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MicroRNA-424/503 cluster involvement in regulation of bovine granulosa cell function and oocyte maturation

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Dedicated to my mother

MicroRNA-424/503 cluster involvement in regulation of bovine granulosa cell function and oocyte maturation

Several microRNA (miRNA) clusters are known to be differentially regulated during follicular development. Previously, it was reported that the miR-424/503 cluster was highly abundant in bovine granulosa cells (bGCs) of preovulatory dominant follicles compared to subordinate counterparts. However, the underlying mechanisms of this miRNA cluster in bGCs functions and oocyte maturation have not been investigated. Here, we aimed to investigate the role of miR-424/503 cluster in bGCs function and oocyte maturation. Target gene validation assay using luciferase reporter showed that SMAD7 and ACVR2A are the direct targets of the miR-424/503 cluster. In line with this, while overexpression of miR-424/503 reduced, inhibition increased the expression of SMAD7 and ACVR2A genes. Furthermore, flow cytometric analysis indicated that overexpression of miR-424/503 cluster enhanced bGCs proliferation by promoting G1 to S phase cell cycle transition. Moreover, knockdown of the miR-424/503 cluster target gene using small interfering RNA also revealed similar phenotypic and molecular alterations when miR-424/503 cluster was overexpressed. Further, increased cell proliferation and downregulation of both miR-424/503 and its target gene with activin A treatment, indicated the presence of negative feedback loop between activin A and the miR-424/503 cluster. Moreover, expression of miR-424/503 was significantly higher at mature cumulus-oocyte complexes (COCs) (MII) compared to immature COCs (GV) in both cumulus and oocytes. Additionally, overexpression of miR-424 enhanced the expression of genes associated with cumulus cell expansion such as EGFR, PTGS2, PTX3 and MAPK1 and also increased the expression of KIT ligand gene associated with oocyte growth. In conclusion, the miR-424/503 cluster regulates bovine granulosa cell proliferation by targeting SMAD7 via activin signalling pathway and enhances the candidate gene expression involved in cumulus cell expansion and oocyte maturation.

Die Beteiligung des microRNA-424/503-Clusters an der Regulation der Rinder-Granulosazellenfunktion und der Oozytenreifung

Es ist bekannt, dass mehrere microRNA (miRNA) Cluster während der follikulären Entwicklung unterschiedlich reguliert sind. In vorherigen Studien wurde bereits berichtet, dass die Expression des miR-424/503-Clusters in Rinder-Granulosazellen (bGCs) von präovulatorischen dominanten Follikeln im Vergleich zu subordinanten Follikeln verstärkt auftritt. Die zugrunde liegenden Mechanismen dieses miRNA-Clusters in bGCs-Funktionen und Oozytenreifung wurden jedoch noch nicht betrachtet. Daher wollten wir in dieser Studie die Funktion des miR-424/503-Clusters in bGCs und der Eizellreifung untersuchen. Ein Target-Gen-Validierungsassay unter Verwendung des Luciferase-Reporter Systems zeigte, dass SMAD7 und ACVR2A die direkten Zielgene des miR-424/503-Clusters sind. In Übereinstimmung damit, während die Überexpression von miR-424/503 reduziert wurde, erhöhte die Inhibierung die Expression der Gene SMAD7 und ACVR2A. Darüber hinaus zeigte die Durchflusszytometrieanalyse, dass die Überexpression des miR-424/503-Clusters die bGCs-Proliferation durch die Stimulierung des G1-zu-S-Phasen-Zellzyklusübergangs verstärkt. Weiterhin ergab die Ausschaltung des miR-424/503-Clusters Zielgene mit siRNA ähnliche phänotypische und molekulare Veränderungen, wie während einer Überexpression des miR-424/503-Clusters. Eine erhöhte Zellproliferation und eine Herunterregulation sowohl von miR-424/503 als auch seiner Zielgene nach Activin A-Behandlung impliziert das Vorhandensein einer negativen Rückkopplungsschleife zwischen Activin A und dem miR-424/503-Cluster. Darüber hinaus war die Expression von miR-424/503 in reifen Kumulus-Oozyten-Komplexen (COCs) (MII) signifikant höher als in unreifen COCs (GV) sowohl in den Kumuluszellen als auch in den Oozyten. Zusätzlich verstärkte die Überexpression von miR-424 die Expression von Genen, die mit der Kumulus-Expansion assoziiert sind, wie z.B. EGFR, PTGS2, PTX3 und MAPK1 und erhöhte auch die Expression des KIT-Ligandengens,

das mit dem Oozytenwachstum assoziiert ist. Zusammenfassend lässt sich sagen, dass der miR-424/503-Cluster die Rinder-Granulosazellproliferation reguliert, indem er SMAD7 über den Activin-Signalweg aktiviert und die Expression von Kandidatengenen für die Kumuluszell-Expansion und Oozytenreifung erhöht.

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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
BAX	Bcl-2-associated X protein
BCL2	B-cell lymphoma 2
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 DANN
CO_2	Carbon dioxide
COC	Cumulus-oocyte complex
CYCB2	Cytochrome C552
CYP11A1	Cholesterol side-chain cleavage enzyme
CYP19A1	Aromatase
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
FOXO	Forkhead box protein O
GV	Germinal vesicle
HAS	Hyaluronan synthases
INHBA	Inhibin beta A subunit
INHBB	Inhibin beta B subunit
IU	International unit
IVM	In vitro maturation
LH	Luteinizing hormone
LNA	Locked nucleic acid
MAPK1	Mitogen-activated protein kinase 1
MII	Metaphase II
mRNA	Messenger RNA
MYC	V-myc avian myelocytomatosis viral oncogene homolog
NURR1	Nuclear receptor related 1 protein
PCR	Polymerase chain reaction
PGR	Progesterone receptor
PGRMC	Progesterone receptor membrane components
PLB	Passive lysis buffer
PTGS2	Prostaglandin-endoperoxide synthase 2
PTX3	Pentraxin 3
qPCR	Quantitative PCR
RBMS1	RNA-binding motif, single-stranded-interacting protein 1
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
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
STAR	Steroidogenic acute regulatory protein
TCM	Tissue culture media
TGF-β	Transforming growth factor beta
UTR	Untranslated region
v	Volume
ZP3	Zonna pellucida glycoprotein 3

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proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded. Adapted from (Winter et al. 2009).

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Chapter 1

General introduction

In the 5th century B.C., Hippocrates suggested that formation of a new life was the result of the reaction of two types of semen one coming from the male (ejaculate) and the other from the female (menstrual blood). Nevertheless, Hippocrates failed to mention any generative role of the ovary. Next observation in this regard came a century later, when Aristotle characterized the ovary as an imperfect vestige of the male testis with no apparent function, and until the mid of sixteenth century, the ovary could not be recognized as a source of eggs. In 1667, for the first time, Niels Steno reported in his treatise that 'the testicles of women are analogous to the ovary.' Interestingly, during the period of these reports a wrong perception prevailed that the follicle itself was the egg, like a bird's egg without a shell. Indeed, the first description of mammalian egg appeared in 1827 by Karl Ernst von Baer from his microscopic study of ovarian vesicles (follicles) in the ovary of a dog, and then for the next 100 years, there were no substantial reproductive studies regarding the dynamics of follicle development (Adams et al. 2008). Later, in 1986, Spicer and Echternkamp tried to define folliculogenesis as the formation of a Graafian follicle from a pool of primordial follicles (non-growing) (Spicer and Echternkamp 1986). Since then, several fact-finding studies revealed many hidden aspects of follicular growth and development, for instance follicular wave and events describing follicular dynamics, role of ovarian hormone and gonadotropins and their interaction with receptors, various growth factors and paracrines, being vital and necessary during follicle development to end up in a competent oocyte. These could be realized and obtained with series of investigations; including morphometric measurements of follicle, in vivo tracking and measurement of follicle with ultrasound guided technique, endocrine level measurements and their functionality, gene expression studies and many more (Spicer and Echternkamp 1986). In line with this, a recently discovered endogenous biomolecule termed

as microRNA (miRNAs), is emerging as an important player in follicular development through posttranscriptional regulation of gene (Bartel 2004; Gebremedhn et al. 2016a; Tesfaye et al. 2016).

The general introduction of this thesis is intended to provide a brief overview of the follicular dynamics, recent advances in genetic regulation of follicular growth through activin signalling pathway and cumulus-oocyte interaction during in vitro oocyte maturation. Further, it sheds insight regarding recent studies conducted in the domain of miRNAs indicating their involvement in governing follicular growth and development. Last part of general introduction highlights research gap, hypothesis and objectives of this study.

1. Folliculogenesis

One of the earlier classic work reported that the bovine pool of primordial follicles remains stable (~133,000 follicles) from birth until about the fourth year of life, which subsequently decline until approximately 3,000 in ovaries of cows 15 to 20 years old, these growing follicles were classified into preantral and antral follicles (Erickson 1966). During the follicular developmental events, primordial follicles continuously enter into the growing pool of follicles, nevertheless the fate of greater than 99% of all follicles entering the growing pool is atresia (Ireland 1987). In an attempt to better describe the folliculogenesis happenings, Hodgen (Hodgen 1982) proposed certain fitting terms; recruitment: a process whereby a cohort of follicles begin to mature in a milieu of sufficient pituitary gonadotropic stimulation to permit progress towards ovulation, selection: the process whereby a follicle avoids atresia and undergoes further development and becomes competent to achieve timely ovulation, dominance: the process whereby a single follicle achieves and maintains its eminence over the other recruited follicles, which undergo atresia (Hodgen 1982). During the course of follicular development towards the ovulatory stage, three features appear to be highly

conserved across all species: 1) the sequence of events (recruitment, selection and dominance); 2) the sequential need for gonadotropins (FSH for recruitment, LH for dominance) and 3) the large variability of numerical parameters (number of waves per cycle, number of follicles per wave) as well as temporal requirements (time of selection, duration of dominance) (Driancourt 2001).

1.1 Bovine follicular waves and follicular sequential events

In 1960, the two waves theory of follicular activity was proposed for bovine estrous cycles, one between day 3 and 12 and the other between day 12 and subsequent estrous (Rajakoski 1960). The hypothesis of two waves per estrous cycle was subsequently supported by several researchers (Swanson et al. 1972; Pierson and Ginther 1984). Contrary to this, Ireland and Roche in 1983 (Ireland and Roche 1983), by measuring follicles and steroid level of blood and follicular fluid, concluded that three follicular waves occur during each estrous cycle, and each resulted in a dominant follicle. Further, advent of ultrasonic imaging provided a means for repeated, direct, noninvasive monitoring and measuring of follicles within the ovary, clarified the nature of bovine folliculogenesis by tracking diameter of follicles (Pierson and Ginther 1984; Fortune et al. 1988; Savio et al. 1988; Sirois and Fortune 1988). Thus during the course, it was observed and concluded that bovine estrous cycle contained two waves (Ginther et al. 1989) to three waves (Savio et al. 1988; Sirois and Fortune 1988) per estrous cycle. Each wave is preceded by a peak in serum FSH concentrations (Adams et al. 1992; Adams et al. 1994) (Figure 1). On average follicular wave emergence in cattle comprises of 7–11 follicles, which is preceded by a rise in FSH, with wave emergence concurrent with the peak (Adams et al. 1992). Patterns of follicle development at different physiological status across species, cattle, sheep and horses suggest that ovaries of all species operate in a wave like fashion unless they are prevented from doing so (Driancourt 2001).

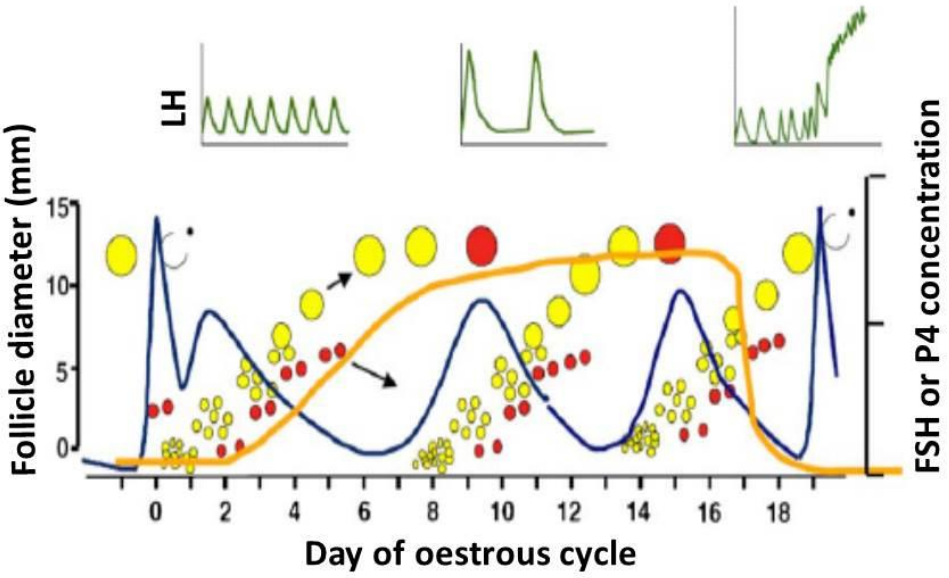

Figure 1. Schematic depiction of the bovine follicular waves. Bovine follicles emerge in wave like fashion where follicle-stimulating hormone (FSH), luteinizing hormone (LH), and progesterone (P4) play key role during the estrous cycle. Each wave of follicular growth is preceded by a transient rise in FSH concentrations. Healthy growing follicles are shaded in yellow while atretic follicles are shaded in red. The pattern of LH secretion pulses in terms of frequency and amplitude during early luteal phase, the mid-luteal phase and the follicular phase is indicated on the top. A surge in LH and FSH concentrations occurs at the onset of estrous and induces ovulation. Adapted from (Forde et al. 2011).

The growing pool of follicle contains primary, secondary and tertiary follicles, whereas the non-growing pool comprises of the primordial follicles (Kanitz 2003). Follicles further can be classified on the basis of gonadotropin dependence. Bovine follicle of 1-3 mm size are gonadotropin dependent follicles, which could be mobilized with exogenous gonadotropins treatment (Driancourt 2001). Usually three to six follicles having a diameter of 4 to 5 mm enter after recruitment into follicular wave (Savio et al. 1988; Sirois and Fortune 1988; Sunderland et al. 1994).

1.1.1 Follicular recruitment

Initiation of gonadotropin-dependent folliculogenesis by a cohort of healthy follicle containing the future preovulatory follicle takes place during a recruitment window (Ginther 2000), which lasts around two days in cattle (Driancourt 2001), where a relationship between size at recruitment and the size at which follicles become gonadotropin dependence exists in most species. Further, growing follicle are selected according to the species-specific ovulation number (Fortune 1994), since post recruitment recruited follicles sorted out into one selected dominant follicles, whereas the rest of the recruited follicles are destined to atresia (Fortune 1994). Atresia seems to be most prevalent as follicles approach the size at which they could be recruited for potential ovulation. Although most follicles become atretic around that stage, a few are recruited into a cohort or wave of follicles that continue to grow beyond the stage at which atresia normally occurs. Largely, there is an agreement that FSH plays key role for inducing recruitment in most mammalian species (Picton et al. 1990; Adams et al. 1992; Sunderland et al. 1994; Driancourt et al. 1995), since an association between FSH surge and recruitment has been demonstrated in cattle (Ginther et al. 1996a) at several physiological stages. This temporal relationship has been observed by blocking endogenous FSH secretion and using exogenous FSH injection (Webb et al. 1999; Crowe et al. 2001). Further, there is a minimal threshold of FSH concentration required to proceed with recruitment, which appears to be variable between animals (Picton and McNeilly 1991). Primarily, FSH is involved to induce aromatase activity within granulosa cells (Saumande 1991). Noticeably, it appears that LH has almost no contribution in the control of recruitment. Hence, recruitment is believed to happen in an endocrine environment where LH pulses frequency are reduced (as in the early-luteal phase), low (as in the mid-luteal phase) or very low (as in the prepubertal stage, postpartum anestrus) (Driancourt 2001), additionally, growth

factor and a paracrine are also held to be involved in the process of recruitment (Gong et al. 1991; Singh et al. 1999).

1.1.2 Follicular selection

During the follicle selection, the dominant one is chosen, leaving the rest of the follicles of the cohort turn out to be subordinate follicles, destined to atresia. It is generally assumed that the largest follicle of the cohort appears to be the one selected for ovulation (Ginther et al. 1996b). The future dominant follicle cannot be identified reliably on the basis of its diameter or estradiol production until the day after the two follicles began to deviate in growth rates (Ginther et al. 1997), which occurs when the two largest follicles have sizes between 8.3 and 7.8 mm in diameter (Kulick et al. 1999; Ginther 2000) with increased blood estradiol and reduced FSH concentrations below threshold for the smaller however not for the largest follicle (Kulick et al. 1999; Ginther 2000). Several lines of evidences support that selection is controlled by endocrine mechanisms, happens under declining FSH concentrations (Evans and Fortune 1997; Austin et al. 2001). Additionally, it is believed that compounds produced by the largest follicle inhibit development of the other follicles of the cohort. Further, it has been noticed that bovine follicles ≤ 3 mm could not suppress FSH; however, follicles having size 3 to 5 mm could suppress FSH (Gibbons et al. 1999; Ginther 2000). Thus, a dominant follicle possesses the features to continuously grow to the largest follicle as dominant follicle and static or reduced growth of the rest of the follicles destined as subordinate follicles. Further, in all species, the selected follicle appears to be the first acquiring LH receptors on granulosa cells; nevertheless, FSH concentration, which occurs 2 to 3 days after recruitment, is a key mechanism in the selection process. In most species, there is a close temporal relationship between selection and the time when FSH levels reach their base. Further, this drop in FSH support is caused by the combined action of inhibin and estradiol. Nevertheless,

it is difficult to obtain single ovulations by only manipulating FSH and LH concentrations, indicating involvement of other regulatory mechanisms, independent of gonadotropin concentrations (Driancourt 2001).

1.1.3 Follicular dominance

The dominant follicle becomes the most important follicle in female reproduction by successfully passing through growth and the development phase of the oocyte, and ultimately celebrating the ovulation event. The dominant follicle behaves differently from the other follicles showing power to escape atresia, and if exposed to the LH surge, results into the corpus luteum. Some dominant follicle become atretic because they mature during the luteal phase and are never exposed to the LH surge (Lucy 2007), while others are redirected through the LH surge toward their ultimate end (i.e., luteinization, ovulation, and differentiation into the corpus luteum (Lucy 2007). The factors that lead to dominance of one follicle, and the mechanisms that suppress the growth of the subordinate follicles are not fully understood (Fortune et al. 1991), nevertheless dominance establishes by negative feedback effects of biomolecules produced by the dominant follicles on circulatory FSH levels. Selection and dominance happen by the virtue of thecal cells to synthesize androgen which subsequently gets aromatized by granulosa cells into estradiol (Fortune 1994). Contrary to follicle selection, which largely depends on FSH, follicular dominance is intricately sensitive to LH. Thus changes in the pattern of pulsatile LH secretion and its interaction with LH receptors located on granulosa cells of the dominant follicle may alter their fate (Sartori et al. 2001). In this regard it is worth to note that levels of estradiol-17 β and aromatase were higher in dominant follicles compared to subordinate follicles. Further, growth of dominant follicle is related with an increment in the ratio of E2:P4 concentration in the follicular fluid, referred as E2-dominated; whereas subordinate follicles cease to grow possessing low E2:P4

ratio, referred as P4-dominated. The destiny of the dominant follicle is governed by presence and functioning of the corpus luteum, since high progesterone levels direct the dominant follicle to atresia by virtue of the negative effect of the progesterone on the pulsatile secretion of LH (Ireland et al. 2000). Interestingly, in this situation, sometime functional dominance is lost between the early and late plateau phases, while the follicle is still morphologically dominant. Further, a decrease in estradiol levels of the dominant follicle of the first follicle wave also supplements loss of dominance leading to new wave of follicle (Sunderland et al. 1994; Ginther et al. 1996a; Mihm et al. 2002). Follicular dominance in mono-ovulatory species such as cattle requires the integration of a number of processes involving both extra-ovarian signals and intra-ovarian growth factors, paracrine and autocrine regulators (Sisco and Pfeffer 2007; Webb and Campbell 2007). Further, in order to better understand molecular aspect of follicular dynamics, recently focus was laid on differentially enriched genes related with follicular dominance (Girard et al. 2015, Hatzirodos et al. 2014) and oocyte competence (Nivet et al. 2013).

1.2 Genetic regulation of bovine folliculogenesis

During the entire course of follicular development, the follicle passes through a series of cellular and molecular changes which are required to attain oocyte developmental competence, which largely happens through the communication between oocyte and its companion cells (Sanchez and Smitz 2012). Indeed, mammalian follicle, consisting of an oocyte surrounded by granulosa and theca cells, represents the basic functional unit of the ovary (Orisaka et al. 2009). Among the follicular cells, granulosa cells are critically indispensable for follicular development, where they undergo a series of morphological and functional changes (Robker and Richards 1998). Further, the sequential, well-controlled transformation from the primordial to antral follicle stage is the result of the differentiation

and proliferation of granulosa cells (GCs) that provide essential and vital inputs in the form of steroid hormones, cytokines, and paracrine and autocrine factors during the process of follicular development (Richards 1994; Huang and Wells 2010; Toda et al. 2012), which is tightly regulated by array of genes (Richards 1994; Robker and Richards 1998; Toda et al. 2012; Nivet et al. 2013; Douville and Sirard 2014). Several transcriptome profiling studies demonstrated the expression patterns of genes in GCs (Nivet et al. 2013) at different phases of antral follicle growth (Douville and Sirard 2014).

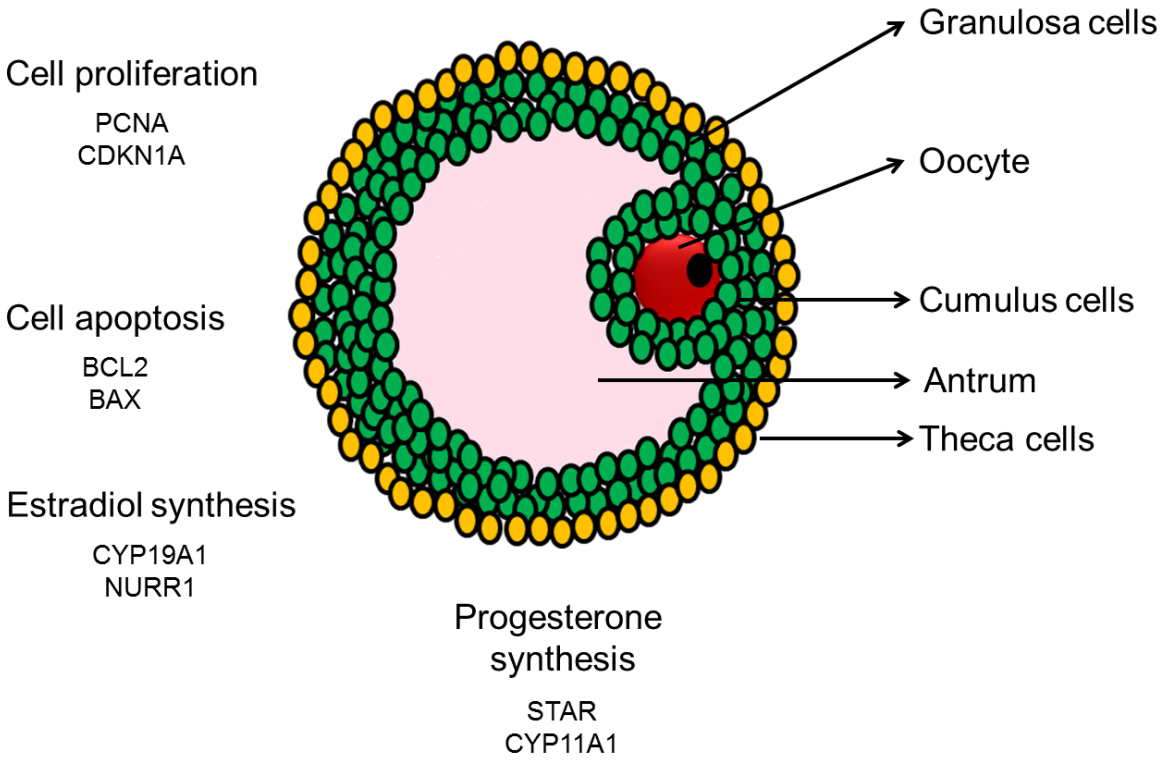

Figure 2. Genetic regulation of folliculogenesis. Certain key genes involved in cell proliferation, cell apoptosis, estradiol synthesis and progesterone synthesis during follicular growth and development.

Accordingly, several genes involved in steroidogenesis (CYP17A1, CYP11A1, HSD3B1, STAR), cell proliferation/the cell cycle (CCND2, PCNA), gonadotropin receptors (LHCGR,

FSHR) and growth factors (GDF9, BMP2, Activins, IGF-I, IGF-II) have been found to be altered depending on the size and stage of follicular development (Robker and Richards 1998; Evans, A C O et al. 2004; Myers et al. 2008; Andreas et al. 2016; Gebremedhn et al. 2016b) (Fig. 2). One of these intercellular communications occurs through a paracrine signalling pathway where intraovarian factors are found to be involved during follicular growth and development. Similarly, activins belonging to the transforming growth factor-β (TGF-β) superfamily regulate follicular development (Li et al. 1995; Thomas et al. 2003; Pangas et al. 2007; McLaughlin et al. 2010). The activin signalling pathway in which an activin dimer binds to activin receptors type II A (ACVR2A) or activin receptors type II B (ACVR2B) leads to phosphorylation of type I receptor. Type I receptor in turn phosphorylates to SMAD2/3 and activated SMAD2/3 forms a heterodimer complex with a common SMAD4. This heterodimer complex then translocates in the nucleus where it activates gene transcription. Further, in this pathway, SMAD7 as inhibitory member blocks the phosphorylation of SMAD2/3 and acts as a negative feedback regulator of activin-signalling pathway (Tsuchida et al. 2009). Activins, ubiquitously present in ovarian follicles are generally synthesized by granulosa cells (Pangas et al. 2007) which act through activin receptors type I (ALK4), IIA and IIB (Activin receptor type IIA/IIB) (Roberts et al. 1993; Cameron et al. 1994). Activins are believed to play autocrine and paracrine role in regulating early follicular development by promoting follicular growth and differentiation (Findlay 1993; Mather et al. 1997; Thomas et al. 2003).

Activins are found to promote the release of FSH from the anterior pituitary (Katayama et al. 1990), which in the presence of insulin and transferrin increases granulosa cell proliferation (Knight and Glister 2003). Further, the dominant follicle selection appears to depend on differential FSH sensitivity amongst the growing cohort of small antral follicles, where activin probably contribute by sensitizing the follicles with the highest ‘activin tone’ to FSH

(Knight and Glister 2003), which could be inhibited by follistatin (Thomas et al. 2003). Further, activin is found to be involved in aromatization, antral cavity formation through reorganization of follicular structure (Findlay 1993; Li et al. 1995; Mizunuma et al. 1999), and have a further intrafollicular role in the terminal stages of follicle differentiation to promote oocyte maturation and developmental competence (Knight and Glister 2003). Like activin, BMP-4 and -7 and BMP-6 are able to enhance estradiol and inhibin secretion from bovine granulosa cells while suppressing progesterone secretion which is involved in delaying follicle luteinization and atresia (Knight and Glister 2003). Further, the oocyte maturation is a complex process requires an integration of several signalling pathways (Takahashi et al. 2006), and interaction between the oocyte and surrounding cumulus cells for proper maturation of oocytes (Buccione et al. 1990; Eppig 2001; Gilchrist et al. 2004; Matzuk et al. 2002).

1.3 Cumulus-oocyte interaction during in vitro oocyte maturation

In vitro oocyte maturation **(**IVM) is an incredibly important and valuable technique by virtue of having great potential in exploiting the oocyte resource within an ovary, and as an alternative to minimize or eliminate the risks and drawbacks associated with intense stimulation of the ovary with gonadotropins (Wrenzycki et al. 2007; Coticchio et al. 2012), which allows to generate mature oocytes ex vivo (Gilchrist and Thompson 2007), involving aspiration of cumulus–oocyte complexes (COCs) from antral follicles, selecting the quality COCs and subsequently culturing them in standard culture conditions until they reach metaphase II (MII). In domestic animals, embryo production from unstimulated ovaries using oocyte IVM has become a routine practice for artificial breeding, cloning and transgenic animal production. Despite all the improvements in oocyte and embryo culture, at the best only 30–35% of immature mammalian COC develop to the blastocyst stage (Wrenzycki et al.

2007). The efficiency of oocyte IVM is limited due to the oocyte's intrinsic developmental competence depending on the biochemical and molecular state that allows a mature oocyte to normally fertilize and develop to an embryo (Gilchrist and Thompson 2007). Completion of oocyte maturation depends on several factors, among these the interaction of the oocyte and somatic cells of the follicle is critical and involves exchange of numerous signals between the two cell types in both directions (Downs 2015). One of the prominent function of the cumulus cells is the channeling of metabolites and paracrine signals through several metabolic pathway to the oocyte to stimulate germinal vesicle breakdown leading to developmental competence to the oocyte (Downs 2015). Indeed, developmental competence of oocyte is acquired after the oocyte becomes meiotically competent and involves an accumulation of transcripts and other factors. It has been observed that acquisition of both nuclear and cytoplasmic maturation is required, since oocytes isolated from early antral follicles could not resume meiosis and failed to progress further than the metaphase I (MI) stage (Eppig 2001). Furthermore, it was also noticed that early embryo development was compromised in the oocytes obtained from small antral follicles compared to the oocytes matured and fertilized obtained from large antral stages (Eppig and Schroeder 1989, Pavlok et al. 1992), indicated that an oocyte that has acquired meiotic competence has not necessarily acquired cytoplasmic maturity.

In antral follicles, the oocyte governs the behaviour of cells in its immediate vicinity, thereby actively regulating its own microenvironment. Since oocyte growth is governed by molecular cues, growth factors, autocrine and paracrine from somatic cells, nevertheless oocytes are not only passive recipients of these signals and biomolecules rather play a key role in the proliferation and differentiation process of granulosa cells. As such, the oocyte establishes and maintains the distinct cumulus lineage of granulosa cells. Presence of this bi-directional communication between oocytes and somatic cells is vital for successful oogenesis (Eppig et

al. 2001; Matzuk et al. 2002; Eppig et al. 2005; Sugiura et al. 2005; Kidder and Vanderhyden 2010; Chang et al. 2016) and occurs through transzonal projections (Macaulay et al. 2015) and gap junctions (Kidder and Mhawi 2002). This process is mediated by connexin 37 which exists between oocytes and granulosa cells in follicles from the primary stage onwards (Veitch et al. 2004; Teilmann 2005). This oocyte-cumulus cell interaction, in general, prevents luteinization of cumulus cells by promoting growth, regulating steroidogenesis and inhibin synthesis, and suppressing luteinizing hormone receptor expression (Gilchrist et al. 2004).

In most mammals, the oocyte in the Graafian follicle is surrounded by tightly packed layers of cumulus cells, forming the cumulus-oocyte complex. During the preovulatory period, cumulus cells change from a compact cell mass into a dispersed structure of cells for the synthesis extracellular matrix, this phenomenon is referred as cumulus expansion (Yokoo and Sato 2004). Cumulus expansion of the COC is necessary for meiotic maturation and acquiring developmental competence (Nevoral et al. 2015) which takes place shortly before ovulation in vivo, as well as during meiotic maturation in vitro (Eppig 1979; Salustri et al. 1989; Chen et al. 1993; Nevoral et al. 2015). Indeed, synthesis of glycosaminoglycan rich in hyaluronic acid (HA) into the extracellular space becomes the basis for cumulus expansion by providing structural component for expanded cumuli (Chen et al. 1993; Han et al. 2006; Yokoo et al. 2010). Furthermore, cumulus expansion is dependent on the stimulation of LH-induced epidermal growth factor (EGF)-like peptides, through the activation of protein kinase A (PKA) which subsequently results in high levels of cAMP produced by somatic cells (Sanchez and Smitz 2012). Moreover, EGF induced response is mediated by the activation of the ERK1/2 pathway, which in turn stimulates the production of prostaglandin E2 (PGE2) via the induction of PTGS2 (Sanchez and Smitz 2012). Cumulative action of these stimuli and enrichment of transcripts leads to mucification and expansion and genes responsible for this

such as HAS2, PTX3 and TNFAIP6 are found to be enhanced in cumulus cells (Varani et al. 2002; Fulop et al. 2003; Ochsner et al. 2003; Ashkenazi et al. 2005). Additionally, cumulus expansion enabling factors (CEEFs) such as transforming growth factor β (TGF- β) superfamily molecules (GDF9, activin A etc.) by signalling through SMAD 2/3 play an important role in cumulus expansion process (Dragovic et al. 2005; Dragovic et al. 2007). For instance, oocyte-derived GDF-9 probably acts on the surrounding cumulus granulosa cells to attenuate estradiol output and promote progesterone and hyaluronic acid production, mucification and cumulus expansion (Knight and Glister 2003).

1.4. MicroRNA as a posttranscriptional regulator of genes

Multiple types of small RNAs have evolved in eukaryotes like molecular switches to suppress unwanted genetic materials and transcripts (Ghildiyal and Zamore 2009; Ishizu et al. 2012; Ha and Kim 2014). Small RNAs, 20–30 nucleotides (nt), associated with Argonaute family proteins (AGO family proteins), are classified into three classes in animals: miRNA, siRNA and PIWI-interacting RNA (piRNA). miRNAs, small noncoding RNAs, ~20-22 nt in lengths, endogenous in nature, regulate gene expression post-transcriptionally by targeting the 3´UTR in sequence specific manner, leading to mRNA degradation or translation inhibition (Ambros 2004; Bartel 2004). Recent studies have shown that miRNAs play crucial role in almost all physiological and pathological function such as developmental processes, tumorigenesis and diseases (McManus 2003; Lu et al. 2008; Winter et al. 2009). The miRNAs, first discovered in *Caenorhabditis elegans,* found in most eukaryotes, are evolutionary conserved in nature (Lee et al. 1993; MacFarlane and Murphy 2010). Indeed, plenty of recent studies have shown a paradigm shift in the understanding of the miRNA biogenesis and their regulatory role in several biological processes (Winter et al. 2009). It is estimated that miRNAs account for nearly 1-5% of the human genome and regulate at least

30% of all protein-coding genes (Lim et al. 2003; Berezikov et al. 2005; Rajewsky 2006). To date, miRBase release 21 database contains 28645 entries representing hairpin precursor miRNAs, expressing 35828 mature miRNA products, in 223 species (http://www.mirbase.org/). Precursors and mature miRNAs of some species have been described in table 1.

Table 1. Number of known miRNAs in certain species

Species	**Mature miRNA**	**Precursor microRNA**
Human (*Homo sapiens*)	2588	1881
Bovine (*Bos taurus*)	793	808
Goat (*Capra hircus*)	436	267
Sheep (*Ovis aries*)	153	106
Pig (*Sus scrofa*)	411	382
Horse (*Equus cabalus*)	690	715
Canine (*Canis familiaris*)	453	502
Chicken (*Gallus gallusI)*	994	740
Nematode (*Caenorhabditis elegans*)	434	250
Common fruit fly (*Drosophila melanogaster*)	466	256

(Source: http://www.mirbase.org/; miRBase release 21)

1.4.1 Biogenesis and regulation of miRNAs

Briefly, miRNA biogenesis a multistep process (Figure 2) including miRNA transcription, its processing by Drosha in the nucleus and Dicer in the cytoplasm, Argonaute loading, and

eventually translational repression, mRNA decay or deadenylation (Ha and Kim 2014). MicroRNA genes encoding miRNAs are transcribed by RNA polymerase into long primary miRNAs (pri-miRNAs) that are processed by the RNaseIII enzyme Drosha and the DiGeorge Syndrome Critical Region 8 (DGCR8) protein (known as Pasha in invertebrates) to produce a characteristic stem loop structure of about 70 base pairs precursor miRNAs (pre-miRNAs) (Lee et al. 2003; Gregory et al. 2004). The pre-miRNAs are subsequently transported from nucleus to cytoplasm with the help of Exportin 5 protein (Knight and Bass 2001; Kim 2004; Zeng and Cullen 2004), where the pre-miRNA hairpin is cleaved by another RNase III enzyme, Dicer in complex with the double-stranded RNA-binding protein TRBP, to yield a mature miRNA as well as a star strand that is degraded (Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001; Winter et al. 2009). The miRNA is then loaded into an Argonaute protein within the RNA-induced silencing complex (RISC), the effector complex that mediates repression of targets. Further, miRNAs can direct the RISC to downregulate gene expression through mRNA cleavage, translational repression or deadenylation (Bartel 2004; Winter et al. 2009) (Figure 2).

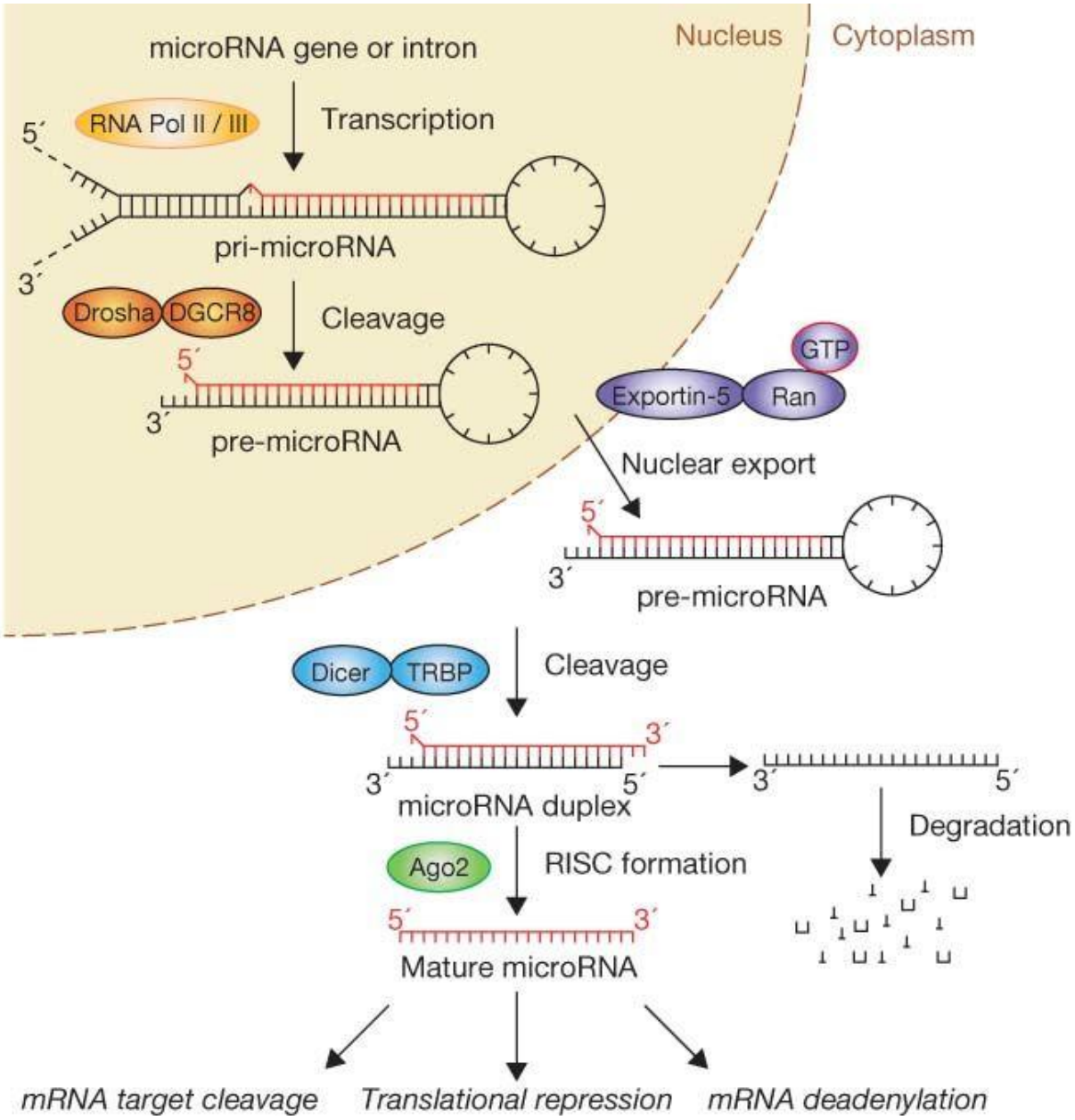

Figure 2. Multiple step miRNA biogenesis starts with the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational

repression or deadenylation, whereas the passenger strand (black) is degraded. Adapted from (Winter et al. 2009).

1.4.2 Role of miRNAs in female reproduction

In one of the notable early reproductive studies, Bernstein (Bernstein et al. 2003)found that Dicer I knockout in mice leads to lethality in early embryonic development, with Dicer1-null embryos depleted of stem cell, this suggested the role for Dicer, and by implication, miRNA machinery, in maintaining the stem cell population during early mouse development. Further, a separate study reported that Dicer1 deficiency resulted in female infertility. This defect in female Dicer deleted mice was caused by corpus luteum insufficiency and resulted from the impaired growth of new capillary vessels in the ovary, which was associated with a lack of miR17-5p and let7b (Otsuka et al. 2008). Recent evidences support the notion that miRNAs play important role in the biology of reproduction; especially, expression profiling, cloning and next generation sequencing have identified the spatiotemporal expression of miRNAs in ovaries of several species (Hossain et al. 2009; Tesfaye et al. 2009; Li et al. 2011; Ling et al. 2013; Salilew-Wondim et al. 2014; Sontakke et al. 2014; Gebremedhn et al. 2015).

1.4.2.1 miRNAs in mammalian folliculogenesis

The initiation of primordial follicle development is essential for female fertility. Recently miRNAs are found to be involved in the initiation of primordial follicle development, miR-145 by targeting TGFBR2 regulated the initiation of primordial follicle development and maintains primordial follicle quiescence (Yang et al. 2013). Down-regulation of miR-145 using an antagomir (AT) decreased the proportion and number of the primordial follicles and increased number of the growing follicles by increasing TGFBR2 expression and activation of SMAD signalling (Yang et al. 2013). In a separate study miR-376a was shown to be

negatively correlated with PCNA mRNA expression in fetal and neonatal mouse ovaries and directly bind to the 3'UTR of PCNA. Further, miR-376a overexpression is significantly increased in primordial follicles and reduced apoptosis of oocytes, which was very similar to those in ovaries co-transfected with miR-376a and siRNAs targeting PCNA which suggested that miR-376a regulates primordial follicle assembly by modulating the expression of PCNA, (Zhang et al. 2014b). Similarly, miR-143 expression increased during primordial follicle formation from 15.5 days post-coitus to 4 days post-partum. MiRNA-143 inhibited the formation of primordial follicles by suppressing pregranulosa cell proliferation and downregulating the expression of genes related to the cell cycle (Zhang et al. 2013). These studies supported the notion that miRNAs could be critical for the formation of primordial follicles and regulating the ovarian development and function.

Recent studies conducted in bovine granulosa cells of subordinate and dominant follicle at day 3, 7 and 19 of the bovine estrous cycle (Salilew-Wondim et al. 2014; Gebremedhn et al. 2015), suggested that the differential expression patterns of miRNAs in granulosa cells could be associated with follicular recruitment, selection and dominance during the early luteal phase of the bovine estrous cycle (Salilew-Wondim et al. 2014). Further, differential enrichment of certain miRNAs in granulosa cells of preovulatory dominant and subordinate follicles supports the potential role of miRNAs in post-transcriptional regulation of genes involved in bovine follicular development (Gebremedhn et al. 2015). These studies provide clues that the dominant follicle endowed with ability to escape atresia might be enriched with certain set of miRNAs, which helps to establish and maintain dominant follicles, and could be future potential molecule to further understand underlying elusive mechanisms during the process of recruitment, selection and dominance in follicular development.

Proliferation and differentiation of granulosa are critical processes for follicular growth and development to produce developmentally competent oocyte by providing essential inputs in

follicular development event by producing steroid hormones, growth factors, cytokines, paracrine and autocrine (Toda et al. 2012). These processes are governed by several molecular cues regulated epigenetically; and miRNAs are one of the different mechanisms. Growing body of evidence supports involvement of miRNAs in follicular growth and development through regulating granulosa cell proliferation, differentiation, apoptosis and steroidogenesis (Sirotkin et al. 2009; Carletti et al. 2010; Sirotkin et al. 2010; Sirotkin et al. 2014; Gebremedhn et al. 2015; Jiang et al. 2015; Wu et al. 2015). In a recent study, miR-17-92 cluster, enriched in bovine granulosa cells derived from subordinate follicle, revealed the involvement of miR-17-92 cluster in bovine granulosa cells proliferation, differentiation and steroidogenesis by targeting PTEN and BMPR2 genes (Andreas et al. 2016). In the same study, overexpression of the miR-17-92 cluster promoted cell proliferation, while reduced differentiation of cells. MicroRNA 383 promoted the expression of miR-320 and enhanced miR-320-mediated suppression of GC proliferation. In polycystic ovarian syndrome (PCOS), miR-93 overexpression promoted cell proliferation and G1 to S transition through targeting CDKN1A (Jiang et al. 2015). Similarly, a recent study showed that miR-22 regulated the expression of SIRT1 in healthy follicles (HF), early atretic follicles (EAF), and progressively atretic follicles (PAF) (Xiong et al. 2016). To investigate the roles of SIRT1 in mouse granulosa cells (mGCs) apoptosis, the endogenous SIRT1 gene in mGCs was knocked down using siRNA specific for SIRT1. MiRNA-22 was increased during follicular atresia and suppressed granulosa cell apoptosis. In addition, knockdown of SIRT1 attenuated apoptosis in mGCs (Xiong et al. 2016). During ovarian follicular development, the majority of follicles undergo atresia at various stages of their development. However, the mechanisms involved in controlling atresia remain unknown. Several miRNA regulate apoptosis in cultured primary granulosa cells of mammalian species (Carletti et al. 2010; Yang et al. 2012; Liu et al. 2014a; Xiong et al. 2016). In one of early miRNA functional study showed, miR-21 blocks apoptosis

in mouse periovulatory granulosa cells. Inhibition of miR-21 induced apoptosis in cultured granulosa cell and decreased ovulation rate, while over expression of miR-21 in in vivo follicular granulosa cells exhibit a decrease in cleaved caspase 3, a hallmark of apoptosis (Carletti et al. 2010). MiRNA-181a was able to inhibit granulosa cell proliferation by targeting Act2A and decreasing PCNA expression (Sirotkin et al. 2010; Zhang et al. 2013) (table 1). Let-7 family (Let-7b/ c/d/g) miRNA decrease proteins related with proliferation and apoptosis (Sirotkin et al. 2010), which was differentially expressed during follicular atresia. Similarly, miRNAs let-7a, let-7b, let-7c, and let-7i were significantly decreased in early atretic and progressively atretic porcine ovary follicles compared with healthy follicles, while let-7g was highly expressed during follicle atresia (Cao et al. 2015). This study suggests involvement of the Let-7 miRNA during cell proliferation, survival, and apoptosis. Granulosa cells of human origin transfected with pre-mir-23a showed decrease in XIAP expression (mRNA and protein level) and caspase-3 protein levels and increase in cleaved caspase-3 protein, along with an increased occurrence of apoptosis (Yang et al. 2012). This study suggests that miR-23a may play important roles in regulating apoptosis via decreasing XIAP expression in human ovarian granulosa cells (Yang et al. 2012). MiR-26b functions as a proapoptotic factor (Lin et al. 2012; Liu et al. 2014a) which was upregulated during follicular atresia, increased the number of DNA breaks and promoted granulosa cell apoptosis by targeting the ataxia telangiectasia mutated gene (ATM) (Lin et al. 2012). Overexpression of miR-26b in follicular bGCs suppressed SMAD4 mRNA and protein levels, resulting in down-regulation of the antiapoptotic BCL-2 gene and the promotion of bGCs apoptosis. In a separate study, it has been observed transforming growth factor beta 1 (TGF-β1) could down-regulates miR-26b expression in porcine granulosa cells (GCs) (Liu et al. 2014a). Similarly, miR-34a promotes granulosa cell apoptosis in pig ovarian follicles by targeting inhibin beta B gene (INHBB) (Tu et al. 2014) while miR-92a inhibits porcine ovarian granulosa cell

apoptosis by targeting SMAD7 gene (Liu et al. 2014b). An independent study demonstrates that miR-513a-3p is involved in the control of the LHCGR expression by an inversely regulated mechanism at the post-transcriptional level. MiR-513a-3p acts as a co-regulator of luteinizing hormone/chorionic gonadotropin receptor gene expression in human granulosa cells (Troppmann et al. 2014). Indeed, several recent studies have indicated miRNAs are functionally involved during follicular growth and development through somatic cell proliferation, differentiation, apoptosis, and also through altering the hormone level produced by somatic cells (Carletti et al. 2010; Sirotkin et al. 2010; Troppmann et al. 2014; Yin et al. 2014; Gebremedhn et al. 2015; Jiang et al. 2015; Pan et al. 2015; Andreas et al. 2016; Gebremedhn et al. 2016b; Xiong et al. 2016) (Table 2).

1.4.2.2 miRNAs in steroidogenesis

Recent studies investigated the indispensable role of miRNAs during steroidogenesis (Xu et al. 2011a; Schauer et al. 2013; Toms et al. 2015; Donadeu et al. 2016), which plays an important role in ovarian follicular development and many reproductive disorders. A recent study showed that overexpression of miR-17-92 cluster increased the progesterone level in the spent media of obtained from bovine granulosa cell culture (Andreas et al. 2016). A separate study showed that overexpression of miR-378 decreased ovarian estradiol production by post-transcriptionally regulating aromatase. Conversely, overexpression of the aromatase 3'-UTR enhanced aromatase expression at the protein level indicated binding of

Table 2. Functionally analyzed miRNAs involved in granulosa cell function and oocyte maturation

Candidate miRNAs	Potential role	Pathways	Target genes	Species	References
miR-181a[1], miR-383[2], miR-145[3], miR-224[4], miR-93[5], miR-183/96/182 cluster[6], miR-17-92 cluster[7], MiR-22	Enhances cellular proliferation	TGF-β[1,2,3] SMAD[4]	ACVR2A[1], RBMS1[2], TGF-β1[2], SMAD4[2], ACVRIB[3], CDKN1A[5] FOXO1[6], PTEN and BMPR2[7], SIRT1[8]	Mouse[1,2,3,4,8] Human[5] Bovine[6,7]	(Jiang et al. 2015; Xiong et al. 2016; Yang et al. 2013; Yao et al. 2010; Yin et al. 2012; Zhang et al. 2013; Gebremedhn et al. 2016b; Andreas et al. 2016)

miR-92a[1], miR26b[2] miR-34a[3], miR-143[4], miR-21[5], miR-23a[6], miR-23a[7], miR-27a[7], miR-181a[8]	Induces cellular apoptosis	TGF-β[1,2, 3,7]	SMAD7[1] ATM[2] SMAD4[2] INHBB[3] SMAD5[7]	Porcine[1,2,3] Mouse[4,5,8] Human[6,7]	(Carletti et al. 2010; Jiang et al. 2015; Lin et al. 2012; Liu et al. 2014a; Tu et al. 2014; Yang et al. 2012; Zhang et al. 2013; Nie et al. 2015)
miR-15a[1], miR-383[2], miR-224[3], miR-133b[4], miR-320[5], miR-132b[6], miR-378-3p[7], miR-873[8], miR-20[8]	Steroidogenesis, promoting estradiol release,[1,2,3,4,5,6,7, 8]	TGF-β[2,3]	RBMS1[2] TGF-β1[2] SMAD4[2] Foxl2[4] E2F1and SF-1[5] Nurr1[6], PGR[7]	Mouse[2,6]	(Dai et al. 2013; Donadeu et al. 2016; Sirotkin et al. 2014; Pan et al. 2015; Toms et al. 2015; Wu et al. 2015; Yao et al. 2010; Yin et al. 2012; Yin et al. 2014)

miR-378[1], Pri-miR-155, pri-miR-222 and pri-Let-7d[2], miR-133b[3]	Cumulus-oocyte interaction and oocyte maturation	NA[*]	HAS2[1], PTGS2[1], CX43[1], and DAMTS1[1] TAGLN2[2]	Porcine[1] Bovine[2] Mouse[3]	(Gilchrist et al. 2016; Pan et al. 2015; Xiao et al. 2014)
miR-126-3p	Development of corpus luteum	NA[*]	TLN2	Bovine	(Dai et al. 2014)

Superscript number relates corresponding particulars

NA[*]: not available

miR-378 within this region and subsequently reducing the binding of this micro-RNA to the endogenous aromatase 3'-UTR (Xu et al. 2011a). Similarly, overexpression of miR-378-3p decreased protein levels and mRNA levels of PGR (progesterone receptor) by regulating its 3'UTR (Toms et al. 2015). Some of miRNAs have been identified like miR-34a and miR-320, whose overexpression could inhibit estradiol release from human granulosa cells and murine ovaries, respectively (Sirotkin et al. 2009; Yin et al. 2012; Yin et al. 2014). These miRNAs could be miRNA of interest to further study the disorders of granulosa cells in relation to steroidogenic capacity. Interestingly, it has been also been noticed that some of the miRNAs namely miR-383 could enhance estradiol production in mouse granulosa cells (table1). Similarly, overexpression of miR-132 regulated Nurr1, that suppresses CYP19A1 expression and therefore enhancement in estradiol synthesis was observed via the translational repression of Nurr1 in ovarian GCs (Wu et al. 2015). Likewise, miR-133b was involved in follicle-stimulating hormone (FSH)-induced estrogen production by inhibiting the Foxl2-mediated transcriptional repression of STAR and CYP19A1 to promote estradiol production (Dai et al. 2013). In a recent study it was suggested that miRNAs can be useful biomarkers of tissue function (Donadeu et al. 2016). In this study, estradiol levels, the estradiol: progesterone (E:P) ratio and CYP19A1 expression were strongly correlated with each other, and identified miR-873 and miR-202 as miRNAs whose levels in follicular tissues can be used as indicators of steroidogenic capacity in bovine (Donadeu et al. 2016). A recent study sheds light regarding the uncontrolled follicular growth observed in polycystic ovary producing excess androgens resulting attenuation of follicular atresia through nuclear and extranuclear signalling pathways by enhancing expression of the miR-125b, which in turn suppresses pro-apoptotic protein expression (Sen et al. 2014). Considering steroidogenesis as one of central figure in estrous cycle, which regulates follicular growth, development and ovulation and additionally involves in many reproductive disorders, therefore whether these

processes are directly or indirectly under control of miRNAs could be much interesting to study more in the future.

1.4.2.3 miRNAs in regulation of cumulus-oocyte communication and oocyte maturation

A growing body of evidences support that apart from the involvement of gene action, oocyte growth and development is also regulated by miRNAs; for instance, higher expression and functional importance of Dicer1 has been identified in the oocyte during folliculogenesis and in mature oocytes (Su and Eppig 2002; Choi et al. 2007; Murchison et al. 2007; Lei et al. 2010). Oocyte competence is acquired during the process of meiosis and in the last stage of maturation. Thus, Dicer and miRNA appeared to play an important role during the process of oogenesis and completion of meiotic division. In striking contrast to Dicer, DGCR88-deficient mouse oocytes matured normally and, when fertilized with wild-type sperm, produced healthy-appearing offspring, even though miRNA levels were reduced to similar levels as Dicer-deficient oocytes; this suggests that miRNA function is globally suppressed during oocyte maturation and preimplantation development (Suh et al. 2010). Moreover, investigation on the expression of miRNAs in immature and in vitro matured bovine oocytes (Tesfaye et al. 2009) and in human oocytes (Xu et al. 2011b) revealed a differential expression of miRNAs (Tesfaye et al. 2009). The expression of seven miRNAs (miR-496, miR-297, miR-292-3P, miR- 99a, miR-410, miR-145 and miR-515-5p) in matured and two miRNAs (miR-512-5p and miR-214) in immature oocyte showed higher abundance by at least 2-fold difference (Tesfaye et al. 2009). Dynamic changes in expression of miRNAs are observed between the oocytes matured with cumulus cells and oocyte matured without cumulus cells. Remarkably, the changes of miRNAs expression were observed in the cumulus cells matured with or without the oocyte cytoplasm (Abd El Naby, W S et al. 2013). Several studies have shown the role of miRNAs in the oocyte and thereby suggested that a

large amount of the maternal genes are one or the other way under the control of miRNAs (Murchison et al. 2007; Tang et al. 2007). In order to further explore the roles of miRNAs in oocyte maturation, identification and characterization of miRNA populations were done in pools of bovine germinal vesicle (GV) oocytes, metaphase II (MII) oocytes, and presumptive zygotes (PZ) (Gilchrist et al. 2016); where, bta-miR-155, bta-miR-222, bta-miR-21, bta-let-7d, bta-let-7i, and bta-miR-190a were found significantly differentially expressed miRNAs. Further, pri-miR-155 was present in MII oocytes, indicating transcription during maturation; however, levels of pri-let-7d decreased during maturation. This study demonstrates that both dynamic and stable populations of miRNAs were present in bovine oocytes and zygotes and extend previous studies supporting the importance of the small RNA landscape in the maturing bovine oocyte and early embryo (Gilchrist et al. 2016). In the final stages of oocyte maturation, minimal transcriptional activity was observed, and regulation of gene expression occurred primarily at the post-transcriptional level (Tscherner et al. 2014). In a separate study, all members of the miR-34 family were found in bovine spermatozoa, while only miR-34a and -34c could be noticed in oocytes and cleaved (2-cell) embryos. Further, miRNA-34c was consistently expressed throughout oocyte maturation and in the embryo. This study suggested that miR-34 miRNAs may be required in developing bovine gametes of both sexes, as well as in embryos, and that primary miR-34b/c processing takes place before the completion of gametogenesis. Individual variation in sperm miR-34 family abundance may offer potential as a biomarker of male bovine fertility (Tscherner et al. 2014). In a recent study it is shown that miR-21 is differentially expressed in the oocyte during meiotic maturation in the pig and inhibition of miR-21 during this process alters PDCD4 protein abundance suggesting posttranscriptional regulatory events involving miR-21 during oocyte maturation may impact subsequent embryonic development in the pig (Wright et al. 2016). In a separate study, the suitability of miRNAs as potential expression normalizers in bovine

oocytes and early embryos, and porcine oocytes was analyzed. The stages examined were bovine oocytes at the germinal vesicle (GV) and metaphase II stages, bovine zygotes, 2, 4 and 8 cell embryos, morulae and blastocysts, as well as porcine cumulus oocyte complexes, GV, metaphase I and II oocytes. MiR-93 and miR-103 were identified as the most stably expressed in bovine samples and miR-26a, miR-191 and miR-93 in porcine samples. The combination of miR-93 and miR-103 is optimal for normalizing miRNA expression for qPCR experiments on bovine oocytes and preimplantation embryos; however, the preferred combination for porcine oocytes is miR-26a, miR-191 and miR-93 (Mahdipour et al. 2015).

1.5 Research gap and hypothesis

Since discovery of miRNAs a very little is understood regarding the functional involvement of miRNAs in female reproduction. As described previously, several individual or cluster of miRNAs have been identified to be involved in follicular development. Similarly, our previous study reported that several clusters of miRNAs have been highly enriched in granulosa cells of preovulatory dominant follicle (Gebremedhn et al. 2015). However, only two cluster of miRNAs have been functionally analyzed in bovine granulosa cells function (Gebremedhn et al. 2016b; Andreas et al. 2016). Further, among several cluster of miRNAs, the miR-424/503 cluster was highly abundant in granulosa cells of preovulatory dominant follicle and has not been studied yet. Taking all these information into account, we hypothesized that microRNA-424/503 cluster members might be playing an important role in bovine granulosa cell function and cumulus cells expansion to support follicular development and oocyte growth.

1.6 Objectives

We tested our research hypothesis with the following two main objectives:

1. To investigate the role of microRNA-424/503 cluster in bovine granulosa cell function.
2. To investigate the effect of microRNA-424/503 cluster modulation in bovine cumulus cell expansion and oocyte maturation in vitro.

Chapter 2

Title: MicroRNA-424/503 Cluster Regulates Bovine Granulosa Cell Function by Targeting SMAD7 Gene through Activin Signalling Pathway

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Abstract

Background

In our previous study, the microRNA-424/503 cluster was found to be highly abundant in bovine granulosa cells (bGCs) of preovulatory dominant follicle compared to subordinate counterpart at day 19 of the bovine estrous cycle. Therefore, this study was aimed to investigate the role of microRNA-424/503 cluster in bGCs function using microRNA gain- and loss-of-function approaches.

Methods

The bovine granulosa cells were collected from ovaries obtained from slaughterhouse. The SMAD7 and ACVR2A genes targeted by miR-424/503 cluster were validated using dual-luciferase reporter assay. The functional role of miR-424/503 cluster members in granulosa cell function was investigated by gain- and loss-of function by overexpressing and inhibiting in vitro cultured granulosa cells using miR-424/503 mimic or inhibitor, respectively.

Results

Luciferase reporter assay showed that SMAD7 and ACVR2A are the direct targets of the microRNA-424/503 cluster members. In line with this, overexpression of microRNA-424/503 cluster members using its mimic and inhibition of its activity by its inhibitor reduced and increased, respectively the expression of SMAD7 and ACVR2A genes. Furthermore, flow cytometric analysis indicated that overexpression of microRNA-424/503 cluster members enhanced bGCs proliferation by promoting G1 to S phase cell cycle transition. Modulation of microRNA-424/503 cluster members also enhanced phosphorylation of SMAD2/3 in the Activin signalling pathway. Moreover, sequence specific knockdown of SMAD7, the target gene of microRNA-424/503 cluster members, using small interfering

RNA also revealed similar phenotypic and molecular alterations observed when microRNA-424/503 cluster was overexpressed. Similarly, to get more insight about the role of microRNA-424/503 cluster in Activin signalling pathway, granulosa cells were treated with activin A. Granulosa cells treated with activin A exhibited increased cell proliferation and downregulation of both microRNA-424/503 members and its target gene, indicated the presence of negative feedback loop between activin A and the expression of microRNA-424/503.

Conclusions

This study suggests that the microRNA-424/503 cluster is involved in regulating bovine granulosa cell proliferation and cell cycle progression by targeting SMAD7 via activin signalling pathway.

Background

The mammalian follicle, consisting of an oocyte surrounded by granulosa and theca cells, represents the basic functional unit of the ovary (Orisaka et al. 2009). The growth of the obligatory gonadotropin-dependent follicle, which is a complex but nevertheless well-coordinated process, occurs in a wave-like fashion, with two to three waves per estrous cycle (Driancourt 2001) followed by ovulation or atresia. Among the follicular cells, granulosa cells are critically indispensable for the growth and maturation of follicles, and they undergo a series of morphological and functional changes (Robker and Richards 1998). The primordial follicle, holding an arrested oocyte at diplotene stage, is enclosed by a single flattened layer of somatic GCs (Buccione et al. 1990). Further, the sequential, well-controlled transformation from the primordial to antral follicle stage is the result of the differentiation and proliferation of GCs that provide essential and vital inputs in the form of steroid hormones, cytokines, and paracrine and autocrine factors during the process of follicular development (Richards 1994; Huang and Wells 2010; Toda et al. 2012), which is tightly regulated by array of genes (Richards 1994; Robker and Richards 1998; Toda et al. 2012; Nivet et al. 2013; Douville and Sirard 2014).

To better understand the genetic regulation of granulosa cell function and their role in follicular development, several transcriptome profiling studies have been conducted to examine the expression patterns of genes in bGCs (Nivet et al. 2013) at different phases of antral follicle growth (Douville and Sirard 2014). Accordingly, several genes involved in steroidogenesis (CYP17A1, CYP11A1, HSD3B1, STAR), cell proliferation/the cell cycle (CCND2, PCNA), gonadotropin receptors (LHCGR, FSHR) and growth factors (GDF9, BMP2, Activins, IGF-I, IGF-II) have been found to be altered in granulosa cells depending on the size and stage of follicular development (Robker and Richards 1998; Evans, A C O et al. 2004; Myers et al. 2008; Andreas et al. 2016; Gebremedhn et al. 2016b). These molecular

cues, which are responsible for follicular development, are under the control of several epigenetic mechanisms, including microRNAs. These small noncoding RNAs, ~20-22 nucleotides in length, are epigenetic regulators that control gene expression post-transcriptionally by targeting the 3´-UTR in a sequence-specific manner leading to mRNA degradation or translation inhibition (Ambros 2004; Bartel 2004).

miRNAs play crucial roles in almost all biological functions, including cell proliferation, differentiation, and apoptosis (Andreas et al. 2016; Gebremedhn et al. 2016b), and are associated with various diseases such as cancer (McManus 2003). Since the discovery of first miRNA (Lin-4) in 1993 (Lee et al. 1993; Bartel 2004), a continuing major challenge has been deciphering the functional aspects of miRNAs in bio-physiology, including mammalian reproduction. Recent evidence supports the involvement of miRNAs in follicular growth and development through the regulation of granulosa cell proliferation, differentiation, apoptosis and steroidogenesis (Sirotkin et al. 2009; Carletti et al. 2010; Sirotkin et al. 2010; Sirotkin et al. 2014; Gebremedhn et al. 2015; Jiang et al. 2015; Wu et al. 2015). However, limited number of studies have validated the role of miRNAs in bovine follicular development (Gebremedhn et al. 2015; Andreas et al. 2016; Gebremedhn et al. 2016b). Recently, we have reported the expression pattern of miRNAs in bovine granulosa cells of subordinate and dominant follicles during the early luteal phase (day 3 and day 7) (Salilew-Wondim et al. 2014) and late follicular phase (day 19) (Gebremedhn et al. 2015) of the bovine estrous cycle and their possible association with follicular recruitment, selection and dominance. In the latter study, of the 64 total differentially expressed miRNAs, the miR-424/503 cluster was significantly enriched in the granulosa cells of preovulatory dominant follicles, which indicated the potential involvement of the miR-424/503 cluster in the follicular development and maturation process. However, the underlying regulatory mechanisms of the miR-424/503 cluster in the function of bGCs have not yet been examined. Recent studies have

demonstrated that the miR-424/503 cluster enhances tumour cell resistance to apoptosis (Zhang et al. 2014a), coordinates the remodelling of the epithelium in the involution of mammary gland (Llobet-Navas et al. 2014), and reverses chemo-resistance via T-cell immune response activation by blocking the PD-L1 immune checkpoint (Xu et al. 2016). Nonetheless, the involvement of the miR-424/503 cluster in reproductive functions remains elusive. An in-silico analysis of miR-424/503 allowed for the identification of the SMAD7 and ACVR2A genes, which are associated with the activin signalling pathway of the TGF-β superfamily members and a known key regulator of follicle development in mammals (Knight and Glister 2006), as potential putative target genes. SMAD7, an inhibitory SMAD, blocks the phosphorylation of SMAD2/3, acts as a negative feedback regulator of the activin signalling pathway (Tsuchida et al. 2009; Gao et al. 2013; Li 2015).The activin signalling pathway, in which an activin dimer binds to activin receptor type II A (ACVR2A) or activin receptor type II B (ACVR2B), is known to result in the phosphorylation of activin type I receptors. The type I receptor in turn phosphorylates SMAD2/3, and activated SMAD2/3 forms a heterodimeric complex with SMAD4 which translocates to the nucleus where it activates transcription. Granulosa cells are the main ovarian source of activins, which have been implicated in various intra-ovarian roles, including germ cell survival and primordial follicle assembly, follicle growth from preantral to mid-antral stages, promotion of granulosa cell proliferation, enhancement of oocyte developmental competence, retardation of follicle luteinization and/or atresia and luteolysis (Knight 1996; Knight and Glister 2001; Knight and Glister 2003; Knight and Glister 2006; Knight et al. 2012). Increasing evidence has shown that several miRNAs, for instance miR-224, let-7g, miR-181a and miR-15a, are involved in regulating granulosa cell function through targeting TGF-β superfamily members (Yao et al. 2010; Zhang et al. 2013; Zhou et al. 2015). In the present study, we demonstrated that miR-

424/503 cluster epigenetically regulates bovine granulosa cell function by targeting SMAD7, and through fine tuning of the activin signalling pathway.

Methods

MicroRNA target gene prediction

The mature sequences of miR-424 and miR-503 were obtained from the miRBase (http://www.mirbase.org/) database. We performed an in silico target prediction for potential putative targets using the miRWalk database (http://www.umm.uniheidelberg.de/apps/zmf/mirwalk/). The miRNA-mRNA binding site prediction in bovine sequences was performed using TargetScan 6.2 (Agarwal et al. 2015). Target gene predictions were considered according to rank based on the predicted efficacy or targeting as calculated using cumulative weighted context++ scores of the sites (Agarwal et al. 2015) and probability of conserved targeting (P_{CT}) (Friedman et al. 2009). Accordingly, among several genes, the SMAD7 (Li 2015) and ACVR2A genes, which are ubiquitously expressed in the ovarian follicle and important in reproductive performance (Matzuk et al. 1995), were selected for functional analysis. The secondary structure of miR-424 and miR-503 was predicted by RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid).

Bovine granulosa cell culture and transfection

Bovine ovaries as sources of bGCs were collected from a local slaughterhouse. Ovaries were transported to the laboratory in warm physiological saline solution (0.9% NaCl). Immediately upon arrival, ovaries were washed three times using a lukewarm (37 °C) saline solution. Then ovaries were rinsed in 70% warm ethanol for 30 seconds, followed by washing three times with saline solution. The follicular fluid containing bGCs were aspirated from healthy small follicle (3-5 mm of diameter) using 20-gauge needle and transferred into 15 ml Falcon tube

(Thermo Fisher Scientific, Germany) containing warm phosphate buffer saline without Ca2+/Mg2+ (CMF-PBS). The cumulus-oocyte complexes were allowed to settle down for 15 minutes. Upper suspension containing floating granulosa cells was transferred into new 15 ml tube and centrifuged at 750x g for 7 minutes. The supernatant was discarded and granulosa cell pellet was re-suspended in one ml red blood cell (RBC) lysis buffer for one minute. Then we added 5 ml DMEM/F-12 Ham (Gibco® Sigma Aldrich Chemie GmbH, Germany) culture medium supplemented with 10% fetal bovine serum (FBS), 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Gibco® Sigma Aldrich Chemie GmbH, Germany), and 100 µg/ml fungizone (Gibco® Sigma Aldrich Chemie GmbH, Germany) to stop the effect of RBC lysis buffer and centrifuged at 500 x g for 5 minutes. The pellet was then washed one or two times using CMF-PBS to remove remaining lysis buffer. The granulosa cell pellet was re-suspended with F12+ culture media (DMEM/F-12 Ham, 10% FBS + 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 100 µg/ml fungizone). Cell viability and concentration were determined by using trypan blue (Gibco® Sigma Aldrich Chemie GmbH, Germany) exclusion method. Finally, a total of 2.0-2.5 x 10^5 bGCs per well were seeded into CytoOne® 24-well plate (Starlab International GmbH, Germany) in the F12+ culture media. The bGCs were cultured in 37 °C with 5% CO_2 in humidified environment. The bGCs were incubated for 48 h to attach and pre-confluent (60-70%) for treatment or transfection purpose. In the culture medium FSH, IGF1 or other factors were not added to avoid its effect on bovine granulosa cell proliferation. In some experiments cells were cultured in the presence of Recombinant Human/Mouse/Rat Activin A (R&D Systems, Minneapolis, MN, USA)

The chemically synthesized miRNA-424-5p mimic and inhibitor, miR-503-5p mimic and inhibitor, and the corresponding negative controls (NC) were used to transfect (Qiagen GmbH, Germany) bGCs. The miRNAs and/or plasmids were diluted in Opti-MEM I reduced-serum medium (Invitrogen). Sub-confluent cultured bGCs (70-80% confluent) were co-

transfected with 500 ng of the wild-type or mutant-construct plasmid and 50 nM individual microRNA mimic or mimic control. For miR-424/503 gain- and loss-of-function analysis, 50 nM individual microRNA mimic, inhibitor or corresponding negative control were co-transfected to sub-confluent cultured bGCs. The transfection was performed using Lipofectamine 2000 transfection reagent (Life Technologies, Germany).

Plasmid construction and luciferase assay

To validate whether the SMAD7 and ACVR2A gene are real targets of the miR-424/503 cluster, fragments of the 3´-UTR of SMAD7 or 3´-UTR of ACVR2A containing the binding sites for miR-424-5p (miR-424) and miR-503-5p (miR-503) (wild type) or with mutations in the seed sequences of miR-424/503 (mutant type) (Fig. 1) were cloned and inserted between the SacI and XhoI restriction sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation, USA). The cDNA from ovarian bGCs was used to amplify the predicted miRNA-mRNA binding site in the 3´-UTR region of the SMAD7 or ACVR2A mRNA. Specific primers and 50-mer mutated oligonucleotides were designed based on bovine SMAD7 (XM_005224232.3) or ACVR2A (NM_174227) mRNA sequences in GenBank (Table S1). The luciferase activity was measured 48 h after transfection using the pmirGLO Dual Luciferase® Reporter Assay System (Promega Corporation, USA) according to the manufacturer's protocol. Firefly and Renilla luciferase activity was detected by measuring the absorbance on a Centro LB 960 Microplate Luminometer (Berthold Technologies GmbH, Germany).

Total RNA isolation and cDNA synthesis

To confirm the expression of target and marker genes in treated bGCs at each stage of the experiment, harvested bGCs were resuspended in lysis buffer, and the subsequent RNA

isolation was performed using an miRNeasy® mini kit (Qiagen GmbH, Germany) following the manufacturer's protocol. Total RNA quantity and purity (260/280 ratios) was measured with a NanoDrop 8000 spectrophotometer (NanoDrop products, USA). After validating the quality and concentration of the RNA samples, the cDNA synthesis was performed using a RevertAid first stand cDNA synthesis kit (Thermo Fisher Scientific, Germany). Briefly, each RNA sample (1 µg) was co-incubated with 1 µl of Oligo $(dT)_{18}$ primer and dH_2O to a total of 11 µl at 65 °C for 5 min and then chilled on ice for 5 min. The reverse transcription was performed in a total mixture volume of 20 µl consisting of 4 µl of 5x Reaction Buffer, 1 µl of RevertAid Reverse Transcriptase, 2 µl of dNTP mix and 1 µl of RiboLock RNase Inhibitor. The reactions were carried out in a thermocycler programmed at 37 °C for 60 min followed by 70 °C for 5 min.

Quantitative PCR analysis

Primers for specific genes were designed using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The details of the primers are described in Table S 2. The specificity of each primer amplicon was confirmed by sequencing the PCR products using a GenomeLab GeXP Genetic Analysis System (Beckman Coulter GmbH, Germany). The quantitative PCR (qPCR) analysis of mRNA was performed in an Applied Biosystem® StepOnePlus™ System (Thermo Fisher Scientific Inc, USA), using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Germany), with the following program: 95 °C for 3 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 1 min. The melting curve was evaluated at the end of the run to observe the specificity of the amplification. The mRNA expression data were analysed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method(Livak and Schmittgen 2001), and β-ACTIN was used as an internal control.

Candidate miRNAs were quantified as described previously(Salilew-Wondim et al. 2014; Gebremedhn et al. 2015). Briefly, cDNA was synthesized using 80 ng of miRNA-enriched total RNA using a miRCURY LNA^{TM} Universal cDNA synthesis kit (Exiqon, Denmark) according to the manufacturer's instructions. The synthesized cDNA was diluted 40x and used for the qPCR analysis of candidate miRNAs using ExiLENT SYBR Green Master mix (Exiqon, Denmark). The thermal cycling program was as follows: initial preheating at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 10 s and 60 °C for 1 min. The specificity of the miRNA amplification was evaluated by melting curve analysis. The 5s ribosomal RNA (5s rRNA) (miRCURY LNA™ Universal RT microRNA PCR) was used as the reference gene primer. The qPCR data were analysed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method(Livak and Schmittgen 2001).

Western blot analysis

Total protein from cultured bGCs was isolated using 1x PLB (Promega Corporation, USA). The total protein concentration was determined using the Bradford method(Bradford 1976). Western blotting was performed as described previously(Andreas et al. 2016; Gebremedhn et al. 2016b). The antibodies used were (Santa Cruz Biotechnology Inc., Germany): anti-ACTR-II2A goat polyclonal antibody (product no. sc-5667), anti-SMAD7 rabbit polyclonal antibody (product no. sc-11392), anti-PCNA rabbit polyclonal antibody (product no. sc-7907), anti-STAR rabbit polyclonal antibody (product no. sc-25806), anti-SMAD2/3 rabbit poly clonal antibody (product no.sct-5678), anti-psmad2/3 rabbit monoclonal antibody (sct-8828) or anti-β-ACTIN mouse monoclonal antibody (product no. sc-47778). At the end of the incubation period, the membrane was washed six times with 1x TBST and incubated with the corresponding donkey anti-goat, goat anti-rabbit, or goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The detection of the

protein signal was then performed using Clarity Western ECL Substrate (Bio-Rad Laboratories). The relative band intensity was determined by ImageJ program (https://imagej.nih.gov/ij/*)*.

Cell proliferation assay

A total of 2 x 10^4 bGCs per well were seeded into a 96-well plate and cultured in F12+ culture medium (DMEM/F-12 Ham, 10% FBS + 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 100 µg/ml fungizone). Individual miR-424/503 mimics, inhibitors or corresponding controls were transfected into sub-confluent cultured bGCs (70-80% confluent). After 48 h of incubation, 10 µl of CCK-8 kit solution (Dojindo EU GmbH, Germany) was added to each well, and the plate was incubated for another 2 h. The optical density (OD) at a wavelength of 450 nm was measured using a Synergy™ H1 Multi-Mode Reader (BioTek Instruments Inc, Germany).

Cell cycle assay

Cultured granulosa cells were transfected with 75 nM miR-424/503 cluster miRNA mimics, inhibitors, or the corresponding negative control (NC) and SMAD7 siRNA, ACVR2A siRNA or NC siRNA. The cells were trypsinized 48 h later and collected in a 15-ml Falcon tube (Thermo Fisher Scientific, Germany), followed by centrifugation at 750 ×*g* for 5 min and washing twice with 1 x CMF-PBS. A minimum of ~1 x 10^6 cells were fixed in ice-cold 70% ethanol at 4 °C overnight. The cells were then centrifuged briefly, and the cell pellets were washed twice with 500 µl of 1x CMF-PBS. The cells were then labelled with 50 µg/ml propidium iodide (PI) and treated with 50 µg/ml RNase. The cells were then incubated at 37 °C for 30 min and processed in a BD LSRFortessa™ flow cytometer (BD Biosciences). The

cell cycle distribution was analysed using ModFit LT software (http://www.vsh.com/products/mflt/index.asp).

Targeted suppression of the SMAD7 and ACVR2A gene using small interfering RNA (siRNA)

Bovine specific LNA™ longRNA GapmeRs (Exiqon, USA) were used to inhibit the expression of SMAD7 (5´-TTCGCAGAGTCGGCTA-3´ and 5´-CGATTTTGCTCCGTA-3´) ACVR2A (5´-GTTACTGGATTCGACG-3´ and 5´-GTTGGTCAGTAATCTA-3´). The transfection of 75 nM SMAD7 siRNA, ACVR2A siRNA or control siRNA was performed as described above. The gene expression analysis and cell proliferation assays were performed as described above.

Data analysis

All the quantitative data are presented as the mean ± standard error (SEM) and at least three biological replicates were used for each analysis. The statistical analysis was performed using GraphPad Prism® 5, version 5.02. The statistical significance between the mean values of two treatment groups was determined using a two-tailed Student's t-test. However, data from more than two treatment groups were analysed using a one-way ANOVA followed by Dunnett's post hoc test. The p-values indicate the statistical significance as described in each figure legend.

Results

SMAD7 and ACVR2A are the direct targets of the miRNA-424/503 cluster

We demonstrated in our previous study that miR-424/503 cluster miRNAs are upregulated in the granulosa cells of preovulatory dominant follicles at day 19 of the estrous cycle.

MicroRNA-424/503 cluster members are transcribed from an intergenic region of chromosome X, which is polycistronic in nature and evolutionary conserved in mammals. The precursors of bta-miR-424-5p (miR-424) and bta-miR-503-5p (miR-503) are 96 bp and 83 bp, respectively. The mature sequences of bta-424-5p and miR-503-5p are CAGCAGCAAUUCAUGUUUUGA and UAGCAGCGGGAACAGUACUG, respectively, which are conserved in other mammalian species. Since miRNAs regulate biological functions by targeting the 3´-UTR of genes post-transcriptionally in a sequence-specific manner, we performed a computational prediction using TargetScan (http://www.targetscan.org) to generate an algorithm to identify putative targets, which revealed that miR-424 and miR-503 target mothers against decapentaplegic homolog 7 (SMAD7) and activin receptor type 2A (ACVR2A). On the other side, miRNA prediction for SMAD7 target gene using TargetScan revealed that miR-424-5p was top predict with 8mer site type (an exact match to positions 2-8 of the mature miRNA (the seed + position 8) followed by an 'A'); while, miR-503-5p showed 7mer-A1 site type (an exact match to positions 2-7 of the mature miRNA (the seed) followed by an 'A'). The validation of the putative target genes, SMAD7 and ACVR2A, of the miR-424/503 cluster was performed by measuring the luciferase activity of an expression vector carrying the 3´-UTR of the SMAD7 and ACVR2A gene, which contains the miRNA binding sites. The luciferase activity in bovine granulosa cells co-transfected with miR-424 and miR-503 mimics and a plasmid vector harbouring the wild-type SMAD7 and ACVR2A 3'-UTR was significantly reduced compared to bGCs transfected with a control for the individual miRNA mimics and the plasmid vector with the wild-type SMAD7 and ACVR2A 3´-UTR ($P < 0.05$; Fig. 2). However, there was no significant reduction in luciferase activity in bGCs co-transfected with individual miRNA mimics or a control and a plasmid vector harbouring a mutated SMAD7 and ACVR2A 3´-UTR sequence.

Modulation of the miRNA-424/503 cluster resulted in differential expression of SMAD7 and ACVR2A in cultured bovine granulosa cells

To obtain further insights into the role of the miR-424/503 cluster in granulosa cell function by regulating the expression of SMAD7 and ACVR2A gene, we transfected cultured bGCs with either the miR-424/503 cluster mimics, inhibitors or the corresponding controls at a concentration of 75 nM. The expression level of the SMAD7 and ACVR2A mRNA was determined by qPCR analysis 48 h after transfection. The transfection of the miR-424/503 cluster mimics resulted in a significant reduction in the relative abundance of SMAD7 ($P < 0.01$) and ACVR2A ($P < 0.05$) mRNA compared to the controls (Fig. 3A). However, the expression of SMAD7 and ACVR2A mRNA was increased in cultured bGCs transfected with miR-424/503 cluster inhibitors. The western blot analysis showed that bGCs transfected with miR-424 cluster member mimic had a reduction in SMAD7 compared to those transfected with controls, however there was only slight change observed for ACVR2A protein expression. The miR-424/503 cluster inhibitors had no significant change in SMAD7 and ACVR2A protein expression compared to those transfected with controls (Fig. 3B).

Overexpression of the miRNA-424/503 cluster members enhanced the proliferation and reduced the differentiation of bovine granulosa cells through activin signalling pathway

MiR-424 mimic transfection in cultured bGCs significantly increased the proliferation activity in cells transfected with a miRNA mimic negative control (Fig. 4A). However, miR-503 had no significant effect on cell proliferation. Conversely, compared to the miRNA inhibitor control, bGCs transfected with miR-424 and miR-503 inhibitors showed no significant differences in proliferation activity. Moreover, the cell proliferation phenotype was accompanied by the higher expression level of the proliferation marker gene PCNA in the bGCs transfected with the miR-424 mimic compared to those transfected with the mimic

control ($P < 0.01$). However, transfection of inhibitors resulted in a slight reduction in the expression of PCNA (Fig. 4B). The PCNA protein expression showed an increasing tendency in bovine granulosa cells transfected with the miR-424/503 cluster mimic (Fig. 4C).

As a marker of differentiation, the expression of the STAR gene was investigated after modulation of the miRNA cluster. Transfection with the miR-424/503 cluster members mimic resulted in a significant reduction in STAR gene expression compared to the negative control (Fig. 4C).These results suggest that miR-424 promotes bovine granulosa cell proliferation and decreases the terminal differentiation of the bGCs, which leads to follicular survival and development.

Further, we investigated the role of miR-424/503 on downstream members of the activin signalling pathway; the western blot results showed that the expression of the SMAD2/3 proteins tended to increase after the overexpression of the miR-424/503 cluster compared to the negative control (4D). Moreover, the overexpression of the miR-424/503 cluster slightly increased the phosphorylated SMAD2/3 protein level (4D). These results indicate that miR-424/503 might be involved in the fine tuning of the activin signalling pathway.

Overexpression of the miRNA-424/503 cluster members changed the cell cycle profile of bovine granulosa cells

To further confirm the proliferation results and to understand the role of the miR-424/503 cluster in modulating the cell cycle of bGCs, we performed flow cytometric analysis after transfection of the miR-424/503 cluster members. The overexpression of the miR-424 mimic resulted in a significant reduction ($P < 0.001$) in the number of cells in G0/G1-phase, while a significant increase ($P < 0.01$) in the percentage of cells in S-phase was observed compared to cells transfected with the mimic negative control (Fig. 5G). This result indicated that the overexpression of miR-424 could increase bovine granulosa cell proliferation by promoting

G1 to S phase cell cycle transition. In contrast, the miR-424/503 cluster inhibitor did not cause any measurable changes in the cell cycle profile of bovine granulosa cells (Fig. 5H).

Effect of sequence specific knockdown of the SMAD7 and ACVR2A gene in bovine granulosa cells

In order to investigate the involvement of SMAD7 in bGCs proliferation and differentiation, and to further validate the regulatory role of the miR-424/503 cluster in SMAD7 expression, we performed an independent experiment by knocking down the expression of SMAD7 using small interfering RNA (siRNA). Bovine GCs transfected with the SMAD7 siRNA showed a significant reduction in the expression of both SMAD7 mRNA ($P < 0.05$) and protein compared to cells transfected with the siRNA negative control (Fig. 6A, 6E). The transfection of bGCs with the SMAD7 siRNA showed significantly enhanced proliferation of bGCs compared to control siRNA-transfected bGCs (Fig. 6B), which was accompanied by noteworthy change in the expression of the marker of proliferation PCNA (Fig. 6C). However, flow cytometric analysis showed reduction ($P < 0.001$) in the number of cells in G0/G1-phase (Fig. 6J), while a significant increase ($P < 0.01$) in the percentage of cells in S-phase (Fig. 6J) was observed compared to cells transfected following knocking down of SMAD7 with siRNA compared to negative control. Further, the suppression of the SMAD7 gene using siRNA had no effect on the expression of the STAR gene (Fig. 6D). Western blot analysis showed that the expression of SMAD2/3 and phosphorylated SMAD2/3 was slightly increased in siRNA-transfected cells compared to cells transfected with the negative control (Fig. 6F).

Similarly, to investigate the possible involvement of ACVR2A in bGC proliferation and differentiation and to further validate the regulatory role of the miR-424/503 cluster in ACVR2A expression, we suppressed ACVR2A using small interfering RNA (siRNA).

Bovine GCs transfected with the ACVR2A siRNA showed a significant reduction in the expression of both ACVR2A mRNA ($P < 0.01$) and protein compared to cells transfected with the siRNA negative control (Fig. 7A, 7E). The transfection of bGCs with the ACVR2A siRNA did not result in any measurable effect on the proliferation of bGCs compared to control siRNA-transfected bGCs (Fig. 7B). Similarly, there was no noteworthy change in the expression of the marker of proliferation PCNA following the suppression of the ACVR2A gene (Fig. 7C). However, western blot analysis showed reduced expression of the PCNA protein (Fig. 7F). Flow cytometric analysis showed no change in G1/S cell cycle transition (Fig. 7J). Further, the suppression of the ACVR2A gene using siRNA had no effect on the expression of the STAR gene (Fig. 7D) Western blot analysis showed that the expression of SMAD2/3 and phosphorylated SMAD2/3 was slightly decreased in siRNA-transfected cells compared to cells transfected with the negative control (7G).

Activin treatment decreased SMAD7 and miRNA-424/503 cluster members expression in bovine granulosa cells

Activin has been implicated in various intra-ovarian roles, including granulosa cell proliferation, follicular luteinization retardation and the recruitment of R-SMADs to activate the activin signalling pathway. Here, we investigated the effect of activin A treatment on the proliferation of granulosa cells and on miR-424/503 cluster expression. We treated bGCs with different doses of activin A (25 ng/ml, 50 ng/ml and 100 ng/ml). We found that activin A treatment increased proliferation of bovine granulosa cells in dose-dependent manner (Fig. 8A). Activin A treatment significantly decreased ($P < 0.001$) the STAR expression compared to control cells (Fig. 8C), which indicates a decrease in bGC differentiation. Further, we also noticed that Activin A treatment in dose dependent manner significantly decreased the expression of SMAD7 ($P < 0.01$; Fig. 8E); however, there was no change in the expression of

ACVR2A (Fig. 8F). Interestingly, we observed a dose-dependent reduction in miR-424/503 expression upon treatment with activin A. miRNA-424 was significantly decreased ($P < 0.05$; Fig. 8G) at the 100 ng/ml dose of activin A. This result indicates that there might be negative feedback loop between activin A and the miR-424/503 cluster.

Discussion

Folliculogenesis is a highly dynamic and well-coordinated process in which granulosa cells support follicular growth and development by producing hormones, autocrine and growth factors, and cytokines (Richards 1994; Toda et al. 2012) under the control of several molecular mechanisms and pathways. Increasing evidence supports the idea that miRNAs are one of the molecular mechanisms that epigenetically regulate the follicular development process (Gebremedhn et al. 2015; Andreas et al. 2016; Gebremedhn et al. 2016b; Maalouf et al. 2016). Several recent studies have shown that miRNAs regulate granulosa cell function by targeting TGF-β superfamily members (Yan et al. 2012; Zhang et al. 2013; Liu et al. 2014b). In our previous study, we demonstrated that the miR-424/503 cluster was upregulated in bovine granulosa cells from preovulatory dominant follicles compared to those derived from subordinate follicle(Gebremedhn et al. 2015), which indicated the potential involvement of the miR-424/503 cluster in granulosa cell function to support ovulation. In the present study, we have demonstrated the role of miR-424/503 in bovine granulosa cell function through targeting SMAD7 and further through coordinating activin signalling pathway. SMAD7 and ACVR2A genes, which are important in reproduction (Matzuk et al. 1995; Abd El Naby, W S et al. 2013; Gao et al. 2013; Li 2015), were identified as a putative targets of the miR-424/503 cluster, and subsequently validated by luciferase assay (Fig. 2). Further, the overexpression of the miR-424/503 cluster reduced the expression of SMAD7 mRNA and protein; however, the overexpression of the miR-424/503 cluster reduced ACVR2A mRNA

and only modest change observed in ACVR2A protein expression. These results suggested that the miR-424/503 cluster members regulate the function of bovine granulosa cells by fine tuning the expression level of SMAD7.

The present study demonstrates that granulosa cell proliferation was significantly increased upon the overexpression of miR-424, which was accompanied by upregulation of the PCNA gene. The evidence of the effect of modulating the expression of the miR-424/503 cluster on granulosa cell proliferation was accompanied by a shift in the proportion of cells from the G0/G1 phase to the S phase of the cell cycle. This finding is consistent with those of several reports demonstrating that miRNAs are essentially involved in the regulation of granulosa cell proliferation (Yao et al. 2010; Liu et al. 2011; Yin et al. 2012; Jiang et al. 2015; Gebremedhn et al. 2016b), which is necessary for follicular growth and creation of the unique micro-environment for oocyte maturation (Maruo 1995). Furthermore, the proliferating granulosa cells in growing follicles depend on growth factors for their survival, which promote the G1 to S phase transition of the cell cycle and prevent apoptosis in granulosa cells with a low-progesterone environment that helps to persist and sustain the dominant follicle (Lucy et al. 1990; Sirois and Fortune 1990; Savio et al. 1993). Interestingly, the present study showed a significant effect of miR-424 on granulosa cell proliferation compared to miR-503. This differential role of miR-424 could be associated with the fact that relative abundance of miR-424 was found to be high compared to miR-503 in granulosa cells of preovulatory dominant follicles in our previous study (Gebremedhn et al. 2015).

Several recent studies have shown that TGF-β superfamily members involved in granulosa cell proliferation are post-transcriptionally regulated by several classes of miRNAs (Yao et al. 2010; Zhang et al. 2013; Wang et al. 2016). Moreover, studies suggested SMAD7 antagonizes TGF-β superfamily, and acts as potential regulator of ovarian function (Gao et al. 2013; Li 2015). In the present study, we demonstrated that SMAD7, which belongs to the

TGF-β signalling pathway, is a direct target of miR-424/503 cluster members. Further, since SMAD7 acts as inhibitor of activin signalling pathway, thus upon inhibition of SMAD7 by targeting miR-424/503 leads to phosphorylation of SMAD2/3. Phosphorylated SMAD2/3 through forming heterodimeric complex with SMAD4, then translocates into the nucleus and consequently forms the SMAD2/3/4 complex along with other co-factors to regulate the transcription of target genes (Tsuchida et al. 2008). Therefore, overexpression of miR-424 leads to reduced expression of SMAD7 (Fig. 3A), resulting in phosphorylation of SMAD2/3, which subsequently activates the activin signalling pathway to enhance cellular proliferation. This suggests that miR-424/503 cluster members are involved in fine tunning of the activin signalling pathway through regulation of the inhibitory effect of SMAD7. Similarly, the cell cycle analysis results showed that overexpression of miR-424/503 resulted significant number of cells to be at S-phase compared to the untreated controls, which further validated proliferation assay of bovine granulosa cells. However, inhibition of both miRNAs did not result any change in cell cycle status of the granulosa cells compared to the untreated controls.

Addition of the activin A in vitro cultured granulosa cells resulted in enhanced proliferation in dose-dependent manner (Fig. 8a), as it has been reported previously (Zhang et al. 2013; Cheng et al. 2014). Similarly, treatment of granulosa cells with varying doses (25, 50, 100 ng/ml) of activin A sharply decreased the mRNA and protein expression of STAR (Fig. 8D). Similar studies also have shown that activin A downregulates STAR expression and progesterone production in granulosa cells through SMAD2/3 phosphorylation (Chang et al. 2015), which ultimately helps to prevent or delay the premature luteinization of bovine granulosa cells (Knight et al. 2012). We showed that similar to activin A, the overexpression of the miR-424/503 cluster members clearly reduced STAR expression, which suggests the

potential role of the miR-424/503 cluster members in the establishment and maintenance of the dominant follicle.

The SMAD pathways are known to be an integral part of a range of biological processes and are errantly activated or inactivated under various biological conditions (Blahna and Hata 2012). SMAD proteins are potentially involved in controlling the transcription of a variety of miRNA genes, and this transcriptional activation of miRNAs has distinct physiological significance (Blahna and Hata 2012). For instance, TGF-β induces both miR-216a and miR-217 in glomerular mesangial cells via SMAD binding elements (SBEs) in the miR-216 promoter (Kato et al. 2009). In contrast, TGF-β-induced Smad3/4 complex binding to the miR-24 promoter inhibits the expression of miR-24 in myoblasts (Sun et al. 2008). Interestingly, activin A treatment reduced the expression of miR-424/503 in a dose-dependent manner, in which a dose of 100 ng/ml, activin A significantly reduced the expression of miR-424 (Fig. 8G). This indicates possibility of negative feedback loop between the miR-424/503 cluster and activin-SMAD2/3 signalling. Here, we proposed a hypothetical model illustrating the involvement of the miR-424/503 cluster members in regulating the activin signalling pathway (Fig. 9). In line with this a recent report by Wang *et al*.(Wang et al. 2016), which showed the presence of a potential feedback loop between miRNAs and the target gene.

Follicular growth and development is a dynamic and complex but nonetheless tightly harmonized process in which activin plays an important role by enhancing the proliferation and reducing the differentiation of granulosa cells (Knight 1996; Findlay et al. 2000; Knight and Glister 2001). Several studies have shown that the dysfunction of granulosa cells may contribute to the abnormal folliculogenesis observed in ovarian pathophysiology (Erickson et al. 1992; Willis et al. 1998; Jakimiuk et al. 2001), although the underlying mechanism remains to be determined. Furthermore, it is also important that granulosa cells should proliferate in controlled manner to avoid granulosa cell tumours, which account for 2-3% of

ovarian malignancies (Malmstrom et al. 1994). Here, we reveal for the first time that the miR-424/503 cluster members are involved not only in the proliferation of granulosa cells but also in the balancing of the activin signalling pathway through regulating the expression of SMAD7 and ACVR2A in bovine granulosa cells. This coordination of the activin signalling pathway through miRNAs might lead to healthy granulosa cells by avoiding any deviation from normalcy, like granulosa cell tumours. Recent studies have shown that miRNA-424 suppresses the expression of SOCS6 in pancreatic cancer (Wu et al. 2013) and inhibits the Akt3/E2F3 axis and tumour growth in hepatocellular carcinoma (Zhang et al. 2014a). Here, we suggest that perturbations in the expression of the miRNA-424/503 cluster members might result in ovarian disorders such as PCOS (polycystic ovarian syndrome) and granulosa cell tumours. Therefore, these results *in toto* suggest that an optimal *miRNA milieu* is required for normal cellular and tissue function. The present study and other related miRNA studies provide insights into the molecular mechanisms underlying the regulation of granulosa cell functions and can facilitate a better understanding of follicular development and the pathophysiology of some reproductive disorders for the improvement of fertility treatments.

Conlclusions

The present study demonstrated that the miR-424/503 cluster members promote granulosa cell proliferation and cell cycle progression activity by fine tuning the SMAD7 gene expression through the activin signalling pathway. Hence, the interplay between the miR-424/503 cluster members and SMAD7 gene might be involved in the complex and coordinated process of bovine follicular development.

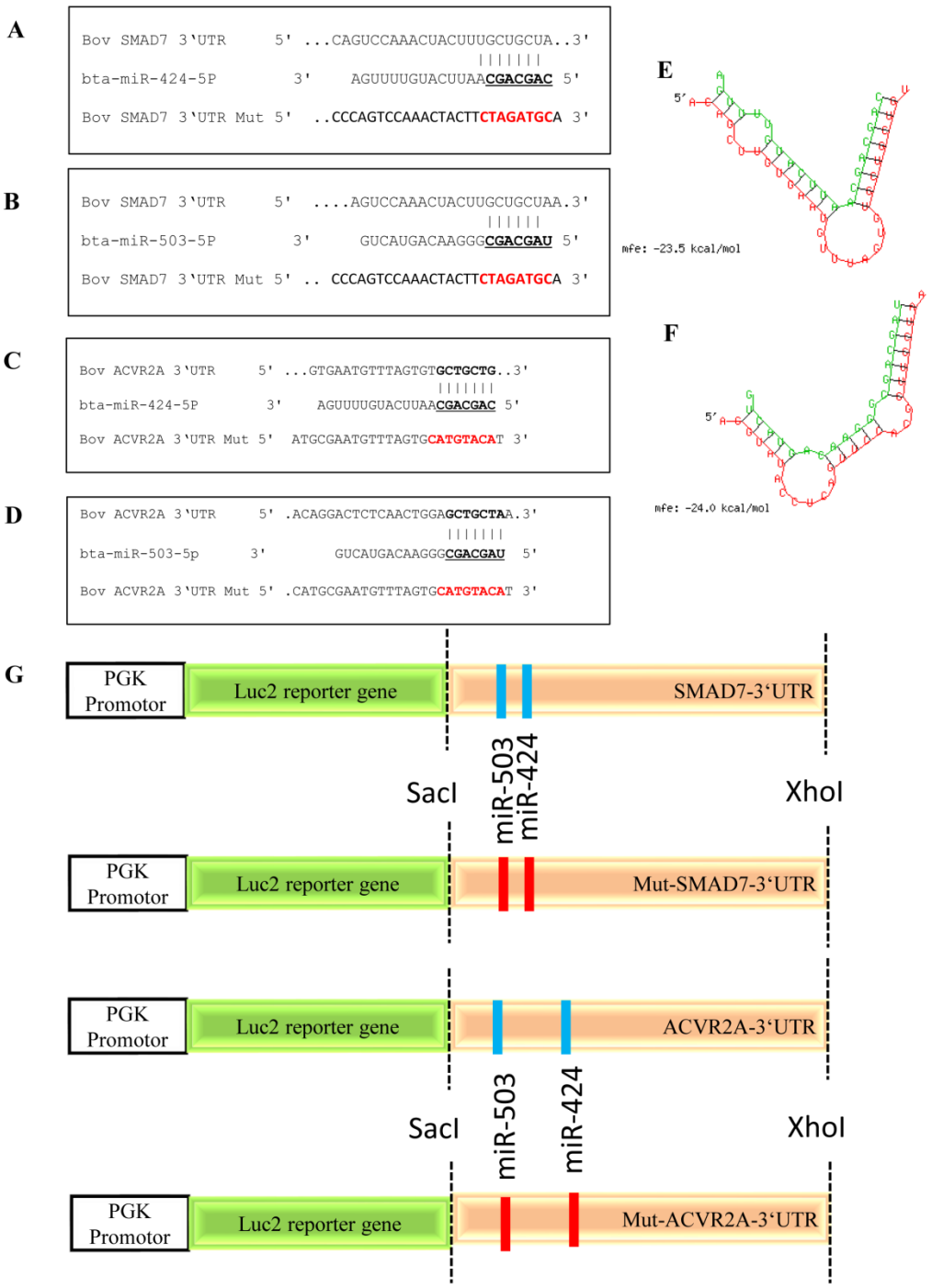

Figure 1. The miRNA-mRNA binding sites in bovine SMAD7 3´-UTR (A, B) and ACVR2A 3´-UTR sequences (C, D) and the minimum free energies of miR-424 (kcal/mol) (E) and miR-503 (F). Bold and underlined letters indicate putative binding sites and mutated regions. Schematic diagram of the reporter constructs containing the putative miRNA-mRNA binding sites of the bovine SMAD7 and ACVR2A 3´-UTR sequences (G).

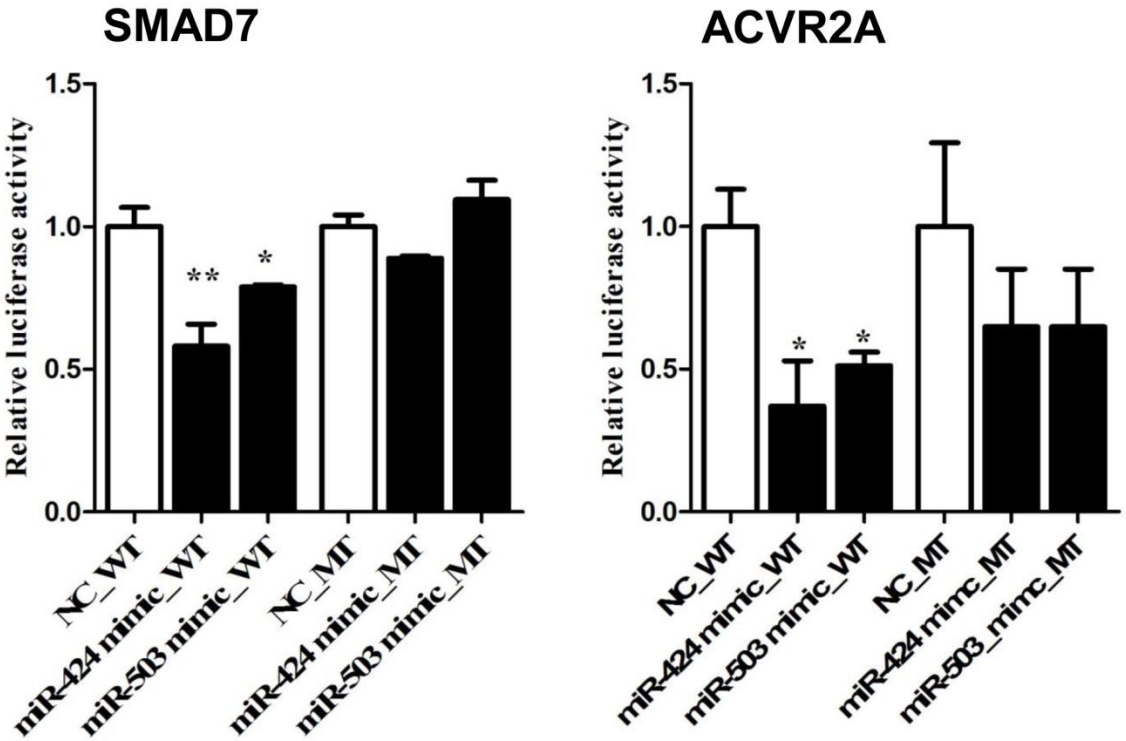

Figure 2. SMAD7 and ACVR2A are the direct targets of the miR-424/503 cluster. Relative luciferase activity in bovine granulosa cells co-transfected with pmiRGLO vector harbouring the wild-type (WT) or mutant (MT) 3´-UTR sequence of SMAD7 or ACVR2A and the corresponding miRNA mimics or negative controls (NC) for miR-424 and miR-503. The data are presented as the mean ± SEM (*$p < 0.05$, **$p < 0.01$).

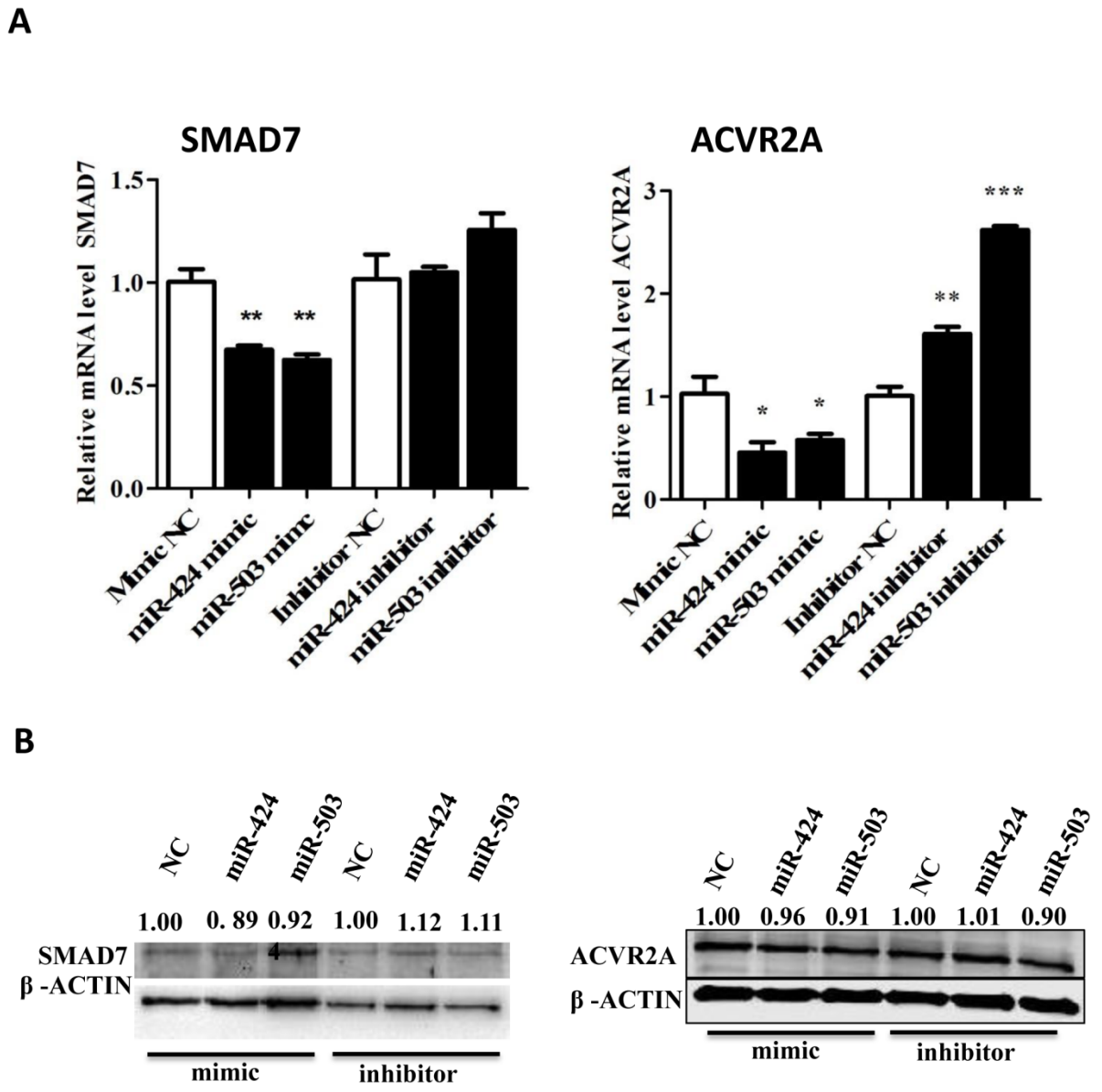

Figure 3. Modulation of the miR-424/503 cluster reduced the expression of SMAD7 and ACVR2A in bovine granulosa cells. Overexpression of miR-424 and miR-503 decreased the SMAD7 and ACVR2A mRNA (A) and protein (B) expression (A) mRNA. The bar graphs indicate the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

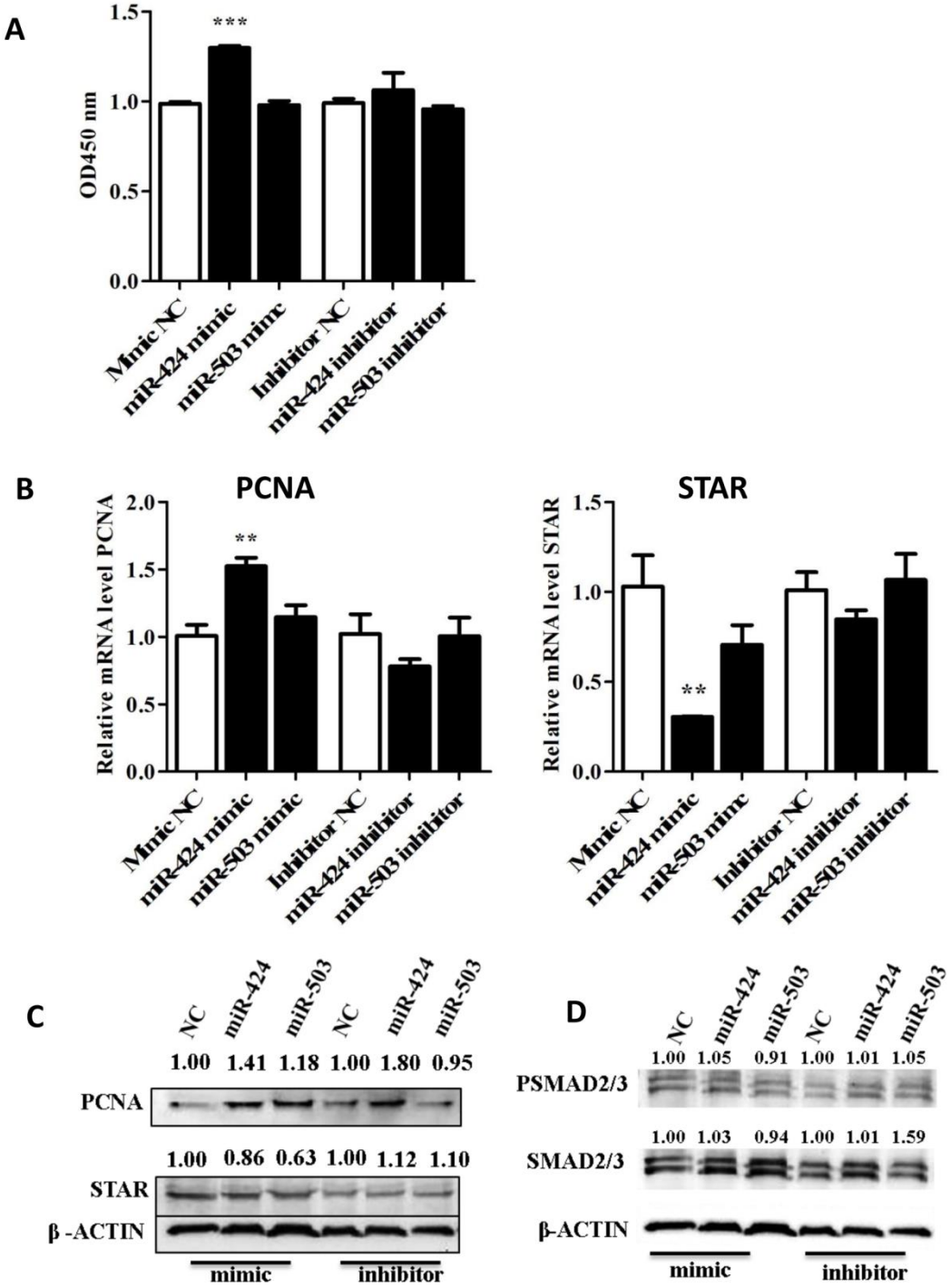

Figure 4. Overexpression of the miR-424/503 cluster increased bovine granulosa cell proliferation. Cell proliferation assay of granulosa cells transfected with miR-424/503 cluster mimics and inhibitors and the corresponding negative control (NC) (A). The mRNA (B) and protein (C) expression level of PCNA and STAR in granulosa cells transfected with miR-424/503 cluster mimics, inhibitors or corresponding NC. The protein expression level of

SMAD2/3 and PSMAD2/3 in granulosa cells transfected with miR-424/503 cluster mimics and inhibitors and the corresponding NC (D). The bar graphs indicate the mean ± SEM (**p < 0.01, ***p < 0.001).

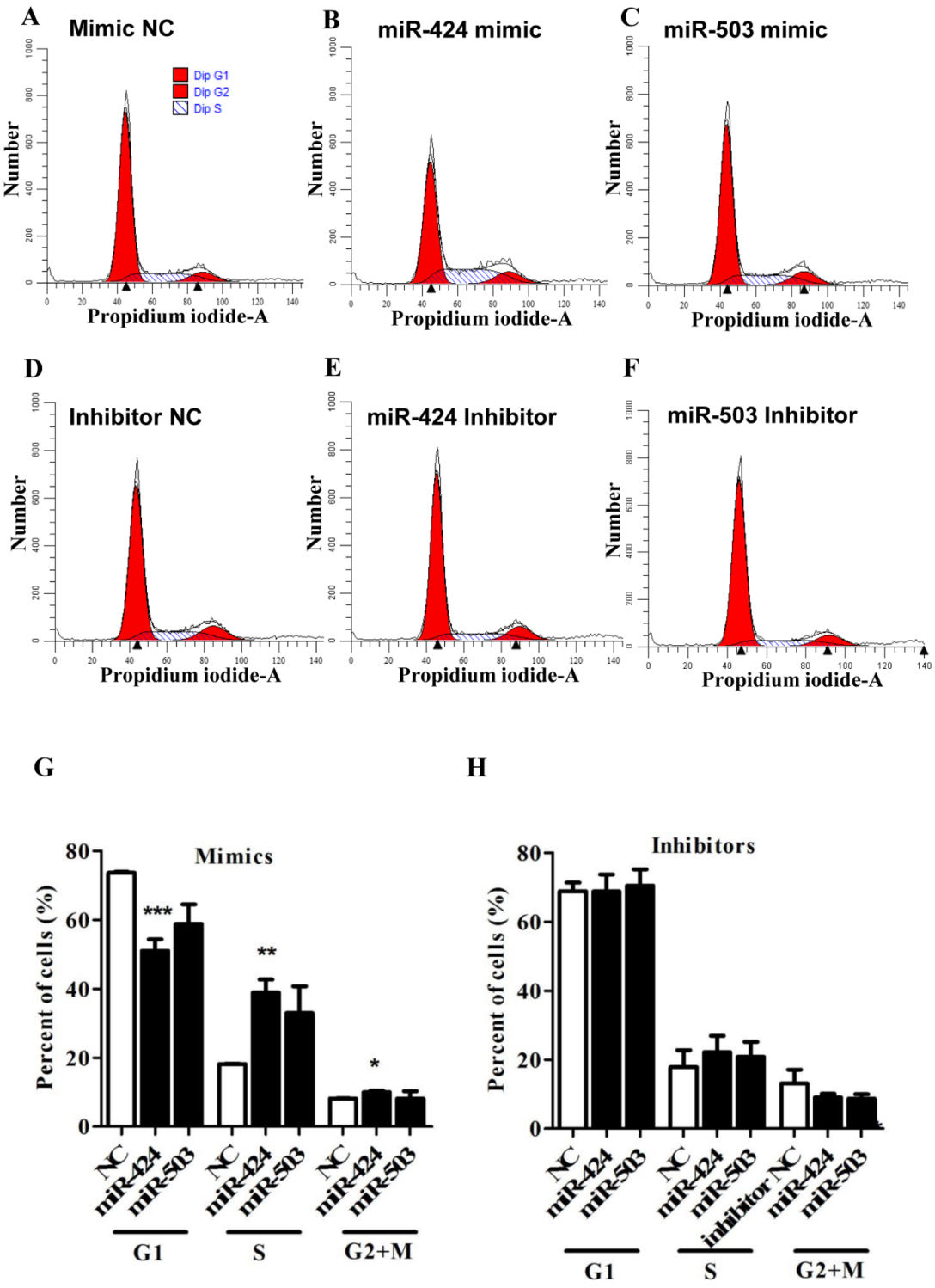

Figure 5. Overexpression of the miR-424/503 cluster enhanced cell cycle progression of bovine granulosa cells. Flow cytometric analysis showing the cell cycle distribution (G1/G0, S and G2/M phases) of propidium iodide (PI)-labeled bovine granulosa cells transfected with the mimic negative control (NC) (A), miR-424 mimic (B), miR-503 mimic (C), inhibitor NC

(D), miR-424 inhibitor (E), or miR-503 inhibitor (F). Histogram showing the percentages of cells in G1/G0, S and G2/M phases of the cell cycle after transfection with miR-424/503 cluster mimics (G), and inhibitors (H). The bar graphs indicate the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

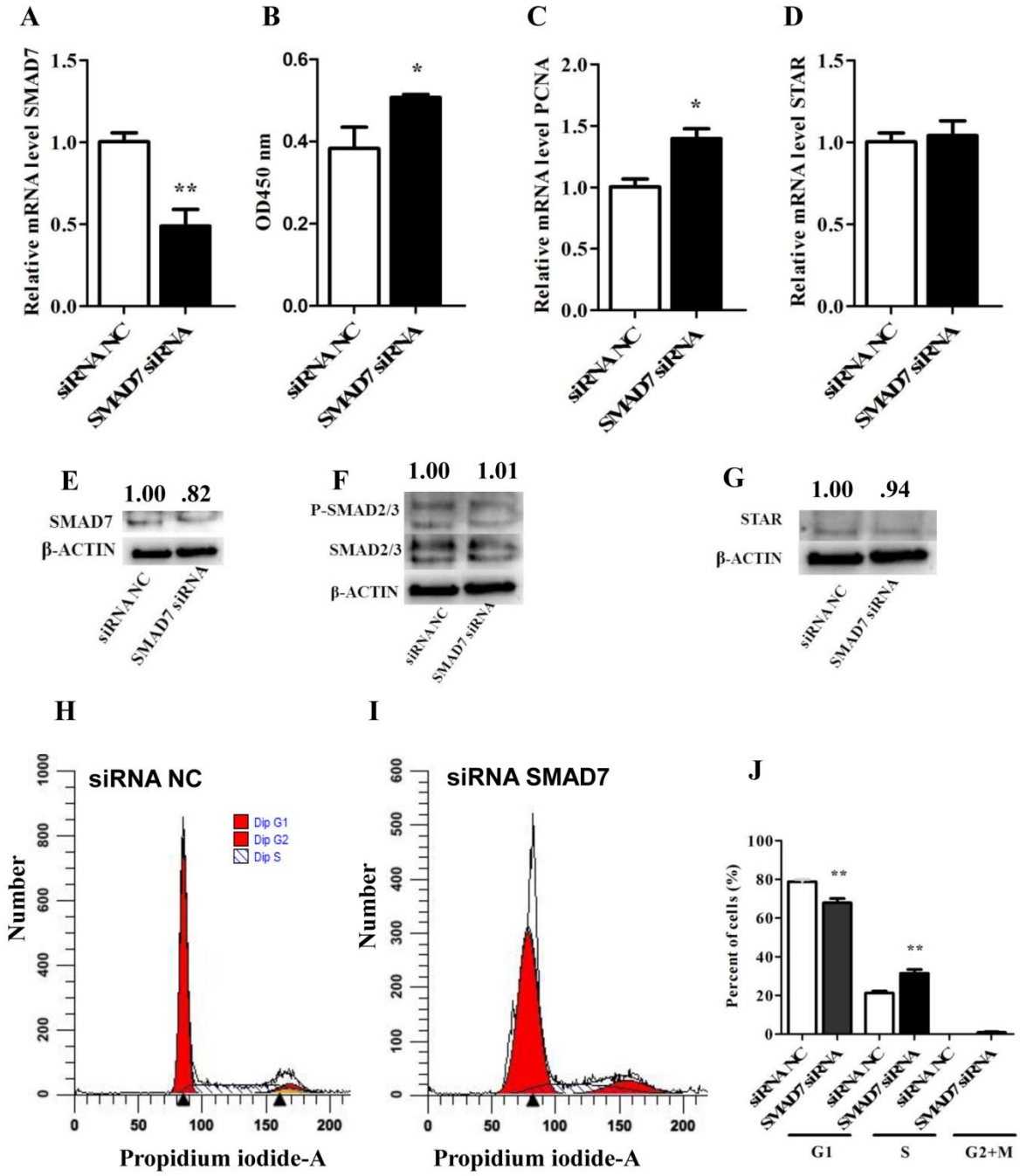

Figure 6. Sequence-specific knockdown of SMAD7 increased granulosa cell proliferation. The mRNA (A) and protein expression level (E) of SMAD7, mRNA level of PCNA (C), mRNA (D) and protein expression level (G) of STAR in granulosa cells transfected with SMAD7 siRNA or siRNA negative control (NC). The proliferation activity in granulosa cells transfected with SMAD7 siRNA (B). The protein expression level of SMAD2/3 and

PSMAD2/3 in granulosa cells transfected with SMAD7 siRNA or siRNA negative control (NC) (F). Flow cytometric analysis showing the cell cycle distribution (G1/G0, S and G2/M phases) of propidium iodide (PI)-labeled bovine granulosa cells transfected with the SMAD7 siRNA (I) or siRNA negative control (NC) (H). Histogram showing the percentages of cells in G1/G0, S and G2/M phases of the cell cycle in cells transfected SMAD7 siRNA or siRNA negative control (NC) (J). The bar graphs indicate the mean ± SEM (**$p < 0.01$).

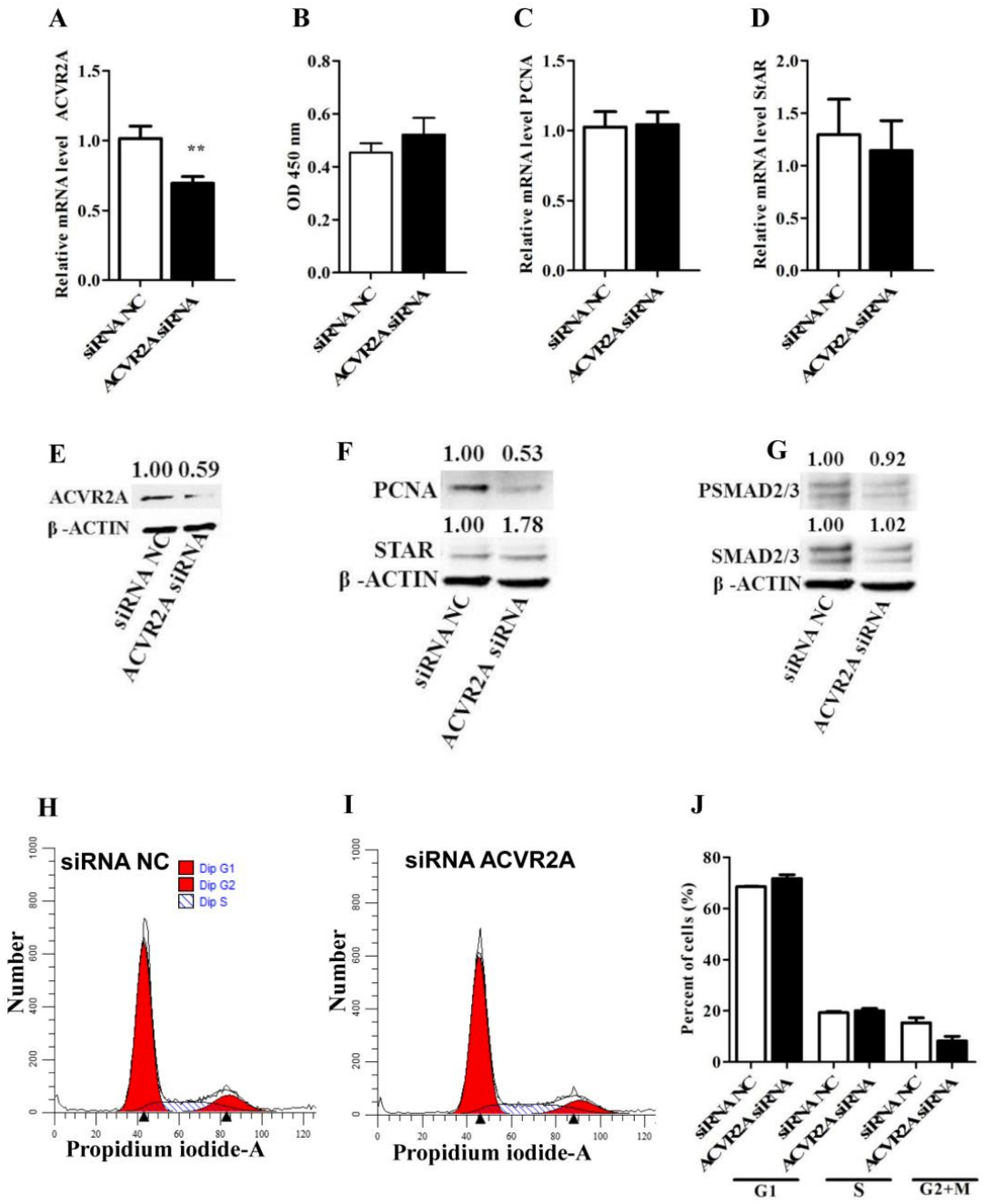

Figure 7. Effect of the sequence-specific knockdown of ACVR2A in bovine granulosa cells. The mRNA (A) and protein level (E) of ACVR2A in bovine granulosa cells transfected with ACVR2A siRNA or siRNA negative control (NC). The proliferation activity in granulosa cells transfected with ACVR2A siRNA or siRNA negative control (NC). (B). The mRNA (C) and protein level (F) of PCNA, and the mRNA (D) and protein level of (F) of STAR in granulosa cells transfected with ACVR2A siRNA or siRNA negative control (NC). The protein expression level of SMAD2/3 and PSMAD2/3 in granulosa cells transfected with

siRNA NC or ACVR2A siRNA (G). Flow cytometric analysis showing the cell cycle distribution (G1/G0, S and G2/M phases) of propidium iodide (PI)-labeled bovine granulosa cells transfected with ACVR2A siRNA (I) or siRNA negative control (NC) (H). Histogram showing the percentages of cells in G1/G0, S and G2/M phases in granulosa cells transfected with ACVR2A siRNA or siRNA negative control (NC) (J). The bar graphs indicate the mean ± SEM (**$p < 0.01$).

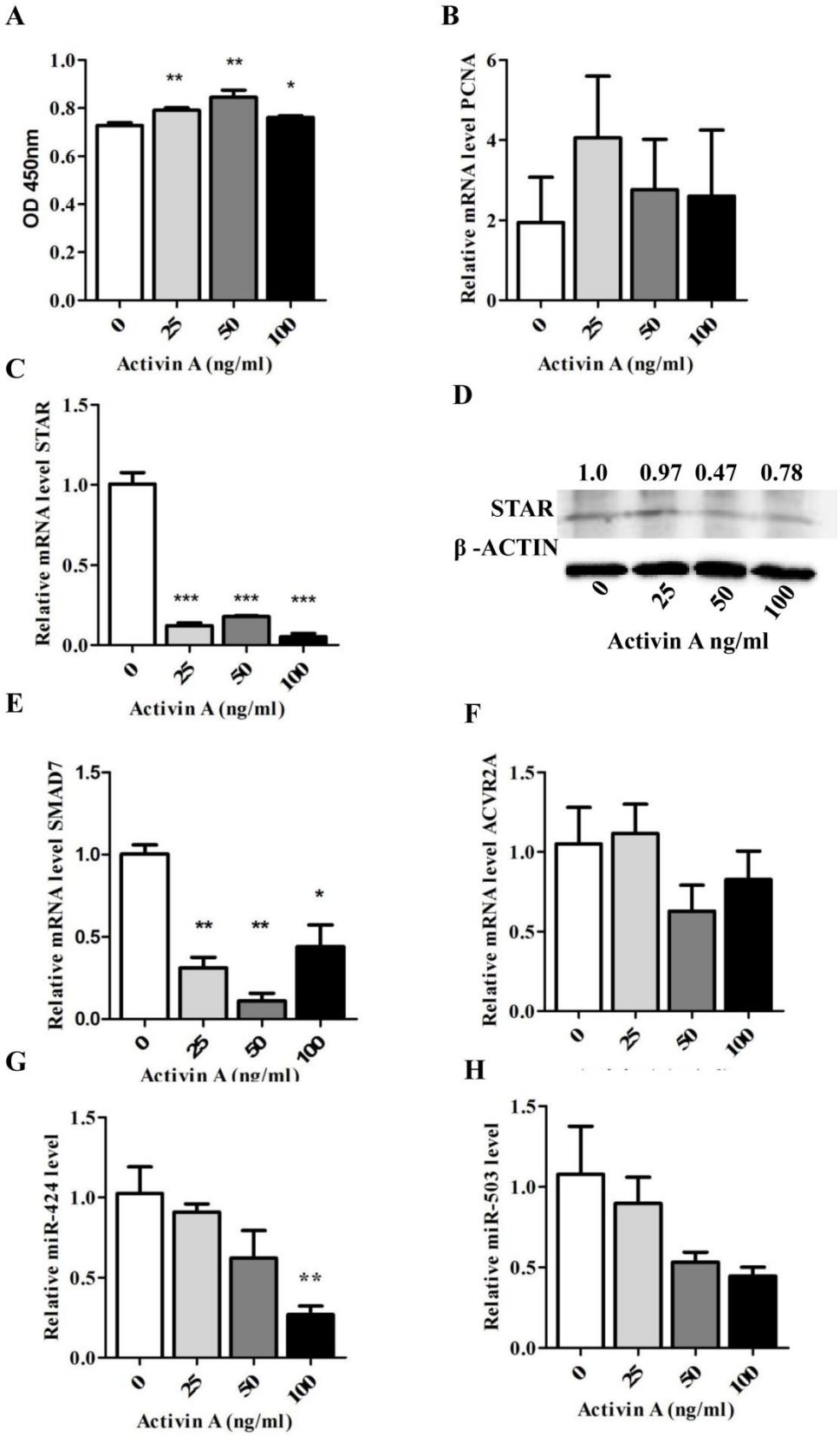

Figure 8. Dose-dependent activin A treatment increased bovine granulosa cell proliferation, reduced the SMAD7 and miR-424/503 expression levels. Cell proliferation assay (A) after

dose-dependent Activin A treatment (Activin A: 25 ng/ml, 50 ng/ml and 100 ng/ml). The relative expression levels of PCNA (B), STAR (C), SMAD7 (E), ACVR2A (F), miR-424 (G) and miR-503 (H) in granulosa cells treated Activin A. The protein expression of STAR The mRNA level of SMAD7 (E) and ACVR2A (F) in granulosa cells treated with treated Activin A (D). The bar graphs indicate the mean ± SEM (**$p < 0.01$, ***$p < 0.001$).

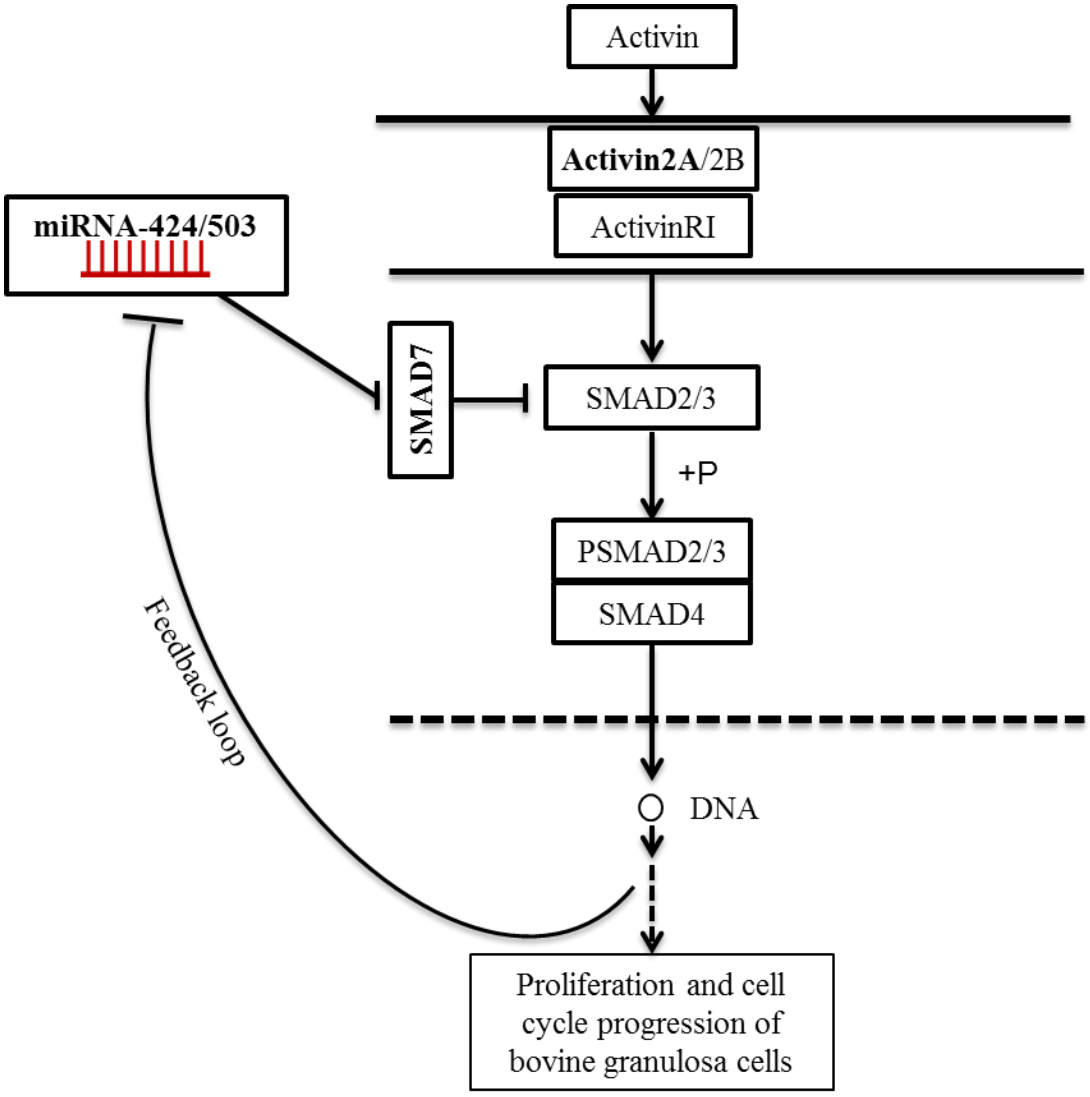

Figure 9. Proposed hypothetical model depicting involvement of miR-424/503 cluster in the activin signalling pathway by targeting SMAD7. Increased expression of miR-424/503 cluster suppresses the expression of SMAD7 which eventually enhances the phosphorylation SMAD2/3 subsequently increasing the granulosa cell proliferation and cell cycle progression. Thus, when granulosa cells are treated with Activin A, the SMAD7 expression is suppressed, but the ACVR2A expression was maintained and leading to enhanced cell proliferation. At the time when the cells reach peak proliferation activity, the miR-424/503 expression was suppressed indicating the presence of negative feed back loop between the expression of miR-424/503 and cell proliferation activity in activin A treated cells.

Table S1. Sequence specific primers used for pmirGLO and 3´-UTR amplification of the SMAD7 and ACVR2A gene harboring binding site for miR-424-5p and miR-503-5p

Accession number	**Genes**	**Primer sequence**	**Size (bp)**
FJ376737	pmirGLO	F: 5´-GCAAGATCGCCGTGTAATTC-3´ R: 5´-CTTTCGGGCTTTGTTAGCAG-3´	107
XM_005224232.3	SMAD7-miR/424-503	F: 5´-GCATGAGCTCAGCAGGCCACACTTCAAACT-3´ R: 5´-ATG**CTCGAG**GGACGAGAAGAAGAAAACCAACC-3	138
NM_174227	ACVR2A-miR-424	F: 5´-GCGAGCTCTTGCATTTGCTGTTGTGTTTCT-3´ R: 5´-TAT**CTCGAG**TAGCAACCGTGGAACTGAGG-3´	201
NM_174227	ACVR2A-miR-503	F: 5´-TAGAGCTCACACACTGAGAAACAGGACTCT-3´ R: 5´-TAT**CTCGAG**CCTTGATTTGGAGAGGGCCA-3´	217

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

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

Accession number	**Genes**	**Primer sequence**	**Product size (bp)**
NM_173979	β-ACTIN	F: 5´-TGTCCACCTTCCAGCAGAT-3´	249
		R: 5´-TCACCTTCACCGTTCCAGT-3´	
NM_001034494	PCNA	F: 5´-CACCAGCATGTCCAAAATAC-3´	192
		R: 5´-CTGAGATCTCGGCATATACG-3´	
NM_174189	STAR	F: 5´-AAATCCCTTTCCAAGGTCTG-3´	204
		R: 5´-ACCAGCATTTCTGCTACTGC-3´	
NM_0011928651	SMAD7	F: 5´-GTGGCATACTGGGAGGAGAA-3´	128
		R: 5´-TTGTTGTCCGAATTGAGCTG-3´	
NM_174227	ACVR2A	F: 5´-CAGAGAAACGAGGCACCAGT-3´	186
		R: 5´-GGCCATCTTTTAGGCCAGGT-3´	

Chapter 3

MicroRNA-424 modulates candidate gene expression during in vitro bovine cumulus cell expansion and oocyte maturation

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(Manuscript under preparation)

Abstract

Recent studies showed involvement of the miRNAs in regulating cumulus cell expansion and oocyte development. In this study, we aimed to investigate, whether miR-424 which was found to be involved in regulating granulosa cell proliferation, could modulate the candidate gene expression related to cumulus cells expansion and oocyte maturation in vitro. We investigated the temporal expression of miR-424 in cumulus cells and oocytes collected of COCs (cumulus-oocyte complexes) from small healthy follicles at immature (germinal vesicle; GV) and mature (metaphase II; MII) stages. Results showed that the miR-424 expression was significantly higher in MII stage COCs compared to the GV stage COCs in both cumulus cells and oocytes. Next, we modulated the miR-424 expression by transfecting the miR-424 mimic, miR-424 inhibitor or corresponding negative controls. Overexpression of miR-424 in cumulus cells enhanced the expression of genes associated with cumulus cells expansion such as; EGFR, PTGS2, PTX3 and MAPK1 compared to those coincubated with the mimic negative control (NC). Moreover, overexpression of miR-424 increased the cell cycle regulator CYCB2 gene in the cumulus cells compared to mimic NC, while the miR-424 mimic transfection resulted in decreased expression of the STAR gene. Further, overexpression of miR-424 did not significantly increased oocyte maturation rate progression from the GV to MII stage. However, overexpression of miR-424 increased KIT ligand expression involved in cytoplasmic maturation of oocytes. Taken together, this study suggests that miR-424 enhances the expression of genes associated with cumulus cell expansion and oocyte maturation, which suggests that miR-424, might be involved in the oocyte maturation through cumulus cell expansion.

Introduction

MicroRNAs (miRNAs) are small (~22 nt), non-coding endogenous RNAs that can play important regulatory roles in animals by targeting mRNAs for cleavage or translational repression (Ambros 2004; Bartel 2004; Bagga et al. 2005). These non coding miRNAs are involved in a range of biological functions and emerging as important regulators in various cellular functions including cell proliferation, differentiation and apoptosis (Hwang and Mendell 2007; Pan et al. 2015). Recently, functional analysis of specific miRNA supports the notion that miRNAs regulate ovarian function primarily through their regulatory actions in ovarian somatic cells (Yin et al. 2012; Zhang et al. 2013; Sen et al. 2014; Pan et al. 2015; Andreas et al. 2016; Gebremedhn et al. 2016b). Further, somatic cells play an important role during oocyte growth, the maintenance of meiotic arrest, the induction of oocyte maturation, and the acquisition of full embryonic developmental competence (Buccione et al. 1990). Cumulus cells are immediate neighbor cells of the oocyte, and by surrounding the oocytes, essentially support the growth and maturation of the oocyte (Buccione et al. 1990; Eppig 1991). The communication between oocyte and cumulus cells is critical and vital for the function of both cell types and the development of oocyte (Eppig 1991; Eppig et al. 2001; Matzuk et al. 2002; Gilchrist et al. 2004; Eppig et al. 2005). Indeed, studies demonstrated that removal of the cumulus cell before in vitro maturation could inhibit oocyte developmental competence (Vozzi et al. 2001; Atef et al. 2005).

In a quest to understand the molecular mechanisms through the transcriptome dynamic between the cumulus cells and oocytes (Assou et al. 2006; Assidi et al. 2010; Regassa et al. 2011; Nivet et al. 2013; Vigone et al. 2013; Assidi et al. 2015) before and after maturation process revealed a spatiotemporal regulation of gene expression. Similarly, we have shown the miRNAs transcript abundance in oocytes (Tesfaye et al. 2009; Abd El Naby, W S et al. 2013) and cumulus cells (Abd El Naby, W S et al. 2013) during in vitro oocyte maturation.

These studies highlighted the involvement of miRNAs by showing differential enrichment in cumulus cells and oocytes. However, targeted deletion of oocyte-specific DGCR8, an enzyme for miRNA biosynthesis, could not adversely affect oocyte maturation (Suh et al. 2010), indicating that the role of miRNA in the oocyte might be limited. Nevertheless, recent studies indicated that miRNAs are involved in oocyte maturation via the support extended through cumulus cells (Pan et al. 2015; Li et al. 2017; Sinha et al. 2017). In our previous studies, we have shown that the miR-424 was differentially expressed in cumulus cells compared to oocytes (Abd El Naby, W S et al. 2013) and was involved in granulosa cell proliferation (Pandey et al. 2016). However, the role of miR-424 in cumulus cell expansion and in vitro oocyte maturation has not been studied yet. In this study we investigated the involvement of miR-424 in cumulus cell expansion and oocyte maturation in vitro.

Materials and methods

Cumulus-oocyte complexes 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 containing a 0.9% saline solution within 2 h after slaughter. The COCs were aspirated from healthy small follicles (2–8 mm 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. 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% estrous 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 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.

miRNA transfection

To investigate the function of miR-424 in oocyte maturation, an equal concentration (50 nM) of miRCURY LNA™ miR-424 mimic, miR-424 inhibitor or corresponding negative controls (mimic NC and inhibitor NC) was transfected into the appropriate well using the Lipofectamine® 2000 reagent according to manufacture's protocol. The transfected COCs were matured in a group of 50 COCs in five-well dishes containing 400 µl maturation media for 22 h at 39 °C in a humidified atmosphere with 5% (v/v) CO_2 in air as described above.

Total RNA isolation and cDNA synthesis

Total RNA of cumulus cells and oocytes was isolated using miRNeasy® mini kit (Qiagen GmbH, Germany) following manufacturer's protocol. The quality and quantity of extracted RNA were determined using NanoDrop 8000 (Thermo Scientific). For gene expression analysis, an 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 miRNA 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-424 expression were performed using iTaq™ Universal SYBR® Green Supermix and ExiLENT SYBR® green master mix, respectively, using Applied Biosystem® StepOnePlus™ thermocycler. The primers for gene expression analysis (Table S1) have been tested using qualitative PCR followed by sequencing analysis using GenomeLab™ GeXP Genetic Analysis System. miRNA primer was 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-424 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-424 expression, respectively.

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 legends.

Results

Temporal expression of miR-424 during in vitro maturation

To further determine the importance of miR-424 which was found to be significantly upregulated in the bGCs of the preovulatory dominant follicle, we investigated the temporal expression of miR-424 in cumulus cells and oocytes. Parts of collected COCs were used as immature (GV) group, while the others were mature (MII). When both cumulus cells and oocytes from both immature and mature groups were compared, we found that the miR-424 expression was significantly higher ($p<0.05$) in both cumulus cell and the oocytes at MII stage compared to GV (Fig.1).

The expression and potential role of miRNA-424 in cumulus cells function

To investigate the role of miR-424 in cumulus cell expansion and oocyte maturation, the COCs were matured in the presence of either mimic or inhibitor of miR-424. Quantitative PCR analysis showed that transfection of the miR-424 mimic resulted in a significant increase ($P<0.05$) of miR-424 expression in both cumulus cells and oocytes compared to negative control. However, transfection of the miR-424 inhibitor had no significant effect on the expression of miR-424 in cumulus cells and oocytes compared to the negative control (Fig. 2).

The effect of miR-424 modulation in the cumulus expansion and oocyte maturation process was assessed based on the expression of genes related to cumulus expansion (EGFR, PTGS2, PTX3 and MAPK1), cell cycle regulator (CYCB2) and cell differentiation (STAR) after treatment with mimic and inhibitors of miR-424 during in vitro maturation. The overexpression of miR-424 increased PTGS2 ($p<0.01$), PTX3 ($p<0.001$), MAPK1 ($p<0.05$) and CYCB2 ($p<0.05$) (Fig. 3A, 3B, 3C, 3E) genes in the cumulus cells compared to those coincubated with the mimic negative control, while the miR-424 mimic transfection resulted

in a decreased expression of STAR gene (Fig. 3F). However, transfection of miR-424 inhibitor had no effect on the expression of cumulus expansion genes except on PTGS2 (Fig. 3A). Further, overexpression of miR-424 in the cumulus expansion process was also assessed based on the expression of HAS gene related to cumulus expansion, aromatase and INHBA genes responsible for steroid synthesis and PGR after treatment with mimic and inhibitors of miR-424 during in vitro maturation. Results revealed that transfection of miR-424 increased the expression of PGRMC ($p>0.05$) and aromatase ($p>0.001$) (Fig. 4B, 4C).

Overexpression of miR-424 promoted oocyte maturation related gene expression

We next investigated whether overexpression of miR-424 could affect oocyte maturation, and gene expression related to oocyte maturation. Results showed that miR-424 transfection did not affect the oocyte maturation rate (Fig. 5A). However, overexpression of miR-424 significantly increased KIT Ligand gene expression involved in cytoplasmic maturation of oocytes compared to negative control (Fig. 5C). Similarly, overexpression of miR-424 significantly increased ZP3 gene, which transcribes in oocytes and its expression developmentally regulates oocytes, compared to negative control (Fig. 5D). However, miR-424 mimic transfection slightly increased in BMP15 expression (Fig. 5B).

Discussion

Interaction between cumulus cells and oocytes is essential for proper maturation of oocytes, fertilization and further embryonic development (Zhou et al. 2016; Kidder and Vanderhyden 2010; Wigglesworth et al. 2013). In the present study, we have shown that miR-424 is abundantly expressed in cumulus cells and oocytes at MII stage, indicating selective increase in the level of miR-424 transcript. Likewise, we have also showed the differential expression of miR-424 between oocytes and the surrounding cumulus cells before and after in vitro

maturation (Abd El Naby, W S et al. 2013). A recent study demonstrated that dynamic expression of several miRNAs between GV and MII stages of oocytes was associated with selective increase in miRNA transcripts (Gilchrist et al. 2016). We found that overexpression of miR-424 enhanced cumulus expansion related genes (PTGS2, PTX3 and EGFR) (Hung et al. 2015; Uyar et al. 2013), which are important during the oocyte maturation process (Hung et al. 2015). This is in accordance with the report that miRNA-224 was found to be involved in the regulation of mouse cumulus expansion by targeting PTX3 (Yao et al. 2014) which plays a key role in the organization of the cumulus oophorus extracellular matrix (Salustri et al. 2004). Further, miR-424 overexpression enhanced the expression of MAPK1, which is required for resumption of meiosis in the oocyte and expansion (mucification) of the cumulus oophorus (Su et al. 2003). Cumulus expansion is dependent on the stimulation of LH-induced epidermal growth factor (EGF)-like peptides, via the activation of protein kinase A (PKA). These stimuli enhance PTX3 (a component of the extracellular matrix of the cumulus oophorus) and other related transcripts (HAS2 and Tnfaip6) involved in the process of expansion in cumulus cells (Varani et al. 2002; Fülöp et al. 2003; Ben-Ami et al. 2006). The increment of the CYCB2 gene expression in cumulus cells could be associated with the evidence that miR-424 promoted granulosa cell proliferation and was found to be upregulated in bovine granulosa cell of pre-ovulatory dominant follicle (Gebremedhn et al. 2015). This was accompanied by a decrease in the expression of differentiation marker gene STAR, which ultimately helps to delay the premature luteinization of bovine somatic cells (Shukovski and Findlay 1990; Knight et al. 2012). These results shed insight that miR-424 might be critically essential during oocyte maturation by involving in granulosa cell proliferation and subsequently cumulus expansion. Recently, few studies have demonstrated the role of miRNAs in oocyte maturation and acquiring developmental competence (Pan et al. 2015b; Wright et al. 2016; Xiao et al. 2014).

Further, it has been reported that the synthesis of steroids was significantly higher in the late preovulatory stage COCs (Vanderhyden and Macdonald 1998), and the presence or absence of steroids during in vitro maturation of oocytes may affect the oocyte quality and its developmental competence both in vivo and in vitro condition (Ali and Sirard 2002; Dieleman et al. 2002). Moreover, as a matter of fact, PGR gene, which is found to be expressed in bovine cumulus cells (Mingoti et al. 2002), plays an important role in cumulus cells through an increased sensitivity to progesterone-induced genes (Li et al. 2004). In accordance with this, we found increased expression of some steroidogenesis-related genes including PGR and aromatase upon the transfection of miR-424 mimic, which might be playing an important role in enhancing the oocyte quality during in vitro oocyte maturation.

In large antral follicles, the KIT-KIT Ligand interaction modulates the ability of the oocyte to undergo cytoplasmic maturation and helps to maximize thecal androgen output (Driancourt et al. 2000). Oocytes regulate granulosa cell kit ligand mRNA levels in a way that is characteristic of the stage of growth and development of the oocyte (Joyce et al. 1999). In this study, we found that overexpression miR-424 increased expression of KIT Ligand gene in oocytes. Further, ZP3 transcribed in oocytes where its expression is developmentally regulated, functions as the sperm receptor at fertilization (Philpott et al. 1987). Our present data demonstrate that the ZP3 gene was found to be increased upon the overexpression of miR-424 mimic. Altogether, the present study gives an insight on the involvement of miR-424 in oocyte maturation by regulating cumulus cell expansion.

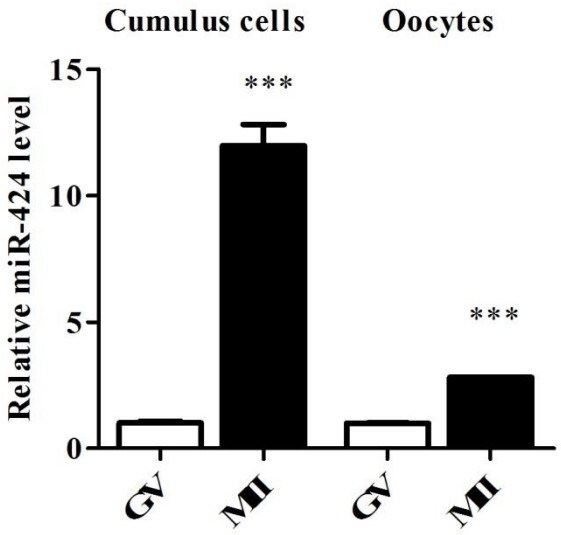

Figure 1. Temporal expression of miR-424 in oocytes and surrounding cumulus cells at immature (GV) and mature (MII) stage. The expression of 5S rRNA was used as internal control. The data are shown as mean ± SEM (n=3; ***$p < 0.001$).

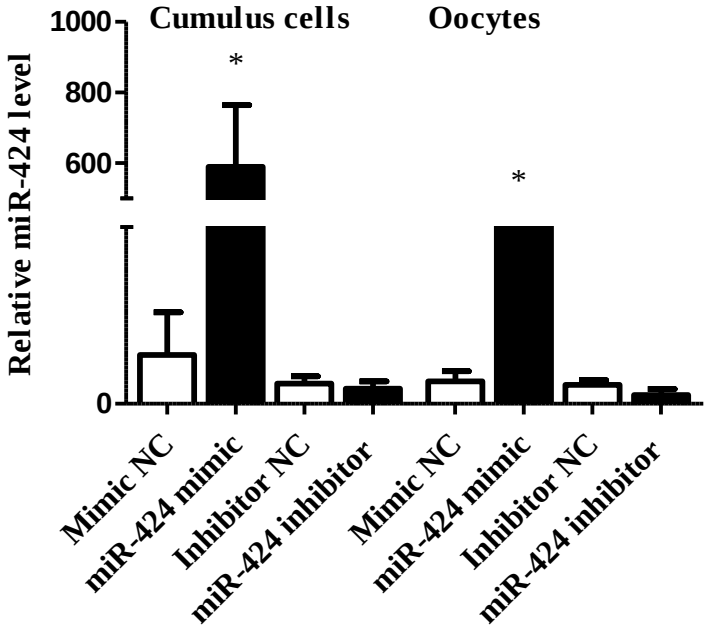

Figure 2. Relative expressions of miR-424 in cumulus cells and oocytes transfected with miR-424 mimic, inhibitor or corresponding negative controls (NC). The expression of 5S rRNA was used as internal control. The data are shown as mean ± SEM (n=3; *$p < 0.05$).

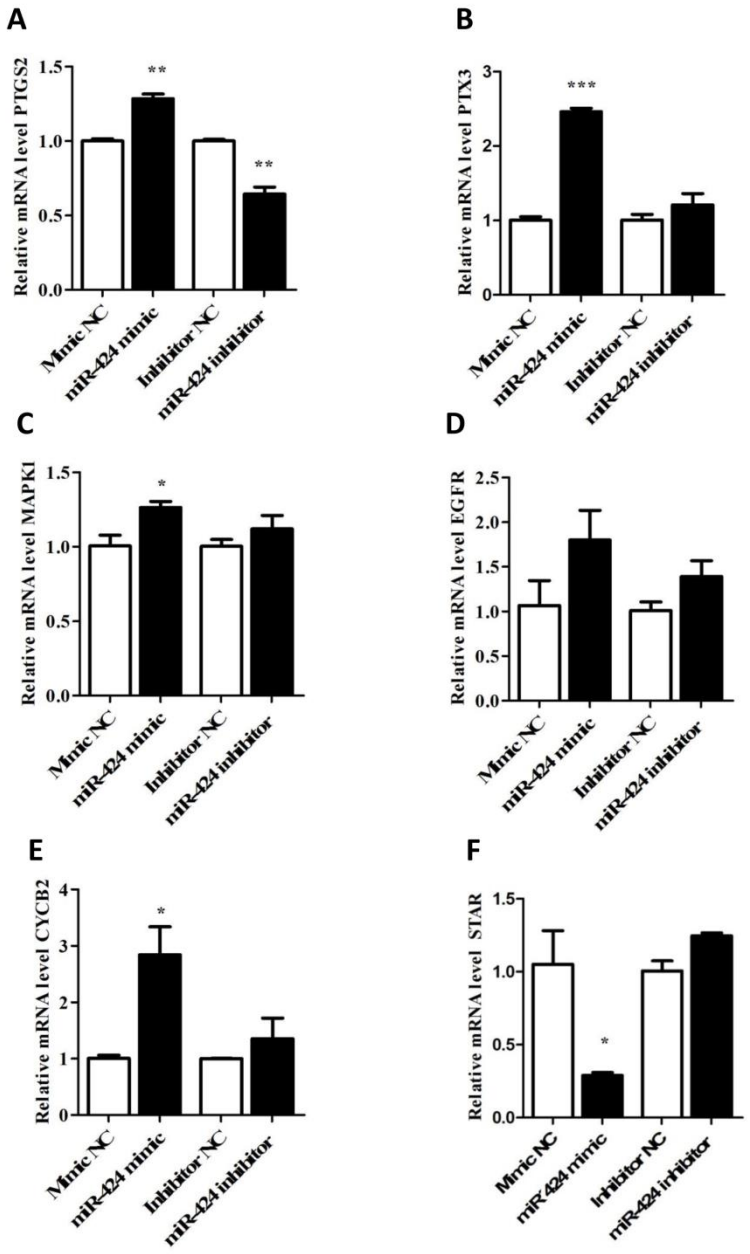

Figure 3. Overexpression of the miR-424 modulated gene expression associated with cumulus cell expansion, cell cycle and cell differentiation. qPCR analysis of cumulus expansion related genes PTGS2 (A), PTX3 (B), MAPK1 (C) and EGFR (D) in cumulus cells transfected with miR-424 mimic, inhibitor or corresponding negative controls (NC). qPCR analysis of cell cycle regulator gene CYCB2 (E) and cell differentiation marker gene STAR (F) in cumulus cells transfected with miR-424 mimic, inhibitor or corresponding NC. The data are presented as the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

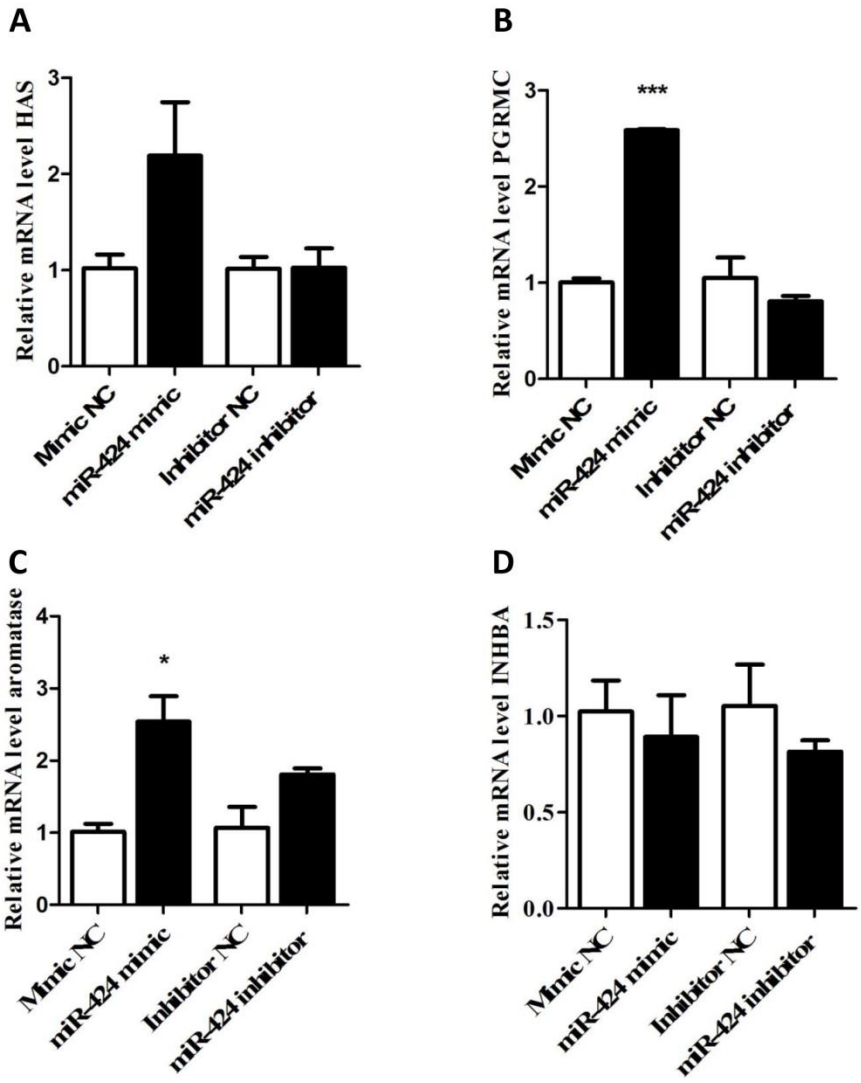

Figure 4. Modulation of miR-424 modulated the genes related to cumulus cell expansion. qPCR analysis of cumulus expansion related genes HAS (A), PGRMC (B), and Aromatase (C) and INHBA (D) in cumulus cells transfected with miR-424 mimic, inhibitor or corresponding negative controls (NC). The data are presented as the mean ± SEM (*$p < 0.05$, ***$p < 0.001$).

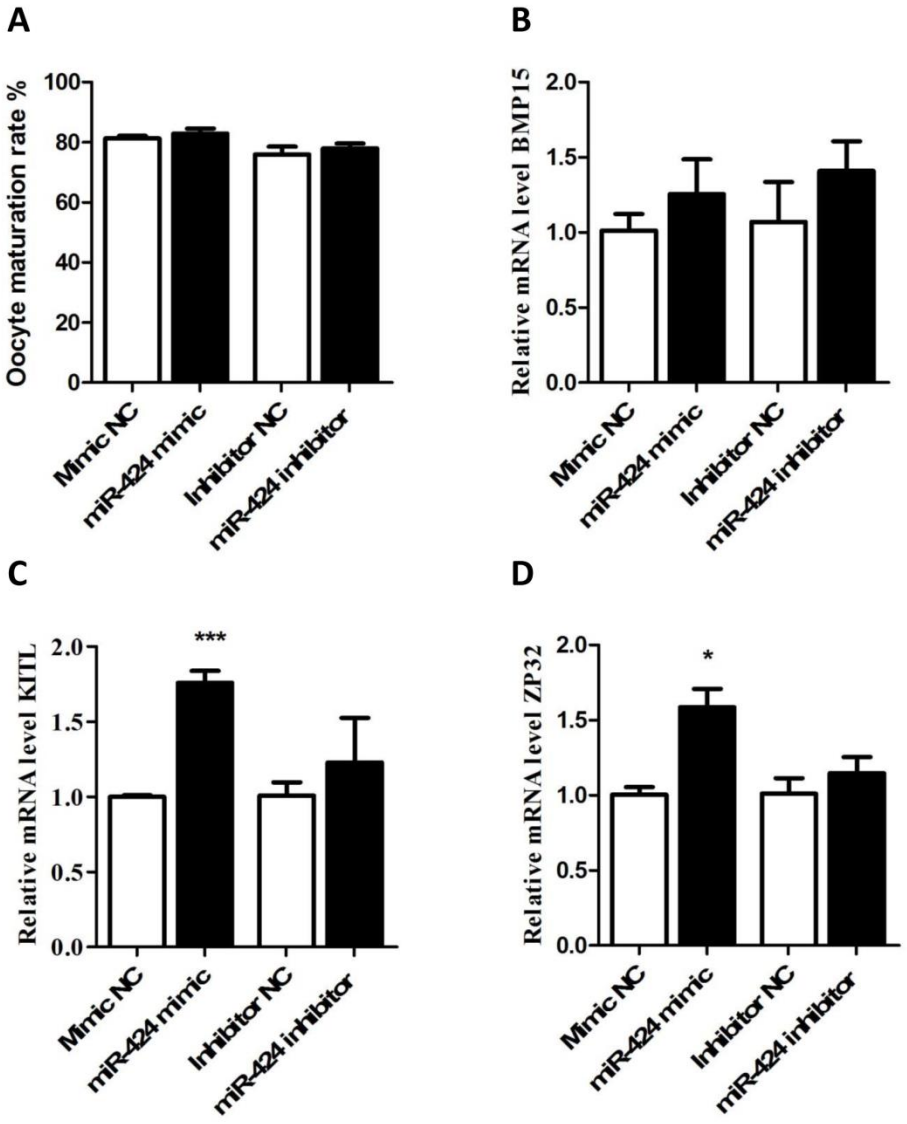

Figure 5. Transfection of the miR-424 mimic modulated the genes associated with oocyte maturation. Maturation rate of oocyte transfected with miR-424 mimic, inhibitor or corresponding negative controls (NC) (A). qPCR analysis of oocyte maturation related genes BMP15 (B), KITL (C), and ZP3 (D) in oocyte transfected with miR-424 mimic, inhibitor or corresponding NC. The data are presented as the mean ± SEM (*$p < 0.05$, ***$p < 0.001$).

Table S1. 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	β-ACTIN	F: TGTCCACCTTCCAGCAGAT	249
		R: TCACCTTCACCGTTCCAGT	
AF080219	CYCB2	F: TGCCACTCTTGTTTGTCCGT	246
		R: GGTTTCGGGTGCTTGTTGAC	
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_001076259	PTX3	F: ACCTGGGATTCAAAGAAAGG	208
		R: CACCCTCCCAGATATTGAAG	
NM_174445	PTGS2	F: CGATGAGCAGTTGTTCCAGA	215
		R: GAAAGACGTCAGGCAGAAGG	
NM_174189	STAR	F: AAATCCCTTTCCAAGGTCTG	204
		R: ACCAGCATTTCTGCTACTGC	
NM_173974.3	ZP3	F: GACCGACAATGCCCTGGTGTAC	214
		R: CTCCCCAGCTACTCACATTGCC	
NM_001075133	PGRMC	F: GCCTTTGCATCTTTCTGCTC	163
		R: ATGAGTATACGCGGGTCCTG	

NM_174375.2	KITL	F: TTTAATCCTCTCGTCCACACTC	164
		R: ATCTCGCTTATCCAACAATGAC	
NM_174305.1	Aromatase	F: TATTGCAAAGCATCCCCAGG	209
		R: CCTTTTTCACCGGGTAGCC	

Chapter 4

General discussion

The bovine dominant follicle has been a focal point in reproductive biology research for the last five decades to reveal the underlying mechanisms that ensure how a single follicle gets recruited, selected, becomes dominant, and eventually ovulates (Lucy 2007). Although, various biological and regulatory mechanisms are known, several key elements that could trigger the selection of one follicle to develop into a dominant follicle leaving rest follicles to regress in cattle remain unclear. This dynamic process is believed to be largely regulated by somatic cells like granulosa cells by producing several hormones, growth factors, autocrine and paracrine factors under the control of an array of genes (Richards 1994; Toda et al. 2012), through several molecular mechanisms and pathways. In this context, the transcript abundance in follicular cells during folliculogenesis indicated the spatiotemporal expression of genes (Nivet et al. 2013; Douville and Sirard 2014; Hatzirodos et al. 2014; Girard et al. 2015; Hatzirodos et al. 2015), which one way or the other, are under control of miRNAs. In line with this, increasing evidence supports the idea that miRNAs are a potential biomolecule that could epigenetically regulate the follicular development process (Gebremedhn et al. 2015; Andreas et al. 2016; Gebremedhn et al. 2016b; Maalouf et al. 2016).

Granulosa cells are the most important somatic cells, being vital and crucial for follicular development; to play this role precisely, the granulosa cell under goes a series of morphological and functional changes, among these changes cell proliferation and differentiation have been the most important cellular activity within the dominant follicle in the late phase of estrous cycle (Manikkam and Rajamahendran 1997; Robker and Richards 1998; Quirk et al. 2004). In our previous study, we demonstrated that the miR-424/503 cluster was upregulated in bovine granulosa cells from preovulatory dominant follicles compared to those derived from subordinate follicle (Gebremedhn et al. 2015), which

indicated the potential involvement of the miR-424/503 cluster in granulosa cell function in the establishment and maintenance of dominant follicle to support ovulation. Nevertheless, the reason why some cluster were highly abundant and what could be the role of these differentially expressed microRNA clusters in the bovine granulosa cells of dominant follicle compared to subdominant one is not known yet. Considering the fact, we believed that the miR-424/503 cluster might be certainly involved in the regulating granulosa cell function by targeting certain genes. Since, underlying mechanisms of the miR-424/503 cluster has not been investigated; hence we aimed to investigate functional involvement of miR-424/503 cluster in bovine granulosa cell function. Accordingly, we predicted target genes, identified potential putative target genes (SMAD7 and ACVR2A) (Matzuk et al. 1995; Gao et al. 2013; Li 2015) and validated the target genes (SMAD7 and ACVR2A) for miR-424/503 cluster. Both the target genes, SMAD7 and ACVR2A, are members of TGF-β superfamily, and recent studies have shown that miRNAs regulate granulosa cell function by targeting TGF-β superfamily members (Yan et al. 2012; Zhang et al. 2013; Liu et al. 2014b).

In the present study, we demonstrated the role of miR-424/503 in bovine granulosa cell function through targeting SMAD7 and further via coordinating activin signalling pathway. SMAD7 and ACVR2A genes, which are important in ovarian function (Matzuk et al. 1995; Gao et al. 2013; Li 2015). Further, this study showed that granulosa cell proliferation was significantly increased upon the overexpression of miR-424, which was further accompanied with upregulation of the PCNA gene. The evidence of the effect of modulating the expression of the miR-424/503 cluster on granulosa cell proliferation was accompanied by a shift in the proportion of cells from the G0/G1 phase to the S phase of the cell cycle. We found this finding consistent with those of several reports indicating that miRNAs are essentially involved in the regulation of granulosa cell proliferation (Yao et al. 2010; Liu et al. 2011; Yin et al. 2012; Jiang et al. 2015; Andreas et al. 2016; Gebremedhn et al. 2016b), which is

necessary for follicular growth and creation of the unique micro-environment for oocyte maturation (Maruo 1995). Here we suggest that miR-424/503 cluster through enhancing granulosa cell proliferation might be involved in establishment and maintenance of the dominant follicle; since studies indicate that proliferating granulosa cells in growing follicles depend on growth factors for their survival, which further promote the G1 to S phase transition of the cell cycle to prevent apoptosis in granulosa cells with a low-progesterone environment that helps to persist and sustain the dominant follicle (Lucy et al. 1990; Sirois and Fortune 1990; Savio et al. 1993). Further, in pursuit to cross validate the results obtained from miR-424/503 modulation, we performed targeted knockdown of SMAD7 and ACVR2A using siRNA. Interestingly, SMAD7 revealed similar phenotypic and molecular alteration as observed when miR-424-5p cluster was overexpressed in bGCs while ACVR2A failed doing so. These results suggests that the miR-424/503 cluster regulates the function of bovine granulosa cells by fine tuning the expression level of SMAD7, and it further suggests that genes merely being target of certain miRNAs could not necessarily qualify to regulate the phenotypes. However, depending on the strength of binding of miRNAs to target genes and context of biochemical function demand of cells are possibly major determinants while regulating the genes and phenotype of cells.

Several recent studies have shown that TGF-β superfamily members are involved in granulosa cell proliferation through post-transcriptional regulation by several classes of miRNAs (Yao et al. 2010; Zhang et al. 2013; Wang et al. 2016). Further, it has also been shown that SMAD7, through antagonizing the TGF-β superfamily, potentially regulate ovarian function (Gao et al. 2013; Li 2015). Our results showed that overexpression of miR-424/503 reduced SMAD7 expression and tended to increase the phosphorylated SMAD2/3. Considering the fact, we suggested that miR-424/503 cluster might be involved in fine tuning of the activin signalling pathway through regulation of the inhibitory effect of SMAD7.

Further, phosphorylated SMAD2/3 through forming heterodimeric complex with SMAD4 translocates into the nucleus, and consequently forms the SMAD2/3/4 complex along with other co-factors to regulate the transcription of target genes (Tsuchida et al. 2008).

Activin as an intraovarian factor plays an important role in follicular development by enhancing the proliferation and reducing the differentiation of granulosa cells and activates activin signalling pathway (Findlay et al. 2000; Knight and Glister 2001; Myers et al. 2008; Knight et al. 2012), we found addition of the activin A in vitro cultured granulosa cells resulted in enhanced proliferation in dose-dependent manner, as it has been reported previously (Zhang et al. 2013; Cheng et al. 2014). Moreover, treatment of granulosa cells with varying doses of activin A decreased the expression of STAR mRNA and protein, which in line with the report that activin A downregulates STAR expression and progesterone production in granulosa cells through SMAD2/3 phosphorylation (Chang et al. 2015), which ultimately helps to prevent or delay the premature luteinization of bovine granulosa cells (Knight et al. 2012). Interestingly, similar to activin A, the overexpression of the miR-424/503 cluster clearly reduced STAR expression, which suggests the potential role of the miR-424/503 cluster in the establishment and maintenance of the dominant follicle. The SMAD pathways are known to be an integral part of a range of biological processes and are errantly activated or inactivated under various biological conditions (Blahna and Hata 2012), and are also potentially involved in controlling the transcription of a variety of miRNA genes having distinct physiological significance (Blahna and Hata 2012). For instance, TGF-β induces both miR-216a and miR-217 in glomerular mesangial cells via SMAD binding elements (SBEs) in the miR-216 promoter (Kato et al. 2009). In contrast, TGF-β induced SMAD3/4 complex binding to the miR-24 promoter inhibits the expression of miR-24 in myoblasts (Sun et al. 2008). Interestingly, activin A treatment reduced the expression of miR-424/503 in a dose-dependent manner which indicated the possibility of a negative feedback

loop between the miR-424/503 cluster and activin-SMAD2/3 signalling. Likewise, presence of a potential feedback loop between miRNAs and the target gene was recently reported (Wang et al. 2016).

Further, several studies have shown that the dysfunction of granulosa cells may contribute to the abnormal folliculogenesis observed in ovarian pathophysiology (Erickson et al. 1992; Willis et al. 1998; Jakimiuk et al. 2001), although the underlying mechanism remains to be determined. Furthermore, it is also important that granulosa cells should proliferate in controlled manner to avoid granulosa cell tumours, which account for 2-3% of ovarian malignancies (Malmstrom et al. 1994). Here, we reveal for the first time that the miR-424/503 cluster is involved not only in the proliferation of granulosa cells but also in the balancing of the activin signalling pathway through regulating the expression of SMAD7 and ACVR2A in bovine granulosa cells. This coordination of the activin signalling pathway through miRNAs might lead to healthy granulosa cells by avoiding any deviation from normalcy, like granulosa cell tumours. Recent studies have shown that miRNA-424-5p suppresses the expression of SOCS6 in pancreatic cancer (Wu et al. 2013) and inhibits the Akt3/E2F3 axis and tumour growth in hepatocellular carcinoma (Zhang et al. 2014a). Here, we suggest with our results that perturbations in the expression of the miRNA-424/503 cluster might result in ovarian disorders such as PCOS (polycystic ovarian syndrome) and granulosa cell tumours. Therefore, these results *in toto* suggest that for normal cellular and tissue function, an optimal *miRNA milieu* is required.

Undoubtedly, the interaction between cumulus cells and oocytes is essential for proper maturation of oocytes, fertilization and further embryonic development (Eppig et al. 2001; Zhou et al. 2016; Matzuk et al. 2002; Gilchrist et al. 2004; Wigglesworth et al. 2013). Recent studies have demonstrated the functional role of specific miRNAs during follicular growth and development through regulating follicular cells function (Yao et al. 2010; Dai et al. 2013;

Zhang et al. 2013; Jiang et al. 2015; Andreas et al. 2016; Gebremedhn et al. 2016b; Wang et al. 2016). However, very little is known about the role of miRNA during oocyte maturation (Yao et al. 2014; Pan et al. 2015b). Our results obtained from miR-424/503 modulation in bovine granulosa cells directed us to further investigate the role of this micoRNA cluster during cumulus expansion and oocyte maturation which has not been revealed yet. In this study, we observed that miR-424 enhanced in cumulus cells and oocytes during in vitro maturation process, which indicated that miR-424, might be involved during cumulus cell expansion and oocyte maturation. This was in consistent with the study that demonstrated dynamic expression of several miRNAs between GV and MII stages of oocytes and increased levels of some selective miRNA transcripts (Gilchrist et al. 2016). In our previous study, we reported that miR-424 was differentially expressed between cumulus and oocytes before and after in vitro maturation (Abd El Naby, W S et al. 2013). Our results revealed that that overexpression of miR-424 enhanced cumulus expansion related genes (PTGS2, PTX3 and EGFR) (Hung et al. 2015); (Uyar et al. 2013) which are important during the oocyte maturation process (Hung et al. 2015). Similarly, in a recent study, microRNA-224 was found to be involved in the regulation of mouse cumulus expansion by targeting PTX3 (Yao et al. 2014), which plays a key role in the organization of the cumulus oophorus extracellular matrix (Salustri et al. 2004). Further, we also found that overexpression of miR-424 enhanced MAPK1, which is required for resumption of meiosis in the oocyte and expansion (mucification) of the cumulus oophorus (Su et al. 2003). Cumulus expansion is dependent on the stimulation of LH-induced epidermal growth factor (EGF)-like peptides, via the activation of protein kinase A (PKA). These stimuli enhance PTX3 (a component of the extracellular matrix of the cumulus oophorus) and other related (HAS2 and TNFAIP6) transcripts involved in the process of expansion in cumulus cells (Varani et al. 2002; Fülöp et al. 2003; Ben-Ami et al. 2006). Recently, few studies have demonstrated the role of miRNAs

in oocyte maturation and acquiring developmental competence (Pan et al. 2015b; Wright et al. 2016; Xiao et al. 2014). The increment of the CYCB2 gene expression in cumulus cells could be associated with the evidence that miR-424 promoted granulosa cell proliferation and was found to be upregulated in bovine granulosa cell of pre-ovulatory dominant follicle (Gebremedhn et al. 2015). This was accompanied by decrease in the differentiation marker gene STAR, which ultimately helps to delay the premature luteinization of bovine somatic cells (Knight et al. 2012; Shukovski and Findlay 1990).

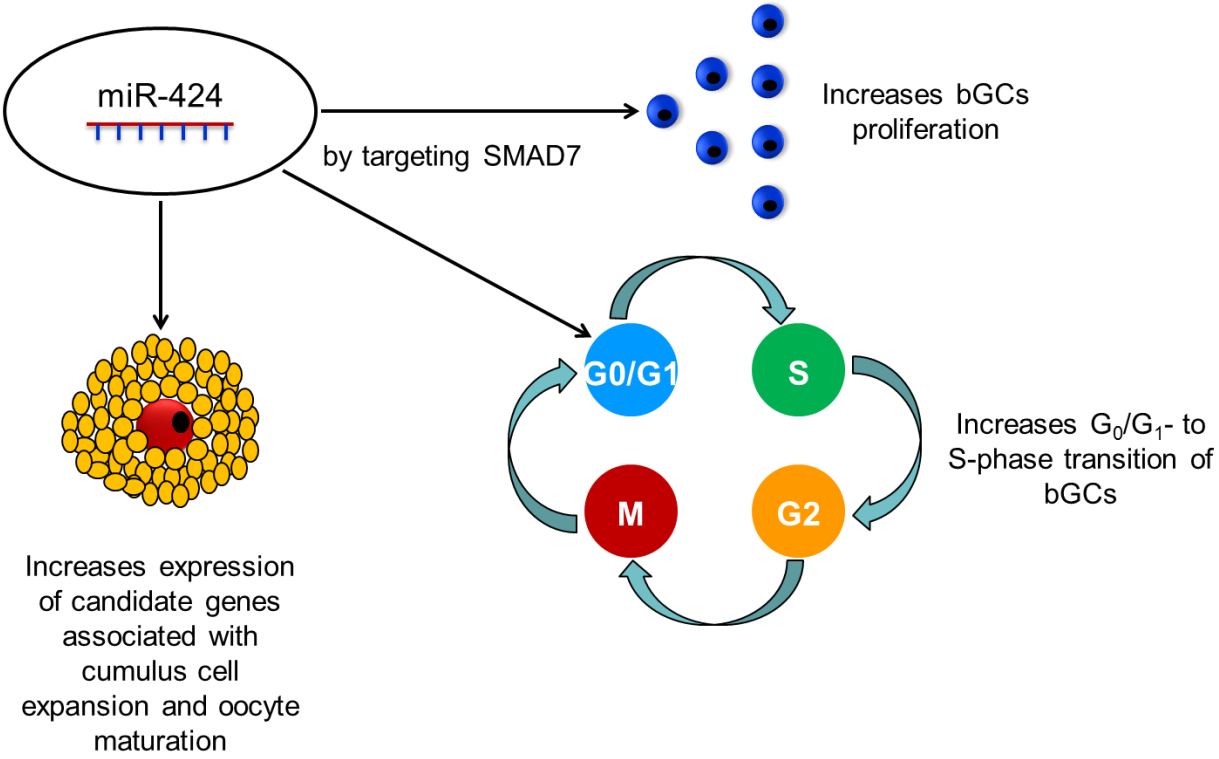

Figure 1. Schematic depiction showing conclusions of the chapter 1 and chapter 2 of the thesis. miR-424 by targeting SMAD7 increases bovine granulosa cell proliferation (bGC) and G0/G1 to S-phase cell cycle transition. miR-424 increases expression of candidate genes associated with cumulus cell expansion and oocyte maturation.

This result gives insight that miR-424 might be critically essential during oocyte maturation by involving in granulosa cell proliferation and subsequently cumulus expansion. In conclusion, this study demonstrates that the miR-424/503 cluster regulates bovine granulosa cell proliferation by targeting SMAD7 via activin signalling pathway, and enhances the candidate gene expression involved in cumulus cell expansion and oocyte maturation (Fig. 1). We believe that the present study would pave the way to further elucidate the molecular mechanisms underlying bovine granulosa cell function and oocyte maturation in vivo, and would facilitate a better understanding of follicular development and pathophysiology of some reproductive disorders associated with granulosa cells for the improvement of fertility treatments.

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5. Published research papers

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ii. Gebremedhn S, **Pandey H O**, Salilew-Wondim D, Hoelker M, Schellander K, Tesfaye D (2016) Dynamics and role of MicroRNAs during mammalian follicular development. Anim Reprod 13,257-263.

iii. **Pandey H O**, Tomar A K S, Triveni D (2013) Comparison of sire evaluation methods in Vrindavani cattle under organized herd condition. Indian J Anim Sci 83,419–422.

iv. Rijasnaz V V, **Pandey H O**, Patel B H M, Tomar A K S, Mondal S K, Singh G (2012) Effect of weaning on postpartum reproductive performance of Murrah buffaloes. LRRD 4,79.

v. Singh S K, Dimri U, Sharma M C, Swarup D, Sharma B, **Pandey H O,** Kumari, P. (2011) The role of apoptosis in immunosuppression of dogs with demodicosis. Vet Immunol Immunopathol 144,87-492.

vi. Srivastava M K, Gaikwad R G, Abdul S, Cahudhary T R, **Pandey H O (**2010) Standardization of glomerular filtration in dog by 99m-tc-dtpa scintigraphy. Indian J Vet Res 19,63-67.

7. Conference abstracts/posters

i. Alemu T W, **Pandey H O**, Gebremedhn S, Salilew-Wondim D, Neuhoff C, Tholen E, Held E, Hoelker M, Schellander K, Tesfaye D (2017) Endoplasmic reticulum and oxidative stress response menchnisms of bovine granulosa cells exposed to heat stress. Vortragstagung der DGfZ und GfT am 20./21. September 2017 in Stuttgart B17.

ii. **Pandey Hari Om**, Salilew-Wondim Dessie, Hoelker Michael, Neuhoff Christiane, Tholen Ernst, , Schellander Karl, Tesfaye Dawit (2016) MicroRNA-424/503 Cluster Orchestrates Bovine Granulosa Cell Function by Regulating Activin Receptor 2A. SSR 2016 System biology of reproduction abstract 208,79-80.

iii. **Pandey H O**, Salilew-Wondim D, Neuhoff C, Tholen E, Hoelker M, Schellander K, Tesfaye D (2016) The role of microRNA-424/503 in bovine granulosa cell function. Vortragstagung der DGfZ und GfT am 20./21. September 2016 in Hanover C02.