cells. This was further confirmed by the opposite phenotypes were observed where the expression of the microRNA 17-92 cluster reduced using miRNA inhibitor. Comparable results in the effect of this microRNA cluster on cell proliferation and differentiation have been reported in various cell types, including ischemic neural progenitor cells of mouse model where overexpression of miR-17-92 cluster increased cell proliferation (Liu et al. 2013) while deletion of miR-17-92 cluster in mice inhibited differentiation of osteoblast cells (Zhou et al. 2014).

Several hours before ovulation, granulosa cells of preovulatory dominant follicle start to express high level of LH receptor and become responsive to the LH surge (Bao et al. 1997). In response to the LH surge, progression of granulosa cells division was dropped while terminal differentiation program was initiated (Hirshfield 1991, Rao et al. 1978, Richards et al. 1986, Richards 1994). During this process, several genes associated with progesterone synthesis, namely CYP11A1 and STAR were found to be increased with subsequent increase in progesterone synthesis by granulosa cells (Baufeld and Vanselow 2013, Chang et al. 2015, Havelock et al. 2004, Zhang et al. 2015). In the present study, spent media analysis was performed to analyze the level of progesterone released by granulosa cells following overexpression or inhibitor of miR-17-92 cluster and we detected a significant elevation in progesterone level in granulosa cells overexpressing miR-20a than other members of the cluster. The opposite was observed in granulosa cells transfected with miR-17-92 cluster inhibitor but these differences were not statistically significant.

The effect of microRNA 17-92 cluster on granulosa cells function through regulation of PTEN and BMPR2 was cross validated by selective knockdown of the two genes using RNA interference technique. In this regard, selective knockdown of PTEN gene in cultured granulosa cells promoted cell proliferation and increased the expression of CCND2 and PCNA genes while the proportion of differentiated cells was reduced. However, as opposed to miR-17-92 cluster mimic transfection, the progesterone profile in spent media and the expression of CYP11A1 and STAR genes were significantly increased. The inhibition of

BMPR2 gene expression in cultured granulosa cells had no effect on proliferation and proportion of differentiated cells, but as observed for PTEN knockdown cells, there was significant increase in progesterone level in spent media and the expression of CYP11A1 and STAR genes in cultured granulosa cells. The elevation of progesterone in spent media in PTEN knockdown cells could be due to suppression of BMP-SMAD signaling pathways (Luo et al. 2015), which was reported to inactivate the FSH-induced progesterone synthesis in rat granulosa cells (Hosoya et al. 2015). All in all, the data of the present study evidenced the involvement of miR-17-92 cluster in bovine granulosa cell proliferation and differentiation by coordinately targeting the expression of PTEN and BMPR2 genes.

Table S1. Sequence specific primers used for 3´-UTR amplification

Accession number	Genes	Primer sequence	Size (bp)
FJ376737	pmirGLO	F: 5´-GCAAGATCGCCGTGTAATTC-3´ R: 5´-CTTTCGGGCTTTGTTAGCAG-3´	107
XM_613125	PTEN-miR-17-5p/20a	F: 5´-TGAGGAGCTCCACAGGGTTTTGACACTTGTTG-3´ R: 5´-CAGT**CTCGAG**TGGTGACAGAACACAAAATGAG-3´	219
XM_613125	PTEN-miR-19a	F: 5´-TGAGGAGCTCCCAATAGATGTCAGCCGTTCC-3´ R: 5´-CAGT**CTCGAG**GCATTATGTGGGACAATTTCTACTG-3´	268
XM_613125	PTEN-miR-92a	F: 5´-TGAGGAGCTCGCTCCTCTTGAACATTTTTCTGC-3´ R: 5´-CAGT**CTCGAG**CCCCACTTTAGTGCACAGTTC-3´	313
NM_001304285	BMPR2-miR-17-5p/20a	F: 5´-GCATGAGCTCCCTCCTACCCCTGCAACAAG-3´ R: 5´-GACT**CTCGAG**GAGACCACTTTTGATACACACACA-3´	199
NM_001304285	BMPR2-miR-19a/92a	F: 5´-GCATGAGCTCCCTGAACGCATCATCTGTTGG-3´ R: 5´-CGGT**CTCGAG**GAGAGAAAGCAAGAAATCAGGTAGC-3	249

Underlined: SacI recognition site (GAGCT|C). Bold: XhoI recognition site (C|TCGAG)

Table S2. Sequence specific primers used for analysis of the relative abundance of genes

Accession number	Genes	Primer sequence	Size (bp)
NM_173979	β-ACTIN	F: 5´-TGTCCACCTTCCAGCAGAT-3´ R: 5´-TCACCTTCACCGTTCCAGT-3´	249
NM_001076372	CCND2	F: 5´-CGACTTCATCGAACACATCC-3´ R: 5´-ATCTTTGCCAGGAGATCCAC-3´	279
NM_001034494	PCNA	F: 5´-CACCAGCATGTCCAAAATAC-3´ R: 5´-CTGAGATCTCGGCATATACG-3´	192
NM_176644	CYP11A1	F: 5´-CGGAAAGTTTGTAGGGGACA-3´ R: 5´-ACGTTGAGCAGAGGGACACT-3´	177
NM_174189	STAR	F: 5´-AAATCCCTTTCCAAGGTCTG-3´ R: 5´-ACCAGCATTTCTGCTACTGC-3´	204
XM_613125	PTEN	F: 5´-TGGGGAAGTAAGGACCAGAG-3´ R: 5´-ATTGCAAGTTCCACCACTGA-3´	172
NM_001304285	BMPR2	F: 5´-GCAAGCACAAGCTCGAATCC-3´ F: 5´-TGGCTGTGAAACATTGGTGG-3´	169

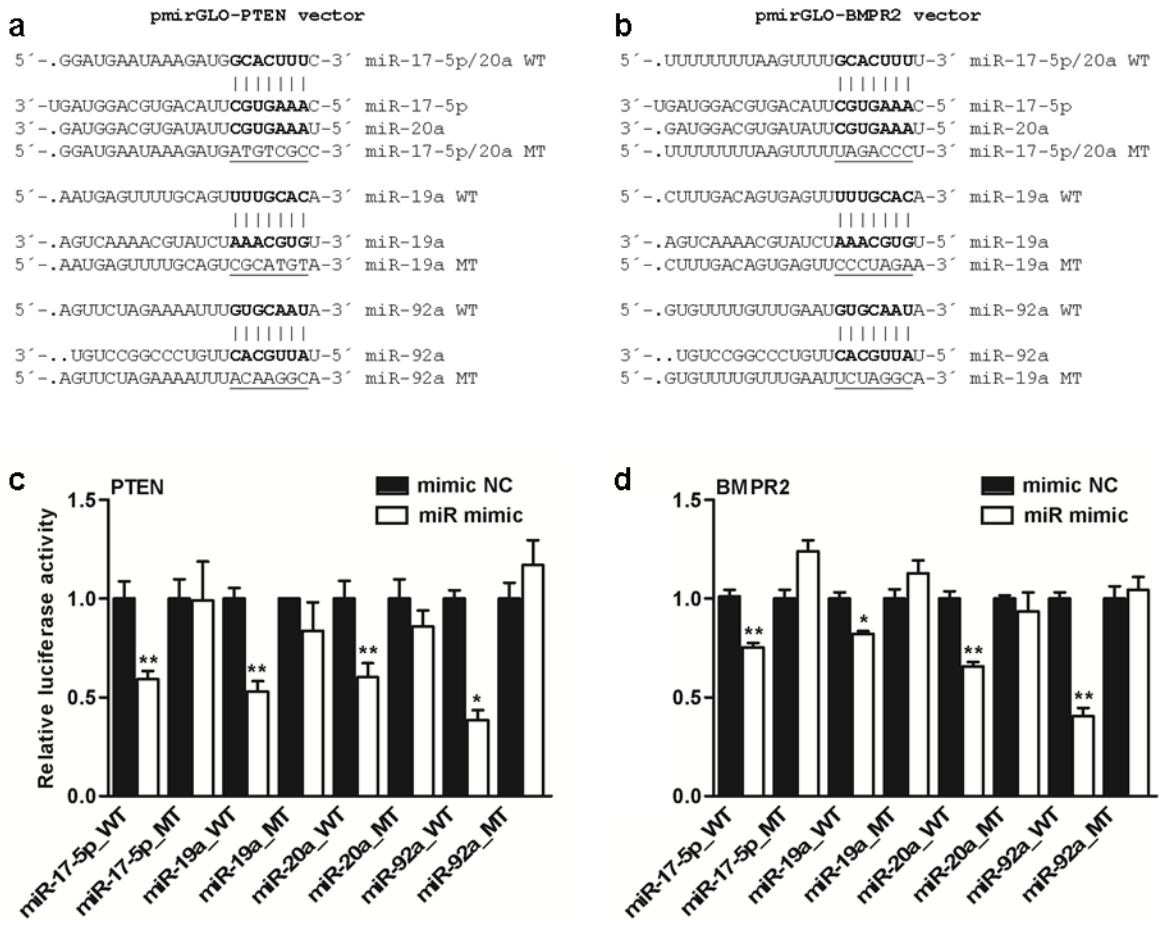

Figure 1. Experimental validation of PTEN and BMPR2 genes as targets of miR-17-92 cluster. Putative miR-17-5p, miR-19a, miR-20a and miR-92a binding sites in bovine PTEN (a) and BMPR2 (b) sequences. The bold faced letters indicated a putative binding site and the underlined sequences indicate the 3′-UTR mutated region to be used as a control. Relative luciferase activity in granulosa cells co-transfected with a vector harboring the wild-type (WT) or mutant (MT) sequences of PTEN (c) and BMPR2 (d) 3′-UTR sequences. Firefly and renilla activity ratio for each constructed plasmid were compared to corresponding mimic NCs. White bars: microRNA mimics. Black bars: mimic NC. Data were shown in mean ± SEM (n=3, $^{*}p<0.05$, $^{**}p<0.01$).

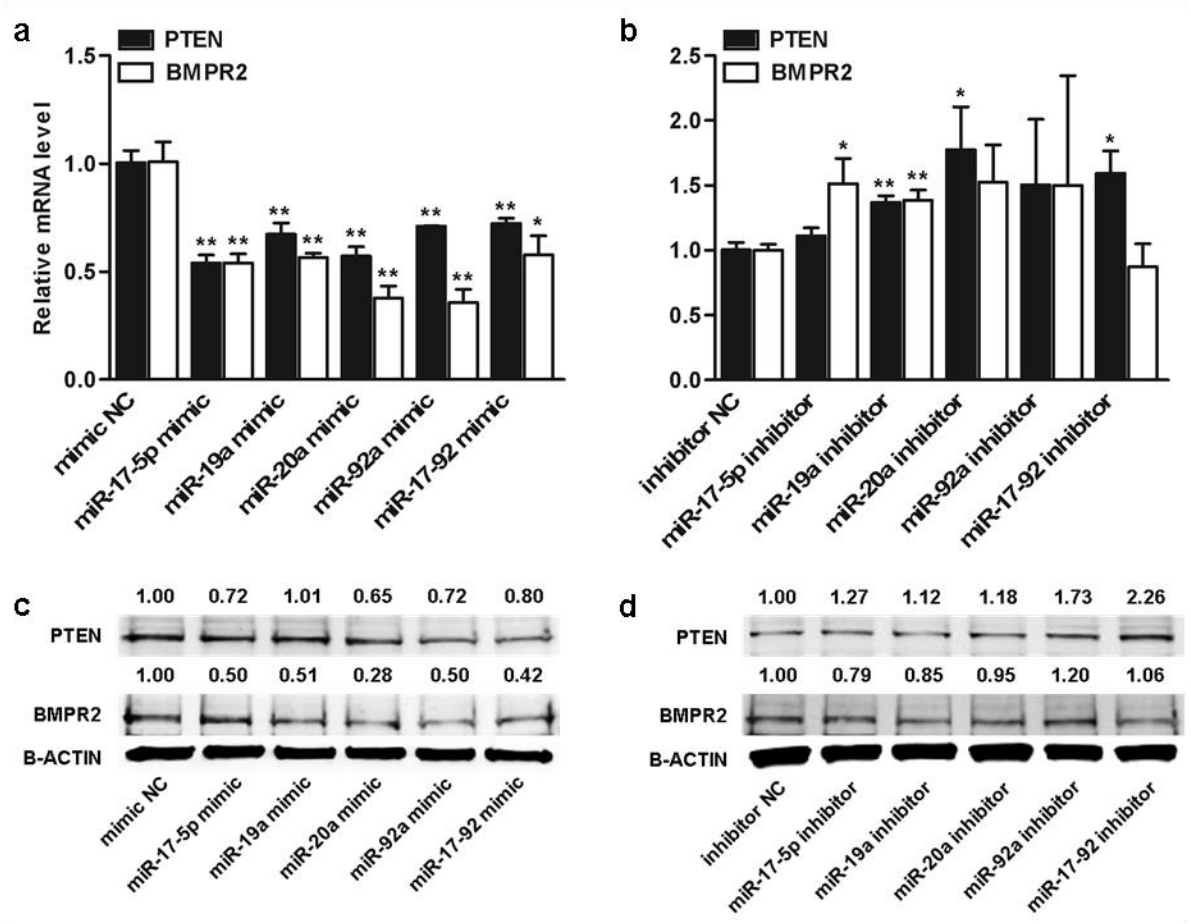

Figure 2. Overexpression or inhibition of miR-17-92 cluster resulted in variation in mRNAs and protein expression of PTEN and BMPR2 genes. The mRNA expression of PTEN and BMPR2 genes in granulosa cells transfected with miR-17-92 cluster mimic (a) and inhibitor (b). Data were shown in mean ± SEM (n=3, $^{*}p<0.05$, $^{**}p<0.01$). The protein expression of PTEN and BMPR2 in granulosa cells transfected with miR-17-92 cluster mimic (c) and inhibitor (d). The numbers on the top western blot figures represent relative protein density. Both mRNA and protein expression levels were compared to negative controls (mimic NC or inhibitor NC) and β-ACTIN was used as an internal control.

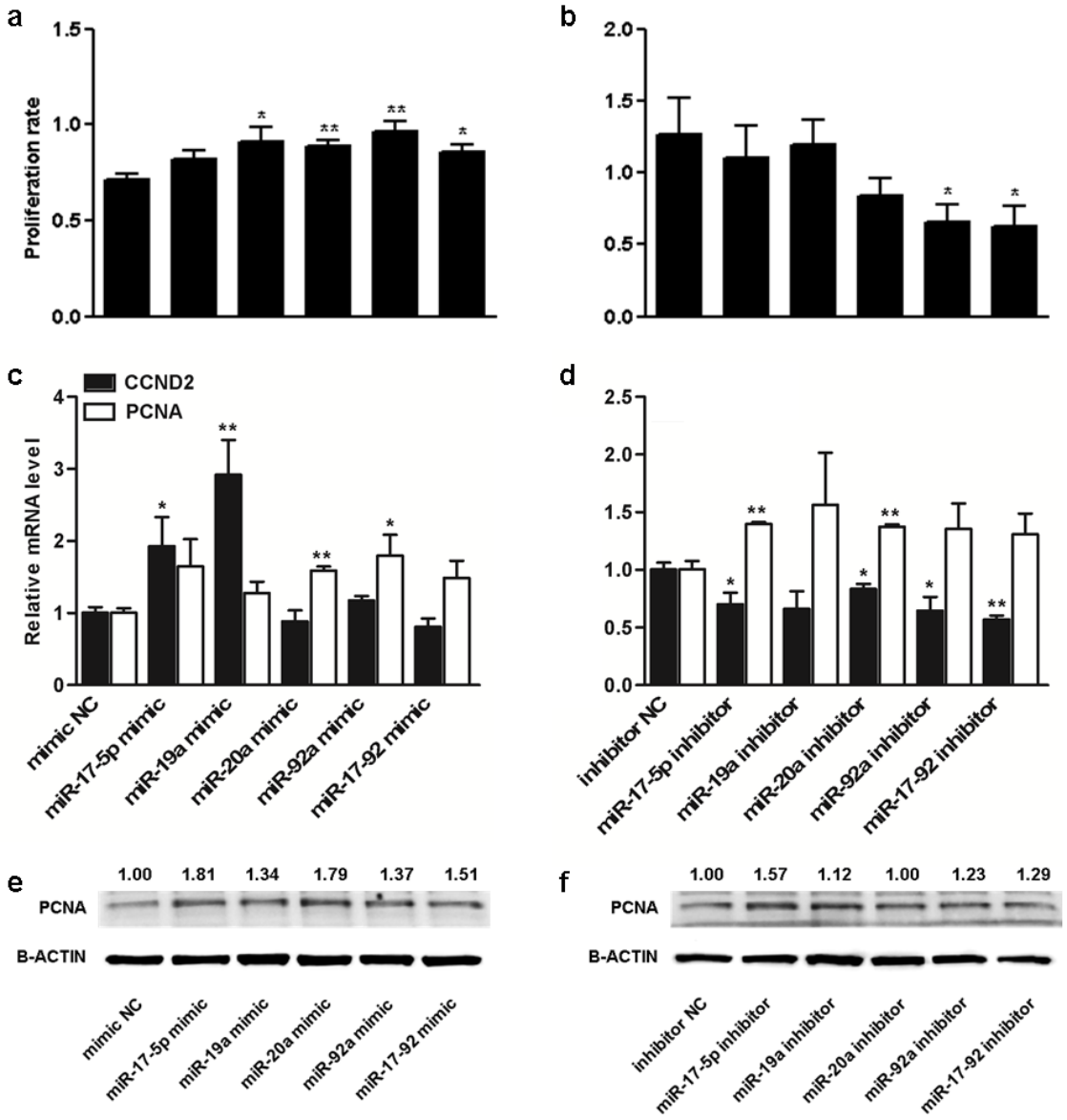

Figure 3. The effect of miR-17-92 cluster overexpression in granulosa cell proliferation and expression of cell proliferation marker genes. The effect of miR-17-92 cluster mimic (a) and inhibitor (b) in granulosa cells proliferation. The mRNA expression of CCND2 and PCNA genes in granulosa cells transfected with miR-17-92 cluster mimic (c) or inhibitor (d). The protein expression of PCNA in granulosa cells transfected with miR-17-92 cluster mimic (e) or inhibitor (f). Data were compared to the corresponding microRNA controls and shown in mean ± SEM (n=3, $^{*}p<0.05$, $^{**}p<0.01$). The numbers on the top western blot figures represent relative protein density. The expression of β-ACTIN was used as internal control in mRNA and protein analysis.

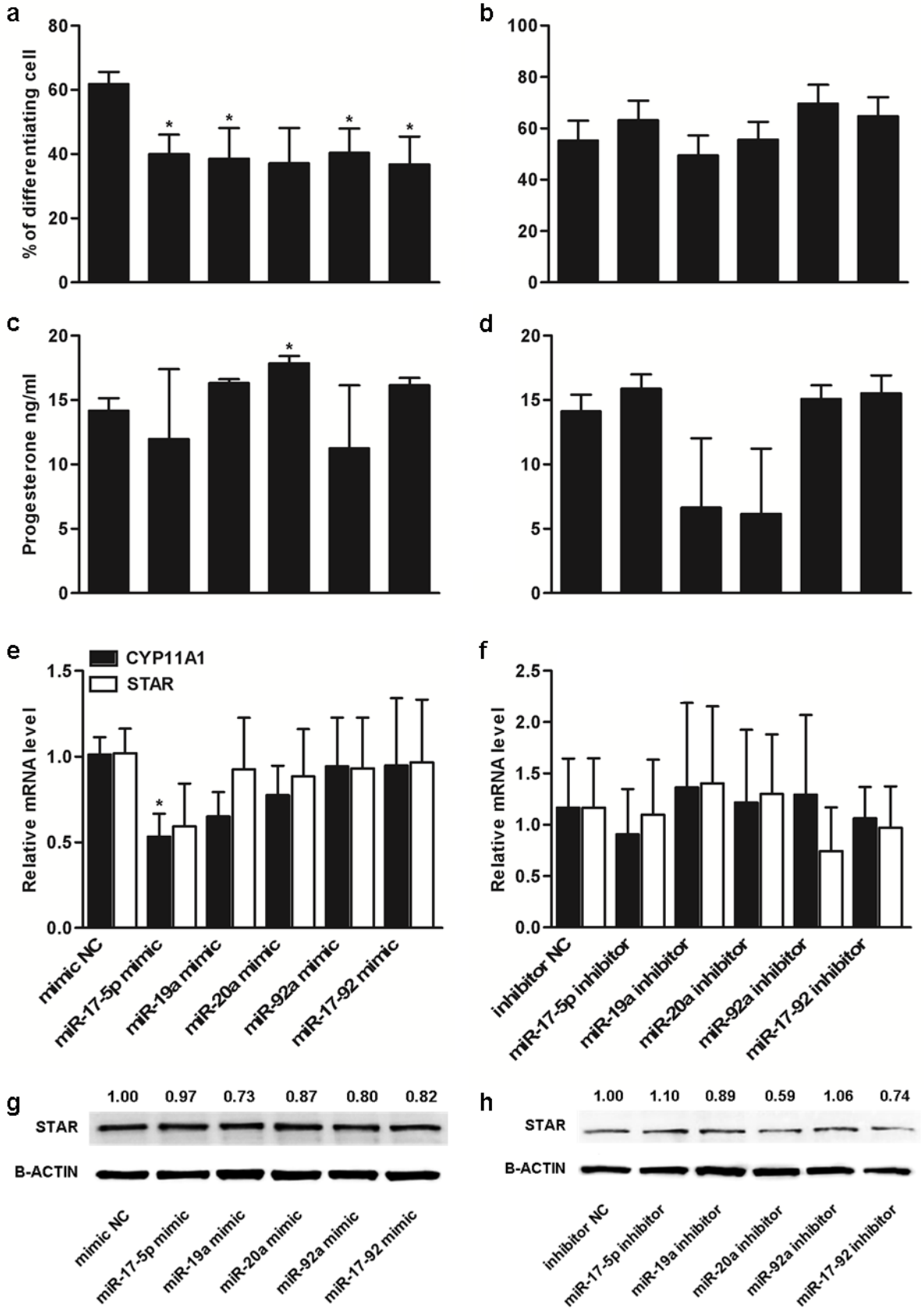

Figure 4. Overexpression or inhibition of miR-17-92 cluster induced partial effect on bovine granulosa cell differentiation and progesterone synthesis. The proportion of differentiated granulosa cell in cultured granulosa cells transfected with miR-17-92 cluster mimic (a) and

inhibitor (b). The progesterone levels measured in spent media of granulosa cell culture transfected with miR-17-92 cluster mimic (c) and inhibitor (d) transfection. The effect of miR-17-92 cluster mimic (e) and inhibitor (f) transfection on the expression of CYP11A1 and STAR genes in the cultured granulosa cells. The protein expression of STAR in granulosa cells transfected with miR-17-92 cluster mimic (g) or inhibitor (h). Data were compared to the corresponding microRNA controls and shown in mean ± SEM (n=3, $^{*}p<0.05$, $^{**}p<0.01$). The numbers on the top western blot figures represent relative protein density. The expression of β-ACTIN was used as internal control in mRNA and protein analysis.

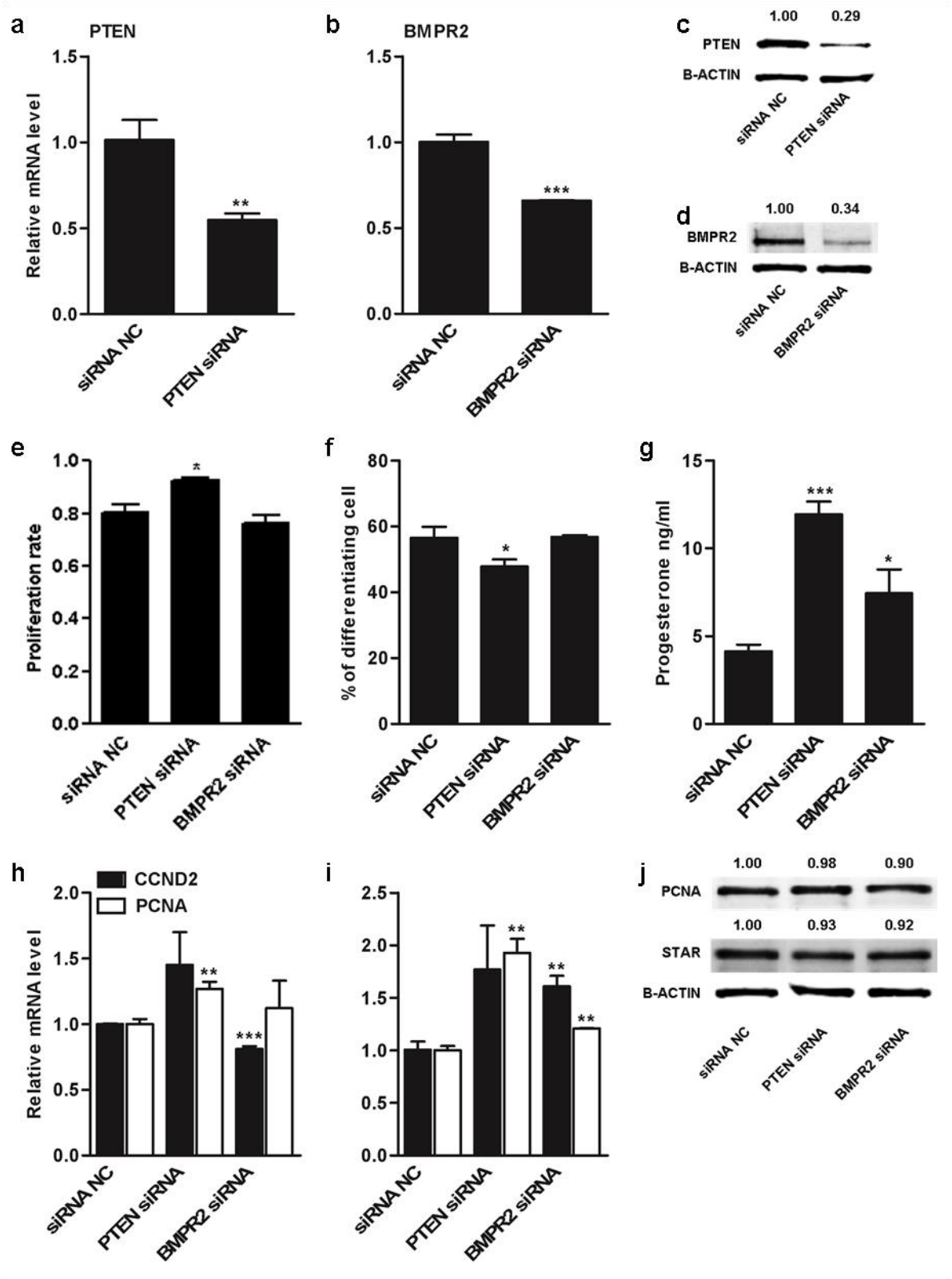

Figure 5. Selective knockdown of PTEN and BMPR2 mRNA in cultured granulosa cells. The relative abundance of mRNA and protein of PTEN (a and c), and BMPR2 (b and d) genes in granulosa cells transfected with siRNA targeting PTEN and BMPR2. The effect of PTEN and BMPR2 knockdown in granulosa cells proliferation (e), differentiation (f), progesterone

synthesis (g), and the mRNA and protein expression of genes associated with cell proliferation, differentiation and progesterone secretion (h, i and j). Data were compared to negative control siRNA (siRNA NC) and presented as mean ± SEM (n=3, $^{**}p<0.01$. $^{***}p<0.001$). The numbers on the top western blot figures represent relative protein density. The mRNA and protein expression were calculated relative to the expression of β-ACTIN.

Chapter 3 (Publication under preparation, to be submitted to Biology of Reproduction)

The miR-20a regulates oocyte maturation through modulation of cumulus cell progesterone synthesis during in vitro maturation by targeting PTEN and BMPR2 genes

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ABSTRACT

Ovulation of the developmentally competent oocyte is an orchestrated process which requires intimate interaction between oocyte and its surrounding cells. The dynamic changes of microRNAs in oocyte and cumulus cells before and after the maturation process revealed the spatiotemporal post-transcriptional gene expression within bovine follicular cells during oocyte maturation process. MiR-20a has been shown to regulate proliferation and differentiation as well as progesterone synthesis in cultured granulosa cells. In the present study, we focus on the function of miR-20a during bovine oocyte in vitro maturation (IVM) process. The maturation of cumulus-oocyte complex was performed at 39 °C in a humidified atmosphere with 5% CO_2 in the air. The expression of miR-20a was investigated in the cumulus cells and oocytes at 22 h post culture. Further, we cultured denuded oocytes and cumulus cells separately to investigate whether the presence of oocytes or cumulus cells will affect the expression of miR-20a in each type of cells. The function of miR-20a was examined by modulation of miR-20a in cumulus-oocyte complex (COCs) during IVM. The cumulus cells, oocytes and spent media were collected 22 h post culture. Oocyte maturation was assessed based on the presence of the polar body. In this study, miR-20a expression in cumulus cells was increased during IVM, while in the oocytes, the expression of this microRNA was decreased. We also found that the absence of oocyte reduced miR-20a expression in cumulus cells, while the absent of cumulus cells increased miR-20a expression in oocyte. The transfection of miR-20a mimic and inhibitor during IVM modulated the expression of miR-20a in cumulus cells but not in oocytes. The overexpression of miR-20a during IVM increased oocyte maturation rate and expression of oocyte maturation marker genes. These findings were consistent with the progesterone level in spent media of COCs and the expression of progesterone synthesis marker genes in cumulus cells. In this study, we also confirmed that PTEN and BMPR2 genes are also targeted genes of miR-20a in cumulus cells. We concluded that modulation of miR-20a expression in cumulus cells regulates oocyte maturation through increasing cumulus cell progesterone synthesis by simultaneously suppression of the expression of PTEN and BMPR2 genes.

Keywords: miR-20a, oocyte maturation, progesterone, PTEN, BMPR2

INTRODUCTION

Oocyte growth development in mammals starts early in the fetal development and is arrested in the diplotene of the prophase stage of first meiosis cleavage within the ovarian follicle. When the follicle is recruited, the oocyte enters the growth phase where it increases in volume, undergoes replication and redistribution of cytoplasmic organelles (Picton et al. 1998). Oocyte meiosis progression and development competence during folliculogenesis is influenced by the local microenvironment formed by companion somatic cells, namely cumulus oophorus (Sanchez and Smitz 2012). The communication between oocyte and its cumulus cells is critical for the development and functions of both cells (Eppig 2001, Gilchrist et al. 2004a, Matzuk et al. 2002). For instance, the removal of cumulus cell before in vitro maturation inhibits oocyte developmental competence (Vozzi et al. 2001). Similar results were obtained when the interaction of both cells were disrupted using gap junction inhibitors (Atef et al. 2005).

In the late stages of follicular development, ovulation is the result of a long and orchestrated process to release the competent oocyte which is fertilizable, followed by normal embryo development and eventually the birth of healthy offspring (Labrecque and Sirard 2014). The transcriptome dynamic in oocyte (Fair et al. 2007, Regassa et al. 2011) and cumulus cells (Assidi et al. 2010, Nivet et al. 2013, Regassa et al. 2011) before and after maturation process revealed a spatiotemporal regulation of gene expression within bovine follicular cells. The differentially expressed genes in oocytes and cumulus cells cultured without their surrounding cumulus cells and oocytes, respectively, indicated the molecular cross-talk between the oocytes and surrounding cumulus cells (Regassa et al. 2011). Similar to the mRNAs, our previous studies revealed the microRNAs transcript abundance in oocytes (Abd El Naby et al. 2013, Tesfaye et al. 2009) and cumulus cells (Abd El Naby et al. 2013) during oocyte maturation.

During in vitro maturation (IVM) of oocyte, bovine cumulus cells are able to produce and to secrete steroid hormones (Mingoti et al. 2002). The inhibition of endogenous steroids production during maturation drastically decreased the percentage of mature oocyte (MII stage) and suppressed cumulus expansion in bovine cumulus-oocyte complexes (COCs) (Pan et al. 2015, Wang et al. 2006). As reported previously by others, progesterone is one of the steroid hormones which is produced and secreted by cumulus cells to support the oocyte meiosis resumption maturation processes (Aparicio et al. 2011, Choi et al. 2001, Ježová et al. 2001, Montano et al. 2009, Nagyova et al. 2011, Nagyova et al. 2012, Shimada et al. 2004c,

van Tol et al. 1996, Yamashita et al. 2003) under stimulation of FSH and LH (Choi et al. 2001, Shimada and Terada 2002, Shimada et al. 2004c, van Tol et al. 1996). The important role progesterone in bovine follicle has been indicated by the higher progesterone level in follicular fluid in follicle with mature oocyte compared to those with immature ones (Grimes and Ireland 1986). In addition, progesterone is believed to promote oocyte maturation in pig (Yamashita et al. 2003), mouse (Jamnongjit et al. 2005) and bovine in a dose-dependent manner (Siqueira et al. 2012) during IVM process. On the other hand, the inhibition of progesterone synthesis had shown a negative effect on cumulus cells expansion, oocyte maturation rate, ovulation rate, and subsequent embryonic development in mouse (Aparicio et al. 2011, Panigone et al. 2008, Siqueira et al. 2012, Sirotkin 1992), porcine (Kawashima et al. 2008, Shao et al. 2003, Shimada and Terada 2002, Shimada et al. 2004c) and bovine (Aparicio et al. 2011, O'Shea et al. 2013, Roh et al. 1988, Shao et al. 2003, Shimada et al. 2004b, Shimada et al. 2004a, Wang et al. 2006).

In our previous work, we have shown that miR-20a is differentially expressed in bovine granulosa cells derived from the different size of follicle at the late phase of estrous cycle (Gebremedhn et al. 2015). The miR-20a overexpression and knockdown of its target genes (PTEN and BMPR2) in culture bovine granulosa cells promoted cell proliferation and suppressed cell differentiation. In addition, the progesterone level in spent media of granulosa cell culture was elevated (Andreas et al. 2016). This finding was supported by the cross-talk between PTEN/PI3K/AKT and BMP-SMAD signaling pathways in progesterone synthesis (Chang et al. 2013, Hosoya et al. 2015, Luo et al. 2015). However, the potential involvement of miR-20a during oocyte maturation has not been reported to date. In the present study, we found that the miR-20a expression during IVM process was increased and decreased in cumulus cells and oocytes, respectively. We also observed that the expression of miR-20a in cumulus cells and oocytes is regulated by the presence or absence their companion cells. Moreover, our experiments provide evidence that oocyte maturation progression during IVM could be triggered by the modulation of miR-20a expression in its surrounding somatic cells.

Materials and Methods

Cumulus-oocyte complexes (COCs) collection and in vitro oocyte maturation

Bovine ovaries, as a source of cumulus-oocyte complexes (COCs), were obtained from a local slaughterhouse and transported to the laboratory in a thermo-flask that contained a 0.9% saline solution within 2 h after slaughter. The COCs were aspirated from healthy small

follicles (2–8 mm of follicle diameter). Good quality and morphologically uniform COCs (oocytes with a homogenous, evenly granulated ooplasm, and surrounded by at least three layers of cumulus cells) were selected in this study. The selected COCs were washed with TCM-199 media before set into culture to obtain matured oocytes or were directly frozen as immature COCs (germinal vesicle; GV). The COCs were cultured in groups of 50 in 400 µl of maturation media (modified parker medium (MPM) supplemented with 12% estrus cow serum and 10 µg/ml Follitropin®) under mineral oil in five-well dishes. Maturation was performed for 22 h at 39 °C in a humidified atmosphere with 5% (v/v) CO_2 in air. Spent media of in vitro maturation medium were collected for progesterone assay. The cumulus cells and oocytes from immature and matured group of COCs were separated by gentle pipetting in TCM-199 media supplemented with hyaluronidase (1 mg/ml). After transferring the denuded oocytes into a new tube containing 10 µl 1x PBS (phosphate buffer saline), the cumulus cells were isolated by gentle centrifugation. The cumulus cells pellet were resuspended using 50 µl lysis buffer (0.8% Igepal, 40 U RNasin and 5 mM DTT). The cumulus cells, oocytes and spent media were snap frozen using liquid nitrogen and stored at -80 °C until further analysis. Matured oocytes (metaphase II stage; MII) were indicated by the presence of first polar body under an inverted microscope. The total numbers of recovered and matured oocytes after in vitro maturation (IVM) were recorded. The maturation rate was calculated from the number of matured oocytes compared to the total number of recovered oocytes.

Cumulus cells and denuded oocytes culture

To investigate the effect of oocyte on cumulus cells microRNA expression and vice versa, cumulus cells and oocytes were cultured in the presence or absence of their companion cells. For this, cumulus cells and oocytes from 50 collected COCs were separated in TCM-199 media supplemented with hyaluronidase (1 mg/ml). The cumulus cells (CCs-Oo) and denuded oocytes (Oo-CCs) were cultured for 22 h in the maturation media at 39 °C in 5% (v/v) CO_2 incubator, as described in the previous section. Denuded oocytes and cumulus cells were collected and stored at -80°C until further analysis. The cumulus cell (CCs+Oo) and oocytes (Oo+CCs) obtained from COCs cultures used as controls.

MicroRNA transfection

To investigate the function of miR-20a in oocyte maturation, the collected COCs were culture in a group of 50 COCs in five-well dishes containing 400 µl maturation media, as described above. The equal concentration (50 nM) of miRCURY LNA™ miR-20a mimic, miR-20a inhibitor or corresponding negative controls (mimic NC and inhibitor NC) was transfected into the appropriate well using Lipofectamine® 2000 reagent. Transfected COCs were cultured for 22 h at 39 °C in a humidified atmosphere with 5% (v/v) CO_2 in air.

Total RNA isolation and cDNA synthesis

Total RNA of cumulus cells was isolated using miRNeasy® mini kit following manufacturer's protocol, while oocyte total RNA extraction was performed using PicoPure® RNA isolation kit. The quality and quantity of extracted RNA were determined using NanoDrop 8000 (Thermo Scientific). For analysis of gene expression, the equal amount of total RNA (100 ng of cumulus cell and 50 ng of oocyte total RNA) were reverse transcribed using RevertAid first stand cDNA synthesis kit (Life Technologies GmbH) according to manufacturer's protocol. For microRNA expression analysis, the cDNA was synthesized from 50 ng and 25 ng of total RNA of cumulus cells and oocytes, respectively, using Universal cDNA synthesis kit (Exiqon) following manufacturer's instructions.

MicroRNA and mRNA quantitative PCR analysis

The quantitative PCR (qPCR) analysis of several candidate genes and miR-20a expression were performed using iTaq™ Universal SYBR® Green Supermix and ExiLENT SYBR® green master mix, respectively, in Applied Biosystem® StepOnePlus™. The primers for gene expression analysis (Table 1) have been tested using qualitative PCR followed by sequencing analysis using GenomeLab™ GeXP Genetic Analysis System, while microRNA primers were purchased from Exiqon. In addition, the specificity of amplification in qPCR processes was indicated by a single melting curve generated at the end of the qPCR protocol. The relative expression of candidate genes and miR-20a were analyzed using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak and Schmittgen 2001). The expression of β-ACTIN and 5s rRNA were used to normalize the candidate genes and miR-20 expression, respectively. In particular, the agarose gel (1.5%) has been used to confirm the miR-20a expression in oocyte samples.

Western blot analysis

The total protein obtained from cumulus cells was extracted using 1x passive lysis buffer (PLB; Promega GmbH) and separated on 4-16% gradient SDS-polyacrylamide gel followed by transferring into Immun-Blot® PVDF Membrane. Transfer protein was performed on Trans-Blot® SD Semi-Dry Transfer Cell. After incubation with 1x Roti®-block solution, the membrane was incubated with anti-PTEN goat polyclonal antibody (1:200 dilution), anti-BMPRII goat polyclonal antibody (1:200 dilution), anti-STAR rabbit polyclonal antibody (1:500) or anti-GAPDH goat polyclonal antibody (1:500 dilution) for overnight, followed by incubation with donkey anti-goat or donkey anti-rabbit IgG-HRP (1:7500 dilution). All antibodies used in this study were purchased from Santa Cruz Biotechnology Inc. Detection of conjugated protein was performed using Clarity™ Western ECL Substrate and captured by ChemiDoc™ XRS+ system.

Progesterone measurement

Progesterone level was measured in spent oocyte maturation media. Prior to measuring the progesterone level, the spent maturation media was diluted 1:1000 in 1x PBS. Progesterone level was measured using a progesterone ELISA kit (ENZO Life Sciences GmbH) according to the manufacturer´s instruction and the 405 nm OD was detected by Synergy™ H1 Multi-Mode Reader.

Data analysis

All quantitative data are presented as mean ± standard error of the mean (SEM). Statistical significance of the data was analyzed using t-test methods (Prism® software version 5.02; GraphPad). The p-values are indicated in the corresponding figure legend.

Results

Temporal expression of miR-20a during in vitro maturation

To investigate the temporal expression of miR-20a in cumulus cells and oocytes, first, we collected COCs from small healthy follicles at the GV stage. Parts of these COCs were used as immature (GV) group, while the others were matured (MII). The cumulus cells and oocytes from both immature and matured groups were separated. The qPCR analysis showed that

miR-20a expression cumulus cell of matured COCs was significantly higher ($p<0.05$) compared to those cumulus cells from GV stage of COCs. Conversely, the expression of this microRNA was lower in oocytes from the matured group (Fig. 1).

The expression of miR-20a in oocytes and cumulus cells in the presence or absence of their companion cells

In addition, in order to investigate whether the expression of miR-20a in oocytes and cumulus cells are affected by the presence or absence of their companion cells, we cultured the cumulus cells and denuded oocytes with the absence of oocyte (CCs-Oo) and cumulus cells (Oo-CCs), respectively. In the present study, we observed that the expression of miR-20a in cumulus cells matured with the presence of oocytes (CCs+Oo) during IVM is relatively higher ($p=0.0543$) than those in cumulus cells cultured without oocyte. On the other hand, the presence of surrounding cumulus cells during IVM resulted in reduction of the miR-20a expression in the oocytes (Fig. 2).

The effect of miR-20a modulation in cumulus cells and oocytes

To investigate the role of miR-20a in oocyte maturation, we first studied the feasibility of miR-20a overexpression or inhibition during IVM using 50 nM of miR-20a mimic or inhibitor. As a negative control, the same amount of mimic or inhibitor negative control (mimic NC or inhibitor NC) was transfected into the COCs culture. The qPCR analysis revealed a significant increase ($p<0.001$) of miR-20a expression in cumulus cells from COCs transfected with miR-20a mimic compared to mimic NC. On the other hand, the transfection of miR-20a inhibitor resulted in decreased ($p<0.001$) cumulus cell microRNA expression compared to the inhibitor NC group (Fig. 3A). However, neither miR-20a mimic nor inhibitor transfection had an effect on the miR-20a expression in the oocytes. We suggest that the expression of miR-20a in oocytes is almost negligible. It was indicated by lower Ct value (beyond 35 cycles) and it was further supported by running electrophoresis of PCR products on 1.5% agarose gel (Fig. 3B).

MiR-20a overexpression during IVM increased oocyte maturation rate

We next studied the effect of miR-20a expression on maturation rate of oocytes. Matured oocyte was assessed by the presence of first polar body under an inverted microscope and the maturation rate was calculated from the number of matured oocytes compared to the total number of recovered oocytes. We observed that miR-20a overexpression during IVM resulted in increased oocyte maturation rate ($p<0.05$). However, the transfection of miR-20a inhibitor had no effect on maturation rate (Fig. 4).

The miR-20a modulation altered expression of oocyte maturation-related genes

To study whether the effect of miR-20a on the maturation rate was accompanied by the changes in oocyte maturation marker gene expression, we analyzed the expression level of genes related to the oocyte competence (INHBA, MAPK1 and PTGS2), cumulus cells expansion (PTX3 and EGFR) and cell cycle regulator (CYCB2) in cumulus cells and denuded oocytes. We found that the inhibition of miR-20a expression during IVM resulted in a decrease of oocyte competence and cumulus expansion-related gene expression (INHBA $p<0.05$; MAPK1 $p<0.05$; PTGS2 $p<0.05$ and EGFR $p<0.01$) in cumulus cells. Conversely, the cumulus cell INHBA, EGFR and CYCB2 gene expression were found to be increased ($p<0.05$) in miR-20a overexpression. In addition, except in oocyte PTGS2 and EGFR gene expression, the miR-20a inhibitor transfection during IVM had no effect on expression of genes related to oocyte competence, cumulus cells expansion and cell cycle regulator. However, the miR-20a overexpression resulted in an increase in oocyte INHBA ($p<0.01$), MAPK1 ($p<0.05$) and PTX3 ($p<0.05$) gene expression (Fig. 5).

MiR-20a enhanced oocyte maturation through cumulus cell-progesterone synthesis

Parallel with the oocyte maturation, the spent media of IVM culture was collected and analyzed for its progesterone level. The progesterone assay revealed that overexpression and inhibition of miR-20a during IVM relatively increased ($p=0.0936$) and decreased ($p=0.0993$) the progesterone synthesis as measured in IVM spent media (Fig. 6A). This result was confirmed by the expression of progesterone synthesis-related genes, namely CYP11A1 and STAR genes (Fig. 6B, C).

The miR-20a regulates oocyte maturation and progesterone synthesis by targeting PTEN and BMPR2 genes in cumulus cells

To confirm whether miR-20a also regulates the expression of PTEN and BMPR2 genes during in vitro oocyte maturation, we next examined the expression of PTEN and BMPR2 in cumulus cells. Here, we found that miR-20a overexpression during IVM tends to reduce the mRNA and protein expression of PTEN and BMPR2 genes in cumulus cell (Fig. 7).

Discussion

The oocyte maturation process is complex and requires an integration of endocrine, paracrine, juxtacrine and autocrine signaling pathways (Takahashi et al. 2006). This process involves an interaction between the oocyte and surrounding cumulus cells. In our previous work, differentially expressed mRNAs (Regassa et al. 2011) and microRNAs (Abd El Naby et al. 2013) in cumulus cells before and after in vitro maturation process indicated the signals released from somatic cells stimulated the meiotic progression and oocyte maturation (Chen et al. 2013). In this study, we observed that the in vitro maturation process resulted in increased miR-20a expression in cumulus cells, but decreased in the oocytes. Similarly, the decrement of miR-20a expression during IVM was also reported in human oocytes (Xu et al. 2011b).

During the oocyte maturation process, it has been postulated that bidirectional communication between those cells is essential for proper maturation of oocytes, fertilization and further embryonic development (Buccione et al. 1990a, Eppig 2001, Gilchrist et al. 2004a, Matzuk et al. 2002). This dialogue is vital for the oocyte to acquire meiotic and developmental competence (Brower and Schultz 1982, Calder et al. 2001, Calder et al. 2005, De La Fuente and Eppig 2001, Eppig 1991, Eppig 2001, Eppig et al. 2002, Matzuk et al. 2002). Moreover, the oocyte-secreted factors (OSFs), such as GDF9 and BMP15 are believed to regulate key cumulus cell functions (Buccione et al. 1990b, Eppig et al. 1997, Eppig et al. 2005, Gilchrist et al. 2001, Gilchrist et al. 2003, Gilchrist et al. 2004a, Gilchrist et al. 2006, Hussein et al. 2005, Joyce et al. 2000, Li et al. 2000, Otsuka and Shimasaki 2002, Sugiura et al. 2005, Tanghe et al. 2002, Vanderhyden et al. 1990). With regard to the transcript abundance, we have shown previously that the presence or absence of oocyte in culture cumulus cells altered several mRNAs (Regassa et al. 2011) and microRNAs (Abd El Naby et al. 2013) expression and vice versa. In the present study, cumulus cells cultured without the presence of oocyte (CCs-Oo) had lower miR-20a expression compared to those cumulus cells with the presence

of oocytes (CCs+Oo). On the other hand, miR-20a expression was higher in oocytes cultured without their cumulus cells (Oo-CCs) compared to those oocytes cultured with the presence of their cumulus cells (Oo+CCs). We suggested that the expression of miR-20a in cumulus cells and oocytes are regulated by the presence or absence of their companion cells.

Within follicle, before LH surge, the oocyte is arrested in the prophase I stage (germinal vesicle; GV) and believed that cumulus cells are set to receive the last major induction of oocyte final maturation (Dieleman et al. 2002). At 6h after LH surge, the germinal vesicle breakdown (GVBD) of the oocyte (Dieleman et al. 1983, Sirard et al. 1989) followed by the increase of steroidogenesis synthesis marker genes in cumulus cell, namely HSD3B2, INHBA, PGR, HPGD, and DHCR24 (Assidi et al. 2010). Previous studies in pig reported that LH and FSH induced oocyte maturation, cumulus cells expansion (Procházka et al. 1991), synthesis of hyaluronic acid (Nagyová et al. 1999) and progesterone production by cumulus cells (Ježová et al. 2001, Nagyova et al. 2011, Nagyova et al. 2012). With regards to microRNA, several studies have been conducted to demonstrate the role of microRNAs in oocyte developmental competence (Pan et al. 2015, Yao et al. 2014). The miR-20a expression analysis revealed that the transfection is restricted to cumulus cells of the COCs. We suggested that several layers of cumulus cell and the thick zona pellucida protect the oocyte from transfection reagent, as reported previously in lentivirus transduction (Pan et al. 2015). In addition, we also observed that miR-20a overexpression during IVM resulted in increased oocyte maturation rate, while no difference was found in maturation rate when miR-20a expression was inhibited. The analysis of oocyte developmental competence related genes revealed that except PTGS2 and CYCB2, the expression of INHBA, MAPK1, PTX3 and EGFR (Assidi et al. 2008, Fülöp et al. 1997, Fülöp et al. 2003, McKenzie et al. 2004, Salustri et al. 2004, Sirois et al. 1992) in both cumulus cells and oocytes were increased when miR-20a was overexpressed. The increment of the CYCB2 gene expression in cumulus cells could be associated with the evidence that miR-20a promoted granulosa cell proliferation (Andreas et al. 2016). Herein, we suggest that miR-20a regulates oocyte maturation and the expression of oocyte developmental competence related genes.

The progesterone synthesis during oocyte maturation process is essential for oocyte meiosis resumption and subsequent oocyte maturation processes (Aparicio et al. 2011, Choi et al. 2001, Ježová et al. 2001, Montano et al. 2009, Nagyova et al. 2011, Nagyova et al. 2012, Shimada et al. 2004c, van Tol et al. 1996, Yamashita et al. 2003). This hormone is secreted by cumulus cells under stimulation of FSH and LH (Choi et al. 2001, Shimada and Terada 2002,

Shimada et al. 2004c, van Tol et al. 1996). The progesterone level was found to be higher in follicular fluid obtained from follicle-enclosed mature oocytes compared to follicle-enclosed immature ones (Grimes and Ireland 1986). Administration of progesterone during in vitro maturation promoted oocyte maturation and induced nuclear maturation in pig (Yamashita et al. 2003), mouse (Jamnongjit et al. 2005) and bovine (Siqueira et al. 2012) in a dose-dependent manner. On the other hand, the inhibition of progesterone synthesis had shown negative effect on oocyte meiosis resumption, cumulus cells expansion, final oocyte maturation, ovulation and number of ovulated oocytes and subsequent embryonic development in mouse (Aparicio et al. 2011, Siqueira et al. 2012, Sirotkin 1992), porcine (Shao et al. 2003, Shimada and Terada 2002, Shimada et al. 2004c) and bovine (Aparicio et al. 2011, O'Shea et al. 2013, Roh et al. 1988, Shao et al. 2003, Shimada et al. 2004b, Shimada et al. 2004a, Wang et al. 2006). Here we showed that progesterone synthesis was increased and decreased in cumulus cells overexpressed and inhibited miR-20a, respectively. This result was accompanied by the expression of progesterone synthesis marker genes, namely CYP11A1 and STAR (Nuttinck et al. 2008). Similarly, the selective knockdown of PTEN and BMPR2 genes in cultured granulosa cells confirmed the role of miR-20a in progesterone synthesis (Andreas et al. 2016). Moreover, we observed that the higher progesterone synthesis in miR-20a overexpressed cumulus cell was consistent with the increase of oocyte maturation rate in miR-20a mimic transfected COCs suggested that miR-20a overexpression in cumulus cells promoted oocyte maturation through increasing cumulus cell progesterone synthesis.

The profiling of microRNA expression in ovarian tissues/cells has been conducted in various species including human (Landgraf et al. 2007), mice (Ro et al. 2007), pigs (Li et al. 2011), cattle (Hossain et al. 2009) and sheep (McBride et al. 2012). Several studies shown the role of specific microRNAs in regulating granulosa cell proliferation, apoptosis and estradiol synthesis (Dai et al. 2013, Jiang et al. 2015, Wang et al. 2016). However, a few report has been reported about the function of microRNA in regulating oocyte maturation (Pan et al. 2015) and cumulus expansion (Yao et al. 2014). Recently, we have shown that miR-20a directly targets PTEN and BMPR2, and miR-20a overexpression in bovine granulosa cells downregulates mRNA expression and protein level of both genes (Andreas et al. 2016). In the present study, we observed the similar decrease in PTEN and BMPR2 gene expression when miR-20a was overexpressed in cumulus cells. Those results suggested that PTEN and BMPR2 genes are direct targets of miR-20a in both types of cells.

In conclusion, our work has demonstrated that modulation of miR-20a expression in cumulus cells regulates oocyte maturation. The miR-20a increased cumulus cell progesterone synthesis by simultaneously suppressed the expression of PTEN and BMPR2 genes. Besides, the expression of several cumulus expansion, oocyte maturation and cell cycle related genes in both cumulus cells and oocytes supported the role of miR-20a during oocyte maturation progression. Our finding offers new insights into how the microRNA expression in oocyte surrounding somatic cells regulates the oocyte developmental competence.

Table 1. List of primers used for candidate genes expression analysis in bovine cumulus cells and oocytes.

GenBank accession number	Genes	Primer sequence (5'-3')	Product length (bp)
NM_173979	B-ACTIN	F: TGTCCACCTTCCAGCAGAT	249
		R: TCACCTTCACCGTTCCAGT	
NM_001304285	BMPR2	F: GCAAGCACAAGCTCGAATCC	169
		R: TGGCTGTGAAACATTGGTGG	
AF080219	CYCB2	F: TGCCACTCTTGTTTGTCCGT	246
		R: GGTTTCGGGTGCTTGTTGAC	
NM_176644	CYP11A1	F: CGGAAAGTTTGTAGGGGACA	177
		R: ACGTTGAGCAGAGGGACACT	
XM_592211	EGFR	F: GACCCGAAAGAACTGGACAT	177
		R: TGTTATATCCAGGCCGACAA	
NM_174363	INHBA	F: GCAAGGTCAACATCTGCTGTA	262
		R: TACAACATGGACATGGGTCTC	
NM_175793	MAPK1	F: GGGCTACACCAAGTCCATCG	249
		R: GCTTTGGAGTCCGCGTTC	

NM_001034494	PCNA	F: CACCAGCATGTCCAAAATAC	192
		R: CTGAGATCTCGGCATATACG	
XM_613125	PTEN	F: TGGGGAAGTAAGGACCAGAG	172
		R: ATTGCAAGTTCCACCACTGA	
NM_001076259	PTX3	F: ACCTGGGATTCAAAGAAAGG	208
		R: CACCCTCCCAGATATTGAAG	
NM_174445	PTGS2	F: CGATGAGCAGTTGTTCCAGA	215
		R: GAAAGACGTCAGGCAGAAGG	
NM_174189	STAR	F: AAATCCCTTTCCAAGGTCTG	204
		R: ACCAGCATTTCTGCTACTGC	

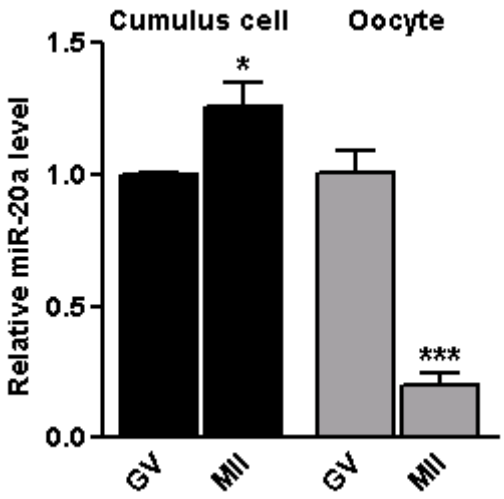

Figure 1. Temporal change of miR-20a expression in cumulus cells and oocytes of immature (GV) and matured (MII) COCs. The expression of 5S rRNA was used as internal control. The data are shown as mean ± SEM (n=3; *p<0.05; ***p<0.001).

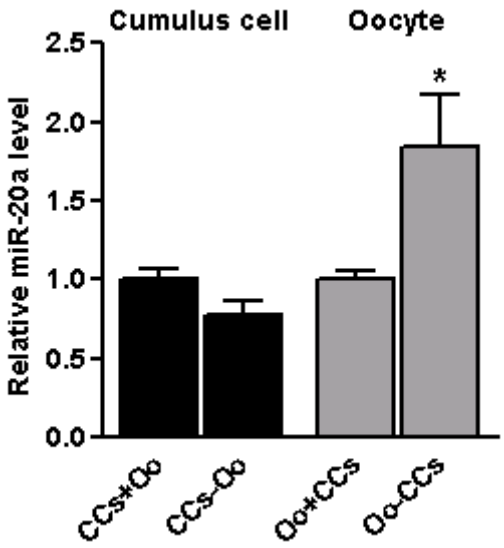

Figure 2. Relative expression of miR-20a in cumulus cells cultured with (CCs+Oo) or without (CCs-Oo) oocytes and in oocytes matured with (Oo+CCs) or without (Oo-CCs) cumulus cells. The expression of 5S rRNA was used as internal control. The data are shown as mean ± SEM (n=3; *$p<0.05$).

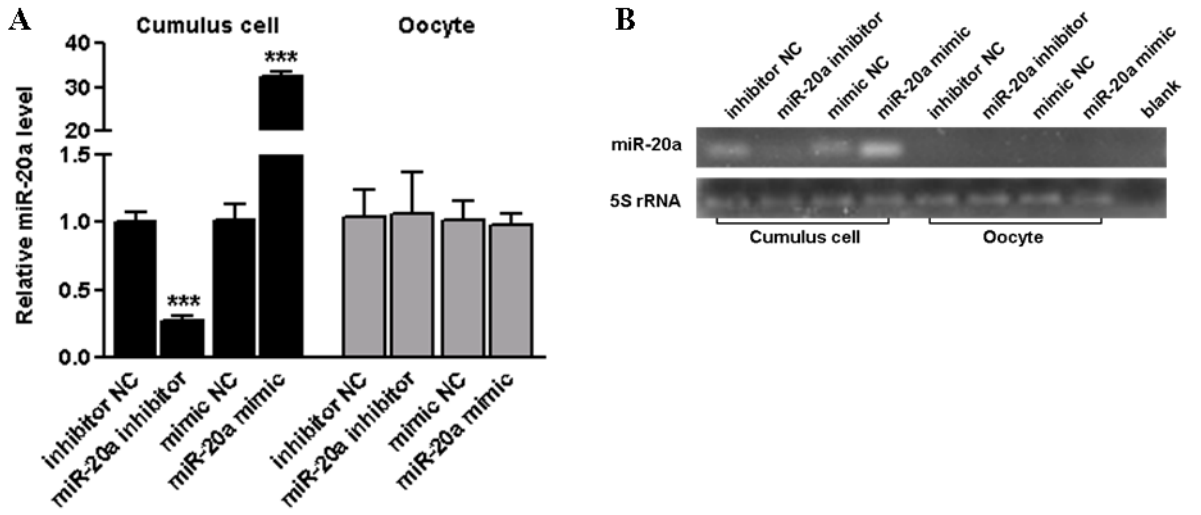

Figure 3. The effect of transfection is restricted in the cumulus cells. The expression of miR-20a in cumulus cells and oocytes transfected with miR-20a mimic, inhibitor and corresponding controls (A). Agarose gel (1.5%) electrophoresis of miR-20a amplification products in cumulus cells and oocytes transfected with miR-20a mimic, inhibitor and corresponding controls (B). The miR-20a expression level was compared to corresponding negative controls (mimic NC or inhibitor NC) and the expression of 5S rRNA was used as an internal control for qPCR analysis. The data are shown as mean ± SEM (n=3; ***$p<0.001$).

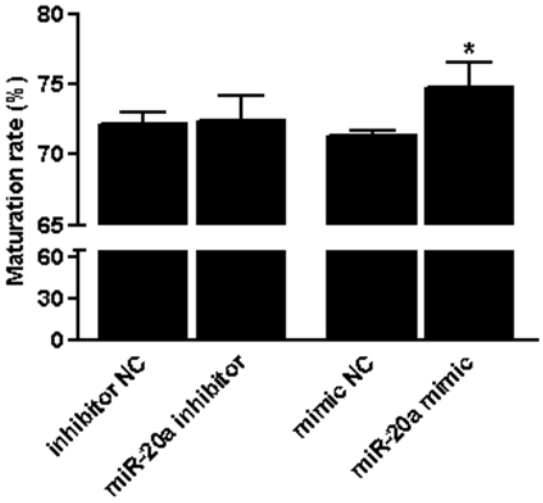

Figure 4. MiR-20a overexpression in cumulus cells increased oocyte maturation rate. The maturation rate was compared to corresponding negative controls (mimic NC or inhibitor NC). Data are shown as mean ± SEM (n=3; *p<0.05).

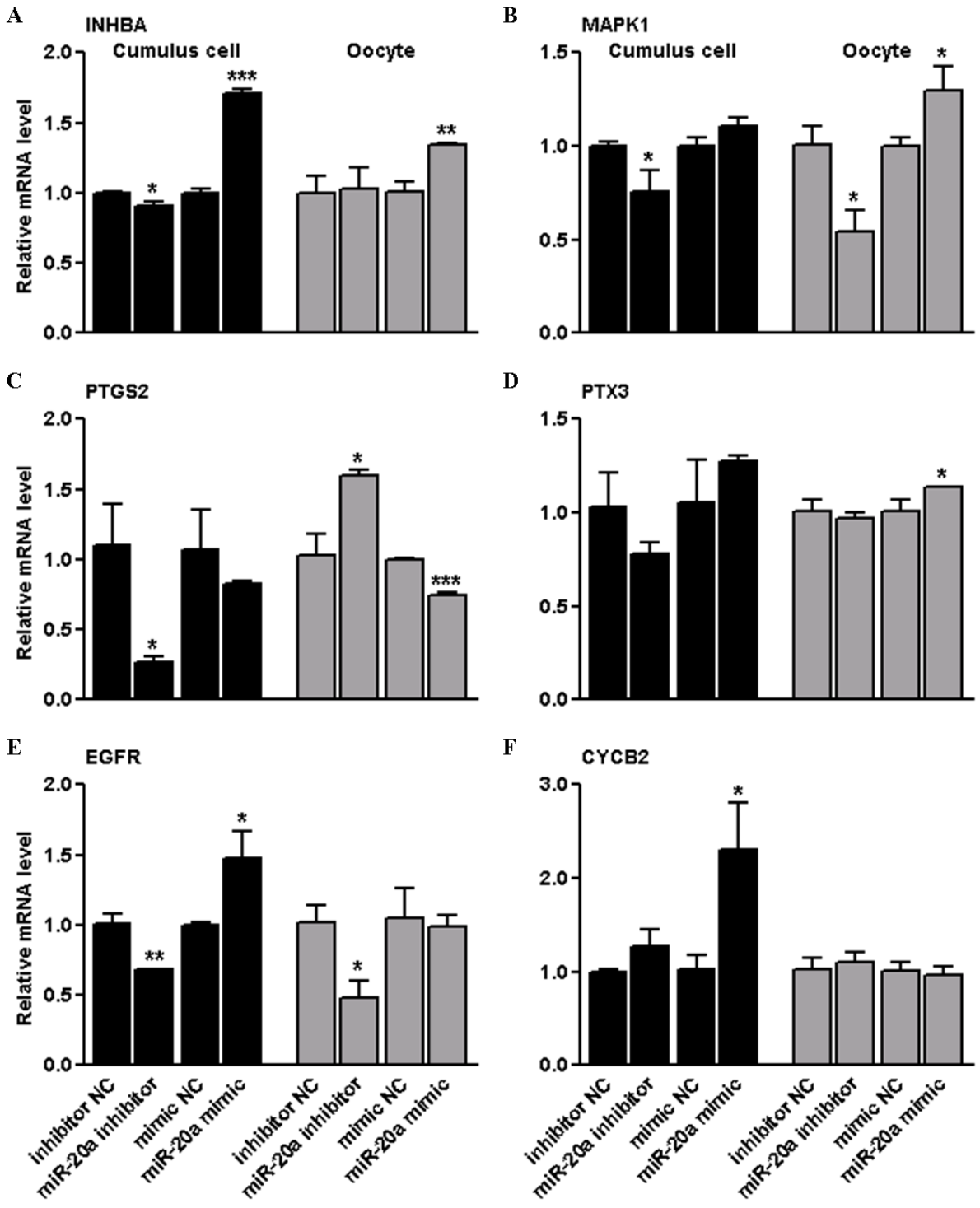

Figure 5. Expression of INHBA (A), MAPK1 (B), PTGS2 (C), PTX3 (D), EGFR (E) and CYCB2 (F) in cumulus cells and oocytes transfected with miR-20a mimic, inhibitor or controls. The expression level of β-ACTIN was used as an internal control. Data are compared to corresponding negative controls (mimic NC or inhibitor NC) and shown as mean ± SEM (n=3; *p<0.05; **p<0.01; ***p<0.001).

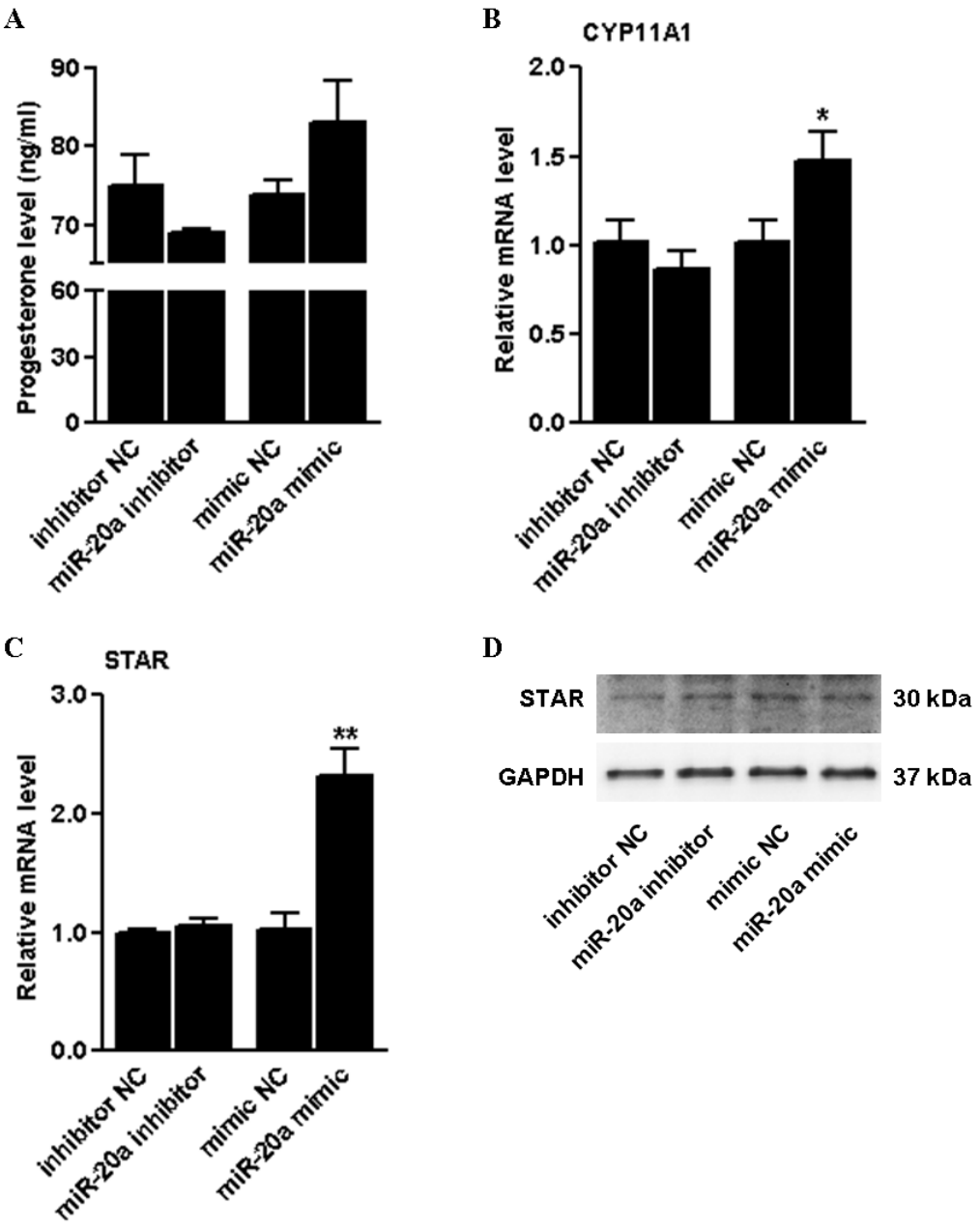

Figure 6. MiR-20a overexpression during IVM elevated cumulus cell-progesterone synthesis. The effect of miR-20a mimic and inhibitor during IVM on progesterone level in spent media (A). The expression of CYP11A1 (B) and STAR (C) mRNA in cumulus cell transfected miR-20a mimic, inhibitor or corresponding controls. Data are shown as mean ± SEM (n=3; *p<0.05; **p<0.01). The STAR protein expression in cumulus cells after miR-20a modulation (D). The expression level of β-ACTIN and GAPDH were used as an internal control in mRNA and protein analysis, respectively.

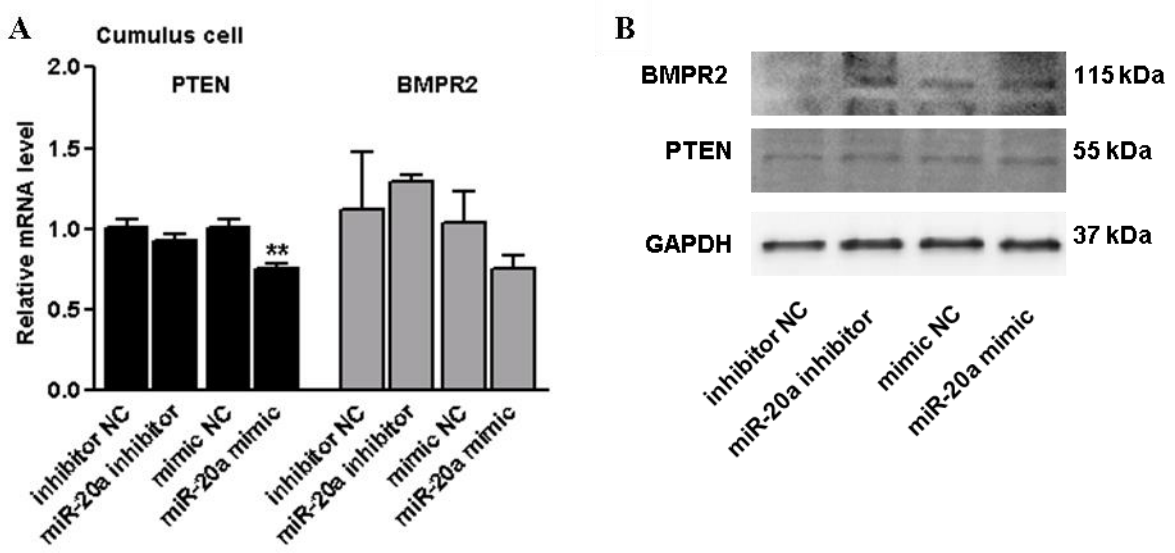

Figure 7. MiR-20a overexpression during in vitro oocyte maturation reduced cumulus cell PTEN and BMPR2 genes expression. The mRNA (A) and protein (B) expression of PTEN and BMPR2 genes in cumulus cells transfected with miR-20a mimic, inhibitor or corresponding controls. Both mRNA and protein expression levels were compared to negative controls (mimic NC or inhibitor NC). The β-ACTIN and GAPDH were used to normalized mRNA and protein expression, respectively. The expression of mRNAs abundance are shown as mean ± SEM (n=3; **p<0.01).

Chapter 4

General discussion

As an important process in the development of competent oocyte, orchestrated physiological processes are believed to occur in granulosa cells during follicular development. Although some biological and regulatory mechanisms are known, several key elements that could trigger the selection of one follicle to develop into dominant follicle while the rest follicles are regressing in cattle remain unclear. The dynamic transcript abundance at mRNA level in follicular cells derived from different size and stages during folliculogenesis indicated the spatiotemporal expression of genes in this process (Douville and Sirard 2014, Girard et al. 2015, Hatzirodos et al. 2014a, Hatzirodos et al. 2014b, Nivet et al. 2013). Comparable to mRNA, we have shown the differential expression of microRNAs, post-transcriptional regulator of genes expression (Ambros 2004, Bartel 2009, Huntzinger and Izaurralde 2011, Smalheiser and Torvik 2005), in granulosa cells derived from dominant and subordinate follicle at day 3, 7 and 19 of estrous cycle (Gebremedhn et al. 2015, Salilew-Wondim et al. 2014). At the late phase of estrous cycle (day 19 of estrous cycle), among others, miR-17-5p, miR-19a, miR-20a and miR-92a, which belong to miR-17-92 cluster, were upregulated in granulosa cells of subordinate follicles compared to dominant ones. However, the function of these microRNAs in follicular development is not known. Therefore, in the present study, we investigated the role of miR-17-92 cluster members during folliculogenesis using bovine granulosa cells under in vitro condition.

Target prediction analysis revealed that miR-17-92 cluster member coordinately regulate several genes involved in PI3K/AKT signaling pathway, FOXO signaling, focal adhesion and hippo signaling pathways which are known to be the key pathways in follicular development and ovulation (Alam et al. 2004, Jagarlamudi et al. 2009, Klusza and Deng 2011, Xiang et al. 2015, Zhang et al. 2010). Among target genes enriched in those pathways, PTEN and BMPR2 were selected for wet lab target validation as those genes have conserved binding sites (seed region) between human and bovine sequences and their potential role in follicular development (Douville and Sirard 2014, Hatzirodos et al. 2014b). The luciferase assay showed that PTEN and BMPR2 genes are direct targets of miR-17-92 cluster members. This result was confirmed by decreased and increased expression of PTEN and BMPR2 genes expression in granulosa cells transfected with mimic and inhibitor of miR-17-92 cluster member, respectively. In addition, PTEN and BMPR2 genes have been known to be involved in follicular growth, ovulation, luteolysis and steroidogenesis (Findlay et al. 2002, Inagaki et al. 2009, Miyoshi et al. 2007, Reddy et al. 2008, Richards et al. 2012, Shimasaki et al. 2004).

Granulosa cell proliferation and differentiation have been reported as the most important cellular activity within dominant follicle in the late phase of estrous cycle (Ali et al. 2001, Manikkam and Rajamahendran 1997). In response to the LH surge, progression of granulosa cells division is reduced while the terminal differentiation program was initiated (Hirshfield 1991, Rao et al. 1978, Richards et al. 1986, Richards 1994), and followed by the increased synthesis and release of progesterone (Baufeld and Vanselow 2013, Chang et al. 2015, Havelock et al. 2004, Zhang et al. 2015). In the present study, we simulated those conditions by modulating the expression of miR-17-92 cluster members in cultured granulosa cell. We observed that cell proliferation was increased while cell differentiation was decreased in miR-17-92 cluster members overexpressing granulosa cells. However, except in miR-20a mimic transfection, there was no difference in progesterone synthesis in granulosa cell transfected with miR-17-92 cluster members. These results were supported by the expression of CCND2 and PCNA genes, and CYP11A1 and STAR genes as marker for granulosa cell proliferation and differentiation, respectively. On the other hand, even not statistically significant, the inhibition of miR-17-92 cluster members in granulosa cell showed an opposite trend compared to those transfected with miR-17-92 cluster members mimic. Similarly, miR-17-92 cluster overexpression increased cell proliferation in mouse ischemic neural progenitor (Liu et al. 2013) while miR-17-92 cluster deletion in mouse model inhibited differentiation of osteoblast cells (Zhou et al. 2014). The role of miR-17-92 cluster member in granulosa cell proliferation and differentiation, as well as progesterone synthesis was cross-validated by selectively knockdown its target genes (PTEN and BMPR2) using siRNA technique. Here, we found that suppression of PTEN and BMPR2 simulated the effect of miR-17-92 cluster members overexpression in cultured granulosa cell proliferation and differentiation. However, the progesterone synthesis was not in agreement with cell differentiation rate result. We suggested that beside reduced the cell differentiation rate, the disruption of BMP-SMAD signaling pathways increased FSH-induced progesterone synthesis by granulosa cells (Chang et al. 2013, Hosoya et al. 2015, Luo et al. 2015).

Besides their role in granulosa cell function, the expression and potential role of miR-17-92 cluster during bovine oocyte maturation is not yet known. Therefore, comparative analysis of the member of miR-17-92 cluster (miR-20a) in oocytes and cumulus cells during oocyte maturation has been performed. In this study, we observed that miR-20a expression increased during IVM in cumulus cells while it decreased in the oocytes. Similarly, the decrement of miR-20a expression during IVM was also reported in human oocytes (Xu et al. 2011b).

The oocyte maturation process is complex and requires an integration of several signaling pathways (Takahashi et al. 2006), and interaction between the oocyte and surrounding cumulus cells for proper maturation of oocytes, fertilization and further embryonic development (Buccione et al. 1990a, Eppig 2001, Gilchrist et al. 2004a, Matzuk et al. 2002). The transcriptome dynamic in oocytes (Fair et al. 2007, Regassa et al. 2011) and cumulus cells (Assidi et al. 2010, Nivet et al. 2013, Regassa et al. 2011) revealed the spatiotemporal regulation of genes expression within bovine follicular cells during oocyte maturation. Moreover, we showed that the presence or absence of oocyte in culture cumulus cells altered several mRNAs (Regassa et al. 2011) and microRNAs (Abd El Naby et al. 2013) expression, and vice versa. In the present study, we cultured cumulus cells and oocytes with the presence or absence of their companion cells. We found that the expression of miR-20a in cumulus cells cultured with oocytes (CCs+Oo) was higher compared to those cumulus cells cultured without oocytes (CCs-Oo). On the other hand, miR-20a expression in oocytes cultured with cumulus cells (Oo+CCs) was lower compared to those oocytes cultured alone (Oo-CCs). We suggest that the expression of miR-20a in cumulus cells and oocytes is affected by the presence or absence of their companion cells.

In order to investigate whether alteration of miR-20a expression plays a role in the oocyte maturation, we modulated miR-20a expression by coculturing COCs with miR-20a mimic or inhibitor during in vitro maturation. The results showed that the expression of miR-20a in cumulus cells was increased and decreased in COCs cocultured with miR-20a mimic and inhibitor groups, respectively. However, the miR-20a expression has not seen detected in the oocytes either in miR-20a mimic or inhibitor cocultured COCs. We suggested that several layers of cumulus cells and the thick zona pellucida protect the oocyte from transfection reagent. Comparable results were obtained in lentivirus-transduced COCs (Pan et al. 2015).

During their developmental process, the oocyte is arrested in the prophase I stage (germinal vesicle; GV) until LH surge occurs (Dieleman et al. 2002). Six hours after LH surge, the germinal vesicle breakdown (GVBD) of the oocyte is started (Dieleman et al. 1983, Sirard et al. 1989) followed by the steroidogenesis in cumulus cell (Assidi et al. 2010). Under in vitro condition, supplementation of LH and FSH in the maturation media induced oocyte maturation, synthesis of hyaluronic acid, cumulus cells expansion and progesterone synthesis in cumulus cells (Ježová et al. 2001, Nagyova et al. 2011, Nagyova et al. 2012, Nagyová et al. 1999, Procházka et al. 1991). With regard to microRNA, several studies have been conducted to demonstrated the association of microRNA with oocyte developmental competence (Pan et

al. 2015, Yao et al. 2014). In the present study, we provide evidenced that miR-20a overexpression during IVM increased oocyte maturation rate. However, inhibition of miR-20a expression had no effect on maturation rate of the oocyte. This result was further confirmed by the expression of oocyte maturation-related genes, namely INHBA, AMPK1, PTGS2, PTX3, EGFR and CYCB2 (Assidi et al. 2008, Fülöp et al. 1997, Fülöp et al. 2003, McKenzie et al. 2004, Salustri et al. 2004, Sirois et al. 1992).

Progesterone is one of the critical hormones during oocyte maturation (Aparicio et al. 2011, Choi et al. 2001, Ježová et al. 2001, Montano et al. 2009, Nagyova et al. 2011, Nagyova et al. 2012, Shimada et al. 2004c, van Tol et al. 1996, Yamashita et al. 2003), being secreted by the cumulus cells under stimulation of FSH and LH (Choi et al. 2001, Shimada and Terada 2002, Shimada et al. 2004c, van Tol et al. 1996). Progesterone supplementation in IVM media has been reported to induce oocyte maturation in pig (Yamashita et al. 2003), mouse (Jamnongjit et al. 2005) and bovine (Siqueira et al. 2012) in a dose-dependent manner. On the other hand, the inhibition of progesterone synthesis resulted in negative effects on oocyte meiosis resumption, cumulus cells expansion, oocyte maturation rate, ovulation rate and subsequent embryonic development in various species (Aparicio et al. 2011, O'Shea et al. 2013, Roh et al. 1988, Shao et al. 2003, Shimada and Terada 2002, Shimada et al. 2004c, Shimada et al. 2004b, Shimada et al. 2004a, Siqueira et al. 2012, Sirotkin 1992, Wang et al. 2006). In the present study we observed that miR-20a overexpression during IVM resulted in increased progesterone synthesis while progesterone synthesis was suppressed when miR-20a expression was inhibited. The analysis of CYP11A1 and STAR genes expression supported the role of miR-20a in progesterone synthesis. The consistent results with oocyte maturation rate suggested that miR-20a overexpression in cumulus cells promoted oocyte maturation through increasing cumulus cell progesterone synthesis.

The role of specific microRNAs in regulating follicular cells function have been studied in the last decade synthesis (Dai et al. 2013, Jiang et al. 2015, Wang et al. 2016). However, less information is available about the role of microRNA during oocyte maturation (Pan et al. 2015). In our previous study, we showed that the PTEN and BMPR2 are direct target of miR-20a by using granulosa cells model (Andreas et al. 2016). In the present study, we found that the overexpression and inhibition of miR-20a resulted in decreased and increased the mRNA and protein expression of PTEN and BMPR2 genes in cumulus cells. These results suggested that miR-20a regulates oocyte maturation and progesterone synthesis by targeting PTEN and BMPR2 genes in cumulus cells.

In conclusion, this work demonstrated the role of miR-17-92 cluster in bovine granulosa cell proliferation and differentiation by coordinately targeting the expression of PTEN and BMPR2 genes. In addition, we also provide evidence that modulation of miR-20a expression in cumulus cells regulates oocyte maturation and progesterone synthesis in cumulus cells.

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