

Aus dem Institut für Veterinär-Biochemie  
des Fachbereichs Veterinärmedizin  
der Freien Universität Berlin

**Characterization of microRNA (miRNA) profiles in selected female reproductive tissues  
of cattle: prediction of fertility associated pathways**

Inaugural-Dissertation  
zur Erlangung des Grades eines  
Doctor of Philosophy (Ph.D.)  
in Biomedical Sciences  
an der  
Freien Universität Berlin

vorgelegt von  
**Sergio Eliseo Palma Vera**  
aus Santiago, Chile

Berlin 2015

Journal-Nr.: 3837

Gedruckt mit Genehmigung  
des Fachbereichs Veterinärmedizin  
der Freien Universität Berlin

**Dekan:** Univ.-Prof. Dr. Jürgen Zentek  
**Erster Gutachter:** Univ.-Prof. Dr. Dr. Ralf Einspanier  
**Zweiter Gutachter:** Univ.-Prof. Dr. Johanna Plendl  
**Dritter Gutachter:** PD Dr. Jennifer Schön

**Deskriptoren (nach CAB-Thesaurus):** cattle, hormones, oestrous cycle, oviducts, endometrium, epithelium, cell culture, in vitro, differentiation, polarization

**Tag der Promotion:** 26.04.2016

*To my family and friends*



## Contents

<b>1. Introduction.....</b>	<b>1</b>
1.1. MiRNA biogenesis and mechanisms of action.....	1
1.2. Principles of miRNA-target interactions .....	2
1.3. Description of selected female reproductive tissues in cattle.....	3
1.3.1. Endometrium.....	3
1.3.2. Oviduct.....	3
1.4. Characterization of miRNAs in bovine endometrium and oviduct .....	4
1.4.1. Endometrium.....	4
1.4.2. Oviduct.....	4
<b>2. Aims of the study.....</b>	<b>5</b>
<b>3. Collection and description of published work.....</b>	<b>6</b>
3.1. Publication 1:.....	6
3.1.1. Follow up studies (data not included in publication 3.1).....	14
3.1.2. Conclusions .....	18
3.2. Publication 2:.....	19
3.2.1. Follow up studies (data not included in publication 3.2).....	27
3.2.2. Conclusions .....	29
<b>4. Discussion.....</b>	<b>31</b>
4.1. A reproducible long term BOEC culture system.....	31
4.2. Insulin effect on cell morphology, gene and miRNA expression of BOECs .....	32
4.3. Applications of the BOEC model.....	33
4.4. Endometrial sample collection and RNAseq strategy .....	33
4.5. <i>In vitro</i> validation of endometrial miRNAs.....	33
4.6. Comparison to reported transcriptomic studies in endometrium.....	34
4.7. Conclusions .....	35
<b>5. Summary.....</b>	<b>37</b>
<b>6. Outlook.....</b>	<b>41</b>
<b>7. References.....</b>	<b>42</b>
<b>8. Publication list.....</b>	<b>46</b>

8.1. First authorship publications .....	46
8.2. Co-authorship publications.....	46
8.3. First authorship presentations (scientific abstracts).....	46
8.4. Co-authorship presentations (scientific abstracts).....	47
<b>9. Acknowledgements .....</b>	<b>48</b>
<b>10. Selbständigkeitserklärung.....</b>	<b>49</b>

## Abbreviations

ACTB	actin, beta
Ago	Argonaute protein
BEND	endometrial cell line
BOECs	oviductal epithelial cells
CDC7L1	cell division cycle 7
CDK2	cyclin-dependent kinase 2
CDKN1B	cyclin-dependent kinase inhibitor 1B
COX-2	cytochrome c oxidase subunit II
cPLA2	phospholipase A2, group IVA
DGCR8	DiGeorge syndrome critical region gene 8
E2F6	E2F transcription factor 6
ESR1	estrogen receptor 1
HGSOC	High grade serous ovarian carcinoma
IFNT	interferon-tau
KEGG	Kyoto encyclopedia of genes and genomes
LPS	lipopolysaccharide (LPS)
MAPK	mitogen activated kinase-like protein
MAPK14	mitogen-activated protein kinase 14
MAPK8	mitogen-activated protein kinase 8
miRISC	miRNA-induced silencing complex
miRNA*	miRNA passenger strand
miRNAs	MicroRNAs
ncRNAs	non-coding RNAs
OVGP1	oviductal glycoprotein 1
PDBU	Phorbol 12,13-dibutyrate
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide
PGF2alpha	Prostaglandin F2 alpha

PGR	progesterone receptor
PKC	protein kinase C
PLA2G4A	phospholipase A2, group IVA (cytosolic, calcium-dependent)
pre-miRNAs	precursor miRNA
pri-miRNAs	primary miRNA
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
RNAseq	RNA sequencing
SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
STIC	serous tubal intraepithelial carcinoma
UTR	untranslated region



## 1. Introduction

MicroRNAs (miRNAs) constitute a large family of short, non-coding RNAs (ncRNAs) (~22 nucleotides long) that are common in single-celled eukaryotes, plant and animal cells. In humans, 2588 miRNAs have already been discovered and in bovine, the amount of identified miRNAs corresponds to 793 (<http://www.mirbase.org/>). MicroRNAs are predicted to control approximately 50% of gene expression in mammals, having roles in physiological as well as in pathological events [1].

Embryonic mortality is one of the main sources of subfertility in cattle, leading to significant economic losses in the dairy industry [2]. Failed implantation is one of the sources of this problem, mainly due to impaired endometrial receptivity and poor embryo quality [3]. Similarly, the oviduct is the first site of contact with the early embryo and provides important factors that directly affect fertility and embryo development [3].

Therefore, in order to improve the current knowledge on the molecular mechanisms driving fertility in cattle, it is necessary to understand the role of miRNAs in these two fertility-defining tissues: oviduct and endometrium.

### 1.1. MiRNA biogenesis and mechanisms of action

MiRNAs play a crucial role in the post-transcriptional regulation of gene expression, mostly involving gene silencing [4]. MiRNAs are processed from precursor molecules (pri-miRNAs), which are either transcribed by RNA polymerase II from independent genes or represent introns of protein-coding genes [1]. As a result, a hairpin is formed and excised by Drosha, an RNase III enzyme, and its cofactor DGCR8 (DiGeorge syndrome critical region gene 8, also known as Pasha), producing the 60–70 nucleotide precursor miRNA (pre-miRNA). The precursor hairpin is exported out of the nucleus by Exportin 5 where another RNase III enzyme, Dicer, processes the precursor into the 21–24 nucleotide duplex miRNA (miRNA/miRNA\*) [5]. One strand of this duplex, representing a mature miRNA, is then incorporated into the miRNA-induced silencing complex (miRISC) [1]. During assembly of the miRISC, the duplex is loaded into the Argonaute protein (Ago), and one of the strands (called the “passenger strand” or miRNA\*) is released and often degraded [4]. However, miRNA\* strands are not always by-products of miRNA biogenesis and can also be loaded into miRISC to function as miRNAs [1].

Consequently, the bound miRNA strand (the “guide strand”) guides miRISC to interact with partially complementary sequences in target transcripts (mostly localized within the 3' untranslated region (UTR)) and triggers mRNA degradation or translation inhibition [4]. MiRNAs can promote mRNA degradation by either of two mechanisms: direct Argonaute catalyzed endonucleolytic cleavage of the target, or deadenylation and exonucleolytic attack

[6]. Direct cleavage by Argonaute only occurs when there is near perfect complementarity between the miRNA and target mRNA, a situation that occurs much more frequently in plants than in mammals [6]. Translational repression is less understood. However, there is accumulating evidence that translational repression of targets is the primary mechanism of miRNAs, occurring even without the need of mRNA degradation [7].

## **1.2. Principles of miRNA-target interactions**

It is accepted that a single miRNA species can regulate hundreds of targets and conversely, several miRNAs can bind to their target mRNAs and cooperatively provide fine-tuning of a single mRNA target expression [8]. Therefore, effective prediction of miRNA-mRNA interactions remains challenging [9]. In general, modeling miRNA targeting consists in 4 main aspects: seed match, conservation, free energy, and site accessibility [10].

**Seed match:** A key specificity determinant for miRNA target recognition is based on Watson-Crick pairing of 5'-proximal "seed" region (nucleotide 2 to 8) in the miRNA to the seed match site in the target mRNA, located in the 3' UTR. It is also claimed that a small subset of miRNAs modulate expression by specifically targeting the 5' UTR and/or coding region of some mRNAs [8].

**Conservation:** Conservation refers to the maintenance of a sequence across species. Conservation analysis may focus on regions in the 3' UTR, the 5' UTR, the miRNA, or any combination of the three. In general, there is higher conservation in the miRNA seed region than in the non-seed region. In a small proportion of miRNA-mRNA target interactions, there is conserved pairing at the 3' end of the miRNA which can compensate for seed mismatches, and these sites are called 3' compensatory sites. In the context of predicting miRNA targets in 3' UTR, conservation analysis may provide evidence that a predicted miRNA target is functional because it is being selected for [10].

**Free energy:** Free energy can be used as a measure of the stability of a biological system. If the binding of a miRNA to a candidate target mRNA is predicted to be stable, it is considered more likely to be a true target of the miRNA. The change in free energy during a reaction is known as  $\Delta G$ . Since reactions with a negative  $\Delta G$  have less energy available to react in the future, they result in systems with increased stability. By predicting how the miRNA and its candidate target hybridize, regions of high and low free energy can be inferred and the overall  $\Delta G$  can be used as an indicator of how strongly bound they are [10].

**Site accessibility:** Site accessibility is a measure of the ease with which a miRNA can locate and hybridize with the mRNA target. Following transcription, mRNA assumes a secondary structure [11], which can interfere with a miRNA's ability to bind to a target site. MiRNA-mRNA hybridization involves a two-step process in which a miRNA binds first to a short

accessible region of the mRNA. The mRNA secondary structure then unfolds as the miRNA completes binding to a target. Therefore, to assess the likelihood that a mRNA is the target of a miRNA, the predicted amount of energy required to make a site accessible to a miRNA can be evaluated [10].

Other features: Target-site abundance is a measure of how many target sites occur in a 3' UTR. Local AU content refers to the concentration of A and U nucleotides flanking the corresponding seed region of the miRNA [10] the accessibility of the target site in the secondary structure of the 3' UTR, as defined by the AU content in the vicinity of the site [12]. GU wobble in the seed match refers to the allowance of a G pairing with a U instead of a C [13]. 3' compensatory pairing refers to base pair matching with miRNA nucleotides 12–17. Seed pairing stability is the calculated free energy of the predicted duplex [14]. Finally, position contribution analyzes the position of the target site within the mRNA [10].

In conclusion, by combining different aspects of miRNA-mRNA interactions, prediction of targets and regulated biological processes can be performed. However, computational implementation of these models leads to a high number of valid outcomes, indicating a dynamic effect of miRNAs in different biological pathways [15].

### **1.3. Description of selected female reproductive tissues in cattle**

#### **1.3.1. Endometrium**

The cow uterus is bicornuate, characterized by having two uterine horns and a small uterine body [16]. From external to internal, the uterus can be divided into three layers: the perimetrium, the myometrium, and the endometrium. The perimetrium is the continuation of the abdominal peritoneum onto the uterus. The myometrium constitutes the muscular layers, which can undergo substantial hypertrophy. The endometrium is the internal epithelial layer of the uterus and is arranged into two distinct regions, caruncular and intercaruncular [17]. Caruncles are small non-glandular protuberances that protrude from the surface of the endometrium [16]. The caruncles join with the cotyledons of the fetal placental membranes to form the placentomes [17]. The intercaruncular endometrium contains glands, whose products are important for the development of the conceptus [18, 19].

#### **1.3.2. Oviduct**

The oviduct consists of the infundibulum, ampulla and isthmus. The infundibulum is the terminal end of the oviduct with a funnel-shaped opening, forming a pocket that captures the newly ovulated oocyte. The surface of the infundibulum is covered with projections called fimbriae that increase the surface of the infundibulum, allowing it to glide over the ovarian surface near the time of ovulation and capture the oocyte. The ampulla occupies one half or more of the oviductal length and merges with the isthmus of the oviduct. The ampulla has a

relatively large diameter, with the internal portions characterized by many fern-like mucosal folds with ciliated epithelium. The isthmus is smaller in diameter than the ampulla. It is connected directly to the uterus and the point of juncture is called the uterotubal junction. The isthmus has a thicker mucosal wall than the ampulla and has fewer mucosal layer folds [16].

#### **1.4. Characterization of miRNAs in bovine endometrium and oviduct**

Dairy production systems have suffered a decline in cow fertility during the last decades. Therefore, one of the greatest challenges of reproductive biologists, nutritionists and geneticists is to gain an understanding of the underlying biology driving low fertility [20]. In this context, studies have been conducted to identify fertility-associated miRNAs.

##### **1.4.1. Endometrium**

A study conducted in cows with subclinical endometritis showed aberrant expression of 23 miRNAs. Predicted targets for these miRNAs were connected to signaling pathways mediating inflammatory responses, cellular proliferation, cell movement, the cell cycle and apoptosis [21]. Ponsuksili *et al.* [22] retrieved endometrial samples on days 3 and 7 of the estrous cycle, before embryo transfer was performed at the next cycle, connecting a set of 11 miRNAs to high endometrial receptivity. The miRNA–mRNA predicted pairs had roles in response to hormonal stimuli and oxidative stress, chromatin organization, miRNA-mediated epigenetic histone changes, cell proliferation, p53 signaling and apoptosis.

##### **1.4.2. Oviduct**

At the point of this review, only one study had been reported exploring the role of miRNAs in cow oviductal function [23]. In this study, bovine oviductal epithelial cells (BOECs) were grown as monolayer *in vitro* for short term and exposed to lipopolysaccharide (LPS). Additionally, the LPS effect on the ability of BOECs to support embryo development was assessed. In the context of miRNA expression, miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215 were suppressed after LPS treatment. The authors conclude that LPS might produce changes in the oviductal transcriptome, leading to a suboptimal environment for embryo development.

## **2. Aims of the study**

The purpose of this study is to characterize miRNA expression in bovine endometrium and oviduct and to predict their role controlling fertility. Specifically, the aims of this study are:

- To characterize miRNAs expressed in bovine endometrium and oviduct.
- To develop cell culture systems for the study of miRNAs *in vitro*.
- To assess *in vitro* the regulation of miRNA expression in response to treatments mimicking physiological conditions.
- To predict the reproductive pathways regulated by miRNAs.

### **3. Collection and description of published work**

The work and findings of this PhD project have been reported in detail in two peer-reviewed publications:

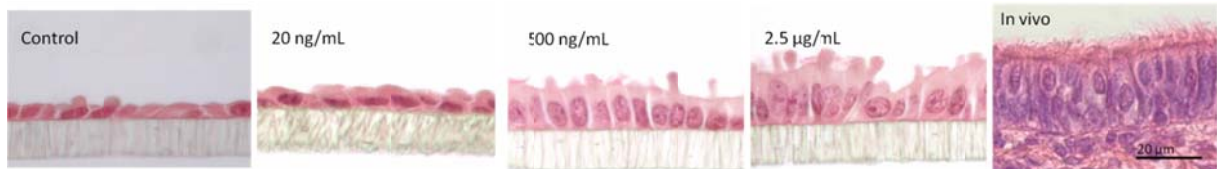
#### **3.1. Publication 1:**

Palma-Vera S, Einspanier R, Schoen J. 2014. Bovine oviductal epithelial cells: Long term culture characterization and impact of insulin on cell morphology. *Reproductive biology* 14(3):206-212. DOI: <http://dx.doi.org/10.1016/j.repbio.2014.04.006>

You have to purchase this part online.

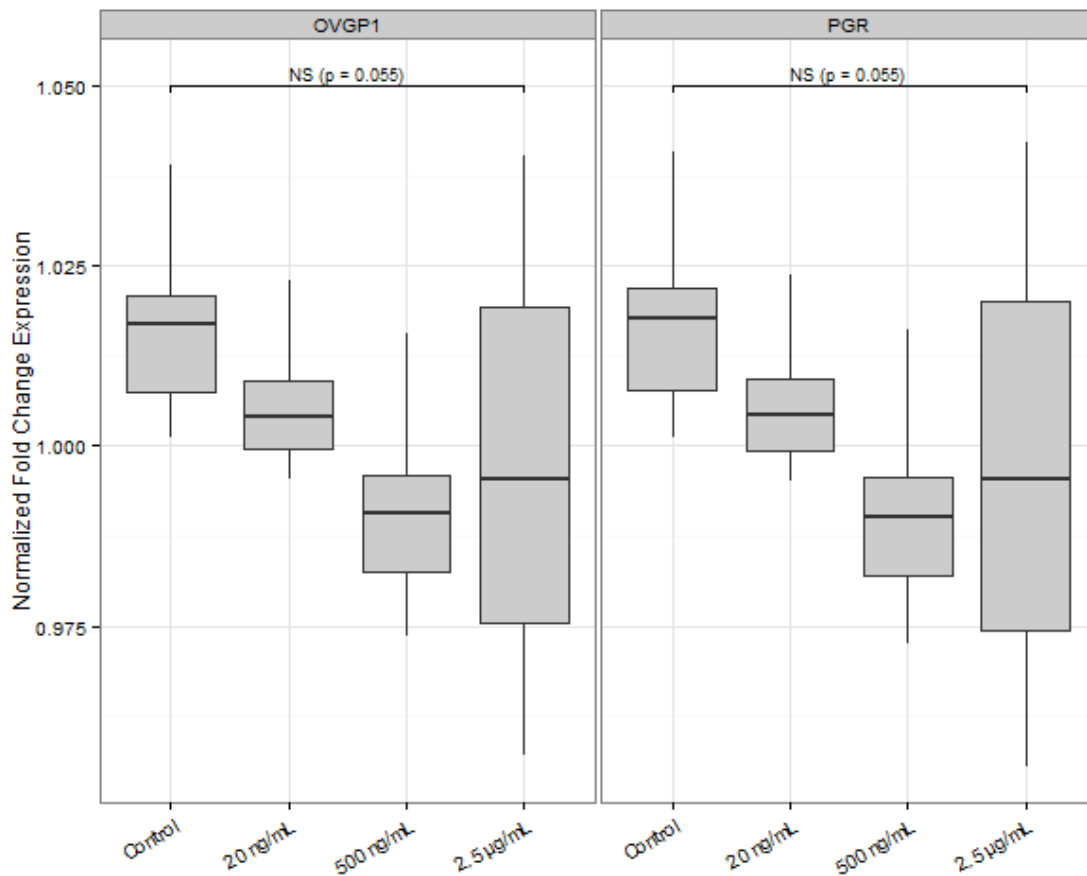
### 3.1.1. Follow up studies (data not included in publication 3.1)

To complement the findings reported in the first publication, the effect of insulin was further studied by including two new doses: 500 ng/mL and 2.5 µg/mL (n = 4). Figure 1 shows a representative microphotograph of BOECs in an air liquid interphase, after 3 weeks of culture. Interestingly, cell morphology was improved by 500 ng/mL and 2.5 µg/mL, compared to 20 ng/mL and control. The 500 ng/mL and 2.5 µg/mL doses induced a phenotype that resembled most closely the *in vivo* features (Fig. 1).



**Figure 1.** Representative microphotographs (haematoxylin/eosin) of primary bovine oviduct epithelial cells cultured for 3 weeks without supplementation (Control) and under three different insulin doses (20 ng/mL, 500 ng/mL and 2.5 µg/mL). As a reference, a section of oviductal tissue is shown.

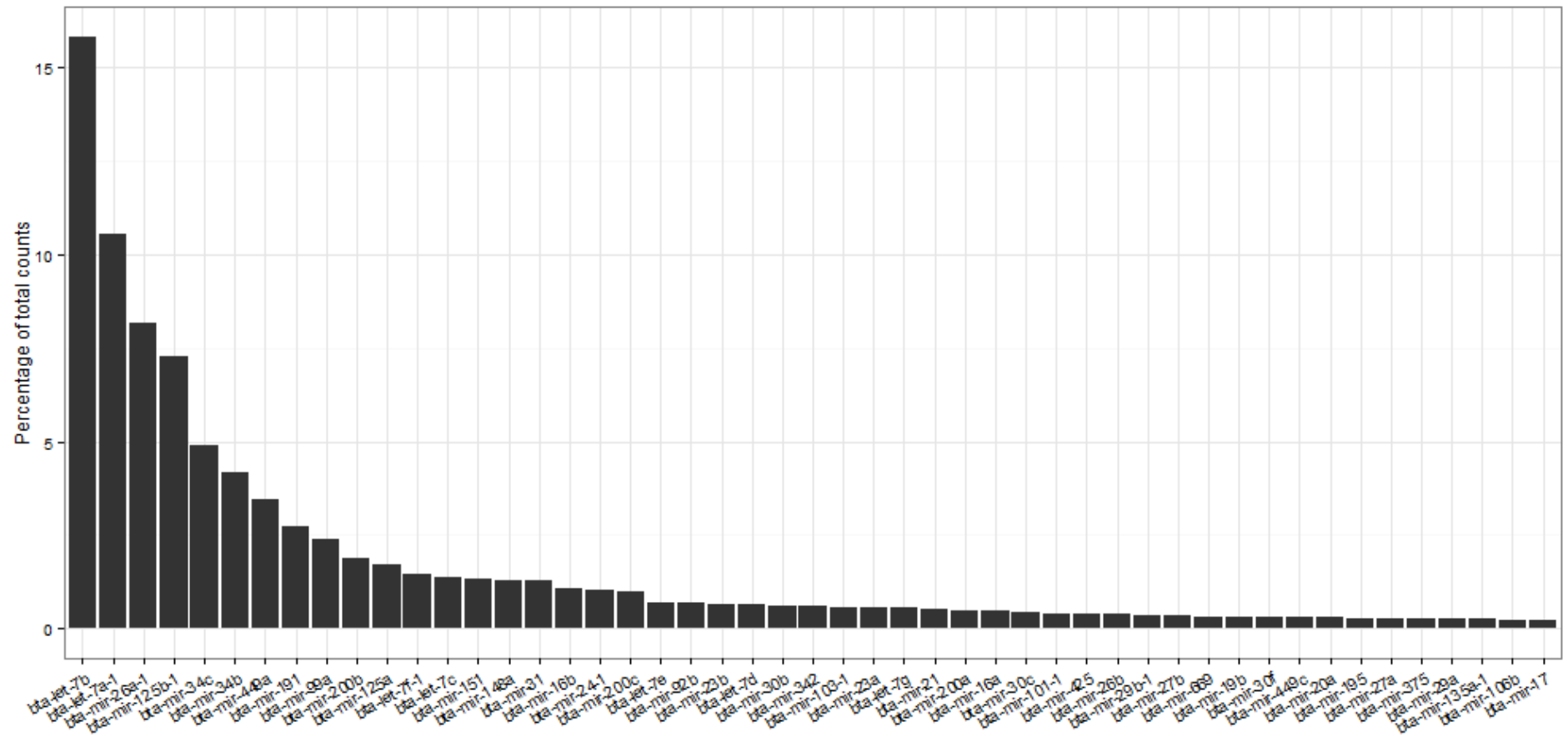
In order to further analyze the effect of insulin on cell differentiation, two markers were tested: oviductal glycoprotein 1 (*OVGP1*) and progesterone receptor (*PGR*) (Fig. 2). Analysis of variance did not find significant differences among treatments, however, there was a strong negative interaction of insulin concentration and mRNA expression of *OVGP1* and *PGR* ( $r_b = -0.43866$ ).



**Figure 2.** Normalized fold changes of *OVGPI* and *PGR* mRNA expression in controls and insulin treated BOECs after 3 weeks of culture. Transcript expression was normalized to a combination of two housekeeping genes (actin, beta (*ACTB*) and succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*)). Values are shown as box plots.

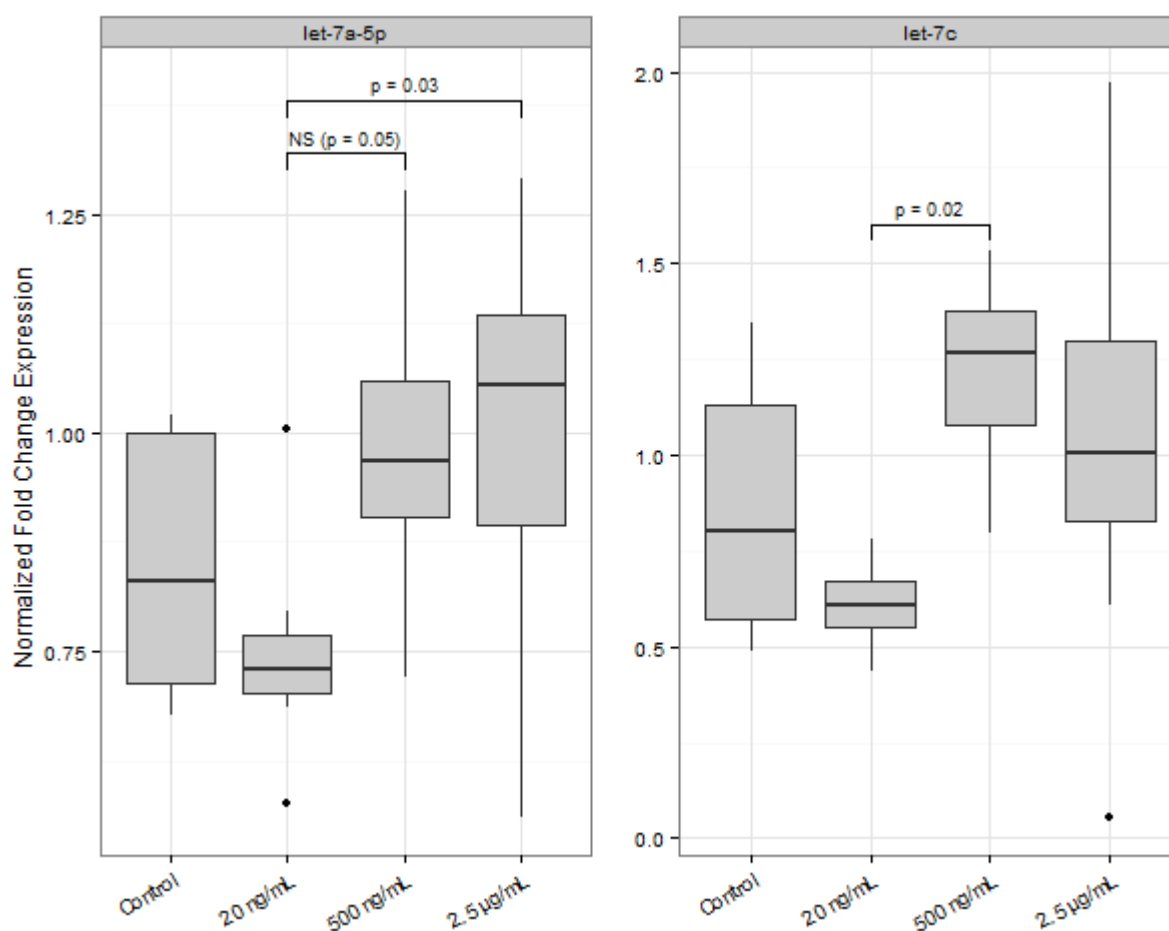
In parallel, the small fraction of RNAs from oviductal tissue, epithelial fraction, of two cows was pooled and sequenced. Figure 3 shows the expression of miRNAs accumulating 90% of total miRNA expression, of which approximately 50% and 70% accumulated in only 5 and 16 miRNAs, respectively.





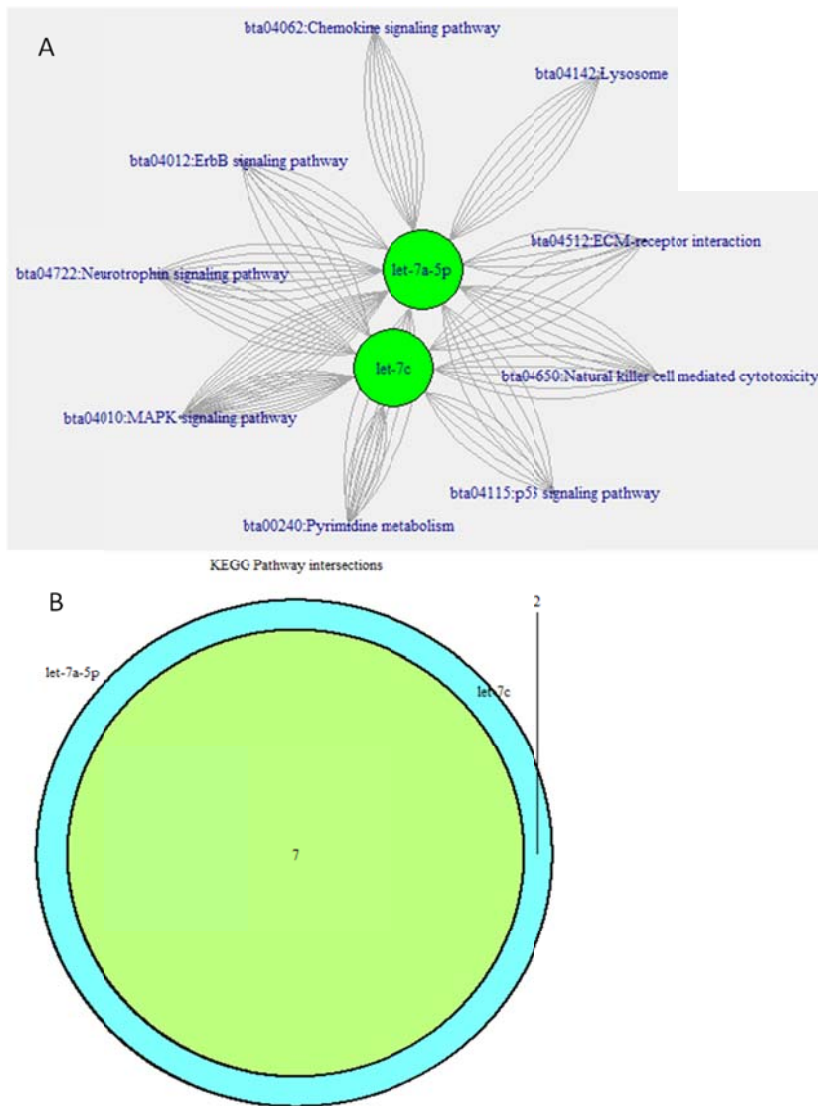
**Figure 3.** Expression of oviductal miRNAs representing 90% of the total expression. Expression values are shown as percentage of the total ncRNAs counts.

*In vivo* detected miRNAs were analyzed *in vitro* in BOECs. The aim was to validate their expression in BOECs and to verify the effect of insulin in miRNA levels. Two miRNAs showed significant regulations. Let-7a-5p and let-7c showed an up-regulation when insulin concentration was increased from 20 ng/mL to 500 ng/mL in the culture medium (Fig. 4).



**Figure 4.** Normalized fold changes for miRNAs let-7a-5p and let-7c expression in controls and insulin treated BOECs after 3 weeks of culture. Expression was normalized to a combination of the three most stable miRNAs (let-7f, miR-26a, miR-151). Values are shown as box plots.

Considering the regulation of let-7a-5p and let-7c as a result of increasing insulin concentration, their targets were predicted and used to search for regulated pathways. Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis indicated that these two miRNAs were highly similar in terms of their predicted biological functions, sharing 7 pathways and leaving only 2 pathways exclusively to let-7a-5p (Fig. 5). The most targeted common pathway was mitogen activated kinase-like protein (MAPK) signaling pathway.



**Figure 5.** A: Network of miRNA predicted targets interactions and enriched KEGG pathways, where the number of connections indicates the amount of miRNA targets assigned to a pathway. B: Venn diagram of intersected KEGG pathways between let-7a-5p and let-7c.

### 3.1.2. Conclusions

BOECs were consistently grown *in vitro* for long terms in an air-liquid interphase system. Even though cells were able to express molecular markers of differentiation, secretory activity and columnar morphology, ciliated cells were not detected.

Additionally, BOECs were highly sensitive to insulin concentrations in the medium. Insulin had large effects on cell morphology and affected the mRNA expression of *OVGP1* and *PGR* and the miRNA expression of let-7a-5p and let-7c.

Target prediction and pathway analysis showed that let-7a-5p, and let-7c shared functions regulating cellular pathways, including MAPK signaling pathway.

### **3.2. Publication 2:**

**Palma-Vera S**, Sharbati S, Einspanier R. Identification of miRNAs in cow endometrium through RNAseq and prediction of regulated pathways. *Reprod Domest Anim*, paper in print.

DOI: <http://dx.doi.org/10.1111/rda.12590>.

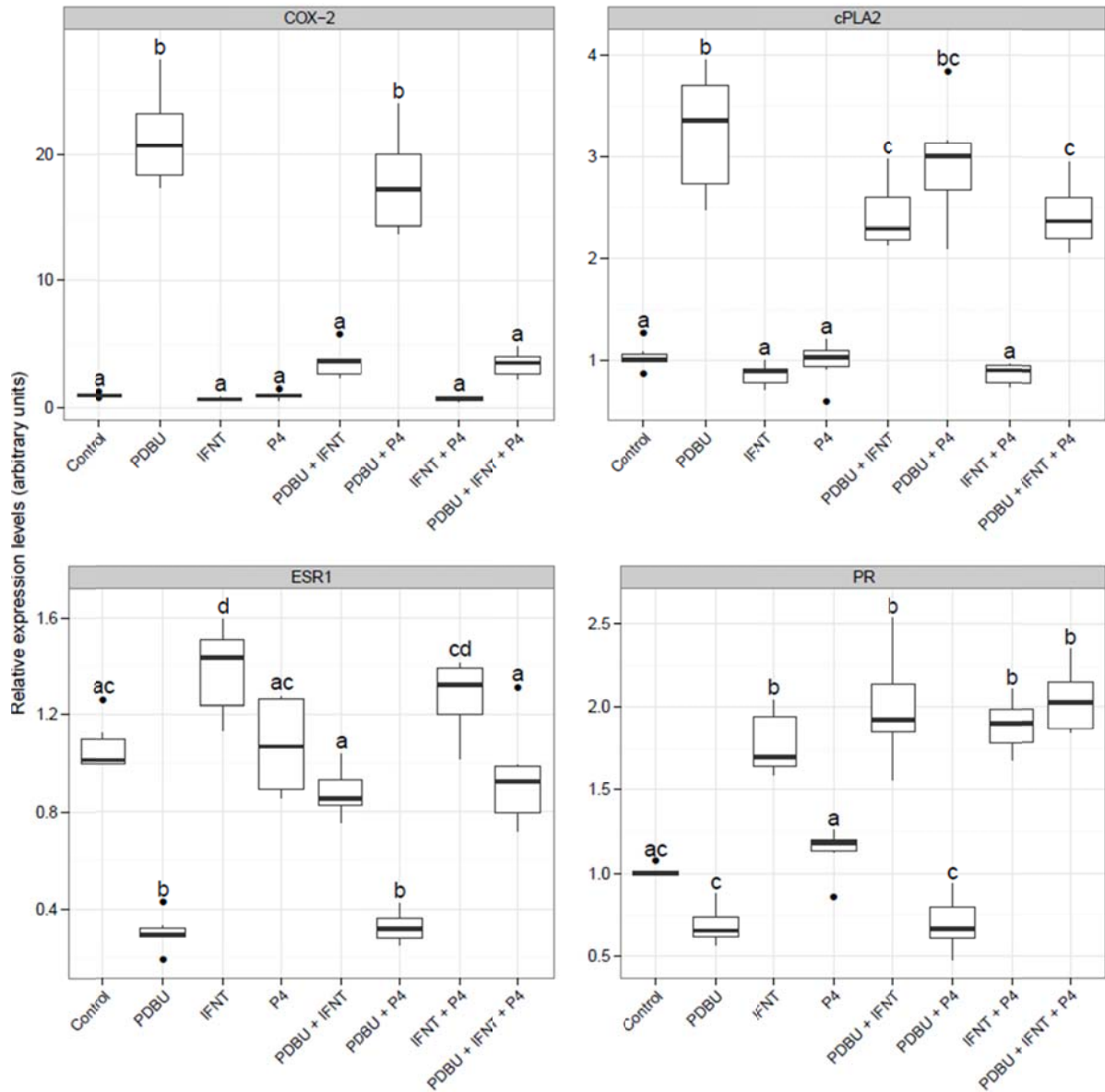
*Reproduction in Domestic Animals* 50, p. 800-806 (2015), ISSN 0936-6768.

You have to purchase this part online.

### 3.2.1. Follow up studies (data not included in publication 3.2)

RNAseq (RNA sequencing) data indicated that endometrial miRNAs are involved in regulation on MAPK signaling pathway. Thus, an *in vitro* system was implemented to study the expression of endometrial miRNAs in a bovine endometrial cell line (BEND). BEND cells are able to respond to Phorbol 12,13-dibutyrate (PDBU), an activator of protein kinase C (PKC) which stimulates MAPK signaling pathway, increasing the production of prostaglandins. This effect is antagonized by embryonic derived interferon-tau (IFNT) [24-29]. Additionally, the effect of progesterone was included, given that this hormone is crucial during embryo implantation [30]. The hypothesis was that endometrial miRNAs predicted to control MAPK signaling pathway might be regulated in BEND cells treated with PDBU, IFNT, progesterone and combinations thereof.

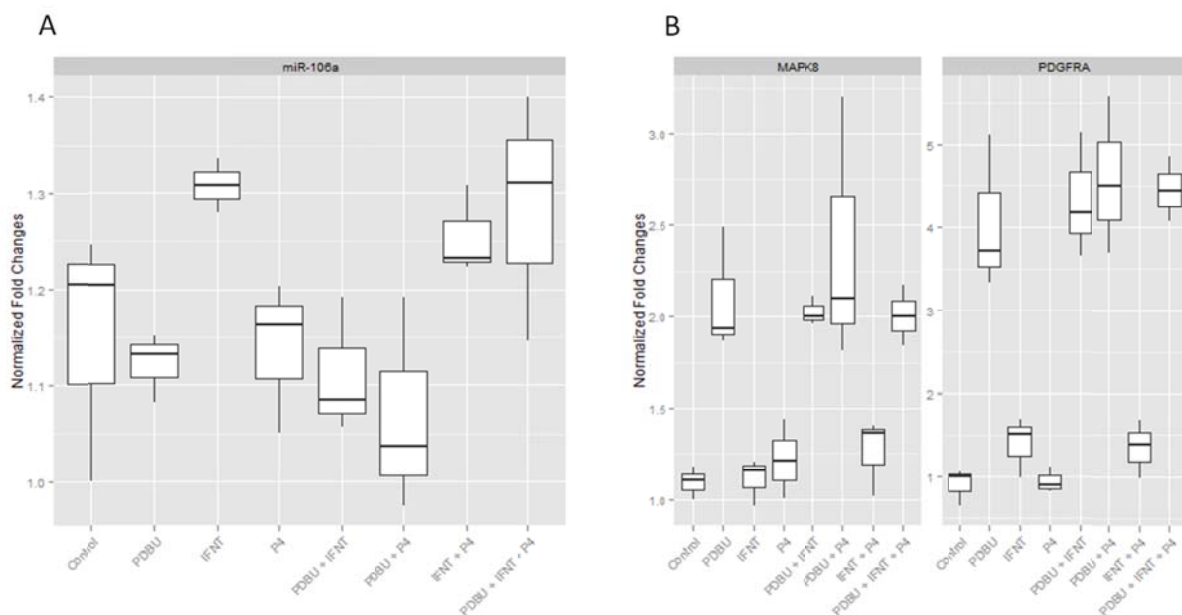
The level of responsiveness to the treatments is shown in figure 6. The expression of functional genes related to the investigated pathway was regulated significantly. Transcription of the enzymes cytochrome c oxidase subunit II (COX-2, *PTGS2*) and phospholipase A2, group IVA (cPLA2, *PLA2G4A*) followed similar patterns, showing increased expression under PDBU and PDBU plus progesterone treatments, which was counter-regulated by the effect of IFNT. However, for *PLA2G4A*, this effect was less severe. Neither IFNT nor progesterone affected the expression of *PTGS2* and *PLA2G4A* when added alone or together. Progesterone also did not interfere with the effect of PDBU on *PTGS2* and *PLA2G4A* expression. Interestingly, IFNT induced expression of *PGR*, which was not antagonized by any of the treatments. Estrogen receptor 1 (*ESR1*) was also induced by IFNT but only when IFNT was added alone. PDBU induced down-regulation of *ESR1* but not of *PGR*.



**Figure 6.** Normalized fold change expression of characteristic genes for BEND cells in response to treatment with PDBU, IFNT, progesterone and their combinations. Transcript expression was normalized to a combination of two housekeeping genes (*ACTB* and *SDHA*). Values are shown as box plots. For each experiment, six biological replicates were used.

Tested miRNAs in endometrial tissue were further validated in BEND cells, with special interest in let-7a-5p. This miRNA was predicted to target genes involved in the MAPK signaling pathway, which regulates the production of prostaglandins by bovine endometrial cells [25]. Therefore, it was interesting to see whether this miRNA would be affected in BEND cells treated with substances known to regulate MAPK signaling pathway [25, 27-29]. The factors IFNT, PDBU, progesterone and their combinations (as described above) were selected as exogenous triggers to analyze potential effects on the expression of the selected miRNA. As a result, the 8 endometrial miRNAs did not show significant variations in

response to the treatments (data not shown). Based on these results, other endometrial miRNAs belonging to the RNAseq data set predicted to target MAPK signaling pathway genes were searched, but whose expression had not been previously validated. Expression analysis demonstrated that miR-106a was approximately 30% up-regulated by IFNT (Fig. 7A). Similarly, up-regulation was induced by IFNT with progesterone and also the combination of PDBU, IFNT and progesterone, leaving PDBU with IFNT as the only case where IFNT did not up-regulate miR-106a. This indicates that PDBU might counter-regulate the effect of IFNT. The expected anti-correlation between miR-106a and its targets, mitogen-activated protein kinase 8 (*MAPK8*) and platelet-derived growth factor receptor, alpha polypeptide (*PDGFRA*) (miRmap score 99.6960 and 92.5068, respectively), was not detected after RT-qPCR (Fig. 7B). However, a similar pattern to that of *PTGS2* and *PLA2G4A* was found, with only one exception: IFNT was not able to counter-regulate the effect of PDBU.



**Figure 7.** Expression of miR-106a (A) and its targets *MAPK8* and *PDGFRA* (B).

### 3.2.2. Conclusions

Endometrial miRNA profiles were characterized across the bovine estrous cycle. Highly expressed miRNAs were predicted to influence cell proliferation, differentiation, transport and metabolism, through gene regulation of MAPK signaling pathway, lysosome and extracellular matrix-receptor interaction. However, not all validated miRNAs, corresponding to the top expressed miRNAs, showed significant regulations across the estrous cycle.

In BEND cells, the top expressed endometrial miRNAs were not regulated by the treatments. Nevertheless, this cell system is highly sensitive to the physiological effects of IFNT counteracting the activation of MAPK signaling pathway.

MiR-106a was up-regulated by IFNT in BEND cells. The control of MAPK signaling pathway by miR-106a could not be correlated to its targets, *MAPK8* and *PDGFRA*, in BEND cells. However, unlike *PTGS2* and *PLA2G4A*, *MAPK8* and *PDGFRA* were not counter-regulated by IFNT under PDBU stimulation.



## 4. Discussion

The present study focused on detecting miRNAs in two key reproductive tissues for bovine fertility: oviduct and endometrium. The approach was based on identifying miRNAs in native tissue, predict their roles *in silico* and analyze their regulation *in vitro*. As a result, miRNAs involved in pathways associated with fertility were detected. These pathways were, for the most part, involved in cell proliferation, differentiation, transport, metabolism and cell defense.

### 4.1. A reproducible long term BOEC culture system

Initially, a bovine oviductal epithelial cell culture system was established. The approach was to induce, *in vitro*, cell differentiation. A differentiated cell culture would be composed of ciliated and secretory cells, preserving their functional *in vivo* features, e.g., response to ovarian steroids. Thereafter, the regulation of *in vivo* found miRNAs was explored to identify key miRNAs involved in oviductal physiology.

In parallel to this study, Gualtieri et al. [31] published a similar system to culture BOECs. They reported that BOECs were able to achieve ciliation and bind to sperm for up to three weeks in an air-liquid interphase culture system. In the work conducted for this dissertation, cells did not preserve ciliation and sperm binding was not tested. However, secretory cells remained in culture in a polarized and long-term manner and exhibited markers of differentiation. One critical difference is found in the isolation method: in the work published by Gueltieri et al. [31], cells were not individualized by enzymatic digestion as in the protocol used for this thesis. Instead, cells were kept as explants before seeding. This lesser degree of detachment between secretory and ciliated cells might explain the difference between both studies, since it is possible that secretory cells act as an anchor for ciliated cells, pulling them down, preserving them in the culture. Indeed, it has been proposed that, whereas ciliated cells are in constant motion, the nonmoving secretory cells attach easier and faster and thus have an advantage to grow [32]. However, culturing explants impedes determining the number of cells that are being seeded, which has a negative impact on standardization and reproducibility. Therefore, even though the related work of this dissertation did not provide the conditions for cultures to exhibit ciliation, it provides a robust basis towards improving and further optimizing this *in vitro* system.

Finally, another two systems have been employed to study the physiology of the cow's oviduct *in vitro*: monolayer and suspension [33, 34]. Monolayer cultures showed rapid loss of cilia after 3-4 days and exhibited secretory granules. In suspension, BOECs did not proliferate nor dedifferentiate, and cells could be grown up to 12 days preserving their *in vivo* features [33]. Compared to these two cell culture systems, an air-liquid interphase provides a more physiological environment for differentiation, since it allows cells to be exposed to nutrients, growth factors and hormones from the basolateral compartment.

#### 4.2. Insulin effect on cell morphology, gene and miRNA expression of BOECs

Initially, three different insulin concentrations were applied to the cultures: 1 ng/mL, 20 ng/mL and 5 µg/mL. The highest and medium insulin concentrations were established based on previous studies [31, 35, 36], and the lowest concentration approximated the physiological levels present in bovine plasma (1 ng/mL [37, 38]). Considering the large gap between 20 ng/mL and 5 µg/mL, two more doses were included later: 500 ng/mL and 2.5 µg/mL. Overall, a dose dependent effect of insulin on cell growth and morphology was observed. The evidence shows that a dose between 500 ng/mL and 2.5 µg/mL leads to a more differentiated cell morphology. This dose dependent effect is most likely a result of the role of insulin in energy storage and cellular growth and differentiation [39].

Interestingly, transcription of two markers of cell differentiation: *OVGPI* and *PGR* showed a strong negative correlation with the level of insulin. This effect has not been previously reported and constitutes an interesting discovery. Accordingly, it has been shown that the reproductive tract of the postpartum lactating dairy cow may be less capable of supporting early embryo development [40] and that concentrations of insulin are lower in lactating cows [41]. This implies that in the animal, *OVGPI* and *PGR* might be sensitive to the circulating levels of insulin. A possible explanation is that since concentrations of insulin are lower in lactating cows [41] which are less able to support early embryo development [40], an up-regulation of *OVGPI* and *PGR* in the phase of low insulin, might have a compensatory mechanism to ensure fertility. Therefore, it is possible that insulin positively influences oviductal cells to support early embryo development.

To this date, only one study has addressed the expression of miRNAs in BOECs *in vitro*, showing that LPS might induce changes in the oviductal transcriptome, leading to a suboptimal environment for embryo development [23]. In this study, a monolayer system was used, where cells were grown for a short term, exposed to LPS and the effect of LPS on the ability of BOECs to support embryo development was assessed. As a result, miR-155, miR-146a, miR-223, miR-21, miR-16 and miR-215 were suppressed after LPS treatment. Out of these regulated miRNAs, all of them were detected in the oviductal tissue used for the work of this dissertation. However, their levels were found at the lowest ranges of expression and were not further studied in the BOEC system.

The results of this work indicate a regulatory effect of insulin on let-7a-5p and let-7c levels, implicating that through these miRNAs, insulin controls cell proliferation and differentiation. The observed effect of insulin on BOEC morphology is most likely to be the result of increase MAPK signaling pathway, as this pathway is activated downstream the insulin receptor signaling cascade [42]. Interestingly, MAPK was one of the most regulated pathways predicted to be targeted by let-7a-5p and let-7c.

### **4.3. Applications of the BOEC model**

The described long term culture system to model the oviductal epithelium *in vitro* is a valuable tool for reproductive biology and to understand pathological states affecting the oviduct. More specifically, two main problems can be approached *in vitro*:

- Animal and human reproduction: long term culture of BOECs in an air-liquid interphase provides a platform to determine hormonal effects on cell function, as well as toxic effects of substances acting on the reproductive tract. Therefore, this system contributes to understand in more depth the biology of the oviductal epithelium.
- Human medicine: High grade serous ovarian carcinoma (HGSOC) is the most lethal gynecological disease in humans. Its detection is usually at late stages when survival chances are poor [43]. HGSOC has been proposed to originate from an occult intraepithelial carcinoma in the fimbrial region of the fallopian tube designated serous tubal intraepithelial carcinoma (STIC) [44]. Even though a similar system has been developed using human specimens [45], human samples are scarce. Therefore, animal models are always necessary in order to reach larger scale results.

### **4.4. Endometrial sample collection and RNAseq strategy**

This study demonstrated a high similarity in miRNA expression profile across the estrous cycle in bovine endometrium. These miRNAs identified were predicted to regulate pathways involved in cell proliferation, differentiation, transport and catabolism. The number of pathways shared by different miRNAs reflects the broad regulatory range of these molecules in the endometrium. The aim of this study was to define an endometrial miRNA profile across the bovine estrous cycle and to predict the role of miRNAs in pathways associated with fertility. For this purpose, the small fraction of RNA was sequenced and analyzed at four key stages of the estrous cycle: post-ovulatory, early luteal, mid-late luteal and pre-ovulatory.

RNAseq is a powerful method to assess overall RNA expression. RNAseq was applied to detect miRNAs present in the endometrium at different stages of the estrous cycle. For this purpose, pools of small fraction RNA samples were prepared and sequenced. The advantage of this approach is the thorough and cost-effective overview of miRNA expression of many individuals at once, where precision depends on the homology of samples, e.g. same estrous cycle stage. The main drawbacks of pooling are the lack of variability within groups, which excludes the possibility to conduct statistical inference, and the high impact of extreme values on measurements. This might explain the fact that some of the miR-Q validated miRNAs did not correlate with their RNAseq profiles.

### **4.5. *In vitro* validation of endometrial miRNAs**

To test for functional regulations *in vitro*, endometrial miRNAs were analyzed using an already established bovine endometrial cell line (BEND). BEND cells have been commonly

used and validated as a model to understand the embryo maternal communication during implantation. IFNT reduces the response of BEND cells upon stimulation with the compound PDBU, an activator of the PKC-MAPK pathway that leads to an increased production of PGF2alpha (Prostaglandin F2 alpha) [25, 27-29]. *In vivo*, high levels of PGF2alpha lead to luteolysis. Therefore, IFNT is able to ensure embryo survival by protecting progesterone production [30].

The mechanism of action of IFNT has been demonstrated to occur most likely before translation and not by interfering directly with proteins of the PKC-MAPK signaling pathway [28]. This is an interesting fact, considering the results produced by the bioinformatic analyses. First, mRNA targets for the most expressed miRNAs were predicted, followed by pathway analysis of each set of targets. As a result, MAPK was one of the most enriched pathways and predicted to be strongly regulated by let-7a-5p. Given this connection between the predictions and BEND cell biology, regulation of let-7a-5p as a result of PDBU and IFNT interactions (including progesterone for its physiological relevance) was a reasonable hypothesis. The interest for this miRNA was additionally supported by the fact that it was the highest expressed miRNA at all stages of the estrous cycle. Other miRNAs validated in tissue were also included, as candidates for reference and normalization. Contrary to the predictions, experimental manipulation of BEND cells did not affect the expression of let-7a-5p, implying that this miRNA is not involved in BEND cell response to IFNT upon PKC-MAPK pathway stimulation and other miRNAs might promote the reduction of PGF2alpha production induced by IFNT.

Nevertheless, another miRNA was shown to be regulated by experimental manipulation of BEND cells. MiRNA-106a belonged to the lowly expressed miRNAs in tissue. Due to this reason, it was not validated in endometrial RNA. However, target prediction and pathway analysis showed its potential role in controlling MAPK signaling pathway, thus, it was tested in BENDs. Evidence showed that miR-106a responds to IFNT alone and in combination with progesterone. This is physiologically relevant, since progesterone is permissive for IFNT activity [30]. On the other hand, when IFNT combined with PDBU were applied, miR-106a expression was not affected, pointing towards a counter-regulation of PDBU over IFNT. Considering that PDBU action is analogous to the activity of oxytocin, e.g. induction of PGF2alpha production, this event parallels the physiology of embryo maternal communication. Therefore, it is possible that miR-106a contributes to the control of endometrial response to IFNT and oxytocin.

#### **4.6. Comparison to reported transcriptomic studies in endometrium**

A previous study showed that subclinical endometritis in cows leads to aberrant expression of 23 miRNAs [21]. Five of those miRNAs (miR-16b, miR-21, miR-126, miR-27a and miR-24) were detected within the 3 highest expressed clusters in the data set. The work conducted in

this dissertation showed that targets predicted for miR-16b were highly enriched in the MAPK signaling pathway, also predicted to be affected by differentially expressed genes in subclinical endometritis through mitogen-activated protein kinase 14 (*MAPK14*).

Ponsuksili *et al.* [46] retrieved endometrial samples on days 3 and 7 of the estrous cycle, before embryo transfer was performed at the next cycle, detecting eleven miRNAs connected to high endometrial receptivity. In comparison, miR-31 was detected within the highly expressed miRNA clusters. This miRNA was predicted to regulate expression of genes belonging to the Toll-like receptor signaling pathway, responsible for detecting microbial pathogens and generating innate immune responses, thus, preserving endometrial health. Finally, transcriptome analysis of endometrial samples comparing high and low fertility heifers during the early to mid-luteal phase of the estrous cycle, identified pathways involved in uterine function and embryo survival [47]. These pathways included metabolic processes that are connected with the results of this dissertation study through nucleotide metabolism. The authors imply the relevance of these mechanisms due to their role in embryo development and endometrial function, acting as precursors of nucleic acids, proteins, osmolites and signaling molecules.

The results of this dissertation are comparable to other reports derived from non-bovine specimens. For example, in human endometrium [48], twelve miRNAs were described to target cell cycle genes to suppress cell proliferation. Out of those 12 miRNAs, miR-29b, miR-200c and miR-31 were detected among the highest expressed bovine endometrial miRNAs and they were also predicted to target genes involved in cell cycle. Specifically, bta-miR-29b and bta-miR-31 targeted cell division cycle 7 (*CDC7L1*) (miRmap score = 97.75) and E2F transcription factor 6 (*E2F6*) (miRmap score = 99.42), respectively, and bta-miR-200c was predicted to target cyclin-dependent kinase inhibitor 1B (*CDKN1B*) (miRmap score = 94.22) and cyclin-dependent kinase 2 (*CDK2*) (miRmap score = 76.16). This overlap might indicate similar control mechanisms in the bovine and human endometrium across the estrous and menstrual cycle, respectively.

#### **4.7. Conclusions**

BOECs grown for long terms in an air-liquid interphase were able to exhibit molecular markers of differentiation and polarization. Cultures were composed of secretory cells, but ciliation was not detected. Additionally, results of this work are the first to report the regulatory effect of insulin on BOEC morphology and expression of *OVGP1*, *PGR*, let-7a-5p and let-7c.

In endometrium, miRNAs were characterized across the estrous cycle, covering key physiological time points, relevant for fertility and endometrial health. The main finding of

this study was the detection of highly homogenous sets of miRNAs, which were predicted to control cell proliferation, differentiation, transport and metabolism

In general, the presented results provide a global characterization of abundant oviductal and endometrial miRNAs, involved in key physiological processes relevant for fertility and health. Additionally, *in vitro* regulated miRNAs were detected in BOECs and BEND cells, setting a base for further exploration of the physiological and pathological roles of miRNAs in bovine fertility.

## 5. Summary

### **Characterization of microRNA (miRNA) profiles in selected female reproductive tissues of cattle: prediction of fertility associated pathways**

Sergio Eliseo Palma Vera

MicroRNAs (miRNAs) are small non coding RNAs, able to bind to mRNA and silence gene expression. They have been described to have roles in physiological, as well as in pathological reproductive processes. Reproductive performance in cattle has been steadily decreasing over the last decades. The reasons for this have not been completely defined, but poor embryo development and implantation are among the main causes. Both processes depend on embryo interactions with the maternal oviduct and endometrium. Therefore, the aim of this study was to characterize the role of miRNAs in these reproductive tissues and to predict their function in the context of fertility.

First, a bovine oviductal epithelial cell culture (BOEC) system was developed. The purpose of this was to establish an *in vitro* model that can resemble the oviductal epithelium. To do this, an air liquid interphase system was implemented and cells were grown for up to six weeks. As a result, cells exhibited polarization and markers of differentiation. However, ciliated cells were not observed. Insulin, a common supplement used to culture BOECs, was detected to significantly impact cell morphology, improving the quality of cultures. Additionally, oviductal miRNAs were characterized in native tissue and then validated in the established air-liquid interphase BOEC system. Among the RNAseq-detected oviductal miRNAs, let-7a-5p and let-7c were found to be regulated by insulin in BOECs. Their targets were mapped to MAPK signaling pathway, a downstream mechanism in the insulin signaling, controlling cell proliferation and differentiation.

Secondly, miRNAs were characterized in endometrial cells. Estrous cycle was divided into four stages: post-ovulatory, early luteal, late luteal and pre-ovulatory phases. RNA samples of four animals were pooled and the miRNA expression pattern analyzed via RNAseq. Results demonstrated a high similarity in miRNA expression profiles across the estrous cycles in bovine endometrium. These miRNAs were predicted to regulate pathways involved in cell proliferation, differentiation, transport and catabolism, among them, MAPK signaling pathway. To test for functional regulations *in vitro*, endometrial miRNAs were analyzed using a commercial bovine endometrial cell line (BEND), where activation of MAPK signaling pathway can be counter-regulated by interferon-tau (IFNT), mimicking the process of embryo implantation. As result, miR-106a responded to IFNT alone and in combination with progesterone, a strong indicator of the relevance of this miRNA for embryo implantation.

In summary, the present study characterized miRNAs expressed in key reproductive tissues for embryo development. Upon validation using *in vitro* systems, regulated miRNAs were detected. In BOECs let-7a-5p and let-7c were regulated by insulin and in BEND cells, miR-106a was regulated by IFNT. Interestingly, in both situations, MAPK signaling pathway plays a central role. Future studies will define the role of miRNAs in the control of oviductal and endometrial function through MAPK signaling pathway.



## **Zusammenfassung**

### **Charakterisierung der microRNA-(miRNA)-Profile ausgewählter Gewebe des weiblichen Reproduktionstraktes von Rindern: Vorhersage fruchtbarkeitsassoziierter Signalwege.**

Sergio Eliseo Palma Vera

MicroRNAs (miRNAs) sind kleine, nicht kodierende RNAs, welche in der Lage sind mRNA zu binden, um die Genexpression zu inhibieren. Es wurde nachgewiesen, dass sie sowohl im physiologischen als auch im pathologischen Reproduktionsgeschehen eine Rolle spielen. Die Reproduktionsleistung bei Rindern hat in den vergangenen Jahrzehnten stetig abgenommen. Die Gründe dafür sind nicht abschließend aufgeklärt, aber Störungen in der Entwicklung und Implantation der Embryonen gehören mit zu den Hauptursachen. Beide Prozesse sind von Interaktionen des Embryos mit dem mütterlichen Eileiter und Endometrium abhängig. Daher war das Ziel der vorliegenden Untersuchung, die Rolle der miRNAs in diesen Reproduktionsgeweben zu charakterisieren und ihre Funktion im Zusammenhang mit der Fruchtbarkeit vorherzusagen.

Zunächst wurde ein System zur Kultivierung von Rinder-Eileiterepithelzellen (BOEC) entwickelt. Es sollte ein in-vitro-Modell etabliert werden, das dem Eileiterepithel möglichst ähnlich ist. Deshalb wurde ein „Air-Liquid-Interphase-System“ angewendet, in dem die Zellen bis zu sechs Wochen kultiviert wurden. Dies bewirkte bei den Zellen Polarisation und weitere Kennzeichen von Differenzierung. Zilienentwicklung konnte allerdings nicht beobachtet werden. Es stellte sich heraus, dass Insulin – ein üblicher Zellkultur-Zusatz für BOECs – die Zellmorphologie Konzentrationsabhängig beeinflusst und in bestimmten Konzentrationen die Qualität der Kulturen verbessern kann. Darüber hinaus wurden die im Eileiter exprimierten miRNAs im nativen Gewebe charakterisiert und dann im etablierten BOEC-Kultursystem validiert. Bei den mittels RNAseq detektierten Eileiter-miRNAs stellte sich heraus, dass *let-7a-5p* und *let-7c* in BOEC von Insulin reguliert werden. Diese miRNAs konnten über ihre Targets mit dem MAPK-Signalweg in Verbindung gebracht werden, einem nachgeschalteten Mechanismus in der Insulin-Signalkaskade, welcher die Zellproliferation und Zelldifferenzierung kontrolliert.

Weiterhin wurden miRNAs in Endometriumszellen charakterisiert. Der Brunstzyklus wurde in vier Stadien geteilt: die postovulatorische Phase, die frühe Lutealphase, die späte Lutealphase und die präovulatorische Phase. RNA-Proben von vier Tieren wurden gesammelt und das miRNA-Expressionsmuster wurde mittels RNAseq analysiert. Die Ergebnisse zeigten, dass die miRNA-Expressionsprofile im Endometrium des Rindes über den Zyklus sehr ähnlich bleiben. Die detektierten miRNAs könnten Pathways regulieren, welche Zellproliferation, Differenzierung, Transport und Katabolismus beeinflussen, unter Ihnen

auch der MAPK-Signalweg. Um funktionale Interaktionen in vitro zu testen, wurden endometriale miRNAs unter Verwendung einer kommerziellen Rinder-Endometrium-Zelllinie (BEND) analysiert. In diesen Zellen kann die Aktivierung des MAPK-Signalweges durch Interferon-tau (IFNT) gegenreguliert werden, was den Prozess der Implantation des Embryos nachahmt. MiR-106a reagierte sowohl auf IFNT als auch IFNT in Verbindung mit Progesteron, was ein starker Indikator für die Relevanz dieser miRNA für die Implantation des Embryos ist.

Zusammenfassend hat die vorliegende Studie gezeigt, dass die entscheidenden Reproduktionsgewebe für die Entwicklung des Embryos miRNAs exprimieren. Nach der Validierung unter Verwendung von in vitro-Systemen, wurden regulierte miRNAs erkannt. In BOECs werden let-7a-5p und let-7c durch Insulin reguliert. MiR-106a wurde von IFNT in BEND-Zellen kontrolliert. Interessanterweise spielte der MAPK-Signalweg in beiden Fällen eine zentrale Rolle. Zukünftige Studien werden zeigen, welche Rolle miRNAs über den MAPK-Signalweg bei der Regulation der Funktion von Eileiter und Endometrium spielen.

## 6. Outlook

In this work, BOECs grown for long terms under an air-liquid interphase were not able to exhibit ciliation. Future studies can be performed to improve the isolation method or growth conditions in order to preserve ciliated cells and resemble more closely the native oviductal epithelium. Nevertheless, in this system, secretory cells were present and mimicked their natural state in the oviductal tissue. Thus, future studies may use this model to understand the function of the oviductal secretory cell in an isolated manner.

The miRNA control of MAPK signaling pathway can be further explored using the *in vitro* systems implemented in this study. BOECs and BEND cells can be employed to validate predicted miRNA-target(s) interactions, not only by detecting negative correlations, but also by specifically knocking down miRNA expression.

The *in vitro* detected miRNA regulations under physiological stimuli provide important data to proceed with *in vivo* experiments in cattle. For this purpose, special consideration must be paid to the experimental design, since *in vitro* experiments are performed under highly controlled conditions. Therefore, animals must also be strictly monitored (hormonal stage, age, energy balance etc.), in order to consistently verify *in vitro* discoveries.

Finally, miRNA-target(s) interactions were performed using predictive algorithms. However, experimentally validated interaction data bases have been developed for model organisms excluding cattle. In the future, it is expected that such data bases will also include bovine data. This will significantly improve the accuracy of the predictions upon validation in biological systems.

## 7. References

- [1] Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews Genetics*. 2010;11:597-610.
- [2] Diskin MG, Morris DG. Embryonic and early foetal losses in cattle and other ruminants. *Reproduction in Domestic Animals*. 2008;43:260-7.
- [3] Leroy JL, Opsomer G, De Vliegher S, Vanholder T, Goossens L, Geldhof A, et al. Comparison of embryo quality in high-yielding dairy cows, in dairy heifers and in beef cows. *Theriogenology*. 2005;64:2022-36.
- [4] Stroynowska-Czerwinska A, Fiszler A, Krzyzosiak WJ. The panorama of miRNA-mediated mechanisms in mammalian cells. *Cellular and Molecular Life Sciences*. 2014;71:2253-70.
- [5] Finnegan EF, Pasquinelli AE. MicroRNA biogenesis: regulating the regulators. *Critical Reviews in Biochemistry and Molecular Biology*. 2013;48:51-68.
- [6] Thomson DW, Bracken CP, Goodall GJ. Experimental strategies for microRNA target identification. *Nucleic Acids Research*. 2011;39:6845-53.
- [7] Wilczynska A, Bushell M. The complexity of miRNA-mediated repression. *Cell Death and Differentiation*. 2015;22:22-33.
- [8] Valinezhad Orang A, Safaralizadeh R, Kazemzadeh-Bavili M. Mechanisms of miRNA-Mediated Gene Regulation from Common Downregulation to mRNA-Specific Upregulation. *International Journal of Genomics*. 2014;2014:970607.
- [9] Witkos TM, Koscianska E, Krzyzosiak WJ. Practical Aspects of microRNA Target Prediction. *Current Molecular Medicine*. 2011;11:93-109.
- [10] Peterson SM, Thompson JA, Ufkin ML, Sathyanarayana P, Liaw L, Congdon CB. Common features of microRNA target prediction tools. *Frontiers in Genetics*. 2014;5:23.
- [11] Mahen EM, Watson PY, Cottrell JW, Fedor MJ. mRNA secondary structures fold sequentially but exchange rapidly in vivo. *PLoS Biology*. 2010;8:e1000307.
- [12] Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nature Reviews Genetics*. 2012;13:271-82.
- [13] Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. *Genes & Development*. 2004;18:504-11.
- [14] Garcia DM, Baek D, Shin C, Bell GW, Grimson A, Bartel DP. Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. *Nature Structural & Molecular Biology*. 2011;18:1139-46.
- [15] Clark PM, Loher P, Quann K, Brody J, Londin ER, Rigoutsos I. Argonaute CLIP-Seq reveals miRNA targetome diversity across tissue types. *Scientific Reports*. 2014;4:5947.

- [16] Senger PL. Pathways to Pregnancy and Parturition. 2 ed. Pullman, WA, USA: Current Conceptions, Inc; 2003.
- [17] Hopper RM. Bovine Reproduction. 1 ed. Iowa, USA: John Wiley & Sons, Inc.; 2015.
- [18] Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, et al. Endometrial glands are required for preimplantation conceptus elongation and survival. *Biology of Reproduction*. 2001;64:1608-13.
- [19] Mansouri-Attia N, Aubert J, Reinaud P, Giraud-Delville C, Taghouti G, Galio L, et al. Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. *Physiological Genomics*. 2009;39:14-27.
- [20] Walsh SW, Williams EJ, Evans AC. A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science*. 2011;123:127-38.
- [21] Hailemariam D, Ibrahim S, Hoelker M, Drillich M, Heuwieser W, Looft C, et al. MicroRNA-regulated molecular mechanism underlying bovine subclinical endometritis. *Reproduction, Fertility, and Development*. 2013;26:898-913.
- [22] Ponsuksili S, Tesfaye D, Schellander K, Hoelker M, Hadlich F, Schwerin M, et al. 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 embryos. *Biology of Reproduction*. 2014;91:24.
- [23] Ibrahim S, Salilew-Wondim D, Rings F, Hoelker M, Neuhoff C, Tholen E, et al. Expression Pattern of Inflammatory Response Genes and Their Regulatory MicroRNAs in Bovine Oviductal Cells in Response to Lipopolysaccharide: Implication for Early Embryonic Development. *PloS One*. 2015;10:e0119388.
- [24] Binelli M, Subramaniam P, Diaz T, Johnson GA, Hansen TR, Badinga L, et al. Bovine interferon-tau stimulates the Janus kinase-signal transducer and activator of transcription pathway in bovine endometrial epithelial cells. *Biology of Reproduction*. 2001;64:654-65.
- [25] Binelli M, Guzeloglu A, Badinga L, Arnold DR, Sirois J, Hansen TR, et al. Interferon-tau modulates phorbol ester-induced production of prostaglandin and expression of cyclooxygenase-2 and phospholipase-A(2) from bovine endometrial cells. *Biology of Reproduction*. 2000;63:417-24.
- [26] Guzeloglu A, Binelli M, Badinga L, Hansen TR, Thatcher WW. Inhibition of phorbol ester-induced PGF<sub>2</sub>α secretion by IFN-tau is not through regulation of protein kinase C. *Prostaglandins & Other Lipid Mediators*. 2004;74:87-99.
- [27] Guzeloglu A, Michel F, Thatcher WW. Differential effects of interferon-tau on the prostaglandin synthetic pathway in bovine endometrial cells treated with phorbol ester. *Journal of Dairy Science*. 2004;87:2032-41.

- [28] Guzeloglu A, Subramaniam P, Michel F, Thatcher WW. Interferon-tau induces degradation of prostaglandin H synthase-2 messenger RNA in bovine endometrial cells through a transcription-dependent mechanism. *Biology of Reproduction*. 2004;71:170-6.
- [29] Godkin JD, Roberts MP, Elgayyar M, Guan W, Tithof PK. Phospholipase A2 regulation of bovine endometrial (BEND) cell prostaglandin production. *Reproductive Biology and Endocrinology*. 2008;6:44.
- [30] Bazer FW, Burghardt RC, Johnson GA, Spencer TE, Wu G. Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. *Reproductive Biology*. 2008;8:179-211.
- [31] Gualtieri R, Mollo V, Braun S, Barbato V, Fiorentino I, Talevi R. Long-term viability and differentiation of bovine oviductal monolayers: Bidimensional versus three-dimensional culture. *Theriogenology*. 2012;78:1456-64.
- [32] Ulbrich SE, Zitta K, Hiendleder S, Wolf E. In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. *Theriogenology*. 2010;73:802-16.
- [33] Walter I. Culture of bovine oviduct epithelial cells (BOEC). *The Anatomical Record*. 1995;243:347-56.
- [34] Rottmayer R, Ulbrich SE, Kolle S, Prella K, Neumueller C, Sinowatz F, et al. A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction*. 2006;132:637-48.
- [35] Reinhart KC, Dubey RK, Mummery CL, van Rooijen M, Keller PJ, Marinella R. Synthesis and regulation of leukaemia inhibitory factor in cultured bovine oviduct cells by hormones. *Molecular Human Reproduction*. 1998;4:301-8.
- [36] Ellington JE, Broemeling LD, Broder SJ, Jones AE, Choker DA, Wright RW. Comparison of fresh and cryopreserved human sperm attachment to bovine oviduct (uterine tube) epithelial cells in vitro. *Journal of Andrology*. 1999;20:492-9.
- [37] McAtee JW, Trenkle A. Metabolic regulation of plasma insulin levels in cattle. *Journal of Animal Science*. 1971;33:438-42.
- [38] Sternbauer K, Luthman J. Insulin sensitivity of heifers on different diets. *Acta Veterinaria Scandinavica*. 2002;43:107-14.
- [39] Cheatham B, Kahn CR. Insulin action and the insulin signaling network. *Endocrine Reviews*. 1995;16:117-42.
- [40] Rizos D, Carter F, Besenfelder U, Havlicek V, Lonergan P. Contribution of the female reproductive tract to low fertility in postpartum lactating dairy cows. *Journal of Dairy Science*. 2010;93:1022-9.

- [41] Maillo V, Rizos D, Besenfelder U, Havlicek V, Kelly AK, Garrett M, et al. Influence of lactation on metabolic characteristics and embryo development in postpartum Holstein dairy cows. *Journal of Dairy Science*. 2012;95:3865-76.
- [42] Bevan P. Insulin signalling. *Journal of Cell Science*. 2001;114:1429-30.
- [43] Vaughan S, Coward JI, Bast RC, Jr., Berchuck A, Berek JS, Brenton JD, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nature Reviews Cancer*. 2011;11:719-25.
- [44] Kurman RJ. Origin and molecular pathogenesis of ovarian high-grade serous carcinoma. *Annals of Oncology*. 2013;24 Suppl 10:x16-21.
- [45] Levanon K, Ng V, Piao HY, Zhang Y, Chang MC, Roh MH, et al. Primary ex vivo cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. *Oncogene*. 2010;29:1103-13.
- [46] Ponsuksili S, Tesfaye D, Schellander K, Hadlich F, Schwerin M, Wimmers K. 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 embryos. *Biology of Reproduction*. 2014.
- [47] Killeen AP, Morris DG, Kenny DA, Mullen MP, Diskin MG, Waters SM. Global gene expression in endometrium of high and low fertility heifers during the mid-luteal phase of the estrous cycle. *BMC Genomics*. 2014;15:234.
- [48] Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. *Biology of Reproduction*. 2010;82:791-801.

## **8. Publication list**

### **8.1. First authorship publications**

**Palma-Vera S**, Einspanier R, Schoen J. Bovine oviductal epithelial cells: Long term culture characterization and impact of insulin on cell morphology. *Reprod Biol*, 2014; 14(3): 206-12.

**Palma-Vera S**, Sharbati S, Einspanier R. Identification of miRNAs in cow endometrium through RNAseq and prediction of regulated pathways. *Reprod Domest Anim*, paper accepted, in print.

### **8.2. Co-authorship publications**

Rojas-Garcia PP, Recabarren MP, Sir-Petermann T, Rey R, **Palma S**, Carrasco A, Perez-Marin CC, Padmanabhan V, Recabarren SE. Altered testicular development as a consequence of increased number of Sertoli cell in male lambs exposed prenatally to excess testosterone. *Endocrine*, 2013; 43(3): 705-13. *\*Work performed before PhD period (University of Concepción, Faculty of Veterinary Sciences, Laboratory of Animal Physiology and Endocrinology).*

Rojas-Garcia PP, Recabarren MP, **Palma S**, Maliqueo M, Carrasco A, Sir-Petermann T, Recabarren SE. Ovarian morphometry and mRNA expression of anti-Müllerian hormone (AMH), follicle stimulating hormone receptor (FSHR) and nuclear factor kappa B (NFkB) in growing follicles of female lambs prenatally exposed to testosterone. *Archivos de medicina veterinaria*, 2014; 46(1): 9. *\*Work performed before PhD period (University of Concepción, Faculty of Veterinary Sciences, Laboratory of Animal Physiology and Endocrinology).*

### **8.3. First authorship presentations (scientific abstracts)**

**Palma Vera S**, Sharbati S, Einspanier R. Cattle endometrium miRNA profiling and prediction of regulated pathways. 47. Jahrestagung Physiologie und Pathologie der Fortpflanzung. February 27-28, 2014. Giessen, Germany. Oral presentation.

**Palma Vera S**, Chen S, Einspanier R, Schoen J. Bovine oviduct epithelium cells *in vitro*: establishment of a polarized long-term culture model. 7. Doktorandensymposium and Dahlem Research School Präsentationsseminar “Biomedical Sciences” des FB Veterinärmedizin. July 13, 2012. Berlin, Germany. Oral presentation.

**Palma Vera S**, Chen S, Einspanier R, Schoen J. Long-term culture of primary bovine oviduct epithelial cells: Impact of insulin and EGF supplementation. Reproductive Biology, Biotechnology, Animal Welfare and Biodiversity Conference. February 27–March 01, 2013. Gdansk, Poland. Poster presentation.



#### **8.4. Co-authorship presentations (scientific abstracts)**

Pawar K, Hanisch C, **Palma Vera SE**, Einspanier R, Sharbati S. Mycobacteria escape autophagy by counter regulating long non-coding RNA MEG3. International Meeting of the German Society for Cell Biology (DGZ). March 24-27, 2015. Cologne, Germany. Oral presentation.

Chen S, **Palma Vera S**, Einspanier R, Schoen J. Long term culture of porcine oviductal epithelium cells: optimization, characterization and validation of an *in vivo*-like culture system. 20. Tagung der Fachgruppe Physiologie und Biochemie der DVG. February 16-18, 2012. München, Germany. Poster presentation.

## **9. Acknowledgements**

During these last years, many people have supported and guided me towards accomplishing my long sought goal of becoming a scientist. Here, I would like to thank some of them.

I would like to start thanking Prof. Dr. Einspanier, my main supervisor. He supported me before the beginning of my studies, when we successfully applied for funding to the DAAD (German Academic Exchange Service) and later, when he provided me the opportunity to perform my PhD project at Freie Universität Berlin, Institute of Veterinary Biochemistry. I greatly appreciate the time and patience he dedicated to the development of this project. His example is one I will follow when the time comes to balance science, administration and family.

Dr. Jennifer Schön and Dr. Soroush Sharbati will always have my gratitude. From early on in my career, they have influenced and motivated me to become a more conscious, creative and independent researcher. Although different in their style, their combined help has always led me to overcome the most difficult obstacles in the path to conclude my PhD project. I have learned valuable lessons from them and I hope that continues over the next years.

My sincere appreciation goes to Prof. Dr. Plendl from the Institute of Veterinary Anatomy. She kindly supervised my progress throughout my PhD and offered her valuable time to review this thesis.

I thank my colleges from the Institute of Veterinary Biochemistry, Freie Universität Berlin for their friendliness and generous help during the course of my project and also to my former colleges in Chile at the University of Concepción, Laboratory of Animal Physiology and Endocrinology for their crucial role in obtaining my DAAD scholarship. I am also thankful to the DAAD for backing my studies in Germany. They do an excellent work providing support to international researchers.

Finally, I would like to thank my family and friends, here in Germany and back in Chile, for their constant support and trust in that I can succeed.

## **10. Selbständigkeitserklärung**

Hiermit bestätige ich, Sergio E. Palma Vera, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 26.08.2015

Sergio Eliseo Palma Vera