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Bc. Kateřina Marcollová

The role of microRNAs in regulation of mammalian oocyte and embryo development

Úloha mikroRNA v regulaci vývoje oocytů a embryí savců

Diploma thesis

Supervisor: MVDr. Radek Procházka, CSc.

Institute of Animal Physiology and Genetics CAS

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Prohlášení

Prohlašuji, že jsem tuto diplomovou práci vypracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Na primární analýze dat v programu QIAGEN CLC Genomics Workbench se podílel Dr. Ahmed Gad, PhD. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Praha, …… …..……

Podpis

Poděkování

Na tomto místě chci především poděkovat svému školiteli, MVDr. Radek Procházkovi, CSc., že mi umožnil práci a vypracování diplomové práce v Laboratoři vývojové biologie AV ČR a všem jeho kolegům. Školiteli děkuji a za jeho odborné znalosti, které mi při konzultacích sdělil. Moc si vážím lidského přístupu, vstřícnosti a trpělivosti během celého období působení v laboratoři. Chci poděkovat Dr. Ahmed Gad, Ph.D. za čas, který mi věnoval při analýze dat. Školiteli i celé rodině chci poděkovat za bezmeznou podporu během celého studia, především během 4-měsíční stáže v Madridu.

Contents

Abstrakt

Komplex kumulárních buněk a oocytu je nezbytný pro savčí reprodukci. Kumulární buňky pouze nevyživují oocyt, ale take představují důležitý komunikační bod pro předávání informací k a od oocytu. Nekódující miRNAs jsou schopny regulavat posttranskripční události a díky tomu mohou posloužit jako vhodný biomarker pro hodnocení stavu buněk. Na základě barvení COCs pomocí vitalní barvičky Lissamine Green B jsme kumulární buňky rozdělelili na více a méně kvalitní skupinu. Navíc jsme začlenili rozdělení podle GV a MII vývojového stadia. Analýza sekvenačních dat neukázala signifikantní rozdíly v DE miRNA z kvalitativně rozdílných stádií. Nicméně, rozdíly v DE miRNA byly detekovány mezi dvěma vývojově rozdílnými stádii. Identifikovali jsme např. ssc-miR-183, ssc-miR-182, a ssc-miR-21-5p jako značně downregulované v porovnání GV a MII stadia. Členové let-7 rodiny (let-7c, let-7a, let-7f-5p), ssc-miR-16, ssc-miR-21-5p a ssc-miR-125a patřily k nejvíce exprimovaným miRNA ve všech vzorcích. Geny, ke kterým se vztahovaly DE miRNA se týkaly především ErbB, TGF-β, MAPK, FoxO, gap junction a cGMP signálních drah. Usuzujeme, že jednotlivé miRNA v kumulárních buňkách pravděpodobně nemohou sloužit jako spolehlivý indikátor kvality oocytu. Na druhé straně, změny v expresi miRNA v kumulárních buňkách během zrání COCs by mohly sloužit jako orientační marker.

Klíčová slova: miRNA, kumulární buňky, rozdílná exprese, kvalita oocytu, prase

Abstract

Cumulus-oocyte complex (COCs) is crucial for mammalian reproduction. Cumulus cells not only nurture the oocyte; they also represent important communicational nodes for mediating information towards and from the oocyte. The non-coding miRNAs can modulate posttranscriptional events they might serve as a useful biomarker for evaluating cell conditions. Based on the COCs staining with vital Lissamine Green B stain we divided cumulus cells into high- and low-quality ones. Furthermore, we implemented division based on the maturation stage, the GV and MII. Sequencing data analysis showed that DE miRNAs from qualitatively different stages do not significantly vary. Nonetheless, significantly DE miRNAs were detected between two developmentally different stages. We identified e.g. ssc-miR-183, ssc-miR-182, and ssc-miR-21-5p to be highly downregulated when comparing GV to MII stage. Among the highly expressed miRNAs from all samples were members of let-7 family (let-7c, let-7a, let-7f-5p), ssc-miR-16, ssc-miR-21-5p, and ssc-miR-125a. Targeted genes by the DE miRNAs were involved in ErbB, TGF-β, MAPK, FoxO, gap junction and cGMP signalling pathways. We conclude that single miRNAs in cumulus cells probably cannot be used as a reliable oocyte quality marker. On the other hand, changes in the miRNA expression in the cumulus cells could serve as indicative markers during COCs maturation.

Keywords: miRNA, cumulus cells, differential expression, oocyte quality, porcine

Abbreviations

Ago	Argonaut protein
AKT	proteinkinase B (synon. PKB)
BMP-15	bone morphogenic protein 15
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCs	cumulus cells
cGMP	cyclic guanosine monophosphate
COCs	cumulus-oocyte complex
DE	differential expression
DGCR8	DiGeorge critical region 8 protein
EGF	epidermal growth factor
ErbB	epidermal growth factor receptor
FGF	fibroblast growth factor
FSH	follicle-stimulating hormone
GDF-9	growth differentiation factor-9
HAS2	hyaluronan synthase 2
hCG	human chorionic gonadotropin
IGF	insulin-like growth factor
KIT	protoonco gene c-KIT
LB	Lissamine Green B
LH	luteinizing hormone
LHR	luteinizing hormone receptor
LIF	leukaemia inhibitory factor
MAPK	mitogen-activated protein kinase
miRISC	miRNA-induced silencing complex
miRNA	micro ribonucleic acid
MII	metaphase II
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline
PCOS	polycystic ovary syndrome
PDE	phosphodiesterase
PI3K	phosphatidylinositol 3-kinase

PKB	protein kinase B
PMSG	pregnant mare’s serum gonadotropin
pre-miRNA	miRNA precursor
pri-miRNA	long primary miRNA molecule
PTGS2	prostaglandin-endoperoxide synthase 2
RAS	rat sarcoma small GTPase
TGF-β	transforming growth factor beta
UTR	untranslated region

1 Introduction

All animals on the Earth have to reproduce for species preservation. Mammalian reproduction is a complex set of biological processes where at the beginning the male and female gamete must fuse. The female gamete – the oocyte – is the biggest mammalian cell and carries half of the genetic and epigenetic information the embryo gets. The oocyte is surrounded by supporting cumulus cells (CCs) that together with the oocyte create a functional unit the cumulus-oocyte complex.

All cells are regulated mainly by the genetic information they have encoded in the nucleus. However, with rising interest in the non-coding part of the genetic information, the miRNAs that regulate the posttranscriptional events make it available to look deeper into cell regulation. This interest is nicely visible in the number of research papers that are published every year.

Taking together the beauty of the oocyte and the undiscovered universe of miRNAs there is a whole new world where we can unveil hidden secrets. As the oocyte develops from the raw germinal vesicle stage to the metaphase II (MII) stage in which it can be fertilized, simultaneously the cumulus cells mature as well. These and other environmental condition changes make a footprint on the miRNA network that can fine-tune metabolic pathways important for development and maturation. Thinking of the uniqueness of the oocyte, using cumulus cells as a biomarker may serve as a non-invasive marker in evaluating the quality of the oocyte.

In this thesis, I want to focus on oocyte maturation, miRNA biogenesis and the role of miRNAs in cumulus cells. The practical part is focused on the different quality and developmental stages of cumulus cells and their differentially expressed miRNAs. In more detail, this work focuses on sequencing data analysis along with biochemical pathways targeted by the expressed miRNAs.

2 Literary overview

Female fertility is a precisely coordinated process where various tissues and organs work together. This complex process results in fertilizable oocytes continuing with embryogenesis, and later with fetal development.

2.1 Structure of ovarian follicle

The female reproductive tract contains a variety of organs. In the ovary, the relationship between the oocyte and the surrounding somatic cells is very intimate. The oocyte itself is unable to create a functional unit, the ovarian follicle, on the other hand, somatic cells cannot substitute the reproductive function of the oocyte. Ovaries at the adult age have follicles of various sizes. The pool of the smallest primordial follicles is already set during embryonic development which is important for the continuity of folliculogenesis throughout the female's reproductive lifespan. Oocyte in this stage is paused in the prophase of the first meiosis and is encased by one flattened layer of somatic cells called granulosa. Reaching the puberty age, periodically repeating cycles of primordial follicles recruitment from the existing pool result in ovulation of one or more oocytes. Primary follicles are recruited from the primordial pool as oocytes grow and the granulosa cells become cuboidal and proliferative. Later, the secondary follicle creates an antrum filled with follicular fluid that differentiates somatic cells into various populations. The oocyte at this time point is covered by *zona pellucida*, the glycoprotein protective sheet, and the oocyte acquires the capacity to resume meiosis again. The largest preovulatory follicle is commonly called the Graafian follicle where *cumulus oophorus* cells penetrate *zona pellucida* and are capable of transmitting molecules straight to the oocyte. Cumulus cells around the oocyte create ~10 layers of protective, nurturing and metabolism-supporting sheet. Inner follicular cells are called mural granulosa cells and many of them are organized as pseudostratified epithelium attached to the basal membrane. The most outer somatic cells in the follicle, so-called theca cells are in the proximity of a rich vascular network and so represent an important communication node. (Zhao and Dean, 2002)*

Until the preantral stage, the follicle is independent of gonadotropic stimulation. However, later the follicle requires follicle-stimulating hormone (FSH) stimulation and every menstrual cycle a cohort of follicles is recruited. The continuous surge of FSH and luteinizing hormone (LH), supported by local estradiol production, causes the selection of one dominant follicle for ovulation that is released into the oviduct, the rest undergoes atresia. (Hillier, 1994*; Matzuk *et al.*, 2002*)

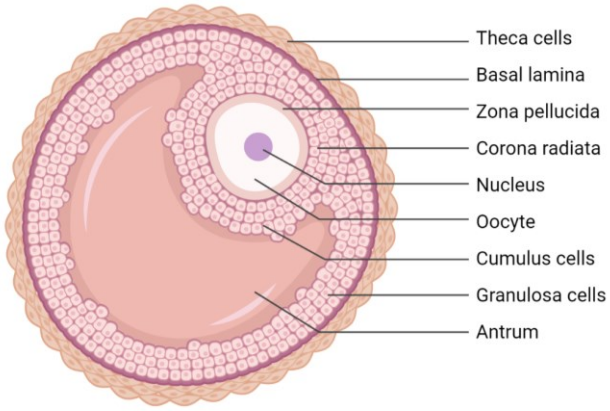


Figure 1 ***Structure of mammalian preovulatory follicle.*** *Oocyte arrested in prophase I is surrounded by layers of cumulus cells that are attached to granulosa cells in one part. Antrum filled with fluid separates somatic cells. Created in Biorender.com*

2.2 Oocyte development

Meiosis, reducing division, serves for the production of haploid gamete. The reduction of homologous chromosomes from two to one is a process of high importance. After fertilization, the fusion of two haploid sets of chromosomes leads to the development of a diploid organism with a complete set of chromosomes. In mammal females, meiosis starts during embryogenesis when the oocyte's DNA duplicates, recombination occurs and is then stopped until puberty. The oocyte grows to approximately 70-120 μm in diameter, depending on the species.

Proper oocyte development relies on bidirectional communication of the oocyte and the surrounding somatic cells via gap junctions. These channels allow exchanging low molecular weight molecules e.g.: cAMP, cGMP, growth differentiation factor-9 (GDF-9), TGF-β, protoonco gene c-KIT (KIT) and bone morphogenic protein 15 (BMP-15). This network of gap junctions units all surrounding cell types with the oocyte into a functional syncytium. Transzonal projections form a communicational bridge that connects cumulus cells with the oocyte via *zona pellucida*. The prophase-arrested oocyte is held in the growth phase mainly due to the increased concentration of cyclic nucleotides. The high concentration of cyclic adenosine monophosphate (cAMP) generated by adenylyl cyclase in the oocyte is essential for the first meiotic arrest.

A high concentration of cyclic guanosine monophosphate (cGMP) is defunding into the oocyte from granulosa cells. The cGMP is effectively blocking phosphodiesterase (PDE)

that would otherwise hydrolyse cAMP in the oocyte and so decrease its concentration which would result in meiotic division resumption.

Reaching puberty under the stimulus of gonadotropin hormones released from the pituitary gland the oocyte restores meiosis. Once the FSH and LH peaks occur the concentration of cGMP in the cumulus cells rapidly decreases, the PDE can hydrolyse cAMP and the oocyte is liberated of the meiotic arrest and continues its cell cycle into M-phase.

The nucleus of the oocyte, the so-called germinal vesicle (GV) first undergoes nuclear membrane breakdown, chromatin condensates and the oocyte passes through the first heterotypic meiotic division, ending with the oocyte in the MII phase and extruded first polar body. These processes are collectively referred to as "oocyte maturation". In most mammals, ovulation occurs in the MII stage. The last step of meiosis, the homotypic division, happens in mammals first when the oocyte is fertilized by sperm in the oviduct and the second polar body is released. (Baudat *et al.*, 2013*; Jaffe and Egbert, 2017)*

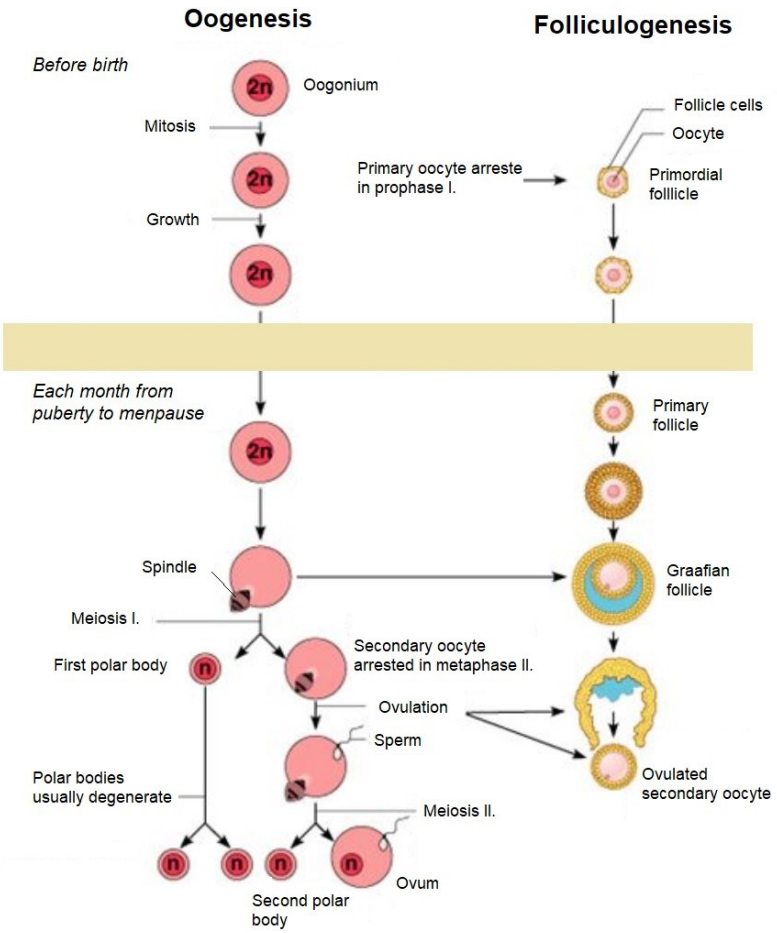


Figure 2 ***Oogenesis in the context of folliculogenesis****. Overtaken and edited from https://images.slideplayer.com/36/10586797/slides/slide_49.jpg*

2.2.1 Cumulus cells

Somatic cells in the proximity of the oocyte undergo dramatic conformational change before ovulation. As it was mentioned previously, surrounding cumulus cells have an important supporting function. Granulosa cells express receptors for luteinizing hormone and the LH signal is transmitted to CCs via EGF-like factors. Importantly, cumulus cells from growing follicles do not have luteinizing hormone receptors (LHR) on their surface. The LHR is expressed on CCs first during the maturation and they react to FSH and EGF-like factors stimuli (Procházka *et al.*, 2009). Through this stimulation, they transmit the information to the oocyte. Cumulus cells also secrete hyaluronic acid, a non-sulphated glycosaminoglycan, in high doses which after hydration expand the intercellular space with mucified matrix. This event is commonly called cumulus expansion. It is mainly due to the increased expression of hyaluronan synthase 2 (HAS2) and genes coding the hyaluronan-binding proteins. Furthermore, it was shown that expression of prostaglandin-endoperoxide synthase 2 gene

(PTGS2) in granulosa and cumulus cells is required for normal ovulation (Eppig, 2001*; Nagyová, Němcová and Camaioni, 2021*). Prochazka and Nemcova (2019) demonstrated that FSH is important for the activation of a central regulator MAPK3/1 in cumulus cells during maturation *in vitro.*

2.3 MiRNAs biogenesis

Lately, the rising interest in post-transcriptional events was caused by the discovery of non-coding RNAs, including small microRNAs (miRNAs). The miRNA pathway provides efficient and selective repression of expressed genes. The biogenesis of miRNAs starts with the transcription of miRNAs genes by either RNA polymerase II or RNA polymerase III that generate long primary miRNA (pri-miRNA) molecules. Pri-miRNAs carry one or more inner stem-loop structures that are later released and trimmed by the complex of RNase II enzyme Drosha and DiGeorge critical region 8 protein (DGCR8) into ∼70-nucleotide (nt) hairpin miRNA precursor (pre-miRNA) (Lee *et al.*, 2003)*.

The pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin-5 and Ran-GTP co-factor where they are subsequently cleaved by RNAse III Dicer enzyme into the product of approximately 22-nt long miRNAs duplex with a precisely defined sequence. Argonaut protein (Ago) with the bound miRNA duplex is loaded into the miRNA-induced silencing complex (miRISC). The miRISC can recognize binding sites within the 3′-untranslated region (UTR) of the target mRNA and the proximity of mRNA and miRNA within miRISC leads to mRNA degradation or repression of protein translation. The miRNA and mRNA rely mainly on the base pairing of the so-called seed region (2-8 nt) principally, the rest shows impaired base paring (Svobodova, Kubikova and Svoboda, 2016)*.

MiRNAs do not have to be generated only by the canonical pathway nonetheless, the non-canonical pathway can bypass the step of Drosha-mediated cleavage. Instead of cleavage, the miRNAs are created by splicing the introns straight from the mRNAs transcript. The conditional knockout of enzymes involved in the miRNA processing proved to be a powerful tool in elucidating the miRNA role in the regulation of female fertility. The Drosha/DGCR8-, Dicer- or Argonaut-knockout mice display severe abnormalities in various reproductive functions including a reduction in ovulation rate, defective spindle formation and chromosome arrangement in oocytes furthermore, there was observed an increase in embryonic lethality and abnormal development of all reproductive organs (Tesfaye et al., 2018)*.

Generally, some miRNAs are circulating in the extracellular environment. These miRNAs were identified in most biological fluids usually associated with extracellular vesicles, lipoproteins and Argonaut proteins but the majority of identified miRNAs are intracellular (Tesfaye *et al.*, 2018)*.

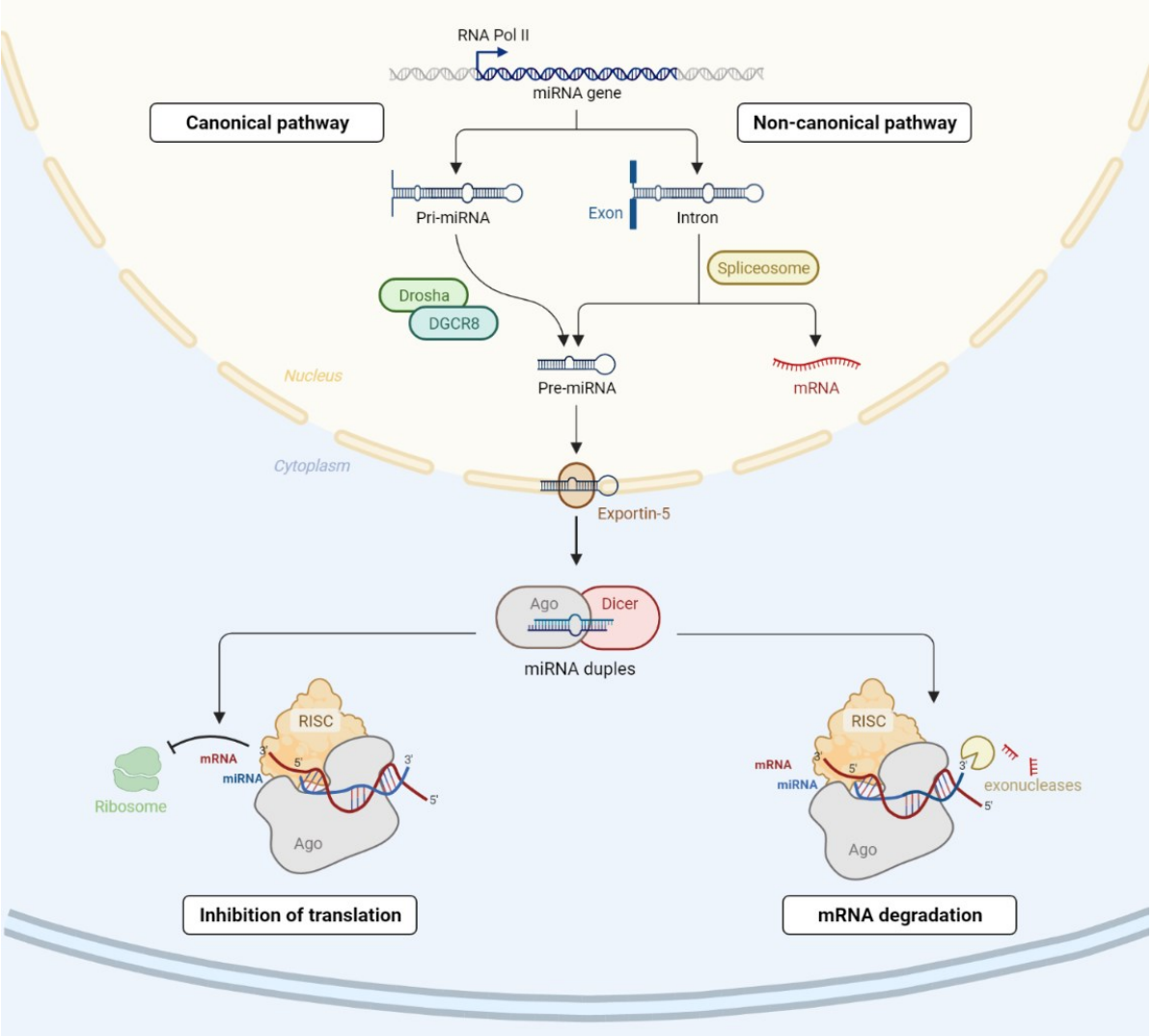


Figure 3 ***Scheme of miRNA biogenesis and its biological outcome.*** *Created in Biorender.com*

2.4 Role of miRNAs during oocyte development

Conditions for proper organism development are achieved not only via hereditary gene sets but also through transcriptional and post-transcriptional regulations. Generally, already 15 years ago it was shown that miRNAs are essential for ordinary mammalian oocyte maturation and early embryonic development (Tang *et al.*, 2007) and that oocyte and cumulus cells exchange messages via secreted factors that lead to a rich conversation (Gilchrist, Lane and Thompson, 2008).

2.4.1 MiRNAs in extracellular vesicles

One part of the miRNA research focuses on extracellular vesicles (EVs). EVs are cell-secreted lipid vesicles that mediate cell-to-cell communication and that are present in a variety of body fluids (Valadi *et al.*, 2007). EVs can be differentiated based on size and origin into apoptotic bodies, microvesicles (100-1000 nm) and exosomes (30-150 nm). All these vesicles can uptake bioactive cargo molecules such as proteins, lipids, DNA fragments and mRNA or miRNAs. In the past few years, EVs and their content gained researchers' attention as potential biomarkers and supplements mimicking *in vivo* conditions (Zaborowski *et al.*, 2015)*.

The size and the diameter of EVs remain the same during the follicle development - from small to medium, and large follicles. What differs is the concentration of EVs in the follicular fluid that is progressively decreasing as the follicle grows (Navakanitworakul *et al.*, 2016).

Circulatory EVs are found in a variety of body fluids. Lopera-Vasquez *et al.*, (2017) demonstrated that EVs derived from isthmus oviductal fluid improve the quality and survival rate of *in vitro* cultured bovine embryos, that were vitrified and later warmed up again. Alcântara-Neto *et al.*, (2020) demonstrated that EVs isolated from porcine oviductal fluid influence decreasing polyspermy when supplemented while fertilization. This indicates that EVs carry some cargo that has the potential to enhance the *in vitro* conditions.

RNA sequencing analysis of EVs from bovine follicular fluid revealed transcripts related to chromatin remodelling and transcriptional regulation. Furthermore, EVs treatment during *in vitro maturation* (IVM) of bovine embryos enhanced the number of blastocysts and there were found more transcripts of genes related to embryonic metabolism and development (da Silveira *et al.*, 2017). Interestingly, co-cultivation of exosomes derived from the follicular fluid can reduce the effect of heat stress (t=41°C) on bovine *in vitro* cultured oocytes and buffer the negative environmental issues (Rodrigues *et al.*, 2019). It is important to note, that the uptake of EVs from the cultivation media occurs via granulosa and cumulus cells that subsequently transmit the EVs to the oocyte by transzonal projections (da Silveira *et al.*, 2017). From this point of view, the EVs and their content can serve as perspective biomarkers.

Extracellular vesicles can intake molecules that reflect physiological conditions. Follicular fluid and blood plasma of hyperstimulated and unstimulated cows showed differences in up- and downregulated miRNAs. (Noferesti *et al.*, 2015; Gad, Sánchez, *et al.*, 2020). Differences after controlled ovarian stimulation were also observed in serum samples

of normal- and hyperstimulated women patients. Poor responders have elevated amount of miR-150-5p, miR-223-3p, let-7d-3p, miR-891a-5p, miR-99a-3p and, miR-200c-5p (Borges Júnior *et al.*, 2019).

In 2014 Diez-Fraile *et al.*, showed that there are differences in miRNA expression in follicular fluid of younger (<31 years) and older (>38 years) women. Hsa-mir-21-5p was present at higher levels in young women whereas hsa-mir-99b-3p, hsa-mir-134 and miR-190b were present at significantly higher levels in the follicular fluid in older women. Potentially targeted genes were involved for example in heparan-sulphate biosynthesis, extracellular-matrix-receptor interaction or 53-signalling pathway, which are pathways important for normal COCs development.

Oestrous physiological changes can be demonstrated either on the EVs miRNA cargo (da Silveira *et al.*, 2014) or on miRNAs dispersed in the follicular fluid (Capra *et al.*, 2020). The progesterone level in follicular fluid changes dramatically during the oestrous cycle. It was observed that DE miRNAs embedded in EVs from low compared to high progesterone group can moderate for example MAPK, Hippo, Cell cycle, FoxO, oocyte meiosis or TGF-β pathways important for biological processes such as proliferation and differentiation. The addition of labelled EVs into IVM showed the uptake of EVs by cumulus cells occurs from the first hour of COCs IVM. Furthermore, it was demonstrated that supplementation of EVs can impact the miRNA profile of cumulus cells (de Ávila *et al.*, 2020)*.

An interesting study led by Matsuno *et al.*, (2019) aimed to compare the mRNA content of EVs from porcine follicular fluid with granulosa cells from the follicle. They found that mRNAs in the EVs have connections to PI3K-AKT and MAPK pathways. Moreover, mRNAs from the EVs were completely different from those found in granulosa cells. It is believed that the majority of the EVs in the follicular fluid originates from granulosa cells. On the contrary, this study showed that the source of EVs can also originate from the oocyte, cumulus, theca cells, or non-ovarian tissues. Taking this into account, miRNAs should be of the same origin as well. These findings were supported by Uzbekova *et al.*, (2020) who focused on the protein cargo of EVs from bovine follicular fluid derived from 3-6 mm follicles. They identified proteins linked to ribosome and protein processing in endoplasmic reticulum pathways.

Body fluids slightly vary and differ depending on what fluid we examine. Ranjbaran *et al.*, (2019)* review that the content of EVs has the potential to influence signalling pathways in cumulus cells such as MAPK, TGF-β, ErbB or Wnt. These pathways were also commonly

shared by exosomal miRNAs derived from women's follicular fluid and plasma (Santonocito *et al.*, 2014).

Inoue *et al.*, (2020) led a complex study on porcine granulosa cells, serum and spent culture medium. miR-17, miR-27, miR-92a, and miR-145 embedded in EVs were differentially expressed in all studied environments comparing small and large follicles. It was shown that the above-mentioned miRNAs' mimics can influence the antrum formation and diameter, together with histone acetylation and chromatin configuration in the oocyte. The developmental ability of oocytes to the blastocyst stage was also improved when using these miRNA mimics. On the other hand, exosome depletion from the follicular fluid worsened all observed parameters.

2.4.2 Cellular miRNAs

MiRNAs are generally found inside the cells. For example, granulosa cells isolated from small and large follicles differ in their miRNA expression (Toms *et al.*, 2021). Search for markers in cumulus cells is of particular interest. It is mainly due to the possibility of having a non-invasive method for evaluating the oocyte's quality without destroying the gamete. It seems that there is a high degree of gene expression conserved in mammalian species. One of the research papers focusing on cumulus cells is from Tong *et al.*, (2014). This comparative miRNA analysis of human *corona radiata* and cumulus cells detected that the miR-let-7 family was the most abundant in both cell populations. The let-7 family has been reported to regulate for example follicular development or oocyte growth and is generally highly expressed in a variety of cell types (Khan *et al.*, 2015; Uhde *et al.*, 2017; Gad *et al.*, 2020)

Another study focusing on human miRNAs was led by Karakaya *et al.*, (2015) who were interested in women undergoing in vitro fertilization-intracytoplasmic sperm injection (IVF-ICSI) and their responsiveness to hormonal stimulation. It was shown that poor responders had upregulated miR-21-5p and miR-let-7f in the cumulus cells when compared to ordinary responders. On the other hand, the miR-150 was significantly downregulated which also correlates with the findings on a murine model (Khan *et al.*, 2015). In mouse ovaries, the mmu-miR-1298 was highly upregulated.

Focusing on women's reproduction polycystic ovarian syndrome (PCOS) is a common endocrine disorder which causes ovulatory dysregulations. Aberrant miRNA expression in a variety of tissues seems to play an important role in PCOS and potential markers are of high

interest in this case (Deswal and Dang, 2020; Gebremedhn *et al.*, 2021). Granulosa cells screening revealed that hsa-miR-3188 and hsa-miR-3235b are significantly upregulated in PCOS patients compared to the control group (Hou *et al.*, 2019).

Already years ago it was shown that the comparison of bovine oocytes during the maturation from GV and MII stages differ in mRNA levels (Fair *et al.*, 2007). In more detail, in bovine cumulus cells, it was shown that miR-21 and miR-155 are among the most highly expressed miRNAs (Uhde *et al.*, 2017). Bovine cumulus cells from GV and MII stages indicate that miR-375 was downregulated in the MII stage and that it can repress the COCs maturation. (Zhang *et al.*, 2019). The gain- and loss-of-function approaches in the bovine model showed that miR-130b is important for cumulus and granulosa cells proliferation and their survival (Sinha *et al.*, 2017). This finding is supported also by the porcine model where the miR-101 targets HAS2 and can regulate cumulus cell expansion during IVM (Luo *et al.*, 2022). Furthermore, the role of miRNAs seems to be highly important for morula and blastocyst formation not only in bovines but also in humans (Bartolucci, Uliasz and Peluso, 2020). Divergently, Green *et al.*, (2018) did not find any significantly DE miRNAs when comparing cumulus cells transcriptome from embryos that resulted in live birth and those that failed to develop.

Regarding the bovine oocyte, there was observed a dramatic decrease in miRNA abundance, namely of miR-205, miR-150, miR-122, miR-96, miR-146a and miR-146b-5p, from 0 h to 22 h after the beginning of maturation. Unlike the oocyte, there was not any decrease observed in cumulus cells. Interestingly, the expression of the same miRNAs in preimplantation embryos copied the typical maternal transcription profile (Abd El Naby *et al.*, 2013). In contrast to the previous study, Kim *et al.*, (2013) observed a reduction in miRNAs (miR-let-7b, miR-let-7c, miR-27a and miR-322) expression which were isolated from mouse granulosa cells in two developmentally distinct stages, the MI and MII stage.

The concentration decrease of miRNAs was also observed during murine oocyte maturation. Kataruka *et al.*, (2020) explain that as the oocyte grows, it lacks to accumulate miRNAs throughout the growing phase in contrast to mRNA accumulation. The oocyte grows in size, the miRNA:mRNA ratio is shifted and consequently, the maternal miRNAs lose their potential to influence the oocyte. For example, the miRNA concentration in the fully grown oocyte is 100-times lower than in somatic cells. Injecting only a few miRNA molecules into the mouse or pig oocytes can restore its function. It appears that this phenomenon is a concentration- and dilution-related issue. On the other hand, porcine ssc-miR-205 and bovine

bta-miR-10b are noteworthy miRNAs that are maintained in high concentration in fully grown oocytes and can effectively suppress gene expression (Kataruka *et al.*, 2022).

BMP15 and GDF-9 are members of the transforming growth factor beta (TGF-β) superfamily and are generally vital for oocyte and cumulus cells communication, mainly for regulating cumulus cells expansion. In bovine cumulus cells, it was shown that miR-375 plays an important role in maintaining the expression level of the BMP receptor. If miR-375 was overexpressed it led to decreased BMP receptor density and cumulus cells proliferation was diminished (Chen *et al.*, 2017). Cumulus cell-oocyte communication can be interrupted by overexpression of miR-224, as the cumulus expansion was interrupted and gene expression associated with cell expansion was decreased, the oocyte was subsequently blocked in the GV stage. Genes that are important for oocyte maturation (GDF-9, BMP15 and ZP3) were shown to be downregulated. A deeper investigation revealed that miR-224 and miR-205 influence the expression of mRNA and protein of PTX3, PTGS2 and HAS2 which are important for cumulus cells development and expansion. *In silico* it was found that miR-205 has a putative matching site present in the 3´UTR of PTX3 mRNA. A significantly lower level of miR-224 was detected in CCs from MII oocytes, as compared to those of the GV stage. When retarding force of the miR-224 was inhibited, CCs expansion increased and oocytes matured into a further stage. However, decreased miRNA expression does not have to occur straight in the oocyte but can be mediated via the paracrine pathway from granulosa and cumulus cells (Li *et al.*, 2016, 2017).

Wright *et al.*, (2016) showed that expression of miR-21 increased 25-fold in cumulus cells during in vitro maturation and it seems that impacts subsequent embryo development in the pig. The miR-21 binding site is present at the 3´UTR of the TIMP3 mRNA. TIMP3 is an inhibitor of metalloproteases that are involved in remodelling the extracellular matrix in COCs. In addition, the investigation of cumulus expansion-related genes indicated that HAS2 was significantly increased after miR-21 transduction (Bo Pan and Julang Li, 2018). Focusing on DE genes in cumulus cells cytoskeleton rearrangement, cell cycle regulation and cell adhesion were among the most overpopulated network associated with cumulus cells competence (O'Shea *et al.*, 2012). However, it is important to note, that the expression profile of oocyte and surrounding cumulus cells may vary (Andreas *et al.*, 2021).

2.4.3 MiRNAs in conditioned media

Conditioned media provide a source of miRNAs that were secreted by cells during culture. Lin *et al.*, (2019) individually cultured bovine embryos and based on the cleavage

speed they showed that the conditioned medium of slowly cleaving embryos was rich in miR-30c and miR-10b. The supplementation of miR-30c mimics resulted in low-quality embryos with increased cell apoptosis. Bovine and human individually cultured preimplantation embryos that later degenerated expressed a special set of miRNAs. These miRNAs were also abundant in culture media where these blastocysts were cultured (Kropp, Salih and Khatib, 2014).

A comparison of spent blastocyst culture media and blastocysts revealed that ~97% of detected miRNAs in the culture media were expressed from trophectodermal cells. Furthermore, miR-20a and miR-30c, detected in the majority of implanted embryos, were involved in cell-to-cell communication, signalling, cell adhesion and cell growth pathways, modulating mainly *PTEN, NRAS, MAPK, MYC, APC, PIK3* and *FOX3* transcripts. Thus, it was proved that conditioned media can serve as a rich miRNA reservoir (Capalbo *et al.*, 2016).

2.4.4 Importance of other non-coding miRNAs

Some studies argue that role of miRNA is not as important as the role of other non-coding RNAs and that the role of miRNA is suppressed during oocyte maturation and further in preimplantation embryos. In the porcine (Liu *et al.*, 2017) and mouse model, it was demonstrated that oocytes lacking DICER show a higher number of misregulated transcripts, promote disordered spindle formation and oocyte apoptosis during IVM or they arrest in the MI stage (Murchison *et al.*, 2007). In other words, DICER is required for the normal completion of meiotic maturation. However, DROSHA and DGCR8 may play a minor role compared to DICER, because deficient mice demonstrated the same phenotype as the wild type. Parameters such as oocyte maturation and preimplantation development were similar and offspring production deviated only slightly from the wild type (Suh *et al.*, 2010)*.

3 Aims

A previously carried out experiment showed that oocytes differ in miRNA expression patterns when derived from COCs from high and low developmental capacity follicles (Gad *et al.*, 2019). MiRNAs are single-stranded RNAs with a length of ~22 nucleotides that can regulate the expansion of cumulus cells and oocyte maturation. There is rising evidence that the expression profile of miRNAs that is present in oocytes and cumulus cells varies and possibly might reflect the oocyte developmental potential and its quality.

Focusing on cumulus cells we want to know, whether there are any differences in miRNA expression dynamics during the maturation of low- and high-quality oocytes. And if any differences are going to be present, we want to know which pathways or molecular functions are affected and how this could influence the oocyte's developmental competence.

4 Materials and methods

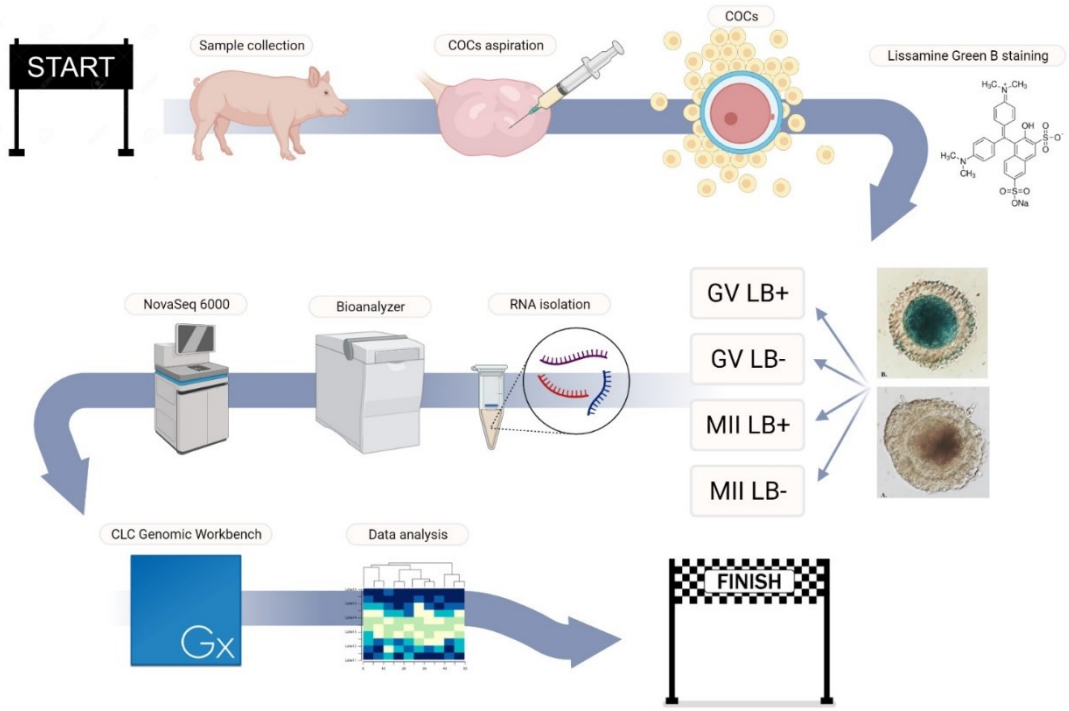


Figure 4 ***Experiment overview.*** *Created in Biorender.com*

4.1 Chemicals, culture media and reagents

All plastic materials were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and all chemicals were purchased from Sigma-Aldrich (Munich, Germany) and Merck (Kenilworth, NJ, USA) unless stated otherwise. All media were prepared fresh and sterilized using 0.22 μm syringe filters.

4.2 Collection of COCs

Ovaries were obtained from premature gilts from a local slaughterhouse. Ovaries were cut off and transported to the laboratory in a thermos at 38°C. Medium-sized follicles of diameter about 3-6 mm were aspirated with a 10ml syringe connected to a 20G needle (Marchal *et al.*, 2002). Follicular content was collected in a test tube and left sediment for 10 min. The sediment was washed twice with PXM-Hepes (*Table 1*) (Yoshioka, Suzuki and Onishi, 2008) and poured into a 90mm Petri dish. COCs were searched under the ZEISS Stemi 508 (Jena, Germany) stereomicroscope and collected with a glass pipette into preheated (38.5°C) PXM-Hepes. COCs selected for the experiment were surrounded by compact multi-layered cumulus cell sheets.

Table 1 ***PXM-Hepes medium composition*** (Yoshioka, Suzuki and Onishi, 2008)

Stock	Component	Supplier	Weight	250ml
Stock A/500ml	NaCl	Sigma S5886	31.5576g	25ml
	KCl	Sigma P5405	3.7275g	
	KH_2PO_4	Sigma P5655	0.2382g	
	$MgSO_4$	Sigma M2643	0.2408g	
Stock B/100ml	$NaHCO_3$	Sigma S5761	2.1003g	5ml
Stock C/100ml	HEPES	Sigma H4034	29.7888g	5ml
Stock E/50ml	Na-pyruvate	Sigma P4562	0.0011g	2.5ml
Stock F/50ml	Ca-lactate	Sigma C8356	0.6166g	2.5ml
Stock G/25ml	Gentamycin	Roth 0233	0.3083g	25µl
BSA		Sigma A7888		0.25g

4.3 Lissamine Green B staining

The groups of 200 selected COCs were located for 10 min in 2 ml 0.5% Lissamine Green B (LB) at room temperature. Stained COCs were washed two times in PXM-Hepes at 38.5°C and examined under the stereomicroscope. Detection of the damaged cytoplasmic membrane was represented by teal ooplasm. Based on the LB staining the COCs were divided into two qualitatively distinct subgroups, stained (LB+; LQ) and stainless (LB-; HQ) meaning low- and high-quality COCs, respectively (Dutta *et al.*, 2016; Bartkova *et al.*, 2020).

4.4 COCs maturation

Selected COCs were washed twice in the FLI maturation medium and cultured in groups of 30-50 oocytes per well in four-well dishes (Nunclon, Roskilde, Denmark) for 40-44h in 0.5 ml of maturation media at 38.5°C in a humidified atmosphere of 5% CO_2. The maturation media, so-called FLI media, was supplemented by human FGF2, human IGF1 and human LIF (*Table 2*) (Yuan *et al.*, 2017).

Table 2 ***FLI medium composition***

Component	Supplier	FLI Medium
TCM199	Sigma, M7528	TCM199
Na-pyruvate	Sigma, P4562	0.2 mM
L-glutamine	Sigma, G8540	6.85 mM
L-Cysteine	Sigma, C7352	0.57 mM
Gentamycin	Roth, 0233	50 µg/ml
BSA	Sigma, A7030	1 mg/ml
PMSG	Prospec[1], HOR-272	10 IU/ml
hCG	Prospec[1], HOR-250	10 IU/ml
EGF	PeproTech[2], AF-100-15	10 ng/ml
human LIF	Merck[3], LIF1005	2 µl/ml
human IGF1	PeproTech[2], AF-100-11	20 ng/ml
human FGF2	Sigma, F0291	40 ng/ml

[1] Prospec, Rehovot, Israel; [2] PeproTech, London, England; [3] Merck Life Science, Prague, Czech Republic

4.5 Cumulus cells collection

The CCs were removed from a group of 20 selected COCs in GV or MII stage by pipetting with a fine glass pipette in a 30 mm Petri dish. The bunch of separated CCs was transferred to a labelled Eppendorf tube, washed in PBS at 38.5°C and shortly centrifuged in a mini-centrifuge at $8x10^3$ RPM. The supernatant was removed and the cells were overlaid with 100 µl of QIAzol Lysis Reagent. Samples were homogenized by vortexing and stored in the freezer at -80°C.

4.6 RNA extraction, RNA quality and integrity assessment

Total RNA was isolated from cumulus cells separated from 20 COCs of four different groups (three replicates each) by commercial kit miRNeasy Micro Kit (Qiagen, Hilden, Germany) according to the given manufacturer's instructions. This protocol is designed to purify total RNA including miRNAs molecules and combines phenol- and membrane-based purification. Cumulus cell lysis was promoted by placing cells of our interest into QIAzol Lysis Reagent which is a phenol/guanidine thiocyanate solution. Chloroform was added to the homogenate that separated into an aqueous and organic phase after centrifugation. The upper aqueous phase was extracted and subsequently adding ethanol the samples were loaded on the RNeasy MinElute spin column where the total RNA binds to the membrane. DNase digestion step was implemented. Adding RNase-free water to the RNAs bound to the membrane were spun into the collection tube. The total yielded solution volume of isolated RNA was 12 µl.

The RNA concentration and integrity were assessed using NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent RNA 6000 Pico kit in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Samples with RIN>8 were used for further analysis.

4.7 Library preparation and sequencing

Library preparation for miRNA next-generation sequencing was done using the QIAseq miRNA Library Kit (Qiagen) according to the company's protocol. This kit allows mature miRNAs library preparation and at the same time, it is minimizing the other small RNAs' background. Library quantification was assessed using the Qubit™ DNA HS Assay Kit in a Qubit 4 Fluorometer (Thermo Fisher Scientific). Library quality assessment was performed using the Agilent DNA High Sensitivity Chip on the 2100 Agilent Bioanalyzer (Agilent Technologies). The libraries were pooled in equimolar ratios and then sequenced in a NovaSeq6000 sequencing instrument (Illumina, Inc., San Diego, CA, USA) as paired-end reads.

4.8 Sequencing data analysis

FASTQ files were generated for each sample using the software bcl2fastq (Illumina Inc., San Diego, CA), and their quality was checked using the FastQC tool version 0.11.9. Data were analysed using the software CLC Genomics Workbench, version 21 (www.qiagenbioinformatics.com). Raw sequencing reads were trimmed based on quality score (Q-score > 30), ambiguous nucleotides (max. two nts allowed), read length (≥15 nucleotides) and removing adapter sequences. Reads were mapped to the porcine (*Sus scrofa*) reference genome (Sscrofa11.1) and annotated against porcine precursor and mature miRNAs listed in the mirBase database (release 22) using the CLC Genomics Workbench RNA-Seq Analysis and Quantify miRNA tools, respectively, applying the default software parameters. Raw expression data were normalized using the trimmed mean of M-values normalization method (TMM normalization) (Robinson and Oshlack, 2010) and presented as TMM-adjusted Counts Per Million (CPM). The CLC Genomics Workbench Differential Expression tool was used for the expression analysis comparison of the two groups. MiRNAs with fold change (FC) ≥ 2, p-adjusted value (FDR < 0.05), and CPM > 5 in the enriched group were considered differentially expressed (DE) (Yoav Benjamini and Yosef Hochberg, 1995).

4.9 Target gene prediction and pathway analysis

DE-miRNAs from porcine were checked on miRBase (https://www.mirbase.org/) for human homologous sequence. The miRWalk tool (http://mirwalk.umm.uni-heidelberg.de/) was used to predict genes targeted by each group of miRNAs. Only validated target genes from miRTarBase (version 8.0), genes predicted by TargetScan (version 8.0) and miRDB (release 6.0) were selected. Using a combination of algorithms can accurate target gene prediction (Hou *et al.*, 2019). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics web-tool 2021 (https://david-d.ncifcrf.gov/) was utilized for ontological classification and pathway analysis (Huang, Sherman and Lempicki, 2009*; Sherman *et al.*, 2022*). Significant pathways were determined from the Kyoto Encyclopaedia of Genes and Genome (KEGG) database (Kanehisa *et al.*, 2017)*, interaction networks of the targeted genes and found pathways were constructed with Cytoscape 3.9.1 (Shannon *et al.*, 2003).

5 Results

5.1 Assessment of COCs quality on Lissamine Green B staining

Cumulus-oocyte complexes were stained with LB according to the protocol. The visually different outcome of stained and unstained oocytes can be seen in *Figure 5*. High-quality (LB-, HQ) oocyte is represented by dark unstained ooplasm, contrasting to low-quality (LB+, LQ) oocyte with teal stained ooplasm.

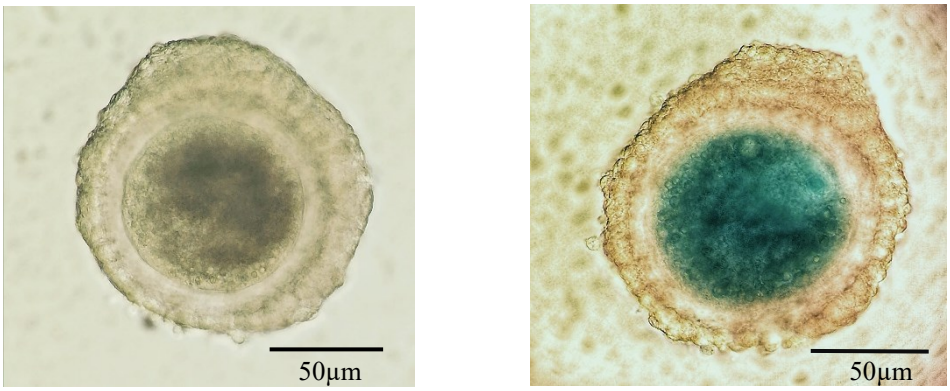


*Figure **1 Lissamine Green B staining** – High-quality (LB-; HQ) oocyte unstained on the left, and low-quality oocyte (LB+; LQ) with stained cytoplasm on the right (Photo RNDr. Alexandra Bartková, PhD.)*

5.2 MiRNA sample quality assessment

All experimental groups were prepared in replicates of five however, only the best tree replicates were sent for sequencing (*Table 3*). This decision was made according to the results from Agilent 2100 Bioanalyzer considering the sample concentration (ng/µl) and RNA Integrity Number (RIN) number. Only samples with RIN>8 were used in this experiment.

*Table 3 **Conversion table of samples' names and ID with corresponding concentration and RIN***

Sample ID	Sample name	Concentration (ng/µl)	RIN
S21_115_01	CCLB+ GV1	2.02	8.10
S21_115_02	CCLB- GV1	1.99	8.70
S21_115_03	CCLB+ MII1	1.24	9.30
S21_115_04	CCLB- MII1	4.79	9.20
S21_115_05	CCLB+ GV2	0.82	9.00
S21_115_06	CCLB- GV2	3.26	8.10
S21_115_07	CCLB+ MII3	1.59	9.50
S21_115_08	CCLB- MII3	1.79	9.40
S21_115_09	CCLB+ GV5	0.99	9.30
S21_115_10	CCLB-GV5	0.72	9.20
S21_115_11	CCLB+ MII5	3.19	9.70
S21_115_12	CCLB- MII5	6.57	9.50

The electrophoretograms (*Figure 6)* from Agilent 2100 Bioanalyzer are showing regions from 20-40 nts where the small peaks represent the isolated miRNAs.

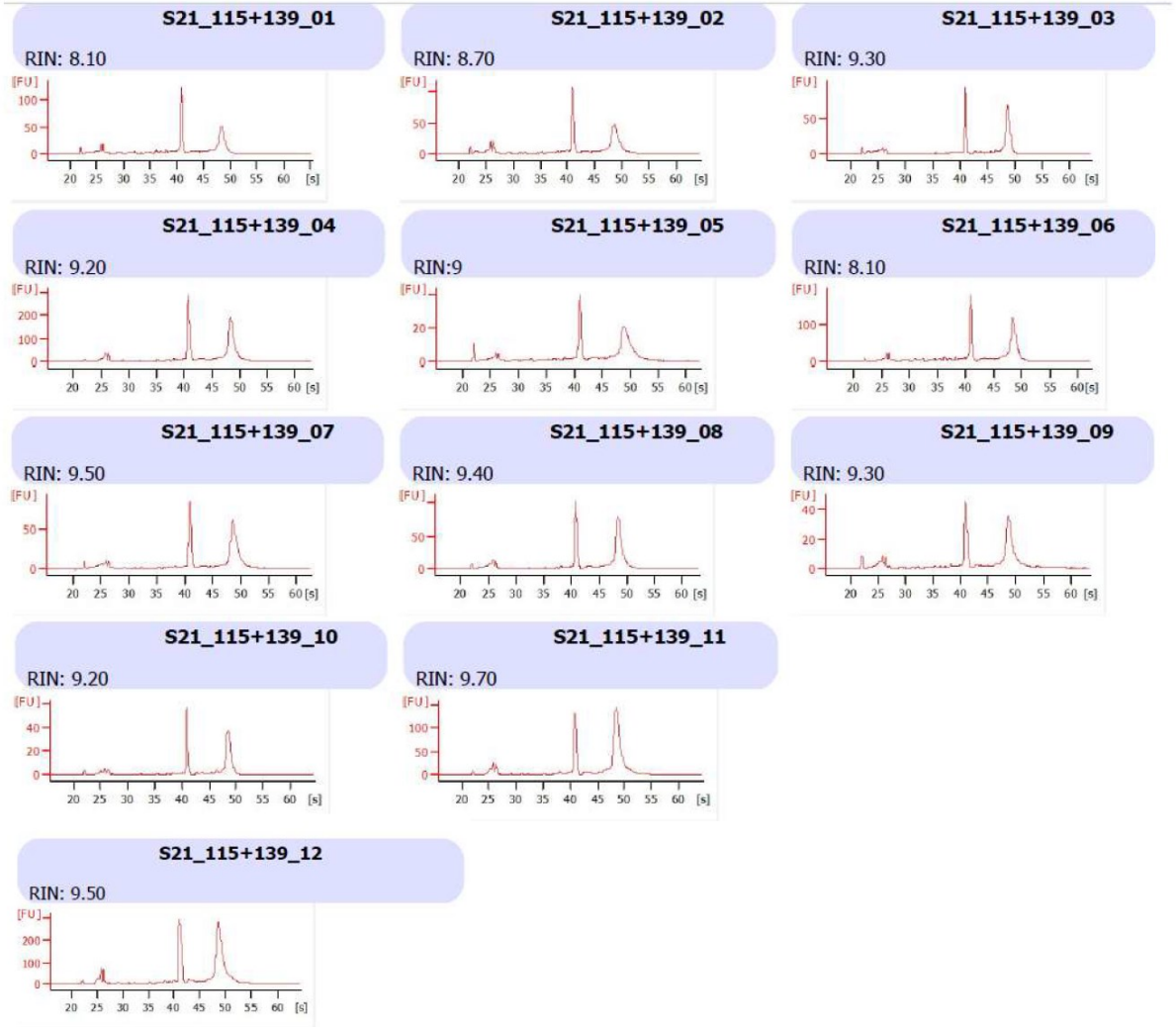


Figure 6 ***Electrophoretograms of samples from Agilent 2100 Bioanalyzer kit***

5.3 MiRNA sequencing data

Libraries of small RNAs were prepared from 12 different CCs samples (3 replicates per group) to find out expressed miRNAs. Raw reads were trimmed from adapters, followed by the length and quality filtering. The average total number of sequenced reads was around 3.4 million and on average nearly 600 000 reads were mapped to the porcine genome, which represents about 16% of total reads. Data for every sample are presented in *Table 4*.

Table 4 ***Summary of sequence reads mapped to the porcine reference genome for all experimental groups***

Sample	Total reads	Mapped reads to miRBase (*Sus scrofa*)	% of total
GVLB+1	3 536 720	762 326	21.55
GVLB-1	3 517 320	705 614	20.06
MIILB+1	3 052 947	192 360	6.3
MIILB-1	2 577 623	637 483	24.73
GVLB+2	2 090 181	172 113	8.23
GVLB-2	5 428 195	813 796	14.99
MIILB+2	2 943 779	241 532	8.2
MIILB-2	2 844 785	266 701	9.38
GVLB+3	3 550 198	222 935	6.28
GVLB-3	4 724 829	482 120	10.2
MIILB+3	1 495 904	285 669	19.1
MIILB-3	5 320 585	2 390 021	44.92
Average	3 423 588.8	597 722.5	16.2

5.4 MiRNAs expression profile

First, defined criteria were set for the evaluation of expressed miRNA. Counts per million mapped reads (CPM) is calculated as a fraction of the number of reads mapped to gene x10^6 to the total number of mapped reads: $\text{CPM}=\frac{\text{number of reads mapped to gene x}10^6}{\text{total number of mapped reads}}$. Only those miRNAs that had CPM>1 and total count value >0 were considered expressed.

The principal component analysis (PCA) shows a multivariate data table as a smaller set of variables in order to observe trends and sample clustering. In our case, the samples are variants of each other based on the expression of each miRNA. In *Figure 6* green dots represent MII samples and GV samples are shown in violet. The quality LB+ and LB- groups are represented by the cross and the plus sign, respectively. In the scheme there is figured the distinct clustering of MII and GV groups however, there is no observable further sub clustering of LB+ and LB-. This analysis shows significant differences between GV and MII samples, on the other hand, there are not any significant differences between LB+ and LB- samples, otherwise there should be visibly separated four groups.

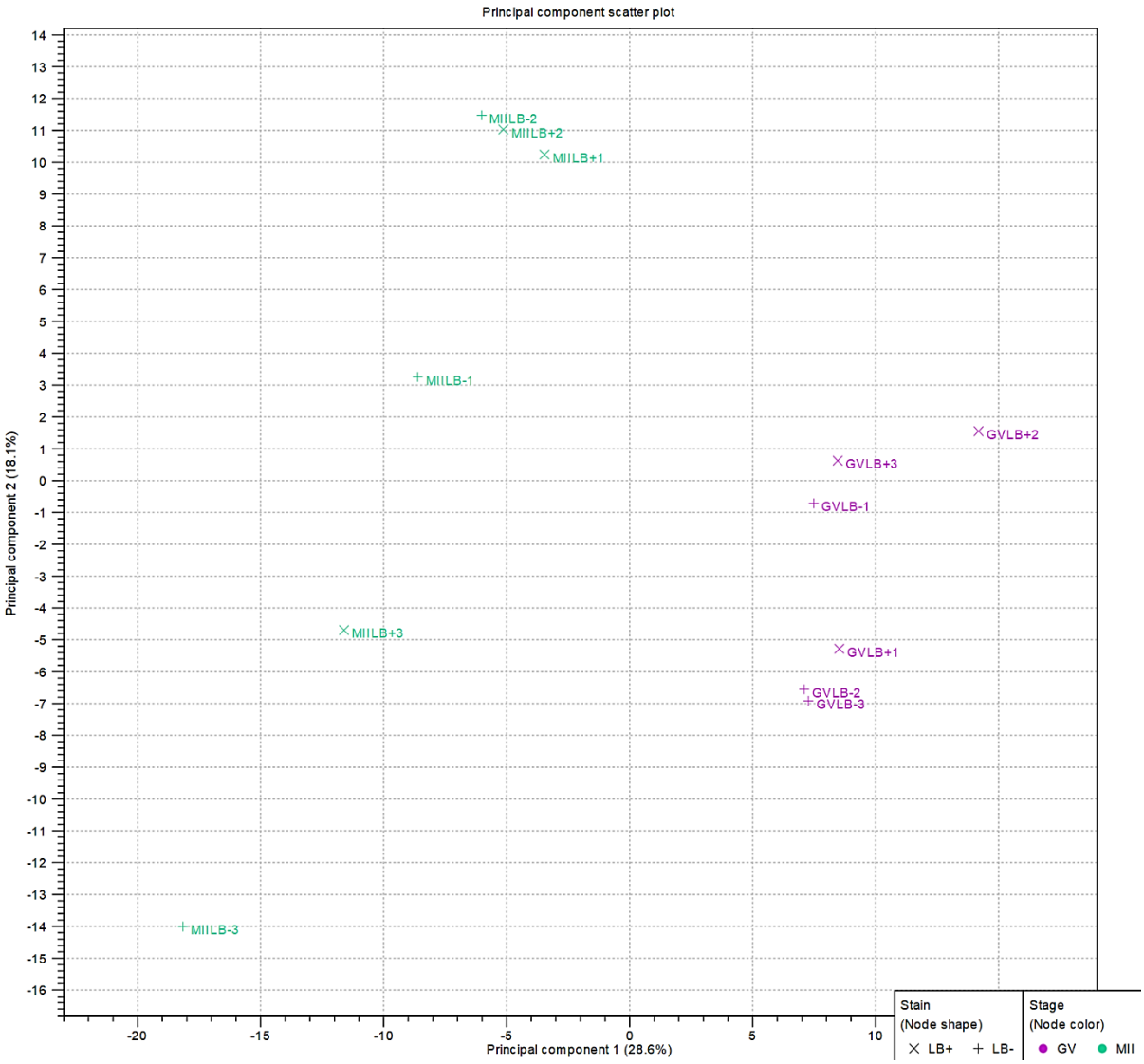
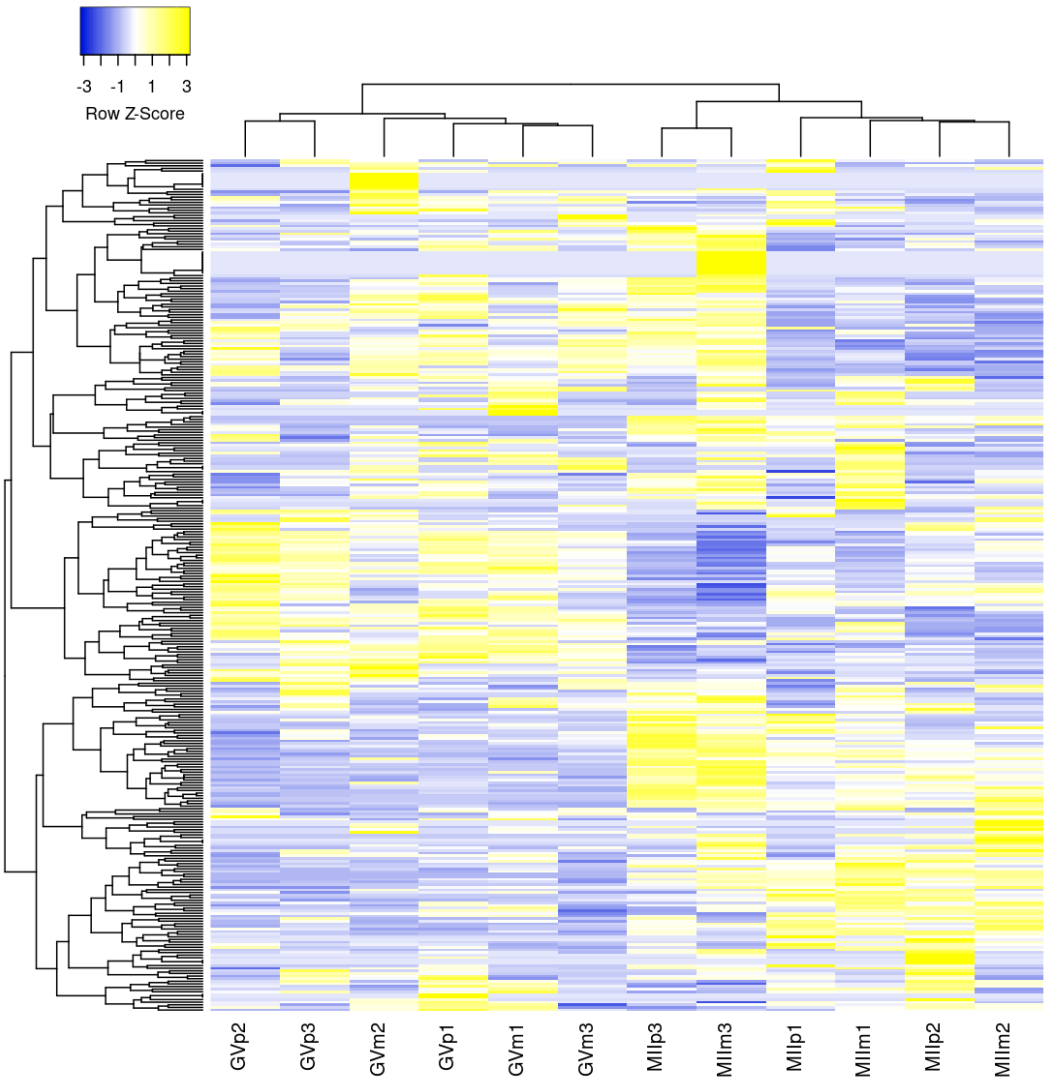


Figure 7 ***Principal component analysis (PCA)*** *– Developmental stages GV and MII are labelled violet and green, respectively. The quality LB+ and LB- groups are represented by the cross and the plus sign, respectively*

A heat map is a data-visualizing tool that is reflecting a studied phenomenon on a two-colour scale. In this case, changes in miRNAs expression in lines with a combination of given samples in columns are used. The main goal of this figure is to show the clustering of samples based on the expression values. In *Figure 7* the bluer the colour the more downregulated sample and the yellower the more upregulated sample. White represents unchanged expression. Furthermore, at the top, the clamps reflect the relationship of all samples. The left and right site is based on their expression pattern observable closeness and similarity of GV



and MII samples, respectively. Unfortunately, samples did not show any further sub clustering based on LB staining.

Figure 7 ***Heat map*** *showing clustering of samples at the top and each line is representing one particular miRNA. Blue and yellow colour mean down- and upregulated states, respectively*

Separation of miRNAs into LB-, LB+, GV and MII groups revealed a total of 214, 189, 202, and 208 miRNAs, respectively. A comparison of qualitatively (*Figure 8*) and developmentally different (*Figure 9*) stages showed 187 identically expressed miRNAs. A comparison of all four experimental groups (GVLB+, GVLB-, MIILB+, MIILB-) showed 187 similarly expressed miRNAs and also exclusively expressed miRNAs are visualized in the Venn diagram (*Figure 10*) and namely enlisted in *Table 5*.

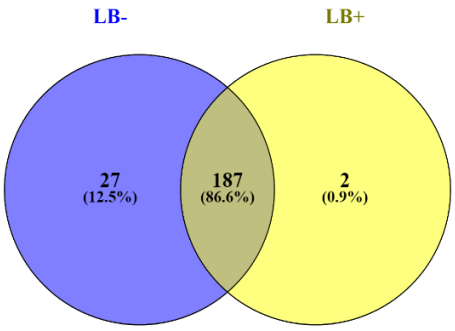


Figure 8 ***Venn diagram showing the number of exclusively and commonly expressed miRNAs between qualitatively different stages***

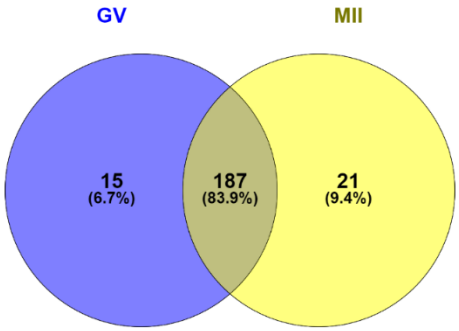


Figure 9 ***Venn diagram showing the number of exclusively and commonly expressed miRNAs between developmental stages***

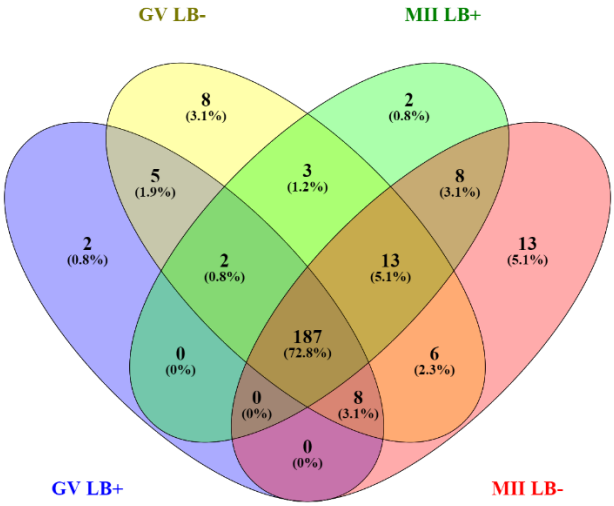


Figure 10 ***Venn diagram comparing all experimental groups***

Table 5 ***Exclusively expressed miRNAs in every experimental group***

Exclusive miRNAs for experimental groups					
GV LB+	2	ssc-miR-196b-5p ssc-miR-1285	**MII LB+**	2	ssc-miR-215 ssc-miR-26b-3p
GV LB-	8	ssc-miR-378b-3p ssc-miR-200b ssc-miR-545-3p ssc-miR-450c-5p ssc-miR-331-5p ssc-miR-545-5p ssc-miR-9860-5p ssc-miR-143-5p	**MII LB-**	13	ssc-miR-20b ssc-miR-381-3p ssc-miR-33b-5p ssc-miR-363 ssc-miR-450c-3p ssc-miR-22-5p ssc-miR-129a-3p ssc-miR-432-5p ssc-miR-382 ssc-miR-365-5p ssc-miR-223 ssc-miR-424-3p ssc-miR-345-5p

The six most abundantly expressed miRNAs from all sequenced samples are presented in *Table 6*. Interestingly, the total reads of these six miRNAs together count for more than half (56.2%) of all miRNA sequence reads. Moreover, three of them are part of the evolutionarily conserved let-7 family, specifically ssc-let-7c, ssc-let-7a, ssc-let-7f-5p are represented by 15.0%, 10.6% and 9.7% of all reads, respectively.

Table 6 ***Most abundantly expressed miRNAs from all samples***

miRNA	% of total reads	Avg. read count	
ssc-let-7c	15.0	79 636	56.2%
ssc-let-7a	10.6	56 263	
ssc-let-7f-5p	9.7	51 288	
ssc-miR-16	9.1	48 400	
ssc-miR-21-5p	7.2	38 504	
ssc-miR-125a	4.6	24 439	

5.5 Differentially expressed miRNAs

Analysis of differentially expressed miRNAs showed that in the high-quality group there were 6 miRNAs and 30 miRNAs significantly up- and downregulated, respectively, in the germinal vesicle compared to the MII group, ($FC \geq 2$, $FDR < 0.05$). On the other hand, the low-quality group revealed only 1 and 16 significantly up- and downregulated miRNAs, respectively, in the germinal vesicle compared to the MII group ($FC \geq 2$, $FDR < 0.05$). These

data are nicely visualized in *Figure 11* and *Figure 12*, and a complete list of all miRNAs is shown in *Table 7*.

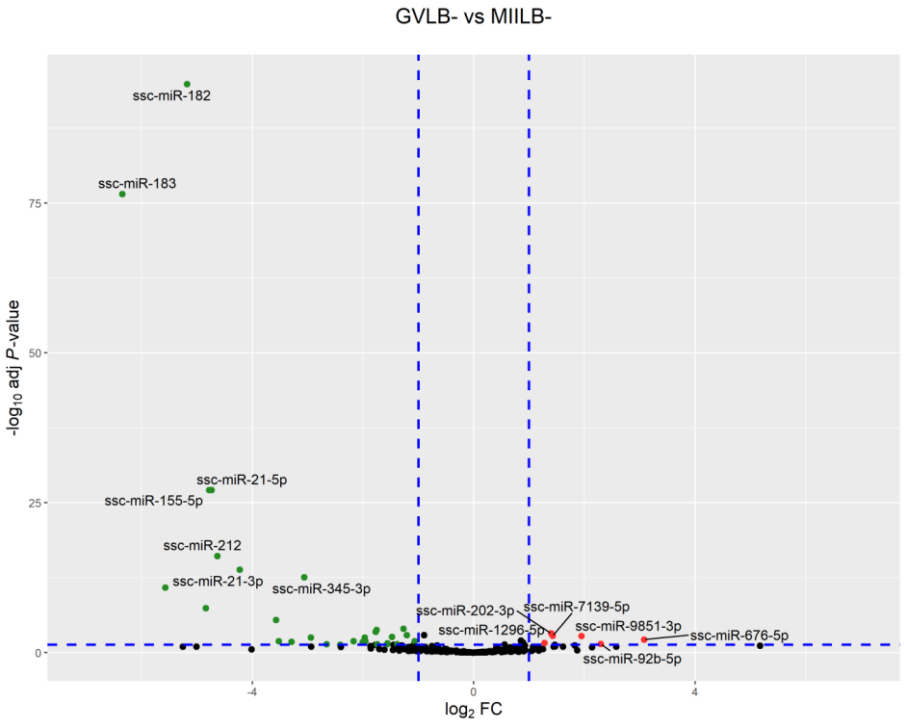


Figure 11 ***Volcano plot presenting up- and downregulated miRNA when comparing GVLB- vs. GVMII-.*** *In this graphical presentation not all dots are labelled, for a complete list see Table 7 below.*

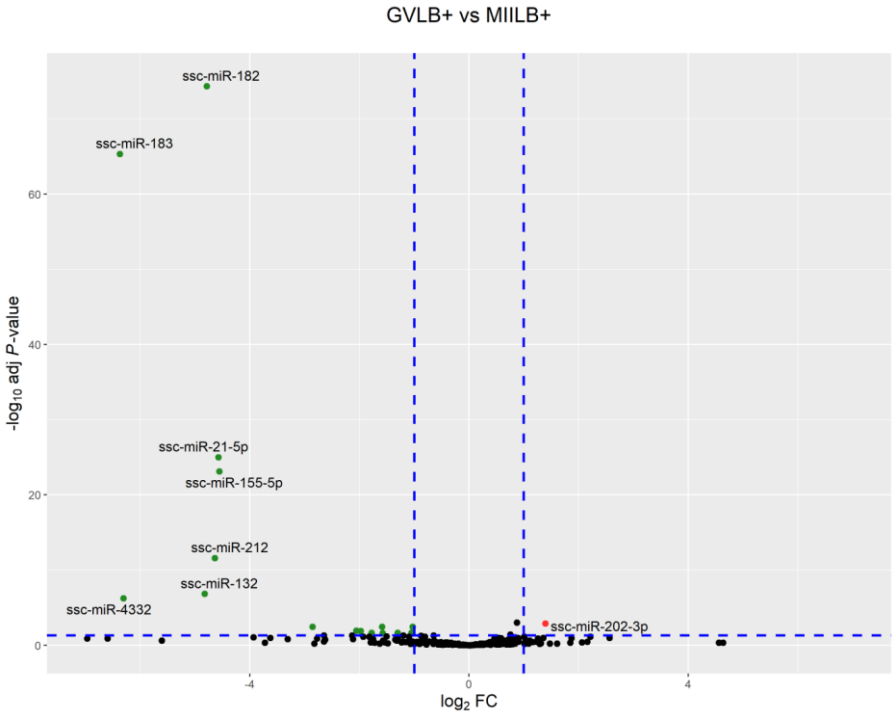


Figure 12 ***Volcano plot presenting up- and downregulated miRNA when comparing GVLB+ vs. GVMII+.*** *In this graphical presentation not all dots are labelled, for a complete list see Table 7 below.*

GVLB- vs. MIILB-		
Upregulated miRNAs	**FC**	**FDR**
ssc-miR-676-5p	8,43	6,61E-03
ssc-miR-92b-5p	4,91	3,38E-02
ssc-miR-9851-3p	3,85	1,79E-03
ssc-miR-7139-5p	2,69	1,56E-03
ssc-miR-202-3p	2,63	6,28E-04
ssc-miR-1296-5p	2,42	2,61E-02
Downregulated miRNAs	**FC**	**FDR**
ssc-miR-6529	-2,10	1,22E-02
ssc-miR-181b	-2,31	1,24E-03
ssc-miR-140-3p	-2,42	1,01E-04
ssc-miR-340	-2,61	4,19E-02
ssc-miR-221-3p	-2,79	2,44E-03
ssc-miR-122-5p	-2,95	2,82E-02
ssc-miR-219a	-2,96	4,19E-02
ssc-miR-1839-5p	-3,35	4,13E-02
ssc-miR-149	-3,38	1,69E-04
ssc-miR-140-5p	-3,43	3,30E-04
ssc-miR-9843-3p	-3,90	1,22E-02
ssc-miR-23a	-3,91	9,43E-03
ssc-miR-27a	-3,92	3,05E-03
ssc-miR-192	-4,03	1,77E-02
ssc-miR-9-1	-4,51	1,29E-02
ssc-miR-205	-5,34	4,90E-02
ssc-miR-127	-6,32	4,06E-02
ssc-miR-365-3p	-7,68	3,05E-03
ssc-miR-345-3p	-8,37	2,71E-13
ssc-miR-4332	-9,82	1,57E-02
ssc-miR-375	-11,50	1,19E-02
ssc-miR-504	-11,89	3,44E-06
ssc-miR-21-3p	-18,74	1,48E-14
ssc-miR-212	-24,79	7,41E-17
ssc-miR-21-5p	-26,64	7,57E-28
ssc-miR-155-5p	-27,50	7,57E-28
ssc-miR-132	-28,71	3,66E-08
ssc-miR-182	-36,29	1,47E-95
ssc-miR-129a-5p	-47,62	1,39E-11
ssc-miR-183	-81,48	2,99E-77

GVLB+ vs. MIILB+		
Upregulated miRNAs	**FC**	**FDR**
ssc-miR-202-3p	2,62	1,24E-03
Downregulated miRNAs	**FC**	**FDR**
ssc-miR-140-3p	-2,04	3,46E-03
ssc-miR-6529	-2,07	2,19E-02
ssc-miR-149	-2,47	2,19E-02
ssc-miR-628-5p	-2,99	2,36E-02
ssc-miR-140-5p	-3,01	3,46E-03
ssc-miR-18a	-3,44	2,19E-02
ssc-miR-27a	-3,93	1,30E-02
ssc-miR-23a	-4,16	1,12E-02
ssc-miR-504	-7,26	3,46E-03
ssc-miR-155-5p	-23,56	7,18E-24
ssc-miR-21-5p	-23,81	9,53E-26
ssc-miR-212	-24,95	2,42E-12
ssc-miR-182	-27,59	4,41E-75
ssc-miR-132	-28,31	1,39E-07
ssc-miR-4332	-79,16	5,52E-07
ssc-miR-183	-82,94	4,48E-66

Table 7 ***DE miRNAs showing the group of high-quality (LB-) and low-quality (LB+)****, within the groups the comparison is based on the maturation stage*

Figure 13 is showing exclusively and commonly expressed miRNAs in all experimental groups. Green and red colours were chosen for up- and downregulated miRNAs, respectively. In this scheme, there is a group of miRNAs that were similarly expressed when considering DE in developmentally and qualitatively distinct stages. Furthermore, a group of the top six expressed miRNAs is pictured.

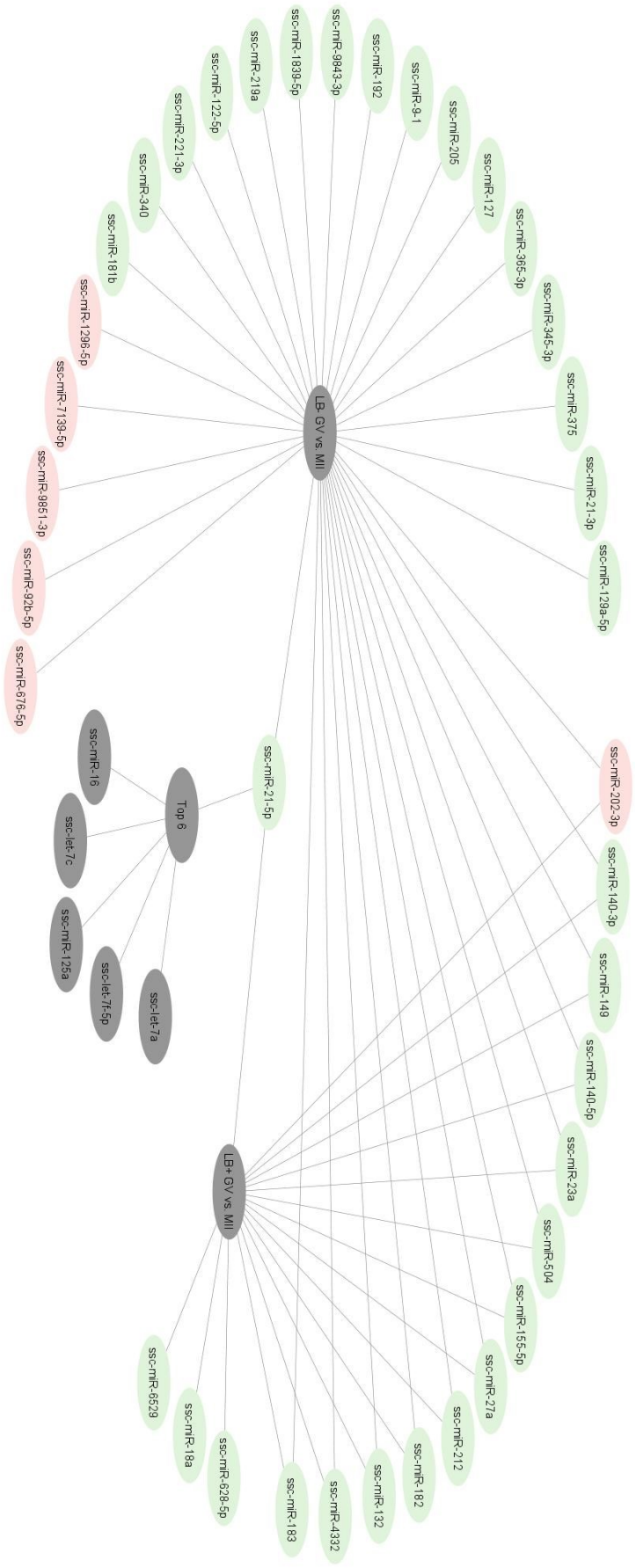


Figure 13 ***miRNA expression*** *scheme showing up—and downregulated miRNAs represented by red and green colours, respectively. Created in Cytoscape.org*

5.6 Human miRNA homologues a target gene prediction

Nowadays, most online software tools for ontological classification are unable to classify porcine miRNAs. Thus, the homologous human miRNAs were found using the miRBase with a maximum of two mismatches in the mature miRNA sequence. A list of compatible human homologues is presented in *Table 8*.

Figure 14 is showing an example of porcine (ssc-miR-129a-5p) and homologous human miRNA (ssc-miR-129a-5p) that was successfully aligned for further analysis. On contrary, *Figure 15* represents an example of porcine miRNA (ssc-miR-7139-5p) that does not correspond to human homologue (hsa-miR-4800-5p) within the set criteria, these miRNAs were eliminated from further analysis.

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
MIMAT0020586	ssc-miR-129b	1	21	1	21	+	105	0.002	Align
MIMAT0000242	hsa-miR-129-5p	1	21	1	21	+	105	0.002	Align

Query: 1-21 ssc-miR-129a-5p: 1-21 score: 105 evalue: 0.002

```
UserSeq          1  cuuuuugcggucuggggcuugc  21
                    |||||||||||||||||||||
ssc-miR-129a-5p  1  cuuuuugcggucuggggcuugc  21
```

Query: 1-21 hsa-miR-129-5p: 1-21 score: 105 evalue: 0.002

```
UserSeq          1  cuuuuugcggucuggggcuugc  21
                    |||||||||||||||||||||
hsa-miR-129-5p   1  cuuuuugcggucuggggcuugc  21
```

Figure 14 ***Example of incompatible homologue alignment***

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue	Alignment
MIMAT0028153	ssc-miR-7139-5p	1	23	1	23	+	115	3e-04	Align
MIMAT0019978	hsa-miR-4800-5p	2	21	1	20	-	64	5.3	Align

Query: 1-23 ssc-miR-7139-5p: 1-23 score: 115 evalue: 3e-04

```
UserSeq          1  ccauuccuucgucugugcacuag  23
                    |||||||||||||||||||||||
ssc-miR-7139-5p  1  ccauuccuucgucugugcacuag  23
```

Query: 2-21 hsa-miR-4800-5p: 1-20 score: 64 evalue: 5.3

```
UserSeq          2  cauuccuucgucugugcacu  21
                    | ||||||| || || ||||
hsa-miR-4800-5p  20 ccuuccuuccucgguccacu  1
```

Figure 15 ***Example of successful homologous miRNAs alignment***

Table 8 ***Human compatible miRNA homologues***

GVLB- vs. MIILB-	GVLB+ vs. MIILB+
Upregulated miRNAs	**Upregulated miRNAs**
hsa-miR-92b-5p	-
hsa-miR-9851-3p	
hsa-miR-1296-5p	**Downregulated miRNAs**
	hsa-miR-6529-5p
Downregulated miRNAs	hsa-miR-628-5p
hsa-miR-181b-5p	hsa-miR-18a-5p
hsa-miR-340-5p	
hsa-miR-221-3p	
hsa-miR-122-5p	
hsa-miR-219a-1-3p	
hsa-miR-192-5p	
hsa-miR-9-5p	
hsa-miR-205-5p	
hsa-miR-127-3p	
hsa-miR-365a-3p	
hsa-miR-375-3p	
hsa-miR-21-3p	
hsa-miR-129-5p	

Gene targets were assessed using the miRWalk database. The upregulated miRNAs in GVLB- vs. MIILB- group target 160 genes when removing duplicates. The downregulated miRNAs in the same group (GVLB- vs. MIILB-) refer to 959 genes when duplicates were removed. There were 299 genes targeted by downregulated miRNAs in GVLB+ vs. MIILB+ group. Since no DE upregulated miRNAs in the GVLB+ vs. MIILB+ group were found, no genes were targeted.

5.7 Ontological classification of differentially expressed genes and related objectives

A list of genes with removed duplicates was submitted into DAVID software. Files with biological processes, cellular components, and molecular functions were downloaded. All records referring to cancer, neuronal development and human diseases were removed, as they were considered irrelevant to *in vitro* experiment we conducted. *Figures 16, 17,* and *18* are showing certain objectives based on the –log(P-value) and the number of genes involved in each term is shown at the end of each bar present.

Biological processes represent the objectives of a particular cell to which it is genetically programmed to achieve. From all the samples *Figure 16* demonstrates 10 top biological processes. It is interesting to note that four of those processes are related to the regulation of transcription. *Figure 17* represents 10 highly targeted cellular components which mean the structural and functional units of the cell. The two top processes are related to the nucleus and nucleoplasm with many genes involved in these processes. On the other hand, molecular functions are processes usually carried out by single machinery in the cell via direct physical contact. In *Figure 18* the highly targeted component was protein binding and among the top 10 related functions were processes for building components which form transcriptional machinery.

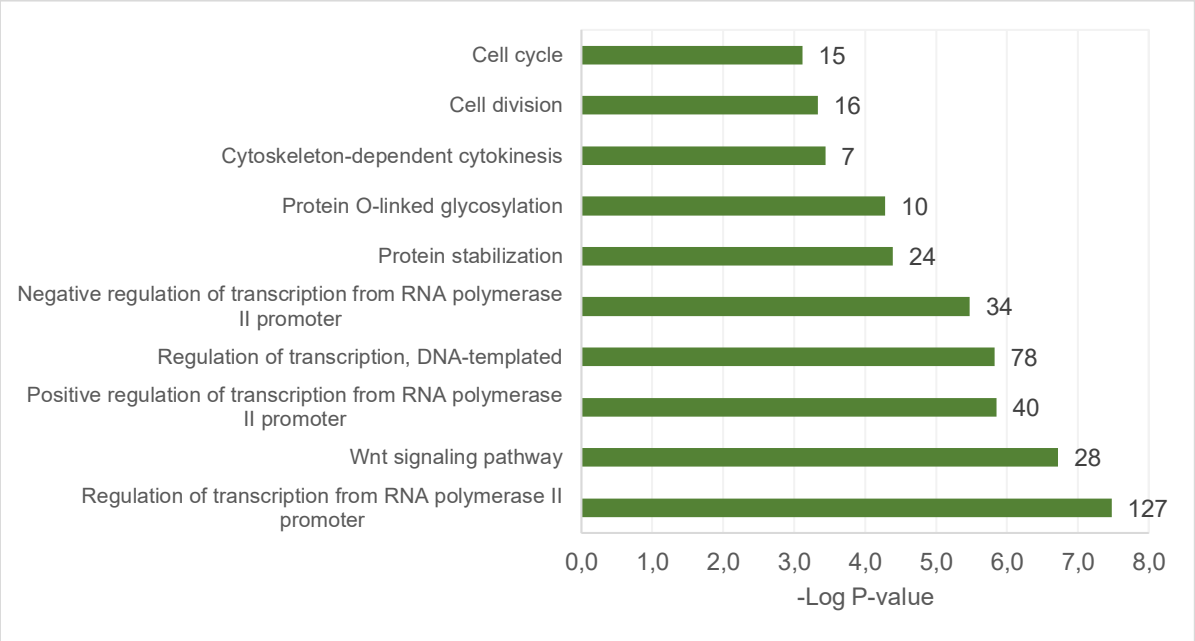


Figure 16 ***Biological processes*** *are presented based on the –log(P-value) and the number of genes involved in each of them is represented by the number at the end of each bar*

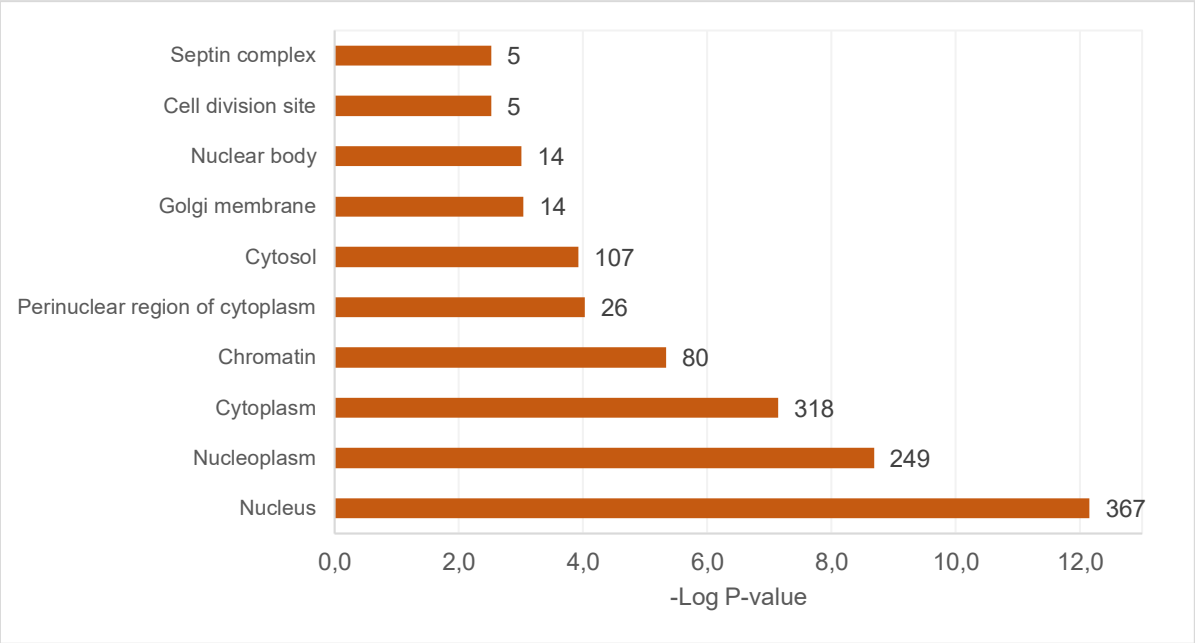


Figure 17 ***Cellular component*** *presented based on the –log(P-value) and the number of genes involved in each of them is represented by the number at the end of each bar*

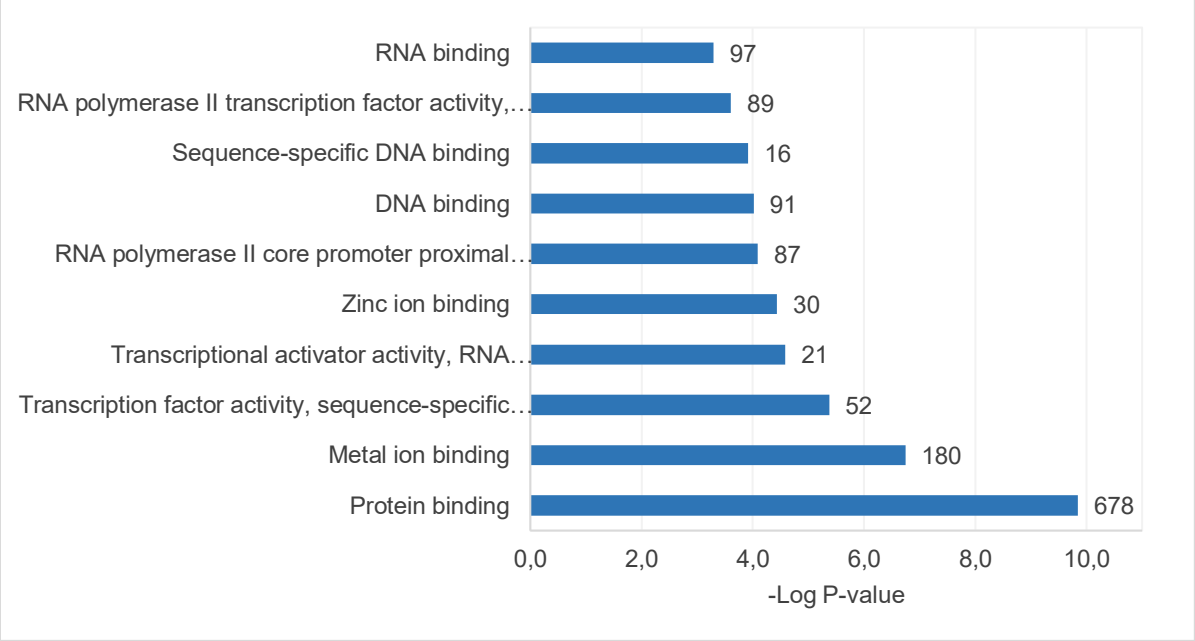


Figure 18 ***Molecular function*** *presented based on the –log(P-value) and the number of genes involved in each of them is represented by the number at the end of each bar*

5.8 Networking and pathway analysis

A list of KEGG pathways was downloaded from the DAVID software. All pathways referring to cancer, neuronal development and human diseases were removed, as they were considered irrelevant to *in vitro* experiment we conducted. MiRNAs upregulated in GVLB- vs. MIILB- relate mainly to ErbB and Wnt signalling pathways. MiRNAs downregulated in GVLB- vs. MIILB- referred to gap junctions and cellular senescence. In the group of downregulated miRNAs in the comparison of GVLB+ vs. MIILB+, the relevant pathways were related to MAPK and neurotrophin signalling. The graphical *Figure 19* is showing all biological pathways related to individual experimental groups.

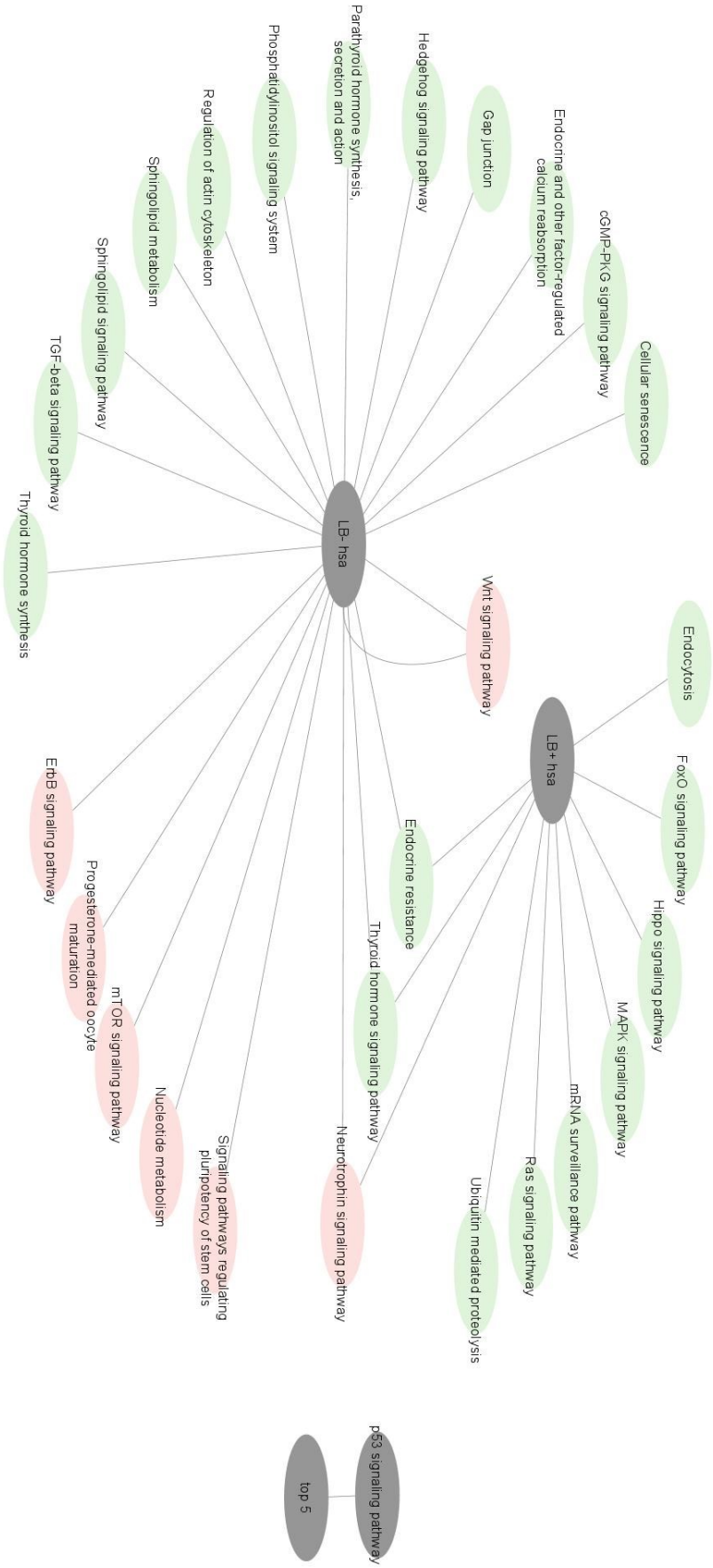


Figure 19 ***Targeted KEGG pathways*** *Up—and downregulated pathways are represented by red and green colour, respectively. Created in Cytoscape.org*

6 Discussion

In this thesis, I identified porcine miRNAs derived from cumulus cells with different developmental and quality stages. Furthermore, I deeper looked into differential miRNA expression and related biological pathways. Primarily, I found that cumulus cells derived from developmentally different oocyte stages, the germinal vesicle and the metaphase II stage, alter in terms of expressed miRNAs. Moreover, cumulus cells isolated from distinct quality COCs coincide with expressed miRNAs. The potential of identified miRNAs lies mainly in the way of using them as non-invasive markers.

Molecular mechanisms that regulate proper oocyte development are not completely clear yet. And understanding these processes could considerably improve IVM and IVF outcomes by enhancing the criteria by which the oocyte quality is assessed. The determining factor for successful IVM, IVF and embryogenesis is predominantly the quality of the oocyte (Blondin and Robert, 2006).

These days, there are several methods used for the evaluation of oocyte quality. They are mainly based on morphology, markers expression or machine learning assistance using oocyte images (Fischer *et al.*, 2021)*. Worldwide used vital stains work on a variety of principles; the LB staining method that can visualize damaged membranes in porcine COCs. One can think that a non-invasive vital stain is harmless. However, the time needed for COCs staining and all procedures conducted in a suboptimal environment can negatively influence the oocyte quality. Therefore, there is a general need for a better non-invasive method for evaluating oocyte quality.

Oocyte cultivation in LIF media does not negatively affect either cleavage or blastocysts yield. However, the cultivation of COCs with LIF increased the expression of miR-21 miR-155 in the oocyte, as well as in cumulus cells (Vendrell-Flotats *et al.*, 2020). This is in line with our results where we observed downregulation of miR-21 and miR-155 when comparing GV vs. MII stages.

MiR-21 is widely studied as it is upregulated in many cancers and tumours, but it seems that it also plays an important role in cumulus cells and preventing apoptosis (Christenson, 2010)*. Bartolucci, Uliasz and Peluso, (2020) state that miR-21-5p was elevated in human cumulus cells of oocytes that developed into blastocysts and therefore it could be considered a reliable quality marker. In our research, we found that members of the let-7 family, namely let-7a, let-7c, let-7f-5p, and miR-16, miR-21-5p and miR-125a were highly expressed

miRNAs in all samples. Gad *et al.*, (2019) postulated that there are groups of miRNAs (miR-205, miR-16, miR-148a-3p, miR-125b and some of the miR-let-7 family) that are highly expressed in porcine oocytes isolated from large or small follicles.

Grossman *et al.*, (2020) showed that miR-125a-3p is synthesized by all somatic cells in the follicle and that the secretion and uptake of this miRNA can be realized by both, cumulus and granulosa cells. Regarding our project, we identified significantly downregulated miR-27a that was reported to promote granulosa cell apoptosis (Tesfaye *et al.*, 2018) and decrease maturation rate follicles (Kim *et al.*, 2013).

The only DE upregulated miR-202-3p in the low-quality group was previously reported to be important for oestrous cycle regulation (An *et al.*, 2021) and during pregnancy (Salas-Huetos *et al.*, 2019)*. Concerning women's health, two identified miRNAs – miR-130, and miR-182 – are DE in patients suffering from PCOS (Hu *et al.*, 2020; Qasemi and Amidi, 2020*). As miR-182 was DE in mural granulosa cells compared to cumulus cells (Andrei *et al.*, 2018) and in the comparison between immature oocyte and their surrounding cumulus cells (Abd El Naby *et al.*, 2013) there might be a possible link between PCOS phenotype and related cell types in the ovary.

Similarly, hsa-miR-92b-5p was found to be expressed in epithelial ovarian cancer (Lopacinska-Joergensen *et al.*, 2022) but more importantly, it was DE between high- and low-receptive cattle (Ponsuksili *et al.*, 2014) and we detected it as one of the highly DE miRNA in the high-quality group.

Significantly DE downregulated was miR-132 in both quality groups. Noferesti *et al.*, (2015) found this miRNA DE in follicular fluid of hyperstimulated vs. unstimulated heifers, furthermore, its expression varied in the comparison between immature oocyte and their surrounding cumulus cells (Abd El Naby *et al.*, 2013). MiR-132 was significantly enriched in the DE in granulosa cells of bovine preovulatory dominant follicle compared to subordinate one (Navakanitworakul *et al.*, 2016) with higher expression in theca cells (Gebremedhn *et al.*, 2015). This matches our results because miR-132 isolated from cumulus cells in the GV stage was significantly downregulated compared to developmentally progressed stage MII. Moreover, this miRNA is involved in meiosis and follicle development (Salilew-Wondim *et al.*, 2020)*.

Inoue *et al.*, (2020) presented miR-183 as a DE miRNA in the follicular fluid isolated from small and large antral porcine follicles, in addition, it was DE in the comparison between

immature oocyte and their surrounding cumulus cells (Abd El Naby *et al.*, 2013). Upregulation of this miRNA was observed in granulosa cells of the bovine preovulatory dominant follicle (Gebremedhn *et al.*, 2015). To this inconsistency, we add that miR-183 is significantly downregulated in the cumulus cells in the GV stage compared to MII.

Set of miRNAs, miR-155, miR-182, miR-122 and miR-212 came out from our analysis as downregulated between GV and MII stage. They are linked with different follicular development stages and quality in a variety of species (Schauer *et al.*, 2013; Donadeu, Mohammed and Ioannidis, 2017; Zou *et al.*, 2020).

(Gad *et al.*, 2019) isolated porcine oocytes from large (3-6 mm) and small (1.5-1.9 mm) follicles. Expression analysis revealed that miR-205, miR-16, miR-148a-3p, miR-125b and some of the miR-let-7 family were among the most expressed miRNAs in both groups. Matching this, we detected miR-16, miR-125a and some of the miR-let-7 family members to be DE as regards the cumulus cells from GV and MII stage. DE miRNAs from our analysis targeted genes that were mainly involved in ErbB, TGF-β, MAPK, FoxO, gap junction and cGMP signalling pathways that match up with the findings of (Gad *et al.*, 2019; Gad *et al.*, 2020). Worth noting is the p53 signalling pathway that was the only significantly targeted pathway by the most abundantly expressed miRNAs. Interestingly, in our analysis, the WNT signalling pathway was found to be up- and downregulated at the same time in the KEGG pathway analysis in the high-quality group. In this term, available online databases for evaluating miRNA and gene expression must be used cautiously.

Cellular miRNAs are not more important than miRNAs isolated from EVs. Follicular fluid from corresponding oocytes that were individually stained with LB, and classified as high- or low-quality was separated in to high- and low-quality follicular fluid. MiRNAs isolated from EVs originating from qualitatively different follicular fluids showed 19 upregulated and 23 downregulated miRNAs. Gad *et al.*, (2022) also enlisted highly expressed miRNAs from both groups. Four of these miRNA, namely miR-let-7c, miR-16, miR-let-7a and miR-let-7f, were similar to the top highly expressed miRNAs in our experiment.

The undoubtful importance of miRNAs in cumulus cells lies for example in the regulation of oocyte maturation via increase of progesterone synthesis in cumulus cells (Andreas *et al.*, 2021), promotion of cellular expansion by facilitating HAS2 expression (Han *et al.*, 2021) or regulating apoptosis (Luo *et al.*, 2019). On the other hand, overexpression of

miR-378 in cumulus cells decreased expansion level, and impaired oocyte meiotic progression (Pan *et al.*, 2015).

According to the analysis, the most targeted biological processes are mainly related to the regulation of transcription. Both, positive and negative regulations are involved. This shows that transcriptional activity changes in cumulus cells isolated from two developmentally different stages and that this activity is regulated by a specific set of miRNAs. Other important processes are linked to cytokinesis, cell division and cell cycle. From the GV stage to the MII stage the oocyte has to restart the cell cycle to be able to extrude the first polar body. This asymmetric cell division process involves orchestrated movements of microfilaments, intermediate filaments and microtubules (Clift and Schuh, 2013)*.

Found miRNAs predictably targeted genes whose molecular function was linked to the binding of all kinds. Highly targeted were the protein and metal ion binding activity and the rest was principally linked to the binding of transcriptional factors and molecules important for transcription. In the cellular component the nucleus, nucleoplasm, cytoplasm and chromatin were cellular parts which were tightly linked to the targeted genes of the miRNAs.

To summarize, miRNAs as molecular entities can positively or negatively regulate and influence important mechanisms leading to proper oocyte and cumulus cell competence. On the one hand, it would be nice to use miRNAs in cumulus cells as reliable biomarkers. Primarily, because the oocyte is an important cell for fertilization itself and hundreds of cumulus cells loosely speaking just hang around. It is important to note that single miRNAs isolated from cumulus cells probably could not be used as effective quality markers of the oocyte as we did not find any significantly DE miRNAs between high- and low-quality cumulus cells. Similar conclusions come from Uhde *et al.*, (2017) who state that there was not any significant difference in miRNA expression from cumulus cells derived from oocytes that became blastocysts versus those that were from non-cleaved oocytes. With this Green *et al.*, (2018) conclude that cumulus cell transcriptome of embryos resulting in live birth compared to sibling embryo that failed to implant does not show significant differences and therefore, cannot be used as a dependable marker.

On the other hand, changes in the miRNA expression in the cumulus cells, while maturing COCs, could serve as a potential quality marker. Therefore, it is needed to further investigate the function of identified differentially expressed miRNAs.

7 Conclusions

The irreplaceable functional unit that is established within the cumulus-oocyte complex consists of the oocyte and somatic cumulus cells. The indispensable role of the oocyte in reproduction is known for years. Despite that, cumulus cells with their nurturing and supporting function gain their popularity mainly in the past few years. This goes hand in hand with the discovery of non-coding RNAs, mainly miRNAs, that can regulate posttranscriptional events and on an epigenetic level can reflect the status of the cell.

Although assisted reproduction techniques are worldwide well standardized in human and farm animal medicine, the outcomes of IVM, IVF and IVC are still not satisfactory. Oocyte quality is an important feature that can be evaluated by different methods. Using next-generation sequencing is an established method for detecting the primary structure of DNA and RNA. Cumulus cells as a closely communicating cell type with the oocyte could serve as an advanced quality biomarker.

Unfortunately, the miRNA expression profile of high- and low-quality cumulus cells seems not to be different enough for being used as a quality marker. On the contrary, differences in the miRNA expression during maturation from the germinal vesicle stage to metaphase II could stand for quality markers as they were presented in this project. To surely confirm this statement, it is important to further study and test the molecular functions of for example DE miRNAs identified in this project in the relationship to reproduction.

8 References

Citations marked with a star (*) in the text represent secondary literature.

Abd El Naby, W.S. *et al.* (2013) 'Expression analysis of regulatory microRNAs in bovine cumulus oocyte complex and preimplantation embryos', *Zygote*, 21(1), pp. 31–51. Available at: https://doi.org/10.1017/S0967199411000566.

Alcântara-Neto, A.S. *et al.* (2020) 'Oviduct fluid extracellular vesicles regulate polyspermy during porcine in vitro fertilisation', *Reproduction, Fertility and Development*, 32(4), p. 409. Available at: https://doi.org/10.1071/RD19058.

An, X. *et al.* (2021) 'Whole Transcriptome Analysis: Implication to Estrous Cycle Regulation', *Biology*, 10(6), p. 464. Available at: https://doi.org/10.3390/biology10060464.

Andreas, E. *et al.* (2021) 'The regulatory role of miR-20a in bovine cumulus cells and its contribution to oocyte maturation', *Zygote*, 29(6), pp. 435–444. Available at: https://doi.org/10.1017/S0967199420000933.

Andrei, D. *et al.* (2018) 'Differential miRNA Expression Profiles in Cumulus and Mural Granulosa Cells from Human Pre-ovulatory Follicles', *MicroRNA*, 8(1), pp. 61–67. Available at: https://doi.org/10.2174/2211536607666180912152618.

de Ávila, A.C.F.C.M. *et al.* (2020) 'Estrous cycle impacts microRNA content in extracellular vesicles that modulate bovine cumulus cell transcripts during in vitro maturation†', *Biology of Reproduction*, 102(2), pp. 362–375. Available at: https://doi.org/10.1093/biolre/ioz177.

Bartkova, A. *et al.* (2020) 'Characterization of porcine oocytes stained with Lissamine Green B and their developmental potential in vitro', *Animal Reproduction*, 17(4), pp. 1–11. Available at: https://doi.org/10.1590/1984-3143-ar2020-0533.

Bartolucci, A.F., Uliasz, T. and Peluso, J.J. (2020) 'MicroRNA-21 as a regulator of human cumulus cell viability and its potential influence on the developmental potential of the oocyte', *Biology of Reproduction*, 103(1), pp. 94–103. Available at: https://doi.org/10.1093/biolre/ioaa058.

Baudat, F. *et al.* (2013) 'Meiotic recombination in mammals: Localization and regulation', *Nature Reviews Genetics*, 14(11), pp. 794–806. Available at: https://doi.org/10.1038/nrg3573.

Blondin, P. and Robert, C. (2006) 'Contribution of the oocyte to embryo quality', 65, pp. 126–136. Available at: https://doi.org/10.1016/j.theriogenology.2005.09.020.

Bo Pan and Julang Li (2018) 'MicroRNA-21 up-regulates metalloprotease by down-regulating TIMP3 during cumulus cell-oocyte complex in vitro maturation', *Molecular and Cellular Endocrinology*, 477(January), pp. 29–38. Available at: https://doi.org/10.1016/j.mce.2018.05.011.

Borges Júnior, E. *et al.* (2019) 'Serum microRNA profiling for the identification of predictive molecular markers of the response to controlled ovarian stimulation', *JBRA Assisted Reproduction*, 24(2), pp. 97–103. Available at: https://doi.org/10.5935/1518-0557.20190070.

Capalbo, A. *et al.* (2016) 'MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment', *Fertility and Sterility*, 105(1), pp. 225-235.e3. Available at: https://doi.org/10.1016/j.fertnstert.2015.09.014.

Capra, E. *et al.* (2020) 'Seasonal effects on miRNA and transcriptomic profile of oocytes and follicular cells in buffalo (Bubalus bubalis)', *Scientific Reports*, 10(1), p. 13557. Available at: https://doi.org/10.1038/s41598-020-70546-5.

Chen, H. *et al.* (2017) 'Regulatory Role of miRNA-375 in Expression of BMP15/GDF9 Receptors and its Effect on Proliferation and Apoptosis of Bovine Cumulus Cells', *Cellular Physiology and Biochemistry*, 41(2), pp. 439–450. Available at: https://doi.org/10.1159/000456597.

Christenson, L.K. (2010) 'MicroRNA control of ovarian function.', *Animal reproduction*, 7(3), pp. 129–133. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21666774%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3111027.

Clift, D. and Schuh, M. (2013) 'Restarting life: fertilization and the transition from meiosis to mitosis', *Nature Reviews Molecular Cell Biology*, 14(9), pp. 549–562. Available at: https://doi.org/10.1038/nrm3643.

Deswal, R. and Dang, A.S. (2020) 'Dissecting the role of micro-RNAs as a diagnostic marker for polycystic ovary syndrome: a systematic review and meta-analysis', *Fertility and Sterility*, 113(3), pp. 661-669.e2. Available at: https://doi.org/10.1016/j.fertnstert.2019.11.001.

Diez-Fraile, A. *et al.* (2014) 'Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of in vitro fertilization', *Human Fertility*, 17(2), pp. 90–98. Available at: https://doi.org/10.3109/14647273.2014.897006.

Donadeu, F.X., Mohammed, B.T. and Ioannidis, J. (2017) 'A miRNA target network putatively involved in follicular atresia', *Domestic Animal Endocrinology*, 58, pp. 76–83. Available at: https://doi.org/10.1016/j.domaniend.2016.08.002.

Dutta, R. *et al.* (2016) 'Non-invasive assessment of porcine oocyte quality by supravital staining of cumulus–oocyte complexes with lissamine green B', *Zygote*, 24(3), pp. 418–427. Available at: https://doi.org/10.1017/S0967199415000349.

Eppig, J.J. (2001) 'Oocyte control of ovarian follicular development and function in mammals', *Reproduction*, 122(6), pp. 829–838. Available at: https://doi.org/10.1530/rep.0.1220829.

Fair, T. *et al.* (2007) 'Global gene expression analysis during bovine oocyte in vitro maturation', *Theriogenology*, 68(SUPPL. 1), pp. S91–S97. Available at: https://doi.org/10.1016/j.theriogenology.2007.04.018.

Fischer, N.M. *et al.* (2021) 'Prognostic value of oocyte quality in assisted reproductive technology outcomes: a systematic review', *F&S Reviews*, 2(2), pp. 120–139. Available at: https://doi.org/10.1016/j.xfnr.2021.03.001.

Gad, A. *et al.* (2019) 'microRNA expression profile in porcine oocytes with different developmental competence derived from large or small follicles', *Molecular Reproduction and Development*, 86(4), pp. 426–439. Available at: https://doi.org/10.1002/mrd.23121.

Gad, A., Murin, M., *et al.* (2020) 'Inhibition of miR-152 during In Vitro Maturation Enhances the Developmental Potential of Porcine Embryos', *Animals*, 10(12), p. 2289. Available at: https://doi.org/10.3390/ani10122289.

Gad, A., Sánchez, J.M., *et al.* (2020) 'Plasma extracellular vesicle miRNAs as potential biomarkers of superstimulatory response in cattle', *Scientific Reports*, 10(1), p. 19130.

Available at: https://doi.org/10.1038/s41598-020-76152-9.

Gad, A. *et al.* (2022) 'Small-extracellular vesicles and their microRNA cargo from porcine follicular fluids: the potential association with oocyte quality', *Journal of Animal Science and Biotechnology*, 13(1). Available at: https://doi.org/10.1186/s40104-022-00723-1.

Gebremedhn, S. *et al.* (2015) 'MicroRNA Expression Profile in Bovine Granulosa Cells of Preovulatory Dominant and Subordinate Follicles during the Late Follicular Phase of the Estrous Cycle', *PLOS ONE*. Edited by M. Zhang, 10(5), p. e0125912. Available at: https://doi.org/10.1371/journal.pone.0125912.

Gebremedhn, S. *et al.* (2021) 'MicroRNA-Mediated Gene Regulatory Mechanisms in Mammalian Female Reproductive Health', *International Journal of Molecular Sciences*, 22(2), p. 938. Available at: https://doi.org/10.3390/ijms22020938.

Gilchrist, R.B., Lane, M. and Thompson, J.G. (2008) 'Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality', *Human Reproduction Update*, 14(2), pp. 159–177. Available at: https://doi.org/10.1093/humupd/dmm040.

Green, K.A. *et al.* (2018) 'Cumulus cell transcriptome profiling is not predictive of live birth after in vitro fertilization: a paired analysis of euploid sibling blastocysts', *Fertility and Sterility*, 109(3), pp. 460-466.e2. Available at: https://doi.org/10.1016/j.fertnstert.2017.11.002.

Grossman, H. *et al.* (2020) 'Pre-ovulatory intercellular regulation of miR-125a-3p within mouse ovarian follicles', *Reproduction*, 159(2), pp. 215–225. Available at: https://doi.org/10.1530/REP-19-0419.

Han, X. *et al.* (2021) 'Expression profiling and function analysis identified new microRNAs regulating cumulus expansion and apoptosis in cumulus cells', *Molecular Human Reproduction*, 28(1), p. gaab070. Available at: https://doi.org/10.1093/molehr/gaab070.

Hillier, S.G. (1994) 'Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis', *Human Reproduction*, 9(2), pp. 188–191. Available at: https://doi.org/10.1093/oxfordjournals.humrep.a138480.

Hou, Y. *et al.* (2019) 'Bioinformatics identification of microRNAs involved in polycystic ovary syndrome based on microarray data', *Molecular Medicine Reports*, 20(1), pp. 281–291. Available at: https://doi.org/10.3892/mmr.2019.10253.

Hu, J. *et al.* (2020) 'The expression of small RNAs in exosomes of follicular fluid altered in human polycystic ovarian syndrome', *PeerJ*, 8(2), p. e8640. Available at: https://doi.org/10.7717/peerj.8640.

Huang, D.W., Sherman, B.T. and Lempicki, R.A. (2009) 'Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources', *Nature Protocols*, 4(1), pp. 44–57. Available at: https://doi.org/10.1038/nprot.2008.211.

Inoue, Y. *et al.* (2020) 'Prediction of major microRNAs in follicular fluid regulating porcine oocyte development', *Journal of Assisted Reproduction and Genetics*, 37(10), pp. 2569–2579. Available at: https://doi.org/10.1007/s10815-020-01909-0.

Jaffe, L.A. and Egbert, J.R. (2017) 'Regulation of Mammalian Oocyte Meiosis by Intercellular Communication Within the Ovarian Follicle', *Annual Review of Physiology*, 79(1), pp. 237–260. Available at: https://doi.org/10.1146/annurev-physiol-022516-034102.

Kanehisa, M. *et al.* (2017) 'KEGG: new perspectives on genomes, pathways, diseases and drugs', *Nucleic Acids Research*, 45(D1), pp. D353–D361. Available at:

https://doi.org/10.1093/nar/gkw1092.

Karakaya, C. *et al.* (2015) 'Poor ovarian response in women undergoing in vitro fertilization is associated with altered microRNA expression in cumulus cells', *Fertility and Sterility*, 103(6), pp. 1469-1476.e3. Available at: https://doi.org/10.1016/j.fertnstert.2015.02.035.

Kataruka, S. *et al.* (2020) 'MicroRNA dilution during oocyte growth disables the microRNA pathway in mammalian oocytes', *Nucleic Acids Research*, 48(14), pp. 8050–8062. Available at: https://doi.org/10.1093/nar/gkaa543.

Kataruka, S. *et al.* (2022) 'Physiologically relevant miRNAs in mammalian oocytes are rare and highly abundant', *EMBO reports*, 23(2). Available at: https://doi.org/10.15252/embr.202153514.

Khan, H.A. *et al.* (2015) 'Identification of miRNAs during mouse postnatal ovarian development and superovulation', *Journal of Ovarian Research*, 8(1), p. 44. Available at: https://doi.org/10.1186/s13048-015-0170-2.

Kim, Y.J. *et al.* (2013) 'MicroRNAs transfected into granulosa cells may regulate oocyte meiotic competence during in vitro maturation of mouse follicles', *Human Reproduction*, 28(11), pp. 3050–3061. Available at: https://doi.org/10.1093/humrep/det338.

Kropp, J., Salih, S.M. and Khatib, H. (2014) 'Expression of microRNAs in bovine and human pre-implantation embryo culture media', *Frontiers in Genetics*, 5(APR), pp. 1–8. Available at: https://doi.org/10.3389/fgene.2014.00091.

Lee, Y. *et al.* (2003) 'The nuclear RNase III Drosha initiates microRNA processing', *Nature*, 425(6956), pp. 415–419. Available at: https://doi.org/10.1038/nature01957.

Li, C. *et al.* (2016) 'BDNF-induced expansion of cumulus-oocyte complexes in pigs was mediated by microRNA-205', *Theriogenology*, 85(8), pp. 1476–1482. Available at: https://doi.org/10.1016/j.theriogenology.2016.01.004.

Li, X. *et al.* (2017) 'MicroRNA-224 delays oocyte maturation through targeting Ptx3 in cumulus cells', *Mechanisms of Development*, 143, pp. 20–25. Available at: https://doi.org/10.1016/j.mod.2016.12.004.

Lin, X. *et al.* (2019) 'Bovine Embryo-Secreted microRNA-30c Is a Potential Non-invasive Biomarker for Hampered Preimplantation Developmental Competence', *Frontiers in Genetics*, 10(APR), pp. 1–15. Available at: https://doi.org/10.3389/fgene.2019.00315.

Liu, W. *et al.* (2017) 'Endo-siRNA deficiency results in oocyte maturation failure and apoptosis in porcine oocytes', *Reproduction, Fertility and Development*, 29(11), p. 2168. Available at: https://doi.org/10.1071/RD16498.

Lopacinska-Joergensen, J. *et al.* (2022) 'Identification of Stably Expressed Reference microRNAs in Epithelial Ovarian Cancer', *In Vivo*, 36(3), pp. 1059–1066. Available at: https://doi.org/10.21873/invivo.12803.

Lopera-Vasquez, R. *et al.* (2017) 'Effect of bovine oviductal extracellular vesicles on embryo development and quality in vitro', *Reproduction*, 153(4), pp. 461–470. Available at: https://doi.org/10.1530/REP-16-0384.

Luo, H. *et al.* (2019) 'Identification of microRNAs in granulosa cells from patients with different levels of ovarian reserve function and the potential regulatory function of miR-23a in granulosa cell apoptosis', *Gene*, 686(October 2018), pp. 250–260. Available at: https://doi.org/10.1016/j.gene.2018.11.025.

Luo, X. *et al.* (2022) 'MicroRNA-101 regulates oocyte maturation in vitro via targeting HAS2 in porcine cumulus cells', *Theriogenology*, 187, pp. 119–126. Available at: https://doi.org/10.1016/j.theriogenology.2022.04.025.

Marchal, R. *et al.* (2002) 'Effect of follicular size on meiotic and developmental competence of porcine oocytes', *Theriogenology*, 57(5), pp. 1523–1532. Available at: https://doi.org/10.1016/S0093-691X(02)00655-6.

Matsuno, Y. *et al.* (2019) 'Characterization of mRNA profiles of the exosome-like vesicles in porcine follicular fluid', *PLOS ONE*. Edited by Y. Taguchi, 14(6), p. e0217760. Available at: https://doi.org/10.1371/journal.pone.0217760.

Matzuk, M.M. *et al.* (2002) 'Intercellular Communication in the Mammalian Ovary: Oocytes Carry the Conversation', *Science*, 296(5576), pp. 2178–2180. Available at: https://doi.org/10.1126/science.1071965.

Murchison, E.P. *et al.* (2007) 'Critical roles for Dicer in the female germline', *Genes & Development*, 21(6), pp. 682–693. Available at: https://doi.org/10.1101/gad.1521307.

Nagyová, E., Němcová, L. and Camaioni, A. (2021) 'Cumulus Extracellular Matrix Is an Important Part of Oocyte Microenvironment in Ovarian Follicles: Its Remodeling and Proteolytic Degradation', *International Journal of Molecular Sciences*, 23(1), p. 54. Available at: https://doi.org/10.3390/ijms23010054.

Navakanitworakul, R. *et al.* (2016) 'Characterization and Small RNA Content of Extracellular Vesicles in Follicular Fluid of Developing Bovine Antral Follicles', *Scientific Reports*, 6(1), p. 25486. Available at: https://doi.org/10.1038/srep25486.

Noferesti, S.S. *et al.* (2015) 'Controlled ovarian hyperstimulation induced changes in the expression of circulatory miRNA in bovine follicular fluid and blood plasma', *Journal of Ovarian Research*, 8(1), p. 81. Available at: https://doi.org/10.1186/s13048-015-0208-5.

O'Shea, L.C. *et al.* (2012) 'Developmental competence in oocytes and cumulus cells: candidate genes and networks', *Systems Biology in Reproductive Medicine*, 58(2), pp. 88–101. Available at: https://doi.org/10.3109/19396368.2012.656217.

Pan, B. *et al.* (2015) 'MicroRNA-378 regulates oocyte maturation via the suppression of aromatase in porcine cumulus cells', *American Journal of Physiology-Endocrinology and Metabolism*, 308(6), pp. E525–E534. Available at: https://doi.org/10.1152/ajpendo.00480.2014.

Ponsuksili, S. *et al.* (2014) 'Differential Expression of miRNAs and Their Target mRNAs in Endometria Prior to Maternal Recognition of Pregnancy Associates with Endometrial Receptivity for In Vivo- and In Vitro-Produced Bovine Embryos1', *Biology of Reproduction*, 91(6), pp. 1–12. Available at: https://doi.org/10.1095/biolreprod.114.121392.

Procházka, R. *et al.* (2009) 'Development of functional LH Receptors on pig cumulus-oocyte complexes cultured in vitro by a novel two-step culture system', *Molecular Reproduction and Development*, 76(8), pp. 751–761. Available at: https://doi.org/10.1002/mrd.21039.

Prochazka, R. and Nemcova, L. (2019) 'Mechanisms of FSH- and Amphiregulin-Induced MAP Kinase 3/1 Activation in Pig Cumulus-Oocyte Complexes During Maturation In Vitro', *International Journal of Molecular Sciences*, 20(5), p. 1179. Available at: https://doi.org/10.3390/ijms20051179.

Qasemi, M. and Amidi, F. (2020) 'Extracellular microRNA profiling in human follicular fluid: new biomarkers in female reproductive potential', *Journal of Assisted*

Reproduction and Genetics, 37(8), pp. 1769–1780. Available at: https://doi.org/10.1007/s10815-020-01860-0.

Ranjbaran, A. *et al.* (2019) 'Exosome-based intercellular communication in female reproductive microenvironments', *Journal of Cellular Physiology*, 234(11), pp. 19212–19222. Available at: https://doi.org/10.1002/jcp.28668.

Robinson, M.D. and Oshlack, A. (2010) 'A scaling normalization method for differential expression analysis of RNA-seq data', *Genome Biology*, 11(3), p. R25. Available at: https://doi.org/10.1186/gb-2010-11-3-r25.

Rodrigues, T.A. *et al.* (2019) 'Follicular fluid exosomes act on the bovine oocyte to improve oocyte competence to support development and survival to heat shock', *Reproduction, Fertility and Development*, 31(5), p. 888. Available at: https://doi.org/10.1071/RD18450.

Salas-Huetos, A. *et al.* (2019) 'The Expression of miRNAs in Human Ovaries, Oocytes, Extracellular Vesicles, and Early Embryos: A Systematic Review', *Cells*, 8(12), p. 1564. Available at: https://doi.org/10.3390/cells8121564.

Salilew-Wondim, D. *et al.* (2020) 'The Role of MicroRNAs in Mammalian Fertility: From Gametogenesis to Embryo Implantation', *International Journal of Molecular Sciences*, 21(2), p. 585. Available at: https://doi.org/10.3390/ijms21020585.

Santonocito, M. *et al.* (2014) 'Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation', *Fertility and Sterility*, 102(6), pp. 1751-1761.e1. Available at: https://doi.org/10.1016/j.fertnstert.2014.08.005.

Schauer, S.N. *et al.* (2013) 'Involvement of miRNAs in equine follicle development', *REPRODUCTION*, 146(3), pp. 273–282. Available at: https://doi.org/10.1530/REP-13-0107.

Shannon, P. *et al.* (2003) 'Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks', *Genome Research*, 13(11), pp. 2498–2504. Available at: https://doi.org/10.1101/gr.1239303.

Sherman, B.T. *et al.* (2022) 'DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update)', *Nucleic Acids Research*, 50(W1), pp. W216–W221. Available at: https://doi.org/10.1093/nar/gkac194.

da Silveira, J.C. *et al.* (2014) 'Regulation of ACVR1 and ID2 by cell-secreted exosomes during follicle maturation in the mare', *Reproductive Biology and Endocrinology*, 12(1), p. 44. Available at: https://doi.org/10.1186/1477-7827-12-44.

da Silveira, J.C. *et al.* (2017) 'Supplementation with small-extracellular vesicles from ovarian follicular fluid during in vitro production modulates bovine embryo development', *PLOS ONE*. Edited by P.J. Hansen, 12(6), p. e0179451. Available at: https://doi.org/10.1371/journal.pone.0179451.

Sinha, P.B. *et al.* (2017) 'MicroRNA-130b is involved in bovine granulosa and cumulus cells function, oocyte maturation and blastocyst formation', *Journal of Ovarian Research*, 10(1), p. 37. Available at: https://doi.org/10.1186/s13048-017-0336-1.

Suh, N. *et al.* (2010) 'MicroRNA Function Is Globally Suppressed in Mouse Oocytes and Early Embryos', *Current Biology*, 20(3), pp. 271–277. Available at: https://doi.org/10.1016/j.cub.2009.12.044.

Svobodova, E., Kubikova, J. and Svoboda, P. (2016) 'Production of small RNAs by

mammalian Dicer', *Pflügers Archiv - European Journal of Physiology*, 468(6), pp. 1089–1102. Available at: https://doi.org/10.1007/s00424-016-1817-6.

Tang, F. *et al.* (2007) 'Maternal microRNAs are essential for mouse zygotic development', *Genes & Development*, 21(6), pp. 644–648. Available at: https://doi.org/10.1101/gad.418707.

Tesfaye, D. *et al.* (2018) 'MicroRNAs: tiny molecules with a significant role in mammalian follicular and oocyte development', *Reproduction*, 155(3), pp. R121–R135. Available at: https://doi.org/10.1530/REP-17-0428.

Toms, D. *et al.* (2021) 'Small RNA sequencing reveals distinct nuclear microRNAs in pig granulosa cells during ovarian follicle growth', *Journal of Ovarian Research*, 14(1), p. 54. Available at: https://doi.org/10.1186/s13048-021-00802-3.

Tong, X.-H. *et al.* (2014) 'Research Resources: Comparative MicroRNA Profiles in Human Corona Radiata Cells and Cumulus Oophorus Cells Detected by Next-Generation Small RNA Sequencing', *PLoS ONE*. Edited by Y. Xue, 9(9), p. e106706. Available at: https://doi.org/10.1371/journal.pone.0106706.

Uhde, K. *et al.* (2017) 'MicroRNA Expression in Bovine Cumulus Cells in Relation to Oocyte Quality', *Non-Coding RNA*, 3(1), p. 12. Available at: https://doi.org/10.3390/ncrna3010012.

Uzbekova, S. *et al.* (2020) 'Protein Cargo of Extracellular Vesicles From Bovine Follicular Fluid and Analysis of Their Origin From Different Ovarian Cells', *Frontiers in Veterinary Science*, 7(November). Available at: https://doi.org/10.3389/fvets.2020.584948.

Valadi, H. *et al.* (2007) 'Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells', *Nature Cell Biology*, 9(6), pp. 654–659. Available at: https://doi.org/10.1038/ncb1596.

Vendrell-Flotats, M. *et al.* (2020) 'In vitro maturation in the presence of Leukemia Inhibitory Factor modulates gene and miRNA expression in bovine oocytes and embryos', *Scientific Reports*, 10(1), p. 17777. Available at: https://doi.org/10.1038/s41598-020-74961-6.

Wright, E.C. *et al.* (2016) 'MicroRNA-21 and PDCD4 expression during in vitro oocyte maturation in pigs', *Reproductive Biology and Endocrinology*, 14(1), p. 21. Available at: https://doi.org/10.1186/s12958-016-0152-2.

Yoav Benjamini and Yosef Hochberg (1995) 'Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing', *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1), pp. 289–300.

Yoshioka, K., Suzuki, C. and Onishi, A. (2008) 'Defined System for In Vitro Production of Porcine Embryos Using a Single Basic Medium', *Journal of Reproduction and Development*, 54(3), pp. 208–213. Available at: https://doi.org/10.1262/jrd.20001.

Yuan, Y. *et al.* (2017) 'Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation', *Proceedings of the National Academy of Sciences*, 114(29), pp. E5796–E5804. Available at: https://doi.org/10.1073/pnas.1703998114.

Zaborowski, M.P. *et al.* (2015) 'Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study', *BioScience*, 65(8), pp. 783–797. Available at: https://doi.org/10.1093/biosci/biv084.

Zhang, J. *et al.* (2019) 'MicroRNA-375 regulates oocyte in vitro maturation by

targeting ADAMTS1 and PGR in bovine cumulus cells', *Biomedicine & Pharmacotherapy*, 118(7), p. 109350. Available at: https://doi.org/10.1016/j.biopha.2019.109350.

Zhao, M. and Dean, J. (2002) 'The zona pellucida in folliculogenesis, fertilization and early development', *Reviews in Endocrine and Metabolic Disorders*, 3(1), pp. 19–26. Available at: https://doi.org/10.1023/a:1012744617241.

Zou, X. *et al.* (2020) 'Comprehensive analysis of mRNAs and miRNAs in the ovarian follicles of uniparous and multiple goats at estrus phase', *BMC Genomics*, 21(1), pp. 1–15. Available at: https://doi.org/10.1186/s12864-020-6671-4.