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Circulating microRNAs as biomarkers of reproductive status in the cow

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List of Abbreviations

BLAST	Basic local alignment search tool
BLAT	Blast-like alignment tool
cDNA	Complementary DNA
ChIP	Chromatin immuno-precipitation
CIDR	Controlled internal drug release
CL	Corpus luteum
Cq	Cycle of quantification
CV	Coefficient of variation
DEFRA	Department for environment, food and rural affairs
DNA	Deoxyribonucleic acid
E2	Oestradiol
EPF	Early pregnancy factor
FDR	False discovery rate
FSH	Follicle-stimulating hormone
GC	Granulosa cells
GnRH	Gonadotrophin releasing hormone
GREL	Gonadal ridge epithelial-like cells
HDL	High density lipoprotein
HPG	Hypothalamus-pituitary-gonad
IFN	Interferon
IGF1	Insulin-like growth factor 1
ISG	Interferon-stimulated glycoprotein
LDL	Low density lipoprotein
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
miRNA	MicroRNA
miRISC	MicroRNA-induced silencing complex
mRNA	Messenger RNA
MVE	Multi-vesicular endosome
nt	Nucleotide
ORF	Open reading frame
P value	Probability value
P4	Progesterone
PAF	Platelet-activating factor
PAG	Pregnancy-associated glycoprotein
PCA	Principal component analysis
PGF _{2α}	Prostaglandin F _{2α}
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPMM	Reads per million mapped
RT	Reverse transcription
RISC	RNA-induced silencing complex

TGF-β	Transforming growth factor beta
TRBP	Transactivation response RNA binding protein
TRUS	Trans-rectal ultrasound
VEGF	Vascular endothelial growth factor

Declaration of Originality

I hereby declare that the work presented in this thesis and the thesis itself have been composed and originated by myself, unless otherwise specified. This work has not been submitted for any other degree or professional qualification.

The research described in Appendices I and II was carried out within a group and I made a substantial contribution to the work including data generation, analysis, presentation and interpretation. The included publications were included with permission from the remaining authors.

Jason Ioannidis

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Research Publications

1. **Ioannidis, Jason** and Donadeu, Xavier. *Circulating microRNA Profiles during the Bovine Oestrous Cycle*. PLoS One. 2016. (Chapter 2)
2. **Ioannidis, Jason** and Donadeu, Xavier. *Circulating miRNA signatures of early pregnancy in cattle*. BMC Genomics. 2016. (Chapter 3)
3. **Ioannidis, Jason** and Donadeu, Xavier. *Changes in circulating miRNAs levels can be identified as early as Day 8 of pregnancy in cattle*. PLOS One. 2017. (Revised, Chapter 4)
4. Donadeu, Xavier, Sontakke, Sadanand and **Ioannidis, Jason**. *MicroRNA indicators of follicular steroidogenesis*. Reproduction, Fertility and Development. 2016. (Appendix I)
5. Donadeu, Xavier, Mohammed, Bushra and **Ioannidis, Jason**. *A miRNA network putatively involved in follicular atresia*. Domestic Animal Endocrinology. 2017. (Appendix II)

Conference Abstracts

1. **Ioannidis, Jason**, Ashworth, Cheryl, Raue, Rudiger and Donadeu, Xavier. *Circulating microRNA for early diagnosis of bovine pregnancy*. International Embryo Transfer Society 41st Annual Meeting, Versailles, France. 2015 (Poster).
2. **Ioannidis, Jason** and Donadeu, Xavier. *Circulating microRNA signatures associated with early bovine pregnancy*. International Embryo Transfer Society 42nd Annual Meeting, Kentucky, USA. 2016 (Oral).
3. **Ioannidis, Jason** and Donadeu, Xavier. *Differential circulating microRNA levels as early as Day 8 of pregnancy in cattle*. Fertility, Edinburgh, UK. 2017 (Poster).

Lay Summary

Poor reproductive performance is a big problem for the dairy cow industry and it affects animal welfare and profits. Reproductive performance can be improved by detecting pregnancy and oestrus in these animals early on and with accuracy. Unfortunately currently available methods do not allow this. Circulating microRNAs (miRNAs) are small RNA molecules in the bloodstream which are thought to act as signals of pregnancy in humans. This work focused on identifying if such miRNA signals exist during early pregnancy and at oestrus in the cow. Using multiple high-throughput methods I identified miR-26a, a miRNA which increases as early as Day 8 of pregnancy in the bloodstream of cows compared to non-pregnant animals. In addition I found that the ratio of miR-26a with miR-205 also increases 7.5-fold during pregnancy. These miRNAs are involved in the control of the immune system, blood formation and metabolism, however their specific roles in this stage of pregnancy must be further investigated. In addition, I identified small increases in the levels of four additional miRNAs (miR-125b, let-7f, miR-145 and miR-99a-5p) at oestrus. Previous research has found that these miRNAs control functions inside the ovary during the oestrous cycle of the cow. Finally, I provide a set of data which can help identify tissue miRNAs that are released in the bloodstream. This can be used to discover new signals of disease in the cow. To this end, I identified miR-802, a new miRNA in the cow bloodstream which is liver-specific. This miRNA may be useful in identifying liver problems in stressed cows. The findings of this work may be useful in the development of diagnostic methods for early pregnancy and oestrus, and are a first step for discovering the function of these miRNAs in cow reproduction.

Abstract

Poor reproductive performance is a major challenge for the bovine dairy industry, with implications for profitability and animal welfare. Early pregnancy diagnosis and accurate oestrus detection can improve reproductive performance through efficient herd management. However currently available methods do not allow this. Circulating microRNAs (miRNAs) have been proposed as non-invasive biomarkers of reproductive status in humans. The hypothesis for this work was that differentially expressed miRNAs in plasma will be detectable during early pregnancy / oestrus, which may provide novel potential biomarkers. Using sequencing and PCR-based platforms I successfully identified and validated increases in miR-26a and the miR-26a / miR-205 ratio as early as Day 8 of pregnancy (max. 7.5-fold) in the plasma of pregnant compared to non-pregnant heifers. These miRNAs are known regulators of immunity, angiogenesis and metabolism, however their specific roles in early pregnancy remain to be investigated. I also identified small but significant increases in the levels of miR-125b, let-7f, miR-145 and miR-99a-5p at oestrus, when compared with the luteal phase of the cycle. These miRNAs have been previously shown to regulate the follicular to luteal transition in the bovine ovary. Finally, I provide a validated high-throughput resource which can help identify potential global biomarkers of tissue function, as shown for the liver-enriched miR-802 in the present results. The findings of this work may be useful in the development of diagnostic methods for early pregnancy and oestrus, and pave the way for the functional characterisation of these miRNAs in bovine reproduction.

1 Background

This section includes a basic introduction to cow reproductive physiology and the fertility issues faced in the dairy industry today. I also present a brief review of what is known about miRNA biogenesis and function as well as the potential use of circulating miRNAs as biomarkers and the technical difficulties involved in quantifying miRNAs. At the end of this chapter I present the hypotheses which drove this work and the objectives I pursued to investigate them.

1.1 Low fertility in modern dairy herds

The bovine dairy industry is one of the largest agricultural industries in the UK. According to data from the Department for Environment, Food and Rural Affairs (DEFRA) the UK cattle dairy herd had a size of 1.8 million heads in 2015 (Blackburn & Harriss, 2013) and low fertility is one of the largest sources of economic loss for the dairy industry (College of Agriculture, 2005b; Diskin & Morris, 2008). Low fertility is a major problem because it increases the incidence of unsuccessful inseminations and extends calving intervals beyond the optimal 365-day guideline. As a result, annual milk sales decrease while veterinary costs and unplanned culling of infertile cows increase. All these events raise concerns for both welfare and economic returns within the industry. It has been estimated that the extension of an ideal 365-day calving interval (based on seasonal calving), which includes a waiting period (not-in-calf) of 90 days, costs between £1.30 and £5.00 per day per animal; this adds up to approximately £500 million per year nationwide, which affects national economy and individual herd owners alike (College of Agriculture, 2005a, 2005b).

Low fertility is indicated by low calving rates at first insemination which average at 40 %, but are measured as low as 34 % of inseminated animals. This average corresponds to a 60 % non-calving rate, 10 % of which is attributable to fertilisation failure, 43 % due to early embryonic death (prior to gestation day 24) and 7 % due to late embryonic death (after gestation day 24, Figure 1-1) (Diskin & Morris, 2008; Diskin, Waters, Parr, & Kenny, 2015). There is a clear link between low fertility and the selection of higher-yielding cattle in recent years, as shown in Figure 1-1, which

are put under high metabolic pressure, leading to fertility problems partially due to altered circulatory progesterone (P4) and/or oestradiol (E2) concentrations both before and during pregnancy (Diskin & Morris, 2008; Inskip, 2004; Sartori, Bastos, & Wiltbank, 2010). These animals also have a compromised immune system and altered metabolism which contribute to low fertility, disease and poor overall performance. However, until the causes of early embryonic death are fully understood and dealt with, the main interest lies in the identification and re-insemination of non-pregnant animals as early as possible after artificial insemination (AI) in order to improve herd productivity (Fricke, 2002). The issue of low fertility in modern dairy herds is introduced further in Chapters 2, 3 and 4.

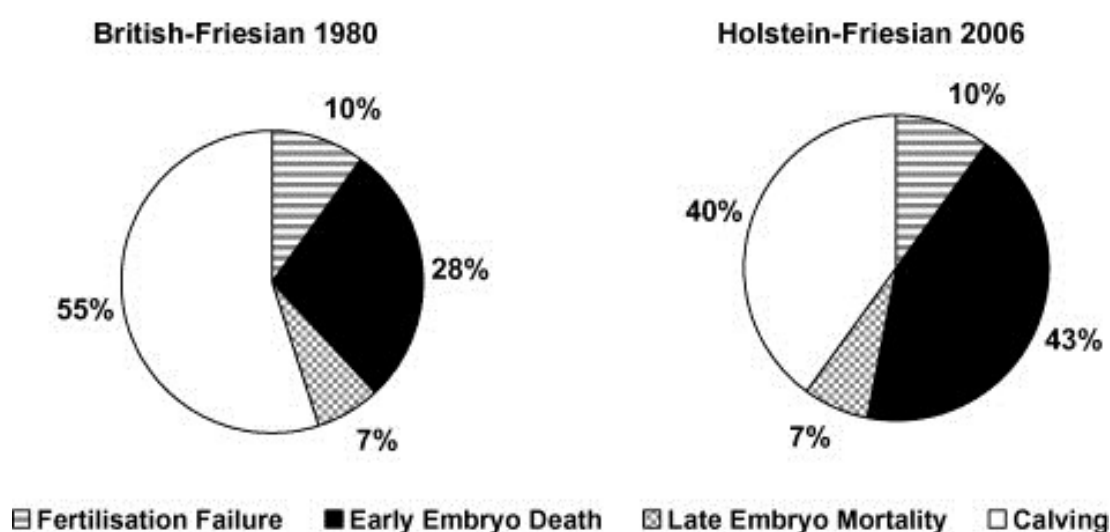


Figure 1-1. Reproductive outcomes in British vs. Holstein-Friesian cows.
Taken from (Diskin, Murphy, & Sreenan, 2006).

In an extension of the low fertility problem, attenuated oestrus expression (especially in the post-partum period) is another major concern in terms of herd reproductive management. Typically, dairy cattle will have resumed follicle-stimulating hormone (FSH) secretion by the fifth day post-partum, triggering a follicular wave which would lead to ovulation approximately 15 days post-partum (Crowe, 2008). The first oestrus after delivery is typically silent (without behavioural signs) and roughly 70 % of dairy cows will go through a shorter cycle following this first ovulation.

Unfortunately high-producing cows are under severe stress due to the metabolic demands of late pregnancy and lactation and the incidence of silent oestrus, anoestrus or attenuated oestrus is considerably higher than a few decades ago, which makes the

identification of animals which are in fertile oestrus difficult for dairy farmers (Dobson, Smith, Royal, Knight, & Sheldon, 2007).

Some significant culprits for abnormal oestrus are under-nutrition, metabolic stress, infection and restriction of movement. Metabolism plays a major role in reproductive performance and studies have shown that sufficient nutrition of high-producing cows is essential to maintain physiological E2 and P4 hormone levels and subsequently, reproductive cyclicity (Dobson et al., 2007). This is mainly because high-producing cattle operate at higher metabolic rates than low-producing cattle and therefore maintain a higher turnover of steroids in the liver. This results in lower levels of circulating E2 and P4, which negatively feedback on the hypothalamus-pituitary-gonad (HPG) axis and affect follicular maturation and oestrus behaviour (Sartori et al., 2010). In addition, there is a clear link between low body condition score and anovulation across studies, with incidence in more than 40 % of cows with a body condition score less than two (Fricke et al., 2014). Finally, oestrus behaviour can be attenuated due to confined housing or infection, both of which are common in some dairy farms (Fricke et al., 2014). All of these issues highlight the need for effective markers of fertile oestrus in modern dairy herds.

During the last two decades farmers, scientists and the public have become increasingly aware of low fertility and the increased incidence of disease and culling in the dairy industry. Social pressure driven by welfare worries and reduced profitability have motivated the industry to address low fertility in dairy herds. Since 2000, more emphasis has been put on improving the robustness of dairy cows, including improved fertility, easier management and reduced disease incidence (Egger-Danner et al., 2015). Many countries have implemented genetic selection programmes which focus on functional traits, including fertility. Research suggests it is possible to improve fertility traits through genetic selection while maintaining the already established high milk yields (Berry, Friggens, Lucy, & Roche, 2016). This is a positive outcome since it makes genetic selection programmes an attractive, sustainable and profitable investment for farmers (Berry et al., 2016). Despite the existence of limited published evidence, it is thought that the decline in dairy cow fertility has been halted and is beginning to reverse, mainly as a result of such

selection programmes and better nutritional management (Berglund, 2008; Egger-Danner et al., 2015). Finally, the effects of genetic selection programmes are additive and their benefits will become more apparent in the following years. In the meantime, research such as this presented in the current thesis may help provide diagnostic tools to better monitor and manage dairy herds.

1.2 Pregnancy detection methods

Because of the poor reproductive performance of modern dairy cows, which was introduced in the previous section, there is considerable interest in identifying non-pregnant animals early after AI and planning their re-insemination to reduce inter-calving intervals. There are a number of methodologies which are currently used to diagnose early pregnancy, including visual observation, trans-rectal ultrasonography (TRUS), serum and milk P4 testing, detection of pregnancy-associated glycoproteins (PAG) and profiling of interferon-stimulated glycoproteins (ISG) in serum or plasma. In the next sub-sections I will briefly introduce each method along with their advantages and disadvantages. All of the methods are summarised in Table 1-1.

Table 1-1. Overview of pregnancy detection methods for dairy cattle.

The methods are sorted by the day of pregnancy at which earliest detection is possible. ‘Common use’ refers to whether each method has been commercialised internationally.

Method	Earliest diagnosis (day of pregnancy)	Common use	Reference
Transrectal palpation	30	Yes	(Balhara, Gupta, Singh, Mohanty, & Singh, 2013)
Transrectal ultrasound	26	Yes	(Pieterse et al., 1990)
Visual observation (oestrus)	20-24	Yes	(Roelofs, Lopez-Gatius, Hunter, van Eerdenburg, & Hanzen, 2010)
Progesterone	21	Yes	(Han, Austin, Rempel, & Hansen, 2006; Pieterse et al., 1990)
Pregnancy associated glycoproteins	21	Yes	(Friedrich & Holtz, 2010; Perenyi, Szenci, Sulon, Drion, & Beckers, 2002)
Circulating DNA	20	No	(Mayer et al., 2012)
Interferon-stimulated genes	18	No	(Han et al., 2006)
Platelet activating factor	2	No	(Stein & O'Neill, 1994)
Early pregnancy factor	0	No	(Morton, 1998)

1.2.1 Common methods

The most common approach for the identification of cows that failed to conceive at first AI is the observation of inseminated cows for signs of oestrus (non-pregnant cows typically return to oestrus on days 20-24 post-AI). This approach can be costly due to the cost of observation staff since several hours of observation are required daily to ensure relative accuracy. Additionally, identification of any non-pregnant animals would be achieved 20-24 days after insemination, which is after the ideal time of re-synchronisation for intensive reproductive management programmes. Such programmes use a single prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection to induce luteolysis and ovulation on Day 20, at the earliest day on which return to oestrus would occur (Lucy, McDougall, & Nation, 2004). A disadvantage that rises from this timing is that if a $PGF_{2\alpha}$ is administered to a misdiagnosed ‘non-pregnant’ animal for the

purpose of re-synchronising it on Day 20, and that animal was actually pregnant, it will result in luteolysis and miscarriage.

Trans-rectal ultrasound (TRUS) requires an experienced practitioner for accurate results and reaches maximum diagnostic accuracy on day 26 post-AI (Pieterse et al., 1990). It is considered the gold standard for diagnosis by researchers and practitioners and is probably the most widely used diagnosis methods. Before the widespread use of TRUS, transrectal palpation was used to detect the amniotic vesicle and chorioallantoic membranes on Day 30 of pregnancy (Balhara et al., 2013). However the window during which these methods are useful is limiting for early pregnancy diagnosis.

Elevated P4 in blood or milk is another indicator of pregnancy; however, in some cases, high P4 levels are detected in the serum and milk of non-pregnant cows, for example because of a persistent corpus luteum (CL), which can result in a false-positive diagnosis. The sensitivity of P4 testing depends on the set threshold concentration, and accurate diagnosis can only be achieved after day 21 post-AI (Han et al., 2006; Pieterse et al., 1990).

PAGs are members of the aspartic proteinase protein family and are expressed by trophoblast cells; they can be detected in the maternal bovine circulation as early as day 21 post-AI (Friedrich & Holtz, 2010; Perenyi et al., 2002). It has been suggested that PAG levels depend on P4 levels, because P4 controls placenta development, which in turn controls secreted PAG levels (Barbato et al., 2013). Their spatial and temporal expression in the placenta varies, and different PAG family members can be used for pregnancy diagnosis, with varying reliability (Perenyi et al., 2002). The most common PAGs are bovine pregnancy-specific protein b (bPSP-B) and bovine pregnancy-associated glycoprotein 1 (bPAG-1). Serum ELISA assays for bPAG-1 have high accuracy from day 30 post-AI (94 % combined specificity and sensitivity), while bPSP-B plasma ELISA assays have a sensitivity of 93.9 % and a specificity of 95.5 % on day 28 post-AI (Friedrich & Holtz, 2010; Romano & Larson, 2010; Sasser, Ruder, Ivani, Butler, & Hamilton, 1986). However, post-partum circulating levels of both proteins do not diminish until day 70 after delivery due to their high stability, which presents a practical problem for field testing (Szenci et al., 1998).

1.2.2 Non-standard methods

ISGs are maternal proteins that are expressed by leukocytes after induction by conceptus-derived interferon-tau (IFN- τ) (Green, Okamura, Poock, & Lucy, 2010). ISG-15 can be used for diagnosis from day 18 post-AI (Han et al., 2006), however, the sensitivity of this method is relatively low at this stage of pregnancy (62%). Another study failed to establish a reliable pregnancy test on day 18 post-AI due to high false-positive rates, and reported higher ISG levels (MX2, OAS2, ISG15) in primiparous cows compared to multiparous cows (Green et al., 2010). Furthermore, ISG levels are affected by viral infections; this prevents reliable diagnosis in infected animals. Finally, a study reported the use of bovine circulating deoxyribonucleic acid (DNA) repeats in pregnancy diagnosis as early as day 20 post-AI; however this method requires a non-standardised transcriptomic analysis and it is not used commercially (Mayer et al., 2012).

A group of molecules are secreted very early from the conceptus and have an effect on the mother; these include platelet-activating factor (PAF), early pregnancy factor (EPF) and IFN- τ , and are reported to assist in the maintenance of pregnancy and placentation (Bazer, Song, & Thatcher, 2012; Ghaffari Laleh, Ghaffari Laleh, Pirany, & Moghadaszadeh Ahrabi, 2008; Morton, 1998). PAF has been detected in the maternal uterine circulation as early as Day 2 of pregnancy, while EPF has been detected as early as 6 hours after fertilisation (Morton, 1998; Stein & O'Neill, 1994). Reports suggest they are expressed in trophoblast cells, however the reliability of their detection has been controversial and they are not used commercially for pregnancy diagnosis (Bazer et al., 2012; Ghaffari Laleh et al., 2008; Morton, 1998; O'Neill, Wells, & Battye, 1990). Taken together, these findings highlight the need for novel early pregnancy diagnostics which are superior in accuracy to the currently available methods and may allow the implementation of advanced reproductive management programmes in modern dairy herds.

1.3 Oestrus detection methods

In addition to the identification of non-pregnant animals, oestrus detection is also critical for the improvement of reproductive performance because it allows farmers to maximise yields by inseminating all fertile animals within a herd. Traditionally,

visual observation is used to identify animals which are at oestrus. However, because of the attenuated oestrus behaviours and silent oestrus and the costs associated with observational detection of oestrus (covered in previous sections), this method is not ideal. Additional methods such as heat mount detectors and activity monitors for oestrus detection have recently become popular in the US and to some extent in the UK.

A recent review compared activity monitors and heat mount detectors and found that activity monitors detected 60 out of 69 true ovulations (87 %) while heat mount detectors identified only 55 true ovulations (80 %) (Fricke et al., 2014). A separate study compared activity monitors, heat mount detectors, visual observation and their combinations. In this study heat mount detectors detected 36-67 % of low-P4 periods while activity monitors detected 59-63 % of low-P4 periods (such periods hypothetically indicating oestrus) (Holman et al., 2011). Visual observation alone detected 57 % of low-P4 periods and it had the least amount of false positive calls (7 %) out of all methods used. Various combinations of these methods performed overall better compared to individual methods. The combination of a heat mount detector with an activity monitor detected 76 % of low P4 periods; however this approach had a 40 % false positive rate. It should be noted that despite the fact that activity monitors are available in the UK, they require a significant financial investment and many farmers choose not to take this approach, opting for heat mount detectors instead. This study concluded that 26 % of low-P4 periods remained undetected by any of the tested methods (Holman et al., 2011). These results highlight the need for novel diagnostic methods of fertile oestrus in dairy cattle, which is examined further in Chapter 2.

1.4 Reproductive physiology of cattle

1.4.1 The oestrous cycle

The development of the bovine ovary begins on Day 30 of gestation with the differentiation of gonadal ridge epithelial-like cells (GREL) from the mesonephric epithelium and the migration and proliferation of stromal cells and primordial germ cells (PGC, migrating from the hindgut) into the early ovary (Figure 1-2) (Smith, Wilhelm, & Rodgers, 2014). From Day 70, the stromal cells will extend outwards

from the medulla (central core) of the ovary into the cortex and separate the germ and GREL cells to form structures known as ovigerous cords (Hummitzsch et al., 2013). These structures are located in the cortex (outer section) of the developing ovary which will increase in size during this stage. From Day 130 onwards, primordial follicles are formed, which consist of flattened granulosa cells (derived from GREL cells) surrounding a single oocyte (Hummitzsch et al., 2013). At this stage, the oocytes proceed through meiosis I and arrest in prophase I, when they are termed primary oocytes. The granulosa cells will further differentiate and develop a cuboidal shape, forming what is known as the primary follicle, containing an arrested oocyte surrounded by a single layer of cuboidal granulosa cells. These primary follicles will continue to grow and acquire layers of granulosa cells (GC) during foetal life. Depending on their developmental stage and layers of granulosa cells the follicles are classified as secondary (two to six GC layers) and tertiary / antral (presence of antrum) (Cohen & Holloway, 2015).

The early stages of follicular development are independent of gonadotrophins. However, once animals begin to produce FSH, follicles enter a gonadotrophin-dependent growth phase which drives a large increase in follicle size (Cohen & Holloway, 2015). The primary oocyte inside the developing follicles will remain arrested in meiosis I during this growth phase, and will only complete meiosis I after ovulation (from puberty onwards). At this stage, the oocyte arrests in meiosis II, and is called secondary oocyte. Meiosis II will only be completed after fertilisation of the oocyte.

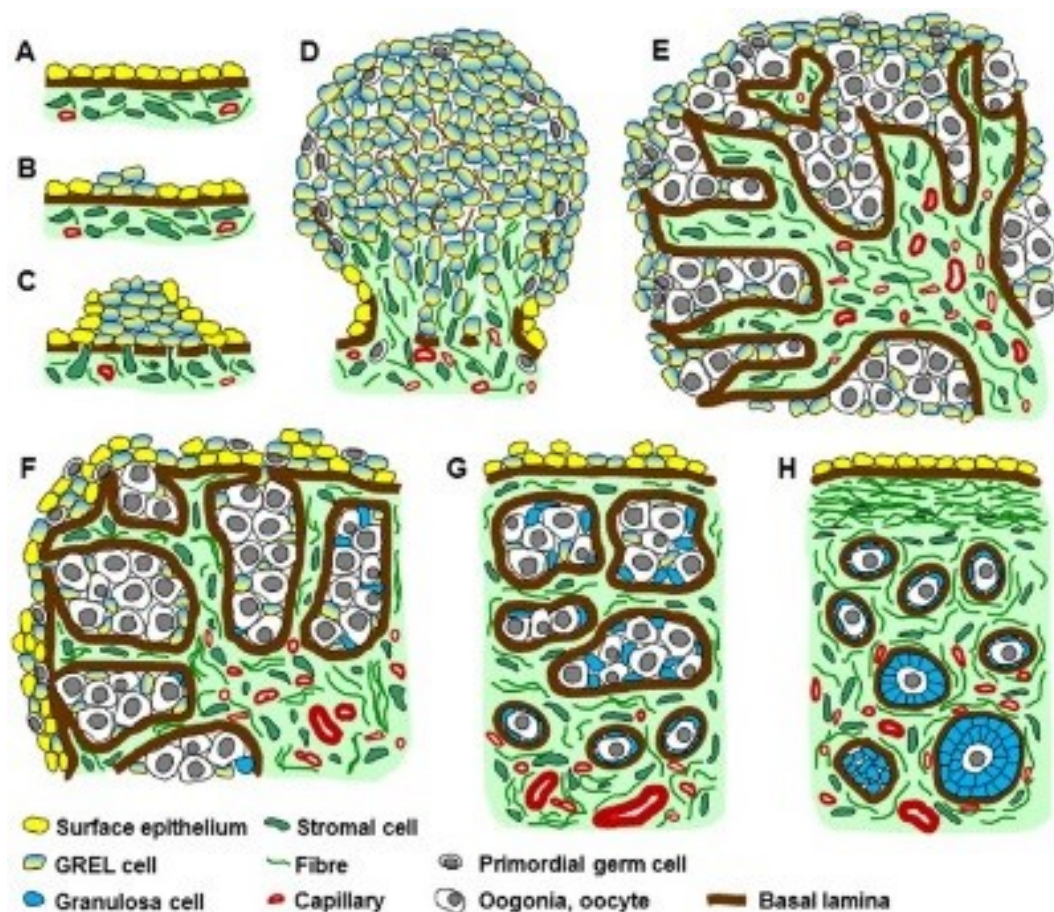


Figure 1-2. Stages of bovine ovarian and follicular development.

a) The mesonephric surface epithelium (yellow cells) prior to the formation of the gonadal ridge. b) Differentiation of the mesonephric surface epithelial cells into GREL cells (yellow-blue cells). c) Proliferation of GREL cells and migration of stromal cells (green) into the gonadal ridge. d) PGCs (grey) migrate into the ridge between the GREL cells. Mesonephric stromal cells and vasculature (red) migrate into the ovary. e) Formation of the ovigerous cords separated by stromal tissue. f) Cortex and medulla compartments are clearly separated. g) The first follicles form, consisting of oocytes surrounded by granulosa cells (blue cells). h) Primordial follicles become activated and commence development into primary and preantral follicles. Adapted from (Hummitzsch et al., 2013).

The majority of young animals experience their first oestrous cycle (resulting in the first LH surge mentioned in the previous paragraph) between 6 and 12 months of age (Forde et al., 2011). The oestrous cycle starts at oestrus, which is marked by the demonstration of oestrus behaviour, such as standing to be mounted, and ends at the next oestrus which typically occurs 21 days later. Two major events occur during this period. Firstly, ovarian follicles grow in size, leading to ovulation of a dominant follicle containing a mature oocyte, the female gamete. Secondly, the uterus

undergoes morphological changes that sequentially facilitate first the deposition and transport of sperm and second the attachment of the early conceptus in case of pregnancy.

At the beginning of the oestrous cycle, briefly after expression of oestrus behaviour, the mature dominant follicle will rupture and release a mature oocyte. The ruptured follicle will then differentiate into the corpus haemorrhagicum and subsequently into the CL, which will be maintained in the ovary for the largest part of the cycle (14 - 18 days). If no pregnancy is established during the luteal phase of the cycle, the CL will undergo luteolysis and regress, allowing a new dominant follicle to mature through to ovulation. The stage that starts with the lysis of the CL is termed follicular phase (4 -5 days) and covers the growth of a new wave of follicles until the ovulation of a dominant follicle, which will mark the beginning of a new oestrous cycle.

In addition to the ovulatory wave of follicles that will give rise to the dominant ovulatory follicle above, cows experience one or two additional waves of follicular growth during the luteal phase of the cycle (Figure 1-3) (Savio, Keenan, Boland, & Roche, 1988). Within each of these follicular waves, a single follicle will be selected as the dominant follicle while the remaining follicles will undergo atresia and regress (atretic follicles). The dominant follicle of the ovulatory wave will respond to an LH surge (explained below) and ovulate whereas dominant follicles from luteal phase waves will not ovulate and undergo atresia instead. This process is tightly regulated by endocrine factors which will be introduced in the following section.

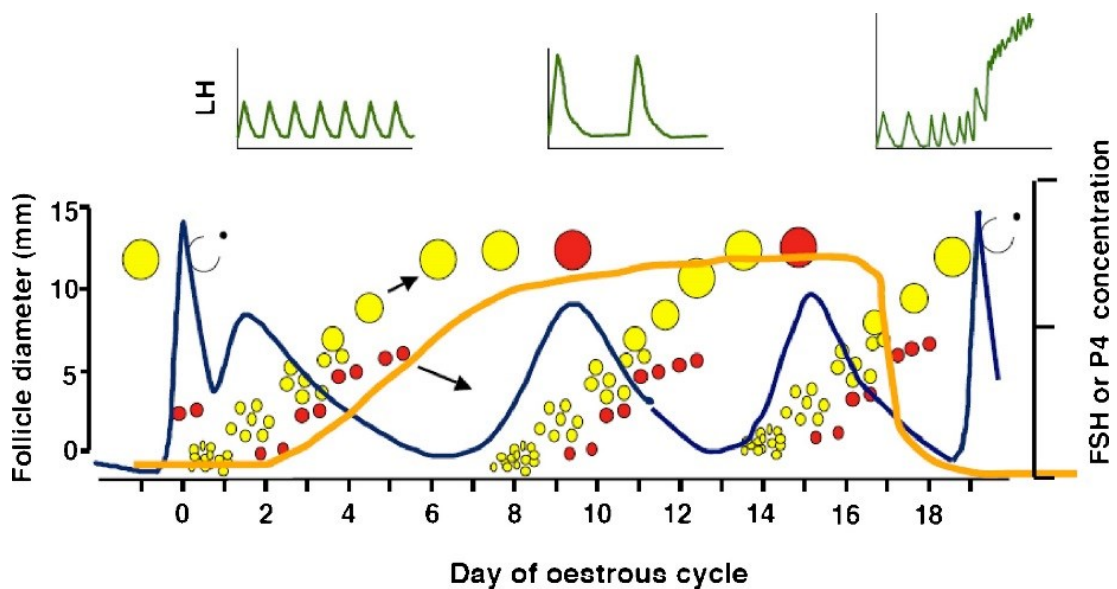


Figure 1-3. Follicular growth during the oestrous cycle.

Levels of follicle-stimulating hormone (FSH, blue), luteinising hormone (LH, green), and progesterone (P4, yellow) are shown. An increase in FSH triggers each wave of growing follicles and dominant follicles become atretic in the presence of a high progesterone background (shaded in red). The LH and FSH surge occurs prior to oestrus and induces ovulation and the secretion of LH during three 8-hour windows is shown (early luteal, mid-luteal and follicular phase, left to right). Taken from (Forde et al., 2011).

Multiple endocrine hormones are secreted from the hypothalamus, the anterior pituitary and the ovaries which control the events that take place during the oestrous cycle. In addition to direct regulation of ovarian processes, these hormones are also involved in positive and negative feedback loops with each other and the resulting regulatory network is termed the hypothalamus-pituitary-gonadal (HPG) axis. In the following lines I will briefly explain the basic roles of gonadotrophin-releasing hormone (GnRH), FSH, LH, E2 and P4 in the oestrous cycle.

The hypothalamus in the brain of the cow secretes GnRH in a pulsatile manner. GnRH reaches the anterior pituitary gland, where it induces the secretion of two gonadotrophins, LH and FSH (Schally et al., 1971). These two hormones access systemic circulation and control ovarian function during the cycle. Specifically, FSH drives the recruitment and growth of the cohorts of antral follicles during the cycle and its concentrations decrease as the follicles grow (Sunderland, Crowe, Boland, Roche, & Ireland, 1994). However, after a dominant follicle has been selected, its

growth becomes less dependent of FSH and comes under the control of LH. LH is secreted in a pulsatile manner and prior to ovulation an increase in GnRH (driven by E2 which is secreted by the dominant follicle) triggers what is known as the LH surge, which consists of high frequency pulses that induce ovulation of the dominant follicle (Forde et al., 2011).

The hormones which are produced in the gonads originate in either the follicle (E2, inhibin) or the CL (P4). An increase in plasma E2 concentration is observed during the follicular phase of the cycle due to the presence of a dominant follicle which secretes large amounts of this hormone. Following ovulation, the concentration of E2 decrease and P4 concentration increases as the CL forms. As mentioned above, E2 is responsible for the induction of the LH surge and it also represses FSH secretion during the late follicular phase of the cycle (Roche, 1996). Progesterone acts on LH through a negative feedback loop, preventing the ovulation of a dominant follicle during the luteal phase of the cycle and leading to atresia of the anovulatory dominant follicle (Sunderland et al., 1994). Finally, at end of the luteal phase and in the absence of maternal recognition of pregnancy the uterus secretes PGF_{2α} which induces the lysis of the CL (Hansel & Convey, 1983).

In addition to these major hormones, there are a number of locally produced (paracrine) growth factors which fine-tune the actions of hormones on the ovary, including insulin-like growth factor 1 (IGF-1), members of the transforming growth factor beta (TGF-β) family, activins, inhibins and follistatin. IGF-1 is the best studied out of these factors and it up-regulates E2; an early increase in its bioavailability is recognised as the first event in the selection of the dominant follicle in the ovary (Lucy, 2007). The role of growth factors in the ovary have been reviewed in a number of publications (Forde et al., 2011; Ginther, 2016; Lucy, 2007).

1.4.2 Early pregnancy

Gestation in cows lasts for 283 days on average. Gestation can be separated in two stages, the embryonic stage (Days 0-42) and the foetal stage (Day 42 - parturition). The embryonic stage includes the development from zygote to blastocyst (Days 1-9), maternal recognition of pregnancy (Day 15), attachment (Days 18-22) and

organogenesis (Days 20-40) (Lemley, Camacho, & Vonnahme, 2014). During the foetal stage organs develop further; also the placenta is fully formed and increases in size. The major events that take place in the first 150 days of pregnancy have been summarised in Figure 1-4. The majority of embryonic loss events occur during the early embryonic stage of pregnancy; this has been reviewed by Diskin et al. and is also illustrated in Figure 1-1 (Diskin & Morris, 2008).

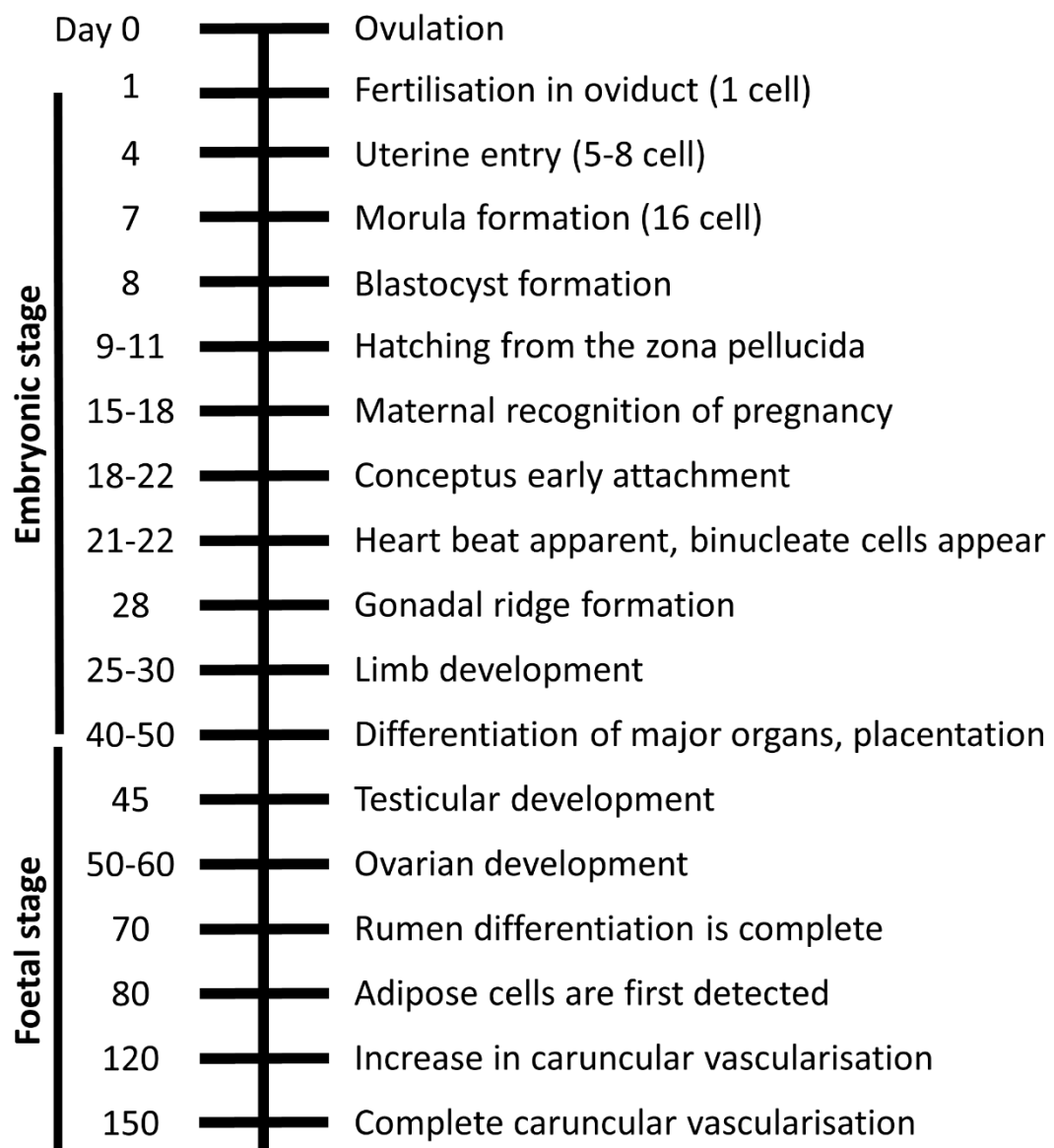


Figure 1-4. Major events within the first 150 days of bovine pregnancy.
This figure has been adapted from (Lemley et al., 2014) using information from (Peters & Ball, 1995).

After fertilisation, the early embryo will remain in the oviduct for 96 hours before moving down the uterine horn (Bazer & First, 1983). Through a number of mitotic divisions, the embryo will progress to the blastocyst stage on Day 8 of pregnancy. Following further divisions and differentiation, the embryo begins to elongate on Day 15 of gestation and takes a filamentous form by Day 20, which is essential for attachment (Bazer et al., 2012). Just prior to the elongation stage (Day 15) the embryo must indicate its presence to the maternal host. The main purpose of maternal recognition of pregnancy is to maintain P4 levels, since this is essential for embryonic development and survival (Mann, Lamming, Robinson, & Wathes, 1999).

IFN- τ is a type-1 interferon which is the signal of maternal recognition of pregnancy in ruminants. It is secreted from trophoblast cells from Day 12 but its expression peaks on Days 15 and 16 of pregnancy. IFN- τ acts on the endometrium by inhibiting the secretion of PGF_{2 α} which is a luteolytic signal and increasing the PGE / PGF_{2 α} ratio, which has a luteotropic effect (Lemley et al., 2014). In addition, IFN- τ modulates numerous ISGs in the bovine endometrium, affecting fatty acid metabolism and prostaglandin precursor synthesis among others (Bauersachs & Wolf, 2015). Interestingly, the amount of IFN- τ and therefore the effectiveness of the anti-luteolytic signal that is secreted depends on the size of the conceptus (Lemley et al., 2014).

Cows have a non-invasive cotyledonary placenta, meaning that circulatory exchange only occurs between uterine structures called caruncles and placental cotyledons. The two structures form placentomes (Figure 1-5) of which there are approximately 100 in each placenta, although this number is quite variable (Schlafer, Fisher, & Davies, 2000). Those are connected by the interplacentome (Figure 1-5), which is an area where nutrient exchange does not occur. Shortly after maternal recognition of pregnancy, the initial stages of attachment of the conceptus take place. By Day 20 trophoblast tissue has adhered to the uterine epithelium and removal of the embryo at this stage will result in uterine damage (Lemley et al., 2014). This adhesion becomes stronger in the following 10 days, and by Day 31 placentomes have been formed and embryonic circulatory function is assumed (Greenstein, Murray, & Foley, 1958; King, Atkinson, & Robertson, 1981; Schlafer et al., 2000). Following its initial

establishment, the bovine placenta will continue to develop and grow, with definitive placentation occurring around Day 40–45 (Peters & Ball, 1995). The placenta will also continue to grow during the foetal stage of pregnancy, throughout the first trimester, increasing placental blood flow to support transfer of nutrients to the foetus.

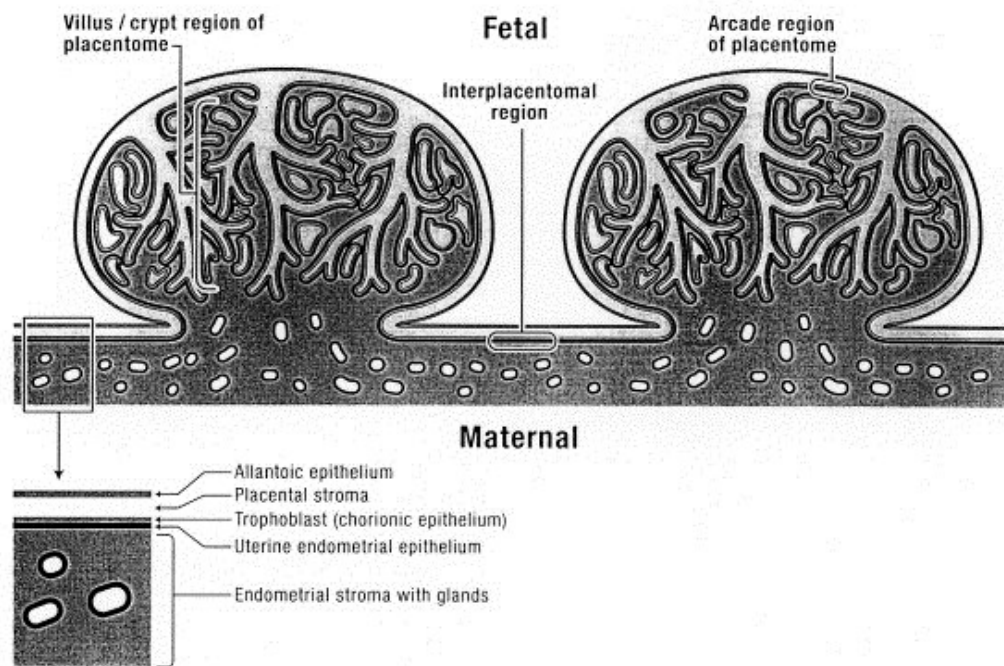


Figure 1-5. Compartments of the bovine placenta.

Diagram features both placentome and interplacentome region and the relevant membranes. Taken from (Schlafer et al., 2000).

1.5 Biogenesis and functions of microRNAs

MicroRNAs are small, non-coding RNA molecules which have a key role in regulating gene transcription in animal and plant cells. The first miRNAs ever discovered were lin-4 and let-7, in *Caenorhabditis elegans*, in 1993 and 2000, respectively (Almeida, Reis, & Calin, 2011; Bartel, 2004). Since then a large number of studies has focused on understanding their evolution, biogenesis and function in organisms. In this section I will briefly review the existing literature about miRNA evolution, biogenesis and the mechanisms of action of miRNAs.

1.5.1 Biogenesis of microRNAs

Evolutionary studies suggest that miRNA loci evolved through a number of routes including gene duplications, transposable element activity, development from existing small RNA loci and gene introns, but also through *de novo* miRNA generation under constant evolutionary pressure (Berezikov, 2011). MiRNA loci are located in coding and non-coding genes and are sometimes under the control of a polycistronic transcription unit, which allows transcription of multiple miRNAs (miRNA clusters) through one control element (Bartel, 2004). Transcription of miRNA loci is commonly performed by RNA polymerase II, which also transcribes mRNA, snoRNA and snRNA, and more rarely by RNA polymerase III, which routinely transcribes other small RNAs (tRNA, rRNA, snRNA) (Bartel, 2004; Winter, Jung, Keller, Gregory, & Diederichs, 2009). Examples of these mechanisms are hsa-miR-21, which is transcribed by Pol II, and members of the human C19MC cluster (hsa-miR-515-1, hsa-miR-517a, hsa-miR-519-1) which are transcribed by Pol III (a result of neighbouring Alu element promoters), as shown in a chromatin immunoprecipitation (ChIP) study (Borchert, Lanier, & Davidson, 2006).

The miRNA primary transcript, termed pri-miRNA, is a long (several kilobases) RNA transcript which includes a 5' cap, a 3' poly-A tail, and a stem-and-loop structure flanked by long single-stranded RNA (ssRNA) ends (Winter et al., 2009). After its transcription, the pri-miRNA is cleaved by Drosha (co-bound by DGCR8) while it is still in the cell nucleus (Figure 1-6) (Winter et al., 2009). This cleavage defines the final sequence of the mature miRNA, because subsequent processing depends on the length of the dsRNA stem, which is determined by this cleavage (Bartel, 2004). The Drosha product is called a pre-miRNA and a nuclear transport receptor protein, exportin 5 (co-bound by GTP-Ran) binds the pre-miRNA through recognition of its stem and 3' overhang, and transports it to the cytoplasm (Winter et al., 2009). MiRNAs which are transcribed from introns (termed miRtrons) are spliced out by normal splicing machinery and skip Drosha processing in an alternative processing pathway which is outside the scope of this section (Winter et al., 2009). These processing steps are summarised in Figure 1-6.

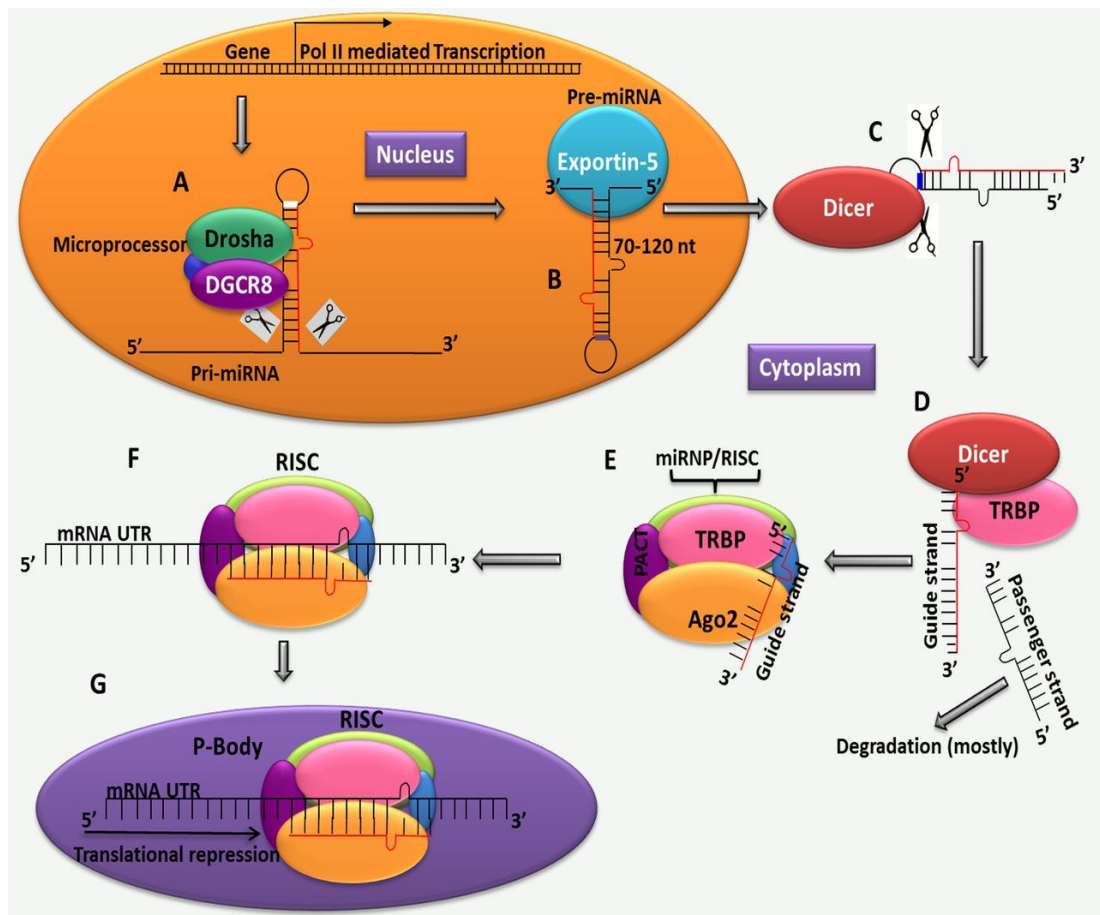


Figure 1-6. The canonical miRNA biogenesis pathway.

MicroRNAs (miRNAs) are transcribed in the nucleus by RNA polymerase II as primary miRNAs (pri-miRNAs). They are then processed into premature miRNAs (pre-miRNAs) by the microprocessor complex (A), followed by export to the cytoplasm by Exportin-5 (B). There, Dicer cleaves the pre-miRNAs into mature miRNA duplexes (C). Then, transactivation response RNA binding protein (TRBP) incorporates the guide strand of the miRNA duplex into the miRNA-induced silencing complex (RISC) (D-E). Finally the mature miRNA is used to target mRNA transcripts, which are eventually channelled into P-bodies (F-G). Figure taken from (Bhaskaran & Mohan, 2014).

Once in the cytoplasm, the pre-miRNA will undergo another cleavage step and this product will be selectively loaded on the silencing complex. Pre-miRNA is recognised by Dicer, an RNase III endonuclease, and its two associated proteins, TAR RNA-binding protein (TRBP) and Ago2 (Figure 1-6). A fourth protein, PACT, joins this complex to form the RISC loading complex (RLC) (Winter et al., 2009). Dicer will then cleave the pre-miRNA to produce a 22-nt duplex with 3' overhangs and 5' phosphates, and TRBP and PACT will dissociate from the RLC (Winter et al., 2009). It has been suggested that miRNA strand loading onto the miRNA-induced

silencing complex (miRISC) depends on thermodynamic stability (Bartel, 2004). Most miRNAs will remain in the cytoplasm after miRISC assembly, however, a few are known to relocate back to the nucleus where they are assumed to have regulatory functions on transcription, for example binding to gene promoters (Krol, Loedige, & Filipowicz, 2010; Winter et al., 2009).

1.5.2 Effector mechanisms of microRNAs

Once the miRISC is formed, the miRNAs are available to base-pair with target mRNAs, resulting in endonucleolytic cleavage, deadenylation and translational repression (Huntzinger & Izaurralde, 2011; Krol et al., 2010). MiRNAs target specific mRNAs, primarily dictated by the conserved miRNA seed sequence located at the 5' end of the mature miRNA (position 2-7), and a large part of miRNAs are expressed in a tissue- and developmental stage-specific manner (Coutinho et al., 2007; Jin, Grant, Stothard, Moore, & Guan, 2009; Lagos-Quintana et al., 2002; Landgraf et al., 2007; Y. Liang, Ridzon, Wong, & Chen, 2007). In addition to the seed sequence, their conserved 3' end (positions 13-16) also contributes to miRNA base-pairing to targets. Targeting occurs primarily on the 3' UTR of the mRNA, and less efficiently on its 5' UTR or the open reading frame (ORF) (Bartel, 2009).

MiRNAs which base pair perfectly to their target will induce endonucleolytic cleavage, cutting approximately 10-11 nucleotides (nt) from the 5' end of the miRNA, in a way similar to siRNAs (Bartel, 2004). However, in animals, perfect complementarity of miRNAs is rare; therefore direct endonucleolytic cleavage is unlikely to take place and to have biological relevance (Bartel, 2004). Studies using transfected miRNA-targeted constructs in HeLa cells suggest that translational repression occurs before, and independently of, mRNA degradation, and materialises through both 1) a reduction in initiation of translation and 2) a reduction in post-initiation translation (Kong et al., 2008; Meijer et al., 2013). Further studies in mouse Krebs-2 ascites cells support the notion that miRNAs first block initiation of translation, prior to any mRNA degradation events (Mathonnet et al., 2007).

However another collection of research suggests that mRNA degradation driven by deadenylation is the main miRNA silencing mechanism. A recent study proposed

that animal miRNAs lead only to mRNA deadenylation, which can result on translational repression or mRNA degradation depending on the stage of development of a given organism (Subtelny, Eichhorn, Chen, Sive, & Bartel, 2014). Supporting this idea, a study on HeLa cells and mouse neutrophils focusing on miR-1, miR-155 and miR-223 suggests that the majority miRNA gene silencing (at least 84 %) is attributable to mRNA destabilisation (H. Guo, Ingolia, Weissman, & Bartel, 2010) and an additional study supports the notion that miRNA actions are primarily deadenylation-driven (C. Y. Chen, Zheng, Xia, & Shyu, 2009). It is clear that there is no consensus on the predominant mechanism of miRNA silencing, however future studies may help clarify the mechanisms of miRNA actions in different conditions. Finally, it should be mentioned that in addition to gene silencing, it has been shown that in some cases miRNAs act as translational activators, by affecting gene promoters and enhancing translational efficiency (Almeida et al., 2011; Krol et al., 2010).

Despite the fact that the mechanism of action of miRNAs is not perfectly clear, it is widely accepted that miRNA interactions with their target genes are more complex than a simplistic ‘one versus one’ approach. An individual miRNA can fine-tune the expression of one or multiple targets and similarly a target can also be regulated simultaneously by multiple miRNAs (Bartel, 2009). Therefore, there are complex networks of miRNA regulation with overlapping targets. In addition, recent studies show that transcription factors regulate groups of miRNAs based on their common targets, effectively amplifying their effect on a given target by up- or down-regulating multiple common regulators of that target (Na & Kim, 2013). Finally, another level of complex control of miRNA activity is the control of their bioavailability through miRNA decay. Studies suggest that miRNAs have variable decay rates; some acute response miRNAs have decay rapidly, while housekeeping miRNAs have much slower decay rates, allowing prolonged effects on their targets (Almeida et al., 2011; Krol et al., 2010).

With the increasing availability of deep sequencing data, it was observed there are non-canonical miRNA sequences which differ slightly from canonical miRNAs in virtually every tissue profiled. These miRNAs are termed isomeric miRNAs, or

isomiRs. They are thought to be the result of variation in the miRNA biogenesis pathway (mainly Drosha and Dicer cleavage) and they are biologically active (Cloonan et al., 2011). Specifically, isomiRs have are loaded onto the miRISC complex with lower efficiency than their canonical counterparts however they have been experimentally shown to repress miRNA targets (Cloonan et al., 2011; Llorens et al., 2013). MicroRNA isomiRs appear to have similar biological roles to the canonical miRNA, and they act in a cooperative manner, typically repressing common targets although less efficiently (Cloonan et al., 2011). An exception is made for isomiRs with changes in the 5' end compared to the canonical miRNAs, in which the seed sequence is affected, resulting in different mRNA targets to the canonical miRNA. Cloonan et al. reported the most abundant types of isomiRs are end-site isomiRs (3' end variants) and shifted isomiRs (frameshift variants), and in some cases these isomiRs are more abundant than the canonical sequence (Cloonan et al., 2011). The abundance of the isomiRs in a sample generally correlates with the abundance of their canonical sequence (Cloonan et al., 2011). In the case of miR-101, which is highly expressed in brain, the start isomiR was biologically active, resulting in 20 % repression of the target EZH2, in contrast to the 40 % observed for the canonical sequence (Llorens et al., 2013).

1.6 MicroRNAs in mammalian reproductive biology

MicroRNAs are involved in virtually all aspects of mammalian physiology and pathophysiology. However the focus of this section will be on the miRNA control of reproduction, specifically the oestrous cycle, the development of the conceptus and the endometrial response to pregnancy. MicroRNAs have been associated with the control of ovarian function, specifically follicular and luteal development. In addition, several studies have identified pregnancy-associated miRNAs and miRNA clusters which are involved in various aspects of placental and embryonic development. Examples of such miRNAs are reviewed here (Diana M. Morales-Prieto, Ospina-Prieto, Chaiwangyen, Schoenleben, & Markert, 2013) and will be introduced in this section and also in Chapters 2, 3 and 4.

1.6.1 MicroRNAs in the oestrous cycle

Several miRNAs have been associated with the physiological changes that occur during the oestrous cycle. In this section I will focus on miRNAs which change during different ovarian states and endometrial miRNAs which change during the oestrous cycle, mainly in association with receptivity. The findings of the studies discussed below are summarised in Table 1-2.

Table 1-2. Summary of miRNAs that regulate the oestrous cycle.

Primarily focusing on studies performed in livestock. 'Expression' indicates the location where each miRNA is expressed and any reported changes in specific conditions. 'Confirmed and predicted targets' includes suggested and/or validated targets for each miRNA as reported in the cited studies. 'Function' lists the processes which are thought to be regulated by each miRNA according to the cited study. 'Species' indicates the species in which each study was conducted. n.d. = not defined.

miRNA	Expression	Confirmed and predicted targets	Function	Species	Study
miR-378	Increase in non-regressed CL	IFNGR1	Pro-apoptotic	Cow	(Ma et al., 2011)
	n.d.	CYP19A1	Steroidogenesis	Pig	(S. Xu, Linher-Melville, Yang, Wu, & Li, 2011)
miR-26b	Increase in follicular atresia	ATM	GC apoptosis	Pig	(Lin et al., 2012)
let-7g	Up in follicular atresia	MAP3K1	Pro-apoptotic	Pig	(Cao et al., 2015; J. Zhou et al., 2015)
miR-34a	Granulosa cells	INHBB	Pro-apoptotic	Pig	(Tu et al., 2014)
miR-10b	Up after hCG treatment in GCs	BDNF	Anti-proliferative	Goat	(Peng et al., 2016)
miR-17-92 cluster	Granulosa cells	PTEN and BMPR2	Proliferation and differentiation	Cow	(Andreas et al., 2016)
miR-92a	Down in atretic follicles	SMAD7	Anti-apoptotic	Pig	(Liu et al., 2014; Quezada, Wang, Hoang, & McGee, 2012)
miR-126-3p	n.d.	FSHR	Pro-apoptotic	Pig	(Du, Li, Pan, & Li, 2016)

miR-183-96-182	Up in granulosa cells	FOXO1	Pro-proliferative	Cow	(Gebremedhn et al., 2016)
miR-31	Up in mid-secretory phase	FOXP3 and CXCL12	Immune tolerance	Cow	(Kresowick, Devor, Van Voorhis, & Leslie, 2014)
miR-382	Up on Day 3 of oestrous cycle	MMP13	Endometrial remodelling	Cow	(Ponsuksili et al., 2014)

Studies in livestock species have shown that ovarian miRNAs regulate cell proliferation, differentiation, apoptosis and maturation in the ovary during the oestrous cycle, as summarised in Table 1-2. An example of a miRNA cluster that is involved in controlling the luteal-to-follicular transition is the miR-183-96-182 cluster. Studies in cow suggest this miRNA cluster controls GC proliferation and apoptosis by targeting FOXO1 ((Gebremedhn et al., 2016) and unpublished data from my group). Another study in cow suggests that the miR-17-92 cluster is involved in GC proliferation, this time by targeting PTEN and BMPR2 (Andreas et al., 2016). A pig study showed that miR-378 is associated with atresia in the CL by targeting interferon-gamma receptor 1 (IFNGR1), while the same miRNA is known to directly target aromatase (CYP19A1) in the cow, blocking steroidogenesis (Ma et al., 2011; S. Xu et al., 2011). For a summary of additional studies regulating ovarian function please refer to Table 1-2.

Other studies have directly or indirectly shown that miRNAs are involved in the changes which occur in the uterus during the oestrous cycle in preparation for pregnancy. A recent study in humans found that miR-31 is elevated in endometrial tissues during the mid-secretory phase of the menstrual cycle, putatively inducing immune tolerance via targeting FOXP3 and CXCL12 in regulatory T-cells (Kresowick et al., 2014). In line with this hypothesis, miR-31 increased in the serum during this stage in women. Other miRNAs which have been involved in endometrial receptivity include miR-494, miR-30b and miR-30d, which target leukaemia inhibitory factor (LIF) among other genes (Altmae et al., 2013). The authors of this study suggested these genes are involved in important gene reprogramming during

the oestrous cycle. A study in cow identified several differentially expressed miRNAs between Days 3 and 7 of the bovine oestrous cycle, including miR-382, miR-486, miR-1290, miR-3437 and miR-1246 (Ponsuksili et al., 2014). Interestingly, miR-382 is a regulator of matrix metalloprotease 13 (MMP13) which is part of the large matrix metalloproteinase (MMP) collagenase family that participates in endometrial remodelling.

1.6.2 MicroRNAs in pregnancy

MicroRNAs also play important roles in pregnancy, specifically in the development of the embryo and the placenta. Perhaps because of their relevance in human pregnancy-related diseases such as pre-eclampsia, some miRNAs and miRNA clusters have been studied in more depth in humans compared to livestock. There are quite extensive reviews on pregnancy-related miRNAs (Diana M. Morales-Prieto et al., 2013; Sadovsky, Mouillet, Ouyang, Bayer, & Coyne, 2015); in this section I will summarise current findings on these miRNAs (see Table 1-3).

Table 1-3. MicroRNAs which are important in pregnancy.

‘Expression’ indicates the location where each miRNA is expressed and any reported changes in specific conditions. ‘Species’ indicates the species in which each study was conducted. ‘Function’ lists the processes which are thought to be regulated by each miRNA according to the cited study. n.d. = not defined.

miRNA	Expression	Species	Function	Studies
miR-196a	Four- to eight-cell embryo	Cow	Folliculogenesis	(Tripurani, Lee, Wee, Smith, & Yao, 2011)
miR-200c	Day 7 embryo	Cow	n.d.	(Castro et al., 2010)
miR-21 and miR-130a	Two- to eight-cell embryo	Cow	Maternal to embryonic transition	(Mondou, Dufort, Gohin, Fournier, & Sirard, 2012)
miR-520, miR-302 and miR-200 family	Embryonic stem cells	Human	n.d.	(Galliano & Pellicer, 2014) (review)
miR-372, miR-720 and miR-302c	Blastocyst	Human	Stem cell pluripotency and embryonic survival	(Galliano & Pellicer, 2014) (review)
miR-96, miR-125a and miR-29	Embryonic stem cells (differentiating)	Human	Trophoectoderm specification	(Viswanathan et al., 2009)
miR-17, miR-19b, miR-517b, miR-29b, miR-125b, miR-195, and miR-378a-5p	Early placenta	Human	Trophoblast proliferation, migration and invasion	(Alpini et al., 2011; Bai et al., 2012; Doridot, Miralles, Barbaux, & Vaiman, 2013; P. Li et al., 2013; L. Luo et al., 2012; Ventura et al., 2013; D. Wang, Song, & Na, 2012)
C19MC cluster	Placenta	Human, mouse	Placental development, antiviral protection, angiogenesis	(Diana M. Morales-Prieto et al., 2013; Mouillet, Chu, & Sadovsky, 2011) (reviews)
miR-379-miR-410	Differentially expressed in pregnancy	Human, mouse	n.d.	(Diana M. Morales-Prieto et al., 2013; Mouillet

cluster (C14MC)				et al., 2011) (reviews)
miR-371- 373 cluster	Embryonic stem cells	Human, mouse	n.d.	(Diana M. Morales-Prieto et al., 2013) (review)
miR-21, miR-101a and miR- 199a	Uterus	Cow	Conceptus implantation (targeting COX- 2)	(Galliano & Pellicer, 2014) (review)
let-7 family, miR-145 and miR- 132	Embryo < 6 weeks	Cow	n.d.	(Galliano & Pellicer, 2014) (review)

A number of studies have focused on the miRNA expression profiles of the developing pre-implantation embryo. A group of miRNAs show increased expression in the early stages of pre-implantation development, from the 2-cell to the blastocyst stage (miR-196a, miR-200c, miR-424, miR-92a and miR-10b) and some are then gradually down-regulated (Castro et al., 2010; Mondou et al., 2012; Tesfaye et al., 2009; Tripurani et al., 2011; Tripurani, Xiao, Salem, & Yao, 2010). Authors have suggested that some of these miRNAs serve the purpose of degrading maternal mRNAs in preparation for the zygotic genome activation as part of the maternal to zygotic transition in early embryogenesis (Tripurani et al., 2011). A range of miRNAs including miR-520, miR-302, the miR-200 family, miR-372, miR-720 and miR-302c are highly expressed in embryonic stem cells and blastocysts (Galliano & Pellicer, 2014).

MiRNAs are also involved in the proliferation of the trophoectoderm, the formation of the trophoblast and the subsequent placentation of the embryo; a number of miRNAs have been linked to trophoectoderm specification (miR-96, miR-125a, miR-29) (Viswanathan et al., 2009). In addition, several miRNAs (miR-17, miR-19b, miR-517b, miR-29b, miR-125b, miR-195, and miR-378a-5p) regulate trophoblast cell migration, immuno-tolerance, and angiogenesis by targeting growth factors involved in these processes, such as VEGF, Nodal and PIGF. (Alpini et al., 2011; Bai et al., 2012; Doridot et al., 2013; P. Li et al., 2013; L. Luo et al., 2012; Ventura et al., 2013; D. Wang et al., 2012). Another significant target of miR-17-92 cluster (including

miR-17 and miR-19b, mentioned above) is CYP19A1, which is involved in the inhibition of trophoblast differentiation (Kumar, Luo, Tudela, Alexander, & Mendelson, 2013). The proliferative nature of the developing placenta has been compared to that of neoplastic tissue; indeed, many miRNA regulators of placentation also have roles in neoplasia, such as miR-125b which is involved in hepatic cancer (Alpini et al., 2011).

Common pregnancy-related miRNA clusters include the primate-specific C19MC cluster, which is one of the largest miRNA clusters ever discovered, producing 58 placenta-specific mature miRNAs which are highly expressed in the trophoblast cells and are thought to control cell proliferation, invasion and differentiation (Mouillet et al., 2011). Another interesting finding is that C19MC members are inducers of antiviral resistance, unveiling a new potential role for this cluster in immunity. The C14MC or miR-379-410 cluster is another large miRNA cluster (63 miRNAs in human) which is also present in cow (Mouillet, Ouyang, Coyne, & Sadovsky, 2015). Interestingly, deletion of this cluster is not detrimental for pregnancy and its function is largely unknown, but it is thought to be involved in embryonic development (Diana M. Morales-Prieto et al., 2013; Mouillet et al., 2015). Finally, the miR-371-373 cluster is highly expressed in embryonic stem cells and its expression declines with the progression of pregnancy; its members are thought to maintain the cell cycle and control cell proliferation (Diana M. Morales-Prieto et al., 2013).

The maternal endometrium also undergoes changes during the early stages of pregnancy in order to promote embryonic survival and attachment. It has been shown that microRNA expression profiles change in the pregnant porcine endometrium compared to non-pregnant endometrium as early as gestation day 20, and a more recent study detected differentially expressed miRNAs in the porcine endometrium between implantation (Day 15) and placentation (Day 26) (Su et al., 2014; Wessels et al., 2013). Differences between receptive and non-receptive endometrium have been reviewed by Galliano and colleagues (Galliano & Pellicer, 2014). Specifically, miR-21, miR-101a and miR-199a are expressed highly in implantation sites, targeting RECK and COX-2. Finally, the same reviewers note the importance of let-7 family

members and their regulators, LIN28, miR-145 and miR-132, in controlling cell proliferation and differentiation in embryonic tissues (Galliano & Pellicer, 2014).

1.7 Circulating microRNAs as biomarkers

1.7.1 Biological importance of circulating microRNAs

In addition to tissues, miRNAs are also present in bodily fluids such as serum, plasma, saliva and urine (Weber et al., 2010). Circulating miRNAs have been characterised as highly stable and resistant to RNAses, extreme pH and freeze-thaw cycles; this is due to the existence of ribonucleoprotein complexes and the inclusion of miRNAs in protective vesicles (Arroyo et al., 2011; X. Chen et al., 2008; El-Hefnawy et al., 2004; Hunter et al., 2008; Turchinovich, Weiz, Langheinz, & Burwinkel, 2011; Valadi et al., 2007; Vickers, Palmisano, Shoucri, Shamburek, & Remaley, 2011). The following section focuses on the carriers of circulating miRNAs, which are summarised in Figure 1-7, their uptake from cells and their potential effects in those cells.

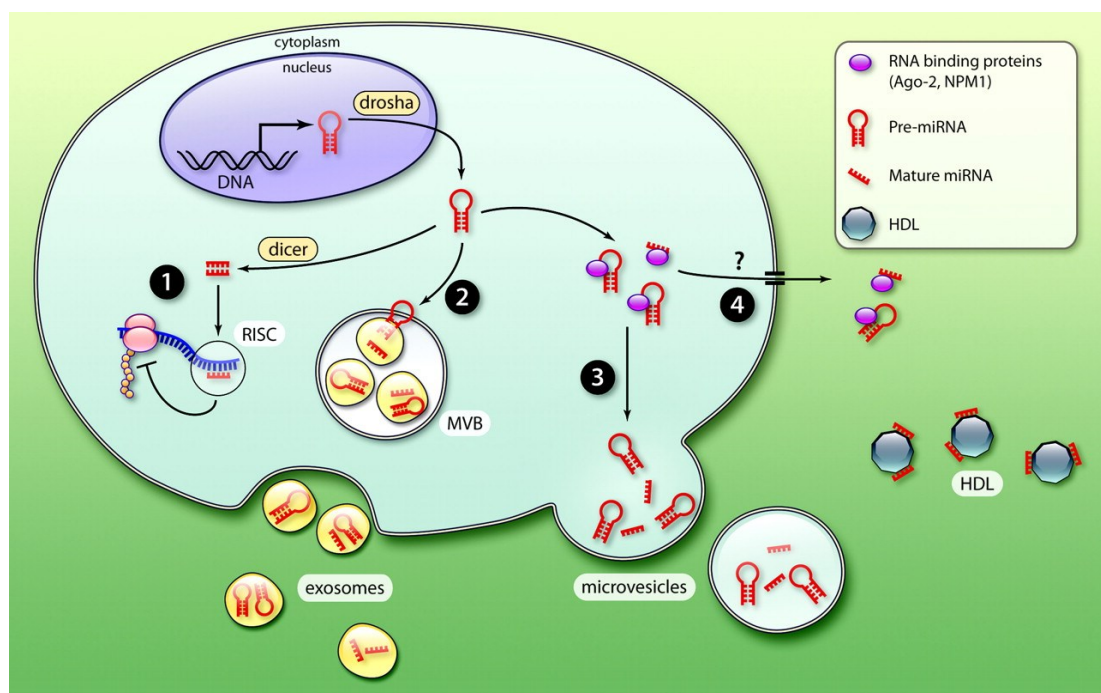


Figure 1-7. Mechanisms of transport of circulating microRNAs.

Once generated, miRNAs can remain in the cell where they target mRNA transcripts as part of the RNA-induced silencing complex (1). Alternatively, they can be released into the extracellular space in exosomes, which form when multi-vesicular bodies (MVB) fuse with the plasma membrane (2). Another mode of extracellular

release is inclusion in microvesicles, which form through budding from the plasma membrane (3). Finally, vesicle-free miRNAs can be found in the circulation, for example bound to protective HDL. This figure has been taken from (Creemers, Tijssen, & Pinto, 2012).

MiRNAs find their way into the extracellular space through multiple pathways. Early work into the origin of extracellular microRNAs identified miRNAs inside cellular vesicles, namely apoptotic bodies (800 nm – 5,000 nm), microvesicles (100 nm – 1,000 nm) and nanovesicles (30 nm – 100 nm) (Valadi et al., 2007; Zernecke et al., 2009). The latter are also known as exosomes. Microvesicles are derived from simple budding of the cell membrane, while exosomes originate from multi-vesicular endosomes (MVEs) which are part of the endocytic pathway (Raposo & Stoorvogel, 2013). MVEs eventually merge with the cell membrane and are released extracellularly; this appears to be an active, ATP-dependent mechanism (Raposo & Stoorvogel, 2013). Studies conducted later also identified miRNAs bound to several proteins in ribonucleoprotein complexes, outside of cellular vesicles (K. Wang, Zhang, Weber, Baxter, & Galas, 2010). Argonaute 2 (AGO2), high density lipoprotein (HDL), low density lipoprotein (LDL) and nucleophosmin 1 (NPM1) are examples of proteins which carry miRNAs in the extracellular space (Turchinovich et al., 2011; Vickers et al., 2011). Finally miRNAs can be released passively as a result of cell lysis (e.g. haemolysis) (Kirschner, Edelman, et al., 2013).

Although most circulating miRNAs originate in body tissues or circulating cells, in recent years some studies reported that plant-derived miRNAs (xenomiRs) could be detected in the circulation in both humans and mice after feeding (J. Yang, Farmer, Agyekum, & Hirschi, 2015; L. Zhang et al., 2012). These findings were seen as controversial and were faced with criticism (Dickinson et al., 2013). The molecular mechanisms through which exogenous miRNAs may find their way in the circulation have not yet been completely elucidated. However this is an exciting area of miRNA biology which, if confirmed, could lead to the development of novel ingestible therapeutic miRNAs.

Another exciting prospect is that foreign or endogenous miRNAs in circulation could potentially be taken up by other cell types and regulate targets in them, acting similarly to hormones. Indeed, reports suggest that extracellular vesicle-bound

miRNAs can be taken up by cells through membrane fusion or via the endocytosis pathway (Janas, Janas, Sapon, & Janas, 2015). Another potential mechanism through which vesicle-contained circulating miRNA could affect gene expression is by binding cell membrane receptors in neighbouring cells, as shown for Toll-like receptors (TLRs) (Fabbri et al., 2012). There is limited evidence for the transfer of protein-associated miRNAs to neighbouring cells. A previous report suggested that HDL-bound miRNAs can be taken up by recipient cells (Vickers et al., 2011) and interestingly a recent study found that protein-bound miR-150 could externally attach to exosomes and essentially 'hitchhike' its way inside T-cells (Bryniarski et al., 2015).

There appear to be multiple pathways allowing extracellular miRNAs to be taken up by cells however research is ongoing in this field. Studies with both vesicle- and protein-associated miRNAs have shown that miRNAs which are taken up by recipient cells can have a quantifiable effect on recipient cell mRNA transcripts. Specifically, Squadrito et al. demonstrated that miRNAs are delivered via exosomes from macrophages to endothelial cells, where they can target reporter constructs (Squadrito et al., 2014). In addition, Zerneck et al. showed that miR-126 secreted in apoptotic bodies from endothelial cells could indirectly modulate CXCL12 levels in vascular cells (Zerneck et al., 2009). Vickers et al. provided evidence that not only vesicle-enclosed miRNAs have functional effects on recipient cells. HDL-bound miRNAs were shown to be capable of down-regulating mRNA targets in hepatocytes (Vickers et al., 2011). Finally, trophoblasts have been shown to confer anti-viral resistance to other cell types through the dissemination of C19MC cluster miRNAs via exosomes (Delorme-Axford et al., 2013). However, despite multiple reports of miRNA-mediated mRNA modulation in recipient cells, it remains unclear if these effects are significant *in vivo*. Williams et al. estimated that the median concentration of all miRNAs in human plasma is in the range of 63 – 250 fM for normal subjects (Williams et al., 2013). However individual miRNAs would only be found at a fraction of these concentrations and it remains to be shown if such amounts of miRNAs could have a quantifiable effect on distant cells systemically *in vivo*.

1.7.2 The biomarker potential of circulating microRNAs

Because of their abundance in the extracellular space, miRNAs have been investigated as potential non-invasive biomarkers. Circulating miRNAs make good biomarker candidates because they regulate key biological processes (F. X. Donadeu, S. Schauer, & S. Sontakke, 2012a; Khoshgoo, Kholdebarin, Iwasiow, & Keijzer, 2013; Prieto & Markert, 2011; Vienberg, Geiger, Madsen, & Dalgaard, 2016), some demonstrate tissue-specific expression (Z. Guo et al., 2014), they are remarkably stable and they are relatively easy to sample and quantify (X. Chen et al., 2008; Mitchell et al., 2008).

So far, more than 100 circulating miRNAs have been proposed as biomarkers of multiple types of human cancer, including gastric, colorectal, hepatocellular, pancreatic, lung, breast and prostate cancer (for comprehensive reviews see (He et al., 2015; Schwarzenbach, Nishida, Calin, & Pantel, 2014)). Circulating miRNAs have also been linked to non-neoplastic diseases such as liver disease, cardiovascular disease, diabetes and neurodegenerative diseases, among others (Arrese, Eguchi, & Feldstein, 2015; Creemers et al., 2012; Grasso, Piscopo, Confaloni, & Denti, 2014; Guay & Regazzi, 2013; Zen & Zhang, 2012). In recent years, both academic and commercial institutes have focused on developing kits to diagnose human cancers using panels of multiple miRNAs (Larne et al., 2013).

An example of disease biomarkers is a panel of three circulating miRNAs (miR-21, miR-210 and miR-486-5p) which was shown to have significant diagnostic value (AUC = 0.86) for distinguishing lung cancer from benign lung tumours (Shen et al., 2011). In another example, seven miRNAs were identified as biomarkers of brain cancer (miR-15b-3p, miR-23a, miR-133a, miR-150-3p, miR-197, miR-497 and miR-548b-5p) in a group of 122 cancer patients (C. Yang et al., 2013). Among non-neoplastic diseases, miR-122 has been proposed as a biomarker of liver disease, including Hepatitis B and C (Haider et al., 2014). Several studies have proposed miR-1 and miR-133a as biomarkers of myocardial infarction; both of these are muscle-enriched miRNAs (Creemers et al., 2012; G. Long et al., 2012; G. K. Wang et al., 2010). Finally, several miRNAs have been identified as diabetes type 2 biomarkers, some of which have been shown to have significant predictive value

(miR-15a, miR-28-3p, miR-29b, miR-126 and miR-223) (Guay & Regazzi, 2013; Zampetaki et al., 2010). In addition to disease, circulating miRNAs have also been linked to physiological states such as intense exercise and pregnancy (Miura et al., 2010; Miura et al., 2014; Mooren, Viereck, Kruger, & Thum, 2014).

In contrast to the extensive literature on human circulating miRNA biomarkers, there is only a limited number of studies which investigated the utility of circulating miRNAs as biomarkers in livestock species. A recent study in pig found that circulating levels of let-7d-3p increased in pigs that were infected with the whipworm *Trichuris suis* (Hansen, Kringel, Thamsborg, Jex, & Nejsum, 2016). The authors suggested that this miRNA may be involved in the blockade of the pathogen's let-7a which downregulated IL-13, leading to an overall upregulation of IL-13 in the system. A similar study in cow profiled serum miRNAs in *Mycobacterium paratuberculosis* infected cattle but found no significant differences between healthy and infected animals (Farrell et al., 2015). A recent publication showed that feed deprivation in chicken plasma resulted in differential expression of many miRNAs, including let-7a-3p, let-7f-5p, miR-19a-3p, miR-30c-5p and miR-21-5p and that different chicken lines responded variably to treatments as shown by differential expression of metabolism-related miRNAs such as miR-122 and miR-204 (Ahanda et al., 2014). Therefore metabolic stress seems to affect the circulating miRNome. Finally, a study in cow found that grazing affected circulating miRNA levels, showing an increase in miR-451 levels; this miRNA is thought to be secreted from the muscles into the bovine circulation due to grazing-related movement (Muroya, Ogasawara, & Hojito, 2015). Taken together, these studies show that circulating miRNAs have the potential to be used as biomarkers in cow and other farm animals, similarly to humans, however there is a clear lack of research in those species.

1.7.2.1 Circulating microRNA biomarkers in reproduction

A number of studies have addressed the involvement of miRNAs in preeclampsia, which is a disease suspected to be brought on by abnormal placentation in women. A recent study showed that human preeclamptic placentas under-express miR-17-92 cluster members such as miR-18a, miR-92a, miR-19b and miR-17, and that their

abundance in plasma reflects this downregulation (for miR-18a, miR-92a and miR-19b) (P. Xu, Zhao, Liu, Wang, Wang, Li, Zhu, Yao, Qiao, et al., 2014). The same study showed that miR-18a causes a reduction in Smad2 protein levels, which in turn reduces trophoblast cell invasiveness, thought to be involved in the pathogenesis of preeclampsia (P. Xu, Zhao, Liu, Wang, Wang, Li, Zhu, Yao, Qiao, et al., 2014). However a more recent study looked at an earlier stage of pregnancy in humans during the first trimester, and found no differences in the serum between healthy and preeclamptic pregnancies (Luque et al., 2014). This study concluded that differences appear later during pregnancy and are not detectable in the first trimester, which takes away some of the diagnostic potential of miRNAs reported in other studies for preeclampsia.

In addition to the potential use of miRNAs as biomarkers of reproductive disease, research has also focused on their suitability as reproductive status and pregnancy biomarkers. A recent study aimed at identifying significant differences in plasma miRNA profiles during the human menstrual cycle, with no success (Rekker, Saare, Roost, Salumets, & Peters, 2013). However, placenta-specific, exosome-enclosed miRNAs have been shown to be secreted by trophoblast cells into the maternal circulation, where they can be detected by RT-qPCR (Donker et al., 2012; S. S. Luo et al., 2009). Both of these studies focused on the C19MC cluster, which is almost exclusively expressed in the trophoblast cells, as mentioned above. In addition, studies have reported significant increases in the circulating levels of placental miRNAs (C19MC members, miR-141, miR-149, miR-299-5p and miR-135b) with increasing gestational age, and the subsequent clearance of these miRNAs from the maternal circulation after delivery (Chim et al., 2008; Gilad et al., 2008; Hromadnikova, Kotlabova, Doucha, Dlouha, & Krofta, 2012; Kotlabova, Doucha, & Hromadnikova, 2011; Miura et al., 2010; Williams et al., 2013).

Studies in human have largely focused on the C19MC miRNA cluster which is primate-specific (Bentwich et al., 2005). Undoubtedly, this cluster is of interest due to the observed anti-viral effect of its members both on expressing cells and on cells that take them up in the form of exosome-enclosed miRNAs (Ouyang, Mouillet, Coyne, & Sadovsky). Differential expression of circulating miRNAs which are

conserved in cattle has also been reported, for example miR-141, miR-149 (Chim et al., 2008; Miura et al., 2010). However, in these human studies, these miRNAs were profiled later in pregnancy (1st trimester onwards), and there is a lack of information on the expression of circulating miRNAs in the first weeks of pregnancy. We think the establishment of an early pregnancy biomarker in cattle with the above characteristics will improve reproductive management in the dairy industry and expand our understanding of early pregnancy loss.

1.7.3 Challenges of quantifying circulating microRNAs

With the explosion of circulating miRNA studies in recent years, there has also been an abundance of reports highlighting technical pitfalls in terms of sample processing, miRNA profiling and data analysis. In this section I will briefly review existing challenges of circulating miRNA profiling including different analysis platforms and their biases as well as suggested methods to control contamination and haemolysis.

Most biomarker studies use either plasma or serum for miRNA profiling, and each has different characteristics; for example serum is missing coagulation factor-related miRNAs and miRNA profiles may be affected by coagulation times (K. Wang et al., 2012). In both plasma and serum studies, appropriate controls must be in place to control for haemolysis and blood cell contamination, both of which have been shown to influence the levels of specific miRNAs in samples (Cheng et al., 2013; Kirschner et al., 2011). A recent study found that the ratio between miR-451 and miR-23a is the most accurate method of haemolysis detection followed by spectrophotometric measurement at 414 nm to quantify oxyhaemoglobin levels (Shah, Soon, & Marsh, 2016). High-speed centrifugation has been shown to effectively eliminate cells, cell debris and platelets from plasma and serum samples (Cheng et al., 2013; Page et al., 2013). Finally, biofluid samples are characterised by low RNA content (Tonge & Gant, 2016) and high levels of enzymatic inhibitors (Al-Soud, Jonsson, & Radstrom, 2000) which can make accurate quantitation problematic.

The most commonly used platforms to profile miRNAs include PCR-based assays, microarrays and small-RNA sequencing platforms. Sequencing platforms suffer from sequence biases which can effectively alter miRNA abundance profiles (Fuchs, Sun,

Zhuang, & Robb, 2015; Hafner et al., 2011; Jayaprakash, Jabado, Brown, & Sachidanandam, 2011), however such biases are thought to affect similar samples equally, allowing for differential expression analysis. A limitation of RT-qPCR and microarray technologies are that prior knowledge of the miRNA sequences is required, limiting the potential for novel miRNA discovery and making it difficult to work with poorly annotated species. In addition using either technology would prevent researchers from analysing isomiRs which may carry biological importance in some cases (L. W. Lee et al., 2010; Llorens et al., 2013). A typical profiling strategy for circulating miRNAs includes the use of high-throughput next generation sequencing platforms to identify differential expression in pooled samples, followed by a validation study utilising PCR technology on individual samples (Zen & Zhang, 2012). Significant sources of bias and technical variability in such studies include the RNA extraction step and the RNA ligation step in sequencing experiments (Kim, Yeo, Kim, Ha, & Kim, 2012; van Dijk, Jaszczyszyn, & Thermes, 2014). The use of RNA carriers during RNA extraction was shown to reduce RNA extraction variation and improve yields and randomisation of the adaptor sequences has been shown to partially normalise sequencing bias (Alon et al., 2011; van Dijk et al., 2014).

Data normalisation has been the subject of multiple recent reports. Microarray and sequencing data typically use cross-sample normalisation which corrects for differences in yields across samples (Trapnell et al., 2013). Traditionally RT-qPCR data are normalised to unique spiked-in miRNAs which allow normalisation for technical variation in the RNA extraction and RT-qPCR (Kroh, Parkin, Mitchell, & Tewari, 2010). However, spike-ins have limitations in that they cannot normalise for differences in miRNA content across biological samples and recently it has been demonstrated that endogenous normalisers as well as global mean normalisation methods perform better in comparison (D'Haene, Mestdagh, Hellemans, & Vandesompele, 2012; Schlosser, McIntyre, White, & Stewart, 2015). Studies in human (Sourvinou, Markou, & Lianidou, 2013) and bovine (Bae et al., 2015) have recently demonstrated the feasibility of utilising endogenous normalisers (optionally in conjunction with exogenous spike-ins) for RT-qPCR data normalisation, although others still consider exogenous normalisers to be better in comparison to endogenous normalisers (Roberts, Coenen-Stass, & Wood, 2014).

A recent study investigated the agreement (concordance) in differentially expressed miRNAs across a range of sequencing, hybridisation and PCR-based miRNA quantification platforms using tissue samples. Perhaps surprisingly, cross-platform concordance averaged at 54.6 %, suggesting that miRNA profiles are largely affected by platform-specific biases (Jensen et al., 2011; Mestdagh et al., 2014). Meta-analyses of both neoplastic and non-neoplastic miRNA biomarker studies have concluded that only a small fraction of published studies are likely proposing valid biomarkers, with many examples of miRNAs being proposed as biomarkers of multiple diseases (Haider et al., 2014; Jarry, Schadendorf, Greenwood, Spatz, & van Kempen, 2014; Leidner, Li, & Thompson, 2013). These findings call for standardisation of study design, methodology and reporting in miRNA research, aiming to increase reproducibility and comparability among studies (Bustin et al., 2009; Witwer & Halushka, 2016).

1.8 Hypotheses

- The differential expression of miRNAs in reproductive tissues during the oestrous cycle will result in distinct miRNA expression patterns in the circulation which will be useful as biomarkers of oestrus in dairy cows (Chapter 2, Objective 1).
- Early pregnancy in cattle will be associated with specific circulating miRNA profiles which will prove useful as diagnostic biomarkers. These miRNAs are expected to be conceptus-derived or conceptus-induced, secreted into the maternal circulation, and will be useful in distinguishing pregnant from non-pregnant cows in the early stages of pregnancy (Chapters 3 and 4, Objective 2).
- Some of the circulating miRNAs detected in cow will originate from individual tissues (tissue-specific) or show enriched expression in a tissue (tissue-enriched), therefore providing novel potential candidates for biomarkers of tissue function (Chapter 5, Objective 3).

1.9 Objectives

- Identify and validate distinct circulating miRNA profiles during oestrus using next-generation sequencing, PCR arrays and RT-qPCR (Chapter 2).

- Identify and validate distinct circulating miRNA profiles during early pregnancy using next-generation sequencing, PCR arrays and RT-qPCR (Chapters 3 & 4).
- Investigate the tissue of origin of circulating miRNAs and attempt to identify potentially novel biomarkers of tissue function using sequencing and RT-qPCR (Chapter 5).

2 Biomarkers of Oestrus

2.1 Chapter Introduction

In this chapter I will present the work that I did to identify potential miRNA-based biomarkers of oestrus in the circulation of dairy heifers. This work was published in 2016 with the title ‘Circulating microRNA profiles during the bovine oestrous cycle’ (Ioannidis & Donadeu, 2016a) and the contents of this chapter have been taken from the published manuscript with some editorial and formatting changes to match the format of the current thesis and avoid repetition.

2.2 Abstract

Up to 50% of ovulations go undetected in modern dairy herds due to attenuated oestrus behaviour and a lack of high-accuracy methods for detection of fertile oestrus. This significantly reduces overall herd productivity and constitutes a high economic burden to the dairy industry. MicroRNAs (miRNAs) are ubiquitous regulators of gene expression during both health and disease and they have been shown to regulate different reproductive processes. Extracellular miRNAs are stable and can provide useful biomarkers of tissue function; changes in circulating miRNA profiles have been reported during menstrual cycles. This study sought to establish the potential of circulating miRNAs as biomarkers of oestrus in cattle.

I collected plasma samples from 8 Holstein-Friesian heifers on Days 0, 8 and 16 of the oestrous cycle and analysed small RNA populations on each Day using two independent high-throughput approaches, namely, Illumina sequencing (n = 24 samples) and Qiagen PCR arrays (n = 9 sample pools, 3-4 samples / pool). Subsequently, I used RT-qPCR (n = 24 samples) to validate the results of high-throughput analyses, as well as to establish the expression profiles of additional miRNAs previously reported to be differentially expressed during reproductive cycles. Overall, I identified four miRNAs (let-7f, miR-125b, miR-145 and miR-99a-5p), the plasma levels of which distinctly increased (up to 2.2-fold, $P < 0.05$) during oestrus (Day 0) relative to other stages of the cycle (Days 8 and 16). Moreover, I identified several hundred different isomiRs and established their relative abundance in bovine plasma.

In summary, my results reveal the dynamic nature of plasma miRNAs during the oestrous cycle and provide evidence of the feasibility of using circulating miRNAs as biomarkers of reproductive function in livestock in the future.

2.3 Background

Failure to adequately identify fertile oestrus in farmed animals is a major contributor to the long-standing problem of low fertility rates in modern production animal systems, particularly in cattle. To illustrate the severity of the problem, current conception rates at first postpartum service in dairy cows are approximately 40 %; according to the UK's Dairy Science Forum (2008), this is estimated to cost the UK dairy industry in excess of £300 million per year through extended calving intervals, in addition to being a major welfare issue through disease and premature culling.

The physiological period of anoestrus following calving is characterised by progressive restoration of the neuroendocrine-reproductive axis to allow adequate maturation of ovarian follicles followed by ovulation before normal oestrous cycles can be re-established (Crowe, 2008). In non-stressed dairy cows ovulatory activity normally begins within 2 to 3 weeks after calving. However, in modern dairy herds, a combination of factors resulting from both genetic selection for production traits and intensive management, including negative energy balance, stress and disease (uterine infection, mastitis, lameness) often disrupt normal neuroendocrine restoration which prevents normal follicle maturation and leads to a delay in the onset of regular, normal-length oestrous cycles (Chagas et al., 2007; Dobson et al., 2007).

Furthermore, behavioural oestrus, physiologically triggered by high E2 levels from mature pre-ovulatory follicles, is significantly reduced under these conditions, both in intensity and duration; this leads to up to 50 % of heats being missed by farm personnel (Heersche & Nebel). Altogether, this leads to a dramatic decrease in breeding efficiency, increased risk of disease through non-productive inseminations, and extended calving intervals.

Periodic visual inspection of cows for behavioural signs of oestrus has been traditionally used to select cows for breeding (Holman et al., 2011). More sophisticated methods include measurement of milk P4, heat mount detectors and

activity monitors; in practice these are frequently combined with visual observation (Holman et al., 2011). Yet, multiple reports agree that approximately 30% of oestrus events may still not be detected even when combinations of these methods are used (Fricke et al., 2014; Holman et al., 2011). Thus, there is growing interest in the development of novel oestrus detection methods to minimise economic losses and boost annual milk production.

MiRNAs are small RNA molecules that play key roles as gene expression regulators in animal tissues (Ul Hussain, 2012). Intracellular miRNAs enter the extracellular space (Mitchell et al., 2008) in the form of stable complexes within protective exosomes (Ge et al., 2014) and / or associated with proteins such as HDL and Ago2 (Arroyo et al., 2011; Vickers et al., 2011). Some miRNAs are tissue-specific and are present in the circulation at quantifiable levels (e.g. placental miRNAs) (Williams et al., 2013). These findings have led to a plethora of studies into the biomarker potential of circulating miRNAs; it is now widely accepted that miRNAs can offer useful circulating biomarkers of multiple diseases such as cancer, heart disease, autoimmune diseases (Haider et al., 2014; He et al., 2015) as well as preeclampsia and other reproductive diseases (Miura et al., 2015; L. Wu et al., 2012). In contrast to the abundant literature focusing on humans, the potential of circulating miRNAs as biomarkers of animal disease and / or productivity traits has not been investigated in depth.

MiRNAs are involved in the regulation of follicular and luteal development (reviewed here (F. X. Donadeu, S. N. Schauer, & S. D. Sontakke, 2012b)) as well as endometrial function (Wessels et al., 2013). Consistent with this, studies using cows and other species have identified numerous miRNAs with expression that changes significantly in association with follicle growth and selection, the follicle-to-luteal transition, and embryo implantation (McBride et al., 2012; Salilew-Wondim et al., 2014; Sontakke, Mohammed, McNeilly, & Donadeu, 2014). Because the reproductive tract is highly vascularised, particularly the ovary, it is reasonable to hypothesise that changes in miRNA levels occurring in those tissues may lead to changes in the levels of circulating miRNAs, which may provide useful oestrous cycle biomarkers. In this study I used next-generation sequencing and PCR-based

platforms to compare miRNA profiles in the circulation of heifers across different stages of a normal oestrous cycle. I identified four circulating miRNAs, the levels of which were significantly higher during oestrus compared to other stages of the oestrous cycle. These findings pave the way towards the development of novel oestrus detection methods.

2.4 Materials and Methods

2.4.1 Experimental animals

Eight Holstein-Friesian heifers (15 - 17 months old) were oestrus-synchronised using Eazi-Breed™ controlled internal drug release (CIDR) Cattle Insert (1.38 g P4 over eight days; Zoetis, USA), Receptal® (0.02 mg buserelin on the day of CIDR insertion; MSD Animal Health, UK) and Estrumate® (0.5 mg cloprostenol seven days after CIDR insertion; MSD Animal Health). Blood was collected on Days 0, 8 and 16 from all animals (Day 0 being the first day of observed oestrus). The stage of the oestrous cycle was confirmed by plasma P4 profiles determined using a Coat-a-Count radioimmunoassay (Siemens Healthcare, Germany). All heifers used in this study were kept under the same housing and feeding conditions and all animal procedures were carried out under the UK Home Office Animals (Scientific Procedures) Act 1986, license 60/4604, and with approval by the Ethical Review Committee, University of Edinburgh and all efforts were made to minimize animal suffering.

2.4.2 Sample collection and processing

Blood was collected in 10 mL K2 EDTA Vacutainer tubes (Becton Dickinson, USA) by jugular venepuncture, using 18G needles (Becton Dickinson) and stored at 4 °C. Within two hours of collection samples were centrifuged at 1,900 g for 10 min at 4 °C to remove blood cells, and then again at 16,000 g for 10 min at 4 °C to remove cellular debris and platelets. The second centrifugation step has been shown to significantly reduce platelet numbers in plasma samples, minimising platelet contamination (Cheng et al., 2013). In addition, haemolysis was controlled for by using absorbance at 414 nm and the 'miR ratio' (difference in quantification cycle (ΔC_q) between miR-451 and miR-23a) as described previously (Ioannidis &

Donadeu, 2016b; Shah et al., 2016). All plasma samples were immediately frozen at -80 °C.

2.4.3 RNA extraction

RNA was extracted from 1.05 mL of plasma using TRIzol LS (Life Technologies, USA), following the manufacturer's protocol. During the RNA extraction protocol, glycogen (180 µg; Sigma-Aldrich, USA) was added to each sample to facilitate the precipitation of RNA, and an exogenous miRNA control, syn-cel-miR-39-3p (0.25 fmol; Qiagen, NL), was spiked into each sample. RNA was re-suspended in 30 µL of RNase-free water and used immediately or frozen at -80 °C.

2.4.4 Small-RNA sequencing analysis

Small-RNA libraries were prepared using the Illumina TruSeq small-RNA sample preparation kit (Illumina, USA) following the manufacturer's protocol. Libraries were submitted to 36-base single-end sequencing using the Illumina HiSeq 2000 platform. The raw sequencing data are available on the GEO database under accession GSE81050. Raw reads were processed using sRNAtoolbox 1.0; initially reads with no adaptor and/or with undetermined bases were removed (Rueda et al., 2015). The bovine genome (assembly UMD 3.1, (Elsik et al., 2016)) was used as reference; trimmed and quality-controlled reads were mapped against mature miRNAs from bovine and human for homologue identification in miRBase 20 (accessed 11/06/2014, (Griffiths-Jones, 2006)) allowing only a one-nucleotide mismatch; novel bovine miRNAs as well as isomiRs were identified at this stage.

Prediction of novel miRNAs was carried out using sRNAbench software, as described previously (Hackenberg, Shi, Gustafson, & Langridge, 2013; Rueda et al., 2015). Briefly, sequencing reads that did not map to known miRNAs were mapped to the bovine genome. Where pairs of read clusters mapped 60 nt apart, the two clusters (putative novel miRNAs) and the connecting sequence were tested for their ability to form a stem-loop (pre-miRNA). I also identified isomiRs of known canonical miRNAs using sRNAbench. Briefly, the procedure maps small RNA reads to pre-miRNA loop sequences, identifying differences in sequence using a step-wise approach (end-variations first, miRNA-body variations next, etc.). To increase

confidence, only sequences present at a minimum of 25 RPMM in more than 75 % of samples analysed were taken as true isomiRs.

After mapping, human and bovine miRNA read counts were merged and normalised to generate a uniform dataset of reads per million mapped (RPM). MiRNAs with less than 25 RPMs in more than 75 % of the samples within each of the experimental groups were excluded, keeping 181 miRNAs for further analysis. Normalised expression levels (RPMs) from sRNAbench 1.0 were log₂ transformed before applying repeated-measures analysis of variance (rmANOVA) using the “ez” package 4.2 (Lawrence, 2013), followed by FDR adjustment using R language 3.02 and RStudio 0.98 (R Core Team, 2013; RStudio Team, 2015). The transformed data were normally distributed as determined by the D’Agostino-Pearson omnibus and Shapiro-Wilk normality tests. Statistical significance was set to FDR < 0.1. Sequencing data are provided in File 2-1.

2.4.5 PCR array analysis

To design my Custom PCR array I took all miRNAs included in the Qiagen Human miRNome™ miScript v. 16.0 PCR array and aligned them using the basic local alignment search tool (BLAST, (Camacho et al., 2009)) to cow miRNA sequences listed in miRBase 19 (accessed 20/02/2013, (Kozomara & Griffiths-Jones, 2011)) in a Linux environment. A total of 308 miRNAs conserved in cow (i.e. with ≤ 2 nt mismatches between human and cow sequences) were included in the 384-well Custom miScript miRNA PCR array (384-well, Qiagen).

Three sample pools from each of Days 0, 8 and 16 (2-3 samples / pool) were analysed. CDNA (10 µL) was synthesised from 2 µL of RNA sample using the miScript II RT kit (Qiagen) in a Whatman-Biometra Thermocycler (Biometra, USA). The arrays were setup according to the manufacturer’s instructions and were analysed on the LightCycler 480 System (Roche, Switzerland). Data analysis was performed using Microsoft Excel (Microsoft Corporation, USA) and R programming using R language 3.02 and RStudio 0.98 (R Core Team, 2013; RStudio Team, 2015). Raw Cq data were initially filtered to remove wells with non-specific amplification as identified by melting-curve analysis. Cq values were normalised using the global

mean expression, which was calculated from miRNAs which were detected in all of the sample pools. The statistical analysis of the transformed normalised data was performed as described for the sequencing data above. The PCR array dataset is provided in File 2-2.

2.4.6 RT-qPCR analysis

CDNA was generated as described above and diluted for use in 10 µL qPCR reactions using Qiagen SYBR Green kits in an Agilent Mx3005P qPCR system (Agilent Technologies, USA). Raw fluorescence data were processed using Agilent MxPro software. A fluorescence threshold of 0.1 was used to determine Cq values for all experiments. The amplification efficiency ranged between 88 % and 109 %, with $R^2 > 0.85$. Data were processed using Microsoft Excel (Microsoft Corporation). Statistical analyses were performed in GraphPad Prism 6 (GraphPad Software, USA) using repeated-measures ANOVA followed by Dunnett's tests or 2-sample t-tests if comparisons involved only two means. MiRNA expression levels were normalised to the levels of the spiked-in cel-miR-39-3p. Statistical significance was set to $P < 0.05$. For selected circulating miRNAs, normalised expression levels were correlated with plasma P4 levels using Spearman's correlation (ρ) in GraphPad Prism 6 (GraphPad Software).

2.4.7 MicroRNA target analysis

Target analysis was carried out using DIANA miRPath 3.0 (Vlachos, Zagganas, et al., 2015). Briefly, experimentally validated miRNA targets were identified by calling DIANA TarBase 7.0 through the miRPath 3.0 interface (Vlachos, Paraskevopoulou, et al., 2015). Targets which were common to two or more of the miRNAs considered were further selected for pathway prediction using miRPath 3.0 selecting the gene intersection analysis method in the tool's interface. Enriched pathways from the KEGG database were exported from the tool along with FDR-corrected p-values. A significance threshold of $P = 0.05$ was used. The predicted pathways (KEGG) are provided in File 2-3.

2.5 Results and Discussion

2.5.1 Sequencing of plasma during the oestrous cycle

I sequenced 24 small-RNA libraries prepared from individual plasma samples collected from 8 non-pregnant heifers on each of Days 0, 8 and 16 of the oestrous cycle in order to identify differentially expressed miRNAs at oestrus (Day 0). On average, I obtained 9.1 million raw sequences from each sample (Table 2-1). The most common read lengths after removing the sequencing adaptors ranged between 20-23 nt, which corresponds to the length of mature miRNAs (Figure 2-1A). On average, 4.3 million high quality reads (47.2 % of raw reads) were mapped to the bovine genome per sample. More than 70 % of these mapped reads corresponded to miRNAs, the majority of which were bovine (as registered in miRBase 20, accessed 11/06/2014 (Griffiths-Jones, Saini, van Dongen, & Enright, 2008)); a much smaller fraction of the reads consisted of human miRNA homologues (0.1 %) and predicted novel miRNAs (0.04 %; Table 2-1). A small percentage of the mapped reads corresponded to other small non-coding structural and regulatory RNAs, such as small nuclear and nucleolar RNA (snRNA and snoRNA, respectively; Figure 2-1B).

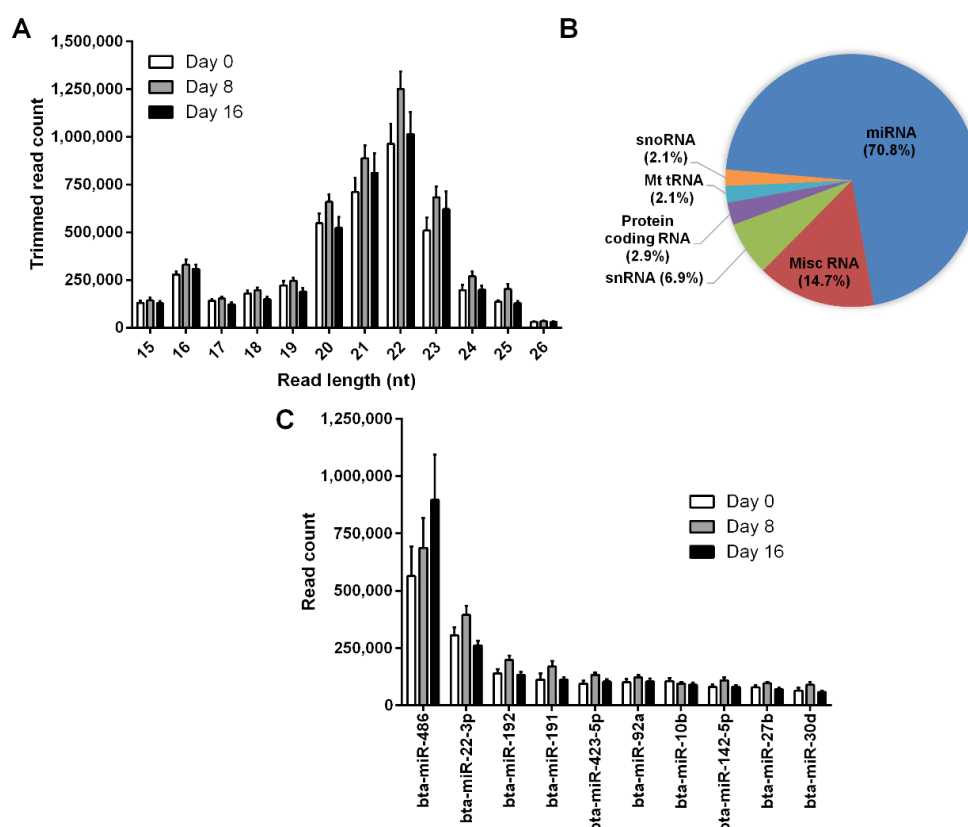


Figure 2-1. Small-RNA sequencing of bovine plasma during the oestrous cycle. Length distribution of trimmed read counts (mean number of reads $\pm$ SEM) for each of Days 0, 8 and 16 of the oestrous cycle ($n = 8$ heifers). (B) Relative abundance (%) of total mapped reads) of different RNA species in bovine plasma. The ‘Misc RNA’ category predominantly includes Y RNA, Vault RNA and RNase P RNA. (C) Ten most abundant miRNAs (mean read counts $\pm$ SEM) in bovine plasma on each of Days 0, 8 and 16 of the oestrous cycle.

Table 2-1. Sequencing endpoints from analysis during the oestrous cycle. Summary showing various endpoints from small-RNA sequencing of plasma samples from Day 0, 8 and 16 of the oestrous cycle ($n = 8$ heifers).

	Day 0	Day 8	Day 16	Mean
Raw reads	9,241,040	9,380,798	8,737,868	9,119,902
Reads with adapter	8,461,507	8,361,521	7,918,324	8,247,117
Reads that passed QC	4,073,814	5,075,814	4,251,624	4,467,084
Reads that mapped to the genome	3,918,066	4,894,047	4,075,763	4,295,959
Total miRNA reads	2,727,909	3,468,900	2,922,572	3,039,794
Bovine miRNA reads	2,724,952	3,465,422	2,920,090	3,036,821
Human miRNA reads	2,957	3,479	2,482	2,973
Novel miRNA reads	1,493	2,034	1,597	1,700

On average, a total of 313 unique miRNAs (296 bovine and 17 human) were detected with more than 10 reads. Among the 10 most abundant miRNAs in plasma (Figure 2-1C), miR-486 (the most abundant) and miR-92a are reportedly expressed primarily in red blood cells (Pritchard et al., 2012), whereas miR-191 is highly expressed in platelets (Ple et al., 2012). Four of the 10 most abundant miRNAs (miR-486, miR-92a, miR-192 and miR-423-5p) were also identified as highly abundant in bovine plasma in another study using Illumina technology (Spornraft, Kirchner, Pfaffl, & Riedmaier, 2015). The partial lack of agreement between the two studies could be explained by the use of different protocols to prepare the sequencing libraries as this has been shown to significantly influence the results of miRNA sequencing, mainly through effects on adaptor ligation (Baran-Gale et al., 2015).

The differential expression analysis included 181 bovine and human miRNAs, all of which were present with more than 25 RPMM in at least 75 % of samples in each experimental group (see Materials and Methods; File 2-1). Principal component analysis (PCA) did not reveal a clear separation of samples according to the day of the oestrous cycle (Figure 2-2A). Upon statistical analysis I detected an effect of day of the oestrous cycle on the expression levels of 20 miRNAs ($P < 0.05$, Table 2-2; Figure 2-2B), however the differences involved were generally small (under 1.6-fold) and were not significant after multiple testing adjustment ($FDR > 0.1$), failing to identify a high-confidence candidate miRNA biomarker of oestrus at this stage of the study.

Table 2-2. Differentially expressed miRNAs during oestrus using sequencing.
Top miRNAs which were up- or down-regulated between different days of the oestrous cycle using small-RNA sequencing.

miRNA	D8/D0	D16/D0	D16/D8	P-value	FDR
bta-miR-199a-5p	0.72	0.62	0.99	0.024	0.405
bta-miR-381	0.71	0.66	1.06	0.008	0.238
bta-miR-125b	0.98	0.68	0.74	0.025	0.405
bta-miR-154c	0.71	0.71	1.07	0.003	0.172
bta-miR-100	0.88	0.72	0.91	0.046	0.405
bta-miR-214	0.78	0.74	1.01	0.002	0.172
bta-miR-199c	0.76	0.74	1.06	0.004	0.172
bta-miR-532	0.81	0.74	0.93	0.005	0.172
bta-miR-2285t	0.86	0.75	0.94	0.040	0.405
bta-miR-99b	0.90	0.75	0.90	0.045	0.405
bta-miR-380-3p	0.76	0.80	1.11	0.025	0.405
bta-miR-222	1.24	0.90	0.76	0.040	0.405
bta-miR-369-3p	0.77	0.79	1.11	0.028	0.405
bta-miR-10b	0.77	0.90	1.19	0.031	0.405
bta-miR-3431	0.79	0.77	1.06	0.035	0.405
bta-miR-224	0.84	0.78	0.97	0.003	0.172
bta-miR-23a	0.87	0.78	0.92	0.010	0.254
bta-miR-23b-3p	0.86	0.79	0.93	0.036	0.405
bta-miR-205	0.81	0.79	1.04	0.045	0.405
bta-miR-155	1.32	0.96	0.76	0.034	0.405

MiRNAs are sorted using the maximum fold-change across all three comparisons. miRNAs with FC > 1.5 are in bold.

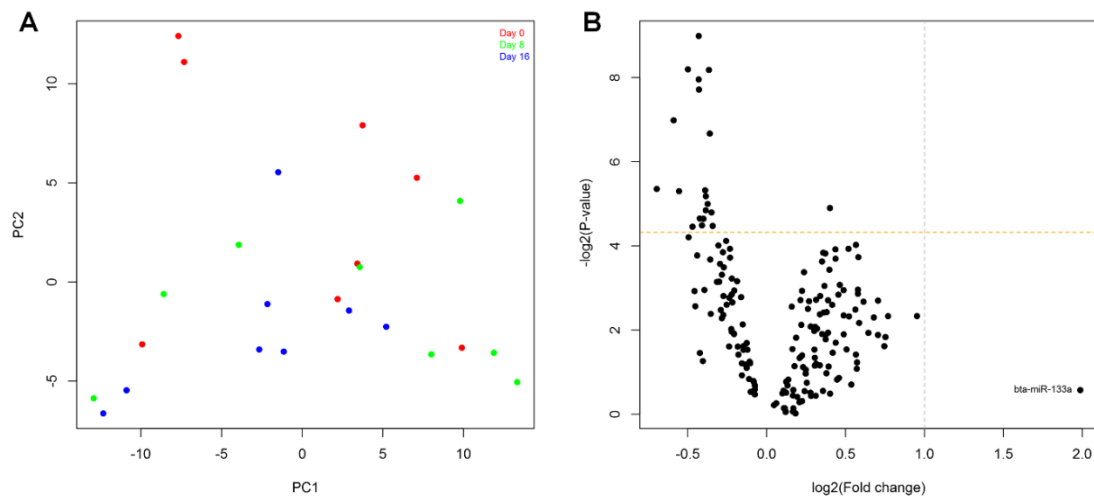


Figure 2-2. Sequencing differential expression analysis during the oestrous cycle.

Analysis of data from Days 0, 8 and 16 of the oestrous cycle ($n = 8$ heifers). (A) PCA plot. (B) Volcano plot showing the largest fold-change between any 2 of the 3 days analysed for each miRNA. The grey dotted line indicates the 2-fold change threshold and the yellow horizontal line indicates $P = 0.05$. The plotted data have been $\log_2$ transformed.

I also used my sequencing data to identify isomiRs in plasma samples using sRNAbench. I identified 655 different isomiRs for a total of 134 ‘canonical’ (miRBase) miRNAs. The majority of the isomiRs (507) involved modifications on the 3’ end of the ‘canonical’ miRNA, while others had modifications on the 5’ end (22 isomiRs) or the middle (13 isomiRs) of the ‘canonical’ miRNA. Ninety-three of the identified isomiRs contained multiple modifications (e.g. 5’ end addition and 3’ end addition). Finally, 20 isomiRs were multiple length variants involving a shift along the pre-miRNA sequence. A list of all the identified isomiRs is provided in File 2-4. Interestingly, the canonical sequence was the most abundant in plasma for only 51 miRNAs (38.1 %). For the remaining miRNAs, the most abundant sequence(s) corresponded to one or more of the identified isomiRs. For some miRNAs, the canonical sequence was expressed at much lower levels than the most abundant isomiR, this difference being ≥ 25 -fold for a total of 21 miRNAs and up to 1044-fold for miR-192 (File 2-4). This indicates that many bovine miRNA sequences listed on miRBase do not actually correspond to the most abundant isoform in plasma, and possibly also other tissues, something which will need to be considered

in future studies. Because of the low abundance of most isomiRs, I did not use these data for differential expression analyses.

2.5.2 PCR array analysis during the oestrous cycle

To complement my sequencing analyses, I used a commercial Custom PCR array platform to profile the expression of 308 bovine miRNA in the same plasma samples. It is usually not feasible to screen a large number of individual samples using PCR arrays, therefore I analysed 3 plasma sample pools (2-3 samples / pool) from each of Days 0, 8 and 16 of the oestrous cycle.

A total of 211 miRNAs were detected at $C_q < 35$ across all samples (Figure 2-3A). The miRNAs which were most abundant in plasma (Figure 2-3B) in my experiment are reportedly expressed at high levels in blood cells, including erythrocytes (miR-451, miR-16b), leukocytes (miR-150, miR-27a, miR-23a) and thrombocytes (miR-223, miR-20a, miR-24) and are putatively released into the plasma through apoptosis, lysis or active shedding (Haider et al., 2014; K. Wang et al., 2012; Willeit et al., 2013). Out of the 20 most abundant miRNAs in each of the sequencing and PCR array datasets, only six (miR-451, miR-486, miR-22-3p, miR-92a, miR-191 and miR-140) were common to both datasets (20 % overlap). A very abundant miRNA in the sequencing dataset (miR-21-5p) could not be compared as it was not included in the PCR array. However, considering the 150 most abundant miRNAs, 104 (70 %) were detected by both platforms; out of the 46 miRNAs which were present only in the sequencing dataset, 35 were not represented on the PCR array, explaining a large part of the apparent lack of overlap in detected miRNAs between the two platforms. Further explanation for differences in the most abundant miRNAs is provided in the form of platform-specific biases involving, for example, sequencing adaptor ligation bias and differences in primer efficiency, which determine the relative abundance for a given miRNA (Fuchs et al., 2015; van Dijk et al., 2014).

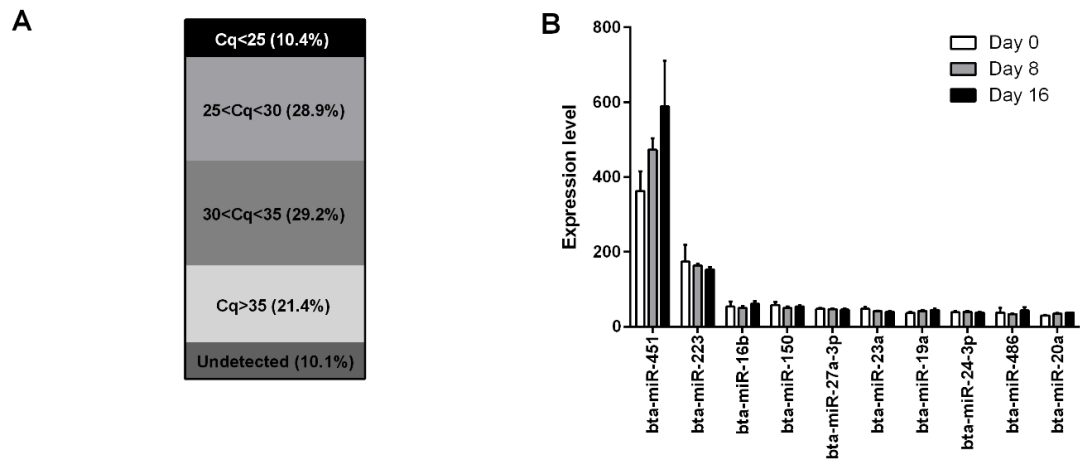


Figure 2-3. PCR array analyses of miRNAs during the oestrous cycle.

(A) Distribution of C_q values. The percentage shown is the number of miRNAs in each category divided by the total number of miRNAs assayed. (B) The expression level (mean $\pm$ SEM of $2^{(40-C_q)}$) of the top 10 miRNAs during each of Days 0, 8 and 16 of the oestrous cycle ($n = 8$ heifers).

PCA did not reveal clear separation of the sample pools according to Day of the oestrous cycle (Figure 2-4A), although sample pools for Day 0 were much more spread than those for Days 8 and 16. After examining the major components in the PCA I could not identify an obvious miRNA or biologically meaningful miRNA group which accounted for the variation within Day 0, although the low number of biological replicates used in the PCR array analyses may have been a contributing factor. A total of 169 miRNAs which had $C_q < 35$ in more than 67 % of samples within each experimental group were used for statistical analyses (File 2-2). Differences in expression across the oestrous cycle (fold-change ≤ 2.1) were identified for 10 miRNAs, although they did not reach significance after multiple testing adjustment ($FDR > 0.1$, Figure 2-4B, Table 2-3). Five of these miRNAs changed by more than 1.5-fold between days (Figure 2-4B, Table 2-3) and only miR-224 was common to both PCR array and sequencing datasets (Tables 2-2 and 2-3).

Table 2-3. Differentially expressed miRNAs during oestrus using PCR arrays.
Top miRNAs which were up- or down-regulated between different Days of the oestrous cycle using PCR arrays.

miRNA	D8/D0	D16/D0	D16/D8	P-value	FDR
bta-miR-224	0.75	0.63	0.86	0.031	0.666
bta-miR-185	1.30	1.51	1.18	0.027	0.666
bta-miR-140	1.57	1.36	0.87	0.028	0.666
bta-miR-455-5p	1.67	1.61	0.99	0.049	0.787
bta-miR-382	0.48	1.00	2.13	0.013	0.556
bta-let-7f	0.71	0.87	1.26	0.012	0.556
bta-miR-20a	1.19	1.27	1.07	0.039	0.732
bta-miR-423-3p	1.38	1.23	0.89	0.032	0.666
bta-miR-106b	1.33	1.39	1.05	0.002	0.311
bta-miR-378	1.46	1.22	0.83	0.013	0.556

MiRNAs are sorted using the maximum fold-change across all three comparisons and miRNAs with FC > 1.5 are shown in bold.

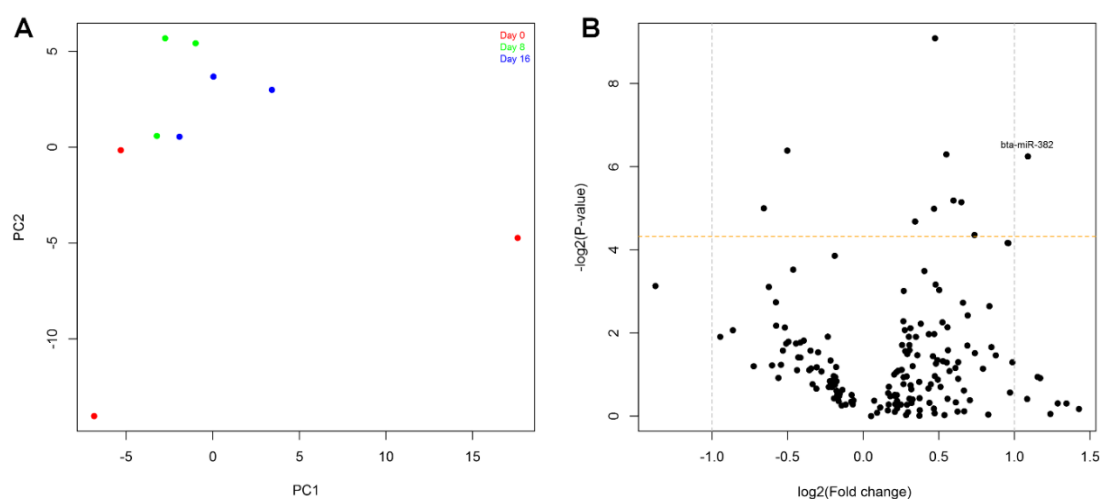


Figure 2-4. Differential expression analysis of PCR array data during the oestrous cycle.

Analysis of data from Days 0, 8 and 16 of the bovine oestrous cycle (n = 8 heifers). (A) PCA plot. (B) Volcano plot showing the largest fold-change between any 2 of the 3 days analysed for each miRNA. The grey dotted lines indicate the 2-fold change threshold and the yellow lines indicate P = 0.05. The plotted data have been log₂ transformed.

2.5.3 RT-qPCR validation of microRNA profiles

Despite the lack of significant differences in miRNA levels after FDR adjustment in either of the sequencing and PCR array datasets, I decided to further analyse some of the differences identified with $P < 0.05$. For this, I performed RT-qPCR on individual plasma samples, which is considered the ‘gold standard’ for validation of high-throughput analyses results due to its relative high accuracy, the ability to factor-in the amplification efficiency for each miRNA and the absence of severe biases which can typically alter miRNA abundance data obtained from sequencing analyses (Fuchs et al., 2015; Mestdagh et al., 2014).

Using RT-qPCR I also profiled additional miRNAs with differential expression in body tissues reproductive cycles according to previous reports (Donadeu et al., 2012a; Fiedler, Carletti, Hong, & Christenson, 2008; Kresowick et al., 2014; McBride et al., 2012; Palma-Vera, Sharbati, & Einspanier, 2015; Salilew-Wondim et al., 2014; Vilella et al., 2015). This was adopted as a complementary approach to identify biologically relevant miRNAs which may have been missed by my high-throughput analyses.

I selected 8 miRNA candidates identified by sequencing (miR-125b, miR-155, miR-199a-5p, miR-381, miR-99b; Table 2-2) or PCR array (let-7f, miR-378, miR-455-5p; Table 2-3) for RT-qPCR analysis. Unfortunately, the plasma levels of miR-199a-5p, miR-381 and miR-99b were too low for accurate profiling by RT-qPCR ($C_q > 35$). Out of the remaining 5 miRNAs, miR-155, miR-378 and miR-455-5p did not change ($P > 0.1$) during the oestrous cycle when analysed by RT-qPCR. In contrast, let-7f levels decreased 2.2-fold ($P < 0.05$) on Day 8 compared to oestrus (Day 0) followed by a non-significant increase in mean levels on Day 16, in agreement with high-throughput data (Figure 2-5A). In addition, for miR-125b, although an overall effect of Day of oestrous cycle was not found, expression levels tended to be higher ($P = 0.08$) during oestrus (Day 0 vs Days 8 and 16 combined). Consistent with these findings, comparison of miRNA and P4 levels during Days 0, 8 and 16 (Figure 2-6) revealed a negative correlation between let-7f and P4 during the oestrous cycle ($\rho = -0.523$, $P = 0.009$).

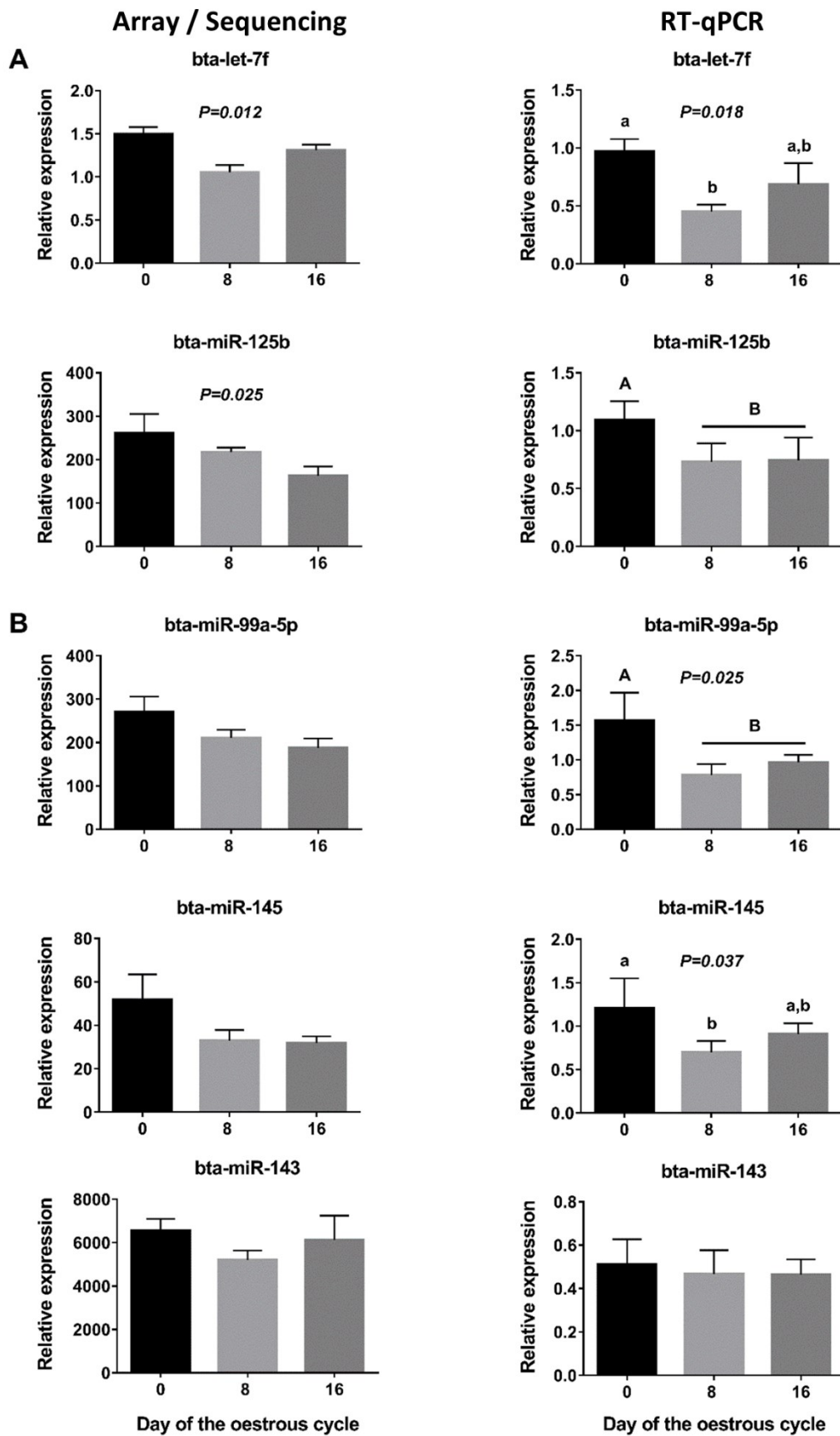


Figure 2-5. RT-qPCR analyses of plasma miRNAs during the oestrous cycle.

(A) RT-qPCR validation (right column) of candidate plasma miRNAs that had been identified as differentially expressed by high-throughput analyses (small-RNA sequencing or PCR array; shown in left column). (B) Expression profiles obtained by RT-qPCR (right column) of miRNAs previously reported to be differentially expressed in the ovary or endometrium during the oestrous cycle. Profiles for the same miRNAs obtained by small-RNA sequencing are shown in the left column. In both (A) and (B) P-values from repeated-measures ANOVA are shown. Mean differences are indicated by different letters; a, b for $P < 0.05$ (Dunnett's tests) and A, B for $P < 0.1$ (t-test); $n = 8$ heifers.

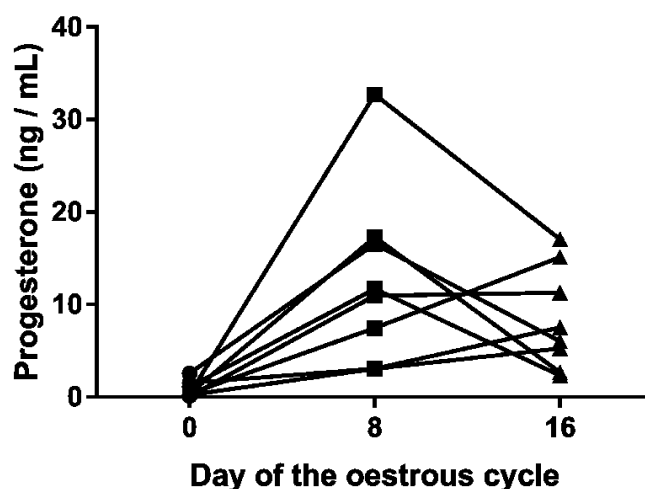


Figure 2-6. Plasma progesterone concentration during the oestrous cycle.

Progesterone concentration in plasma samples collected on Days 0, 8 and 16 of the oestrous cycle from synchronised cycling cows (Day 0 = day of oestrus). Progesterone concentration in plasma was determined using the Coat-a-Count radioimmunoassay (Siemens).

Thus, overall, 2 out of 5 miRNAs identified by either sequencing or PCR array were validated successfully by RT-qPCR. A recent study involving all major vendors of miRNA profiling technologies (Mestdagh et al., 2014) assessed different quantitative miRNA gene expression platforms (hybridisation, sequencing and RT-qPCR) and found concordance in differentially expressed miRNAs to be surprisingly low between quantification platforms (54.6 % on average). Based on this, the RT-qPCR validation rates observed in my study were not lower than expected, especially when considering 1) the difficulty of profiling miRNAs in plasma and 2) the magnitude of the differences I attempted to validate.

Following this, I profiled additional miRNAs which we and others previously found to be differentially expressed in ovarian (Donadeu & Schauer, 2013; Fiedler et al., 2008; McBride et al., 2012; Sontakke et al., 2014) or endometrial (Kresowick et al., 2014; Palma-Vera et al., 2015; Vilella et al., 2015) tissues across different stages of the reproductive cycle, but did not come up as significantly different between days of the oestrous cycle in my high-throughput analyses. I selected 8 miRNAs that were reported to be upregulated (miR-145, miR-143, miR-99a-5p) or downregulated (miR-155 and miR-142-3p, miR-132, miR-378) in follicular relative to luteal tissues of ruminants (McBride et al., 2012; Sontakke et al., 2014) or differentially expressed in endometrium during the human menstrual cycle (miR-31) (Kresowick et al., 2014; Palma-Vera et al., 2015; Vilella et al., 2015). These analyses revealed significant changes in the plasma levels of both miR-99a-5p and miR-145 during Days 8 and 16 (Figure 2-5B) of the oestrous cycle compared to Day 0, in agreement with differences reported between ovarian follicular and luteal tissues during oestrous cycles (McBride et al., 2012). Accordingly, the circulating levels of these miRNAs were negatively correlated with plasma P4 levels ($\rho = -0.407$; $P = 0.049$ and $\rho = -0.404$; $P = 0.05$ for miR-99a-5p and miR-145, respectively; P4 levels are shown in Figure 2-6), similarly to let-7f above. Higher mean levels of those two miRNAs during oestrus (Day 0) were also apparent from high throughput data, but they were not significant (Figure 2-5B, left column). These results underline the advantages of combining different analytical approaches for identification of changes in circulating miRNA levels, particularly when such changes are expected to be modest.

Two previous studies analysed circulating miRNA profiles during human menstrual cycles; one reported no changes (Rekker et al., 2013) and the other reported an increase in miR-31 levels during the secretory phase of the cycle (Kresowick et al., 2014). Differences in reproductive physiology between species and in the analytical platforms that were used could very well account for the variable results between studies, especially if changes in miRNA levels in circulation across reproductive cycles are small. Another study recently reported differences in plasma miRNA levels (including miR-26b-4p, miR-125b and miR-99a-3p) between naturally cycling heifers and heifers treated with FSH to induce ovarian hyper-stimulation, but did not

report differences in plasma miRNA expression during natural oestrous cycles (Noferesti et al., 2015).

The origin and function of the miRNAs identified as increasing in plasma during oestrus is not known. A reasonable assumption is that they may result, at least partially, from changes in miRNA expression in the ovary, particularly given that mature follicles and especially CLs are highly vascularised structures that may contribute significantly to miRNA populations in the systemic circulation. In support of this, all 4 miRNAs that were expressed at higher levels on Day 0 than Day 8 of the oestrous cycle (let-7f, miR-125b, miR-99a-5p and miR-145) are also known to be expressed at higher levels in pre-ovulatory follicles than in CLs of ruminant ovaries, consistent with their role in the follicle-to-luteal transition (McBride et al., 2012). On the other hand, although some of the miRNAs analysed in this study are known to increase in expression in the ovary during the luteal phase (e.g. miR-132; (Fiedler et al., 2008; Salilew-Wondim et al., 2014; Schauer, Sontakke, Watson, Esteves, & Donadeu, 2013)), they were not higher in plasma on Days 8 or 16 relative to Day 0 of the oestrous cycle. Moreover, the 4 miRNAs identified in my study to be differentially expressed during the oestrous cycle (let-7f, miR-125b, miR-99a-5p and miR-145; Figure 2-5) are indeed expressed naturally not only in the ovary but also (at lower levels) in many other tissues (Landgraf et al., 2007; Mishima et al., 2008), the relative contribution of which to circulating levels is not known. It is also worth pointing out that I have not been able to accurately quantify in plasma the gonad-specific miRNA, miR-202, despite this miRNA being present at relatively high levels in both follicular cells and follicular fluid (Donadeu & Schauer, 2013; Sontakke et al., 2014), a finding that, in the absence of any other evidence, argues against the ready transfer of at least some miRNAs from follicular tissues at levels that can be robustly quantified in the circulation.

Finally, miRNA target analysis identified predicted KEGG pathways simultaneously targeted by two or more of the miRNAs identified in this study (miR-125b, let-7f, miR-99a-5p and miR-145), and those included ECM-receptor interaction, p53 signalling, Hippo signalling, Thyroid hormone and Cell cycle pathways (File 2-3). However, the precise origin of these plasma miRNAs will need to be elucidated

before meaningful conclusions about their role during the oestrous cycle can be drawn from these data.

In summary, the changes in miRNA levels during the bovine oestrous cycle identified in this study likely reflect changes in expression in tissues other than or in addition to the reproductive tract, a possibility that should be further investigated.

2.6 Conclusions

Using a combination of small-RNA sequencing and RT-qPCR I have identified for the first time a subset of miRNAs with changing levels in plasma during the bovine oestrous cycle. Specifically, I identified an increase (up to 2.2-fold) in the levels of let-7f, miR-125b, miR-99a-5p and miR-145 during oestrus. These may reflect changes in miRNA expression in reproductive and/or other body tissues possibly involved in regulating cyclic reproductive activity. In addition, I have characterised and provide a list of miRNA isoforms together with their relative abundance in bovine plasma, which will provide valuable information for future studies. My results pave the way towards exploring the role of circulating miRNAs as biomarkers of reproductive function in livestock. Future improvements in nucleic acid profiling technology may allow more accurate measurement of low-abundance miRNA levels in circulation and the identification of additional miRNA candidate biomarkers.

3 Early Pregnancy Biomarkers

3.1 Chapter Introduction

In this chapter I profiled miRNAs in the circulation of pregnant heifers on Days 16 and 24 of pregnancy and in non-pregnant heifers in order to identify potential biomarkers of early pregnancy in these animals. This work was published in 2016 with the title ‘Circulating miRNA signatures of early pregnancy in cattle’ (Ioannidis & Donadeu, 2016b) and the manuscript text is included in this chapter with minor changes to avoid repetition.

3.2 Abstract

Low fertility remains a leading cause of poor productivity in dairy cattle. In this context, there is significant interest in developing novel tools for accurate early diagnosis of pregnancy. MicroRNAs (miRNAs) are short RNA molecules which are critically involved in regulating gene expression during both health and disease. MiRNAs have been shown to regulate ovarian function, uterine receptivity, embryonic development and placental function. Circulating miRNAs can provide useful biomarkers of tissue function and disease; importantly, differential miRNA profiles have been linked to pregnancy and preeclampsia in humans. This study sought to establish the potential of circulating miRNAs as biomarkers of early pregnancy in cattle.

I applied Illumina small-RNA sequencing to profile miRNAs in plasma samples collected from 8 non-pregnant heifers on Days 0, 8 and 16 of the oestrous cycle and 11 heifers on Days 16 and 24 of pregnancy. I sequenced a total of 46 samples and generated 9.2 million miRNA reads per sample. There were no differences in miRNA read abundance between any of the pregnant and non-pregnant time-points ($\text{FDR} > 0.1$). As a complementary approach, I analysed sample pools (3-4 samples / pool) corresponding to Days 0, 8 and 16 of the oestrous cycle and Day 24 of pregnancy ($n = 3$ pools / group) using Qiagen PCR arrays. A total of 16 miRNAs were differentially expressed ($\text{FDR} < 0.1$) in plasma between pregnant and non-pregnant animals. RT-qPCR validation using the same plasma samples confirmed that miR-26a was differentially upregulated on Day 16 pregnant relative to non-

pregnant heifers (1.7-fold; $P = 0.043$), whereas miR-1249 tended to be upregulated in Day 16 pregnant heifers (1.6-fold; $P = 0.081$). Further validation in an independent group of heifers confirmed an increase in plasma miR-26a levels during early pregnancy, which was significant only on Day 24 (2.0-fold; $P = 0.027$).

Through genome-wide analyses I have successfully profiled plasma miRNA populations associated with early pregnancy in cattle. I have identified miR-26a as a potential circulating biomarker of early pregnancy.

3.3 Background

As of December 2014, there were approximately 9.7 million cattle in the UK (Gardiner, 2015). It is estimated that low fertility and suboptimal management of dairy herds cost £500 million in lost productivity annually (College of Agriculture, 2005a, 2005b). This problem is currently maintained by the chronic selection and use of high-yielding dairy cows. Profitable milk production depends on regular calving, the target being that every cow produces one calf a year. An important limitation to meeting this target is that, although conception rates remain very low (less than 50% at first service), tools for accurate early detection of pregnancy (within 3 weeks) are not available, leading to prolonged inter-calving intervals and significant losses in milk production. In light of this, the veterinary and dairy sectors have a particular interest in the development of novel biomarkers of early pregnancy.

MicroRNAs (miRNAs) have recently emerged as promising diagnostic biomarkers with high clinical potential. MiRNAs are short, non-coding RNA molecules which are centrally involved in post-transcriptional control of gene expression (Ha & Kim, 2014). Different roles of miRNAs in the reproductive system have been proposed including the development of ovarian follicles and the CL (McBride et al., 2012; Sontakke et al., 2014), uterine cyclicity and establishment of pregnancy, and embryonic development (Forde et al., 2012; Kresowick et al., 2014; Su et al., 2014; Wessels et al., 2013). In the early developing bovine conceptus, the levels of some miRNAs including miR-496 and miR-125a vary greatly, suggesting a role in the maternal-to-zygotic transcriptional transition (Tsfaye et al., 2009). Furthermore, several miRNAs including miR-27a and miR-92b are differentially expressed during

the development of the placenta, where they have been associated with trophoblast differentiation and vascularisation (Doridot et al., 2013; Su, Zhao, Zhu, & Yu, 2010). Finally, let-7 and miR-125b among other miRNAs have been shown to control mammary gland development and lactation (Y. Zhou et al., 2014).

MiRNAs are naturally secreted from cells into body fluids where they remain in relatively stable protein or lipid complexes and can be easily quantified (Mitchell et al., 2008; Moldovan et al., 2014; Turchinovich et al., 2011; Vickers et al., 2011). This, combined with the fact that some miRNAs are tissue or developmental stage specific presents the opportunity to use miRNAs as non-invasive biomarkers of tissue function associated with a variety of physiological states (e.g. pregnancy) and diseases (e.g. neoplasia, cardiovascular disease, osteoarthritis, sepsis) (Creemers et al., 2012; Kosaka, Iguchi, & Ochiya, 2010). Indeed, miRNA-based platforms are currently being used for clinical diagnosis of various types of human cancer (Creemers et al., 2012; Kosaka et al., 2010). Despite this, there are still limitations associated with measurement of circulating miRNA levels using existing technologies, which are derived from the presence of enzymatic inhibitors in serum and plasma, the low RNA content in bio-fluids, haemolysis and other cell contamination, and the need for unbiased procedures for normalisation of miRNA expression data (Chevillet, Lee, Briggs, He, & Wang, 2014; Moldovan et al., 2014).

There is promising evidence of the potential of miRNAs as biomarkers of pregnancy. The circulating levels of miRNAs belonging to the primate- and placenta-specific C19MC cluster increase with gestational age, while levels of C14MC cluster miRNAs increase in the first trimester and decrease in later pregnancy (Prieto & Markert, 2011; Williams et al., 2013). Furthermore, the C19MC cluster miRNAs, miR-516-5p, miR-518b, miR-520a and miR-525, are detectable in the human maternal circulation as early as 12 weeks of gestation (Kotlabova et al., 2011). In addition, the circulating levels of some C19MC miRNAs are significantly correlated with placental weight (Miura et al., 2014), consistent with their secretion from the developing placenta. Recent studies in humans have also identified miR-141 and miR-149 as pregnancy-associated circulating miRNAs; circulating levels of miR-141 significantly increase during gestation whereas the levels of both miRNAs decrease

after delivery (Chim et al., 2008). In sheep, miR-30c, miR-132, miR-379, miR-199a-3p and miR-320 are differentially expressed in serum on Days 30 or 60 of pregnancy (Cleys et al., 2014). There is limited information on pregnancy-related miRNAs in livestock and, to my knowledge, the levels of circulating miRNAs during early pregnancy have not been previously published for any domestic species.

The aim of this study was to profile miRNA levels in the plasma of cattle during Days 16 and 24 of pregnancy and to identify miRNA signatures that could be potentially used for diagnosis of early pregnancy. I present miRNA profiling results generated using two independent approaches, Illumina small-RNA sequencing and Qiagen PCR array.

3.4 Materials and Methods

3.4.1 Experimental animals and sample collection

During March-April of 2013, 24 cycling Holstein-Friesian heifers (14-17 months old) were oestrus-synchronised using the same treatment protocol as in Chapter 2. After 48 hours (Day 0) animals were either sham-inseminated (non-pregnant group; n = 8) or inseminated with frozen semen (pregnant group; n = 16). Animals were inseminated or sham-inseminated again 24 hours later. Blood was collected on Days 0, 8, 16 from all animals. Additional samples were collected on Day 24 from the pregnant group. Pregnancy was confirmed twice on Day 35 and Day 60 by TRUS. Five animals failed to become pregnant after insemination and were excluded from the study. Blood was collected from all animals and processed as described in Chapter 2, under the same UK Home Office licence and ethics approval.

3.4.2 RNA extraction

RNA was extracted from 1.05 mL of plasma using the same protocol as in Chapter 2.

3.4.3 Small-RNA sequencing

Small-RNA sequencing was carried out as described in Chapter 2, with the following differences. After initial data processing and generation of normalised reads, miRNAs detected with less than 25 RPMs in more than 75 % of the samples in each of the experimental groups were excluded, keeping 178 miRNAs for further analysis. The average within-animal expression of each miRNA in the non-pregnant

group was calculated (mean of Days 0, 8 and 16) and used for further analyses. Normalised expression levels (RPMs) were $\log_2$ transformed before applying two-sample t-tests on the data, followed by an FDR adjustment of the P values using R programming. The same normality tests were carried out as in Chapter 2, and comparisons made were NP16 vs P16 (a direct comparison between non-pregnant and pregnant groups on Day 16), NP vs P16 and NP vs P24.

3.4.4 PCR array analysis

The PCR arrays were run as described in Chapter 2, with the following modifications. Three pools of 3 to 4 samples each, from each of Day 0, Day 8 and Day 16 for non-pregnant animals, and from Day 24 pregnant animals (12 pools in total) were analysed. Following the initial data clean-up, miRNAs which had Cq > 35 in more than 75 % of samples per experimental group were removed from the dataset. The mean expression across non-pregnant time-points (Days 0, 8 and 16) was used for analyses, as for the sequencing data, resulting in 3 data-points for analyses from each of pregnant and non-pregnant groups (NP vs P24). The statistical analysis of the transformed normalised data was performed as described for the sequencing data in the current chapter.

3.4.5 RT-qPCR analysis

Results of high-throughput analyses (small-RNA sequencing or PCR arrays) were validated by RT-qPCR on the same plasma samples used for sequencing (n = 8 heifers / group) using the protocols described in Chapter 2 with the following modifications. The amplification efficiency generally ranged between 85 % - 115 %, with $R^2 > 0.85$. Specifically, data were $\log_2$ transformed and tested for normality as described for the small-RNA sequencing data, and Dunn's multiple comparison tests (non-parametric) were used to generate P values for the comparisons of miRNA levels between non-pregnant (NP) and pregnant groups (P16 or P24).

3.4.6 Validation of results in an independent group of heifers

During November 2015, 16 cycling, 14-17 month old Holstein-Friesian heifers were oestrus-synchronised and inseminated as described above. Blood samples were collected on Days 0 (before insemination), 16 and 24 and processed for RT-qPCR

analysis as described above. Samples were analysed for 11 animals confirmed pregnant on Day 35.

3.5 Results and Discussion

3.5.1 Optimisation of microRNA profiling in bovine plasma

I deemed it important to introduce sample quality control measures and optimise my quantification methodology before proceeding with profiling miRNAs in the bovine circulation, in order to address common problems in circulating miRNA quantification which might bias my study, such as low RNA yields, enzymatic inhibition and haemolysis.

I tested three different commercial kits for extraction of RNA from bovine plasma, namely, miRNeasy mini (Qiagen, Netherlands), miRNeasy plasma (Qiagen), and TRIzol LS (Life Technologies, United Kingdom). Using 200 μ L of bovine plasma, the TRIzol LS protocol was more efficient than the column-based kits as determined by RT-qPCR quantification of spiked-in exogenous cel-miR-39-3p (Figure 3-1A). An added advantage of the TRIzol LS protocol is that it allows scaling-up the extraction volume; for my experiments I decided to use 1.05 mL of bovine plasma which yielded a mean of 9.5 ± 0.8 ng of RNA (Figure 3-1B). This yield is similar to that reported for bovine plasma in another study (8.6 ng / mL), but lower than the mean yield reported for human plasma (25 ng / mL) (Spornraft et al., 2015; Williams et al., 2013).

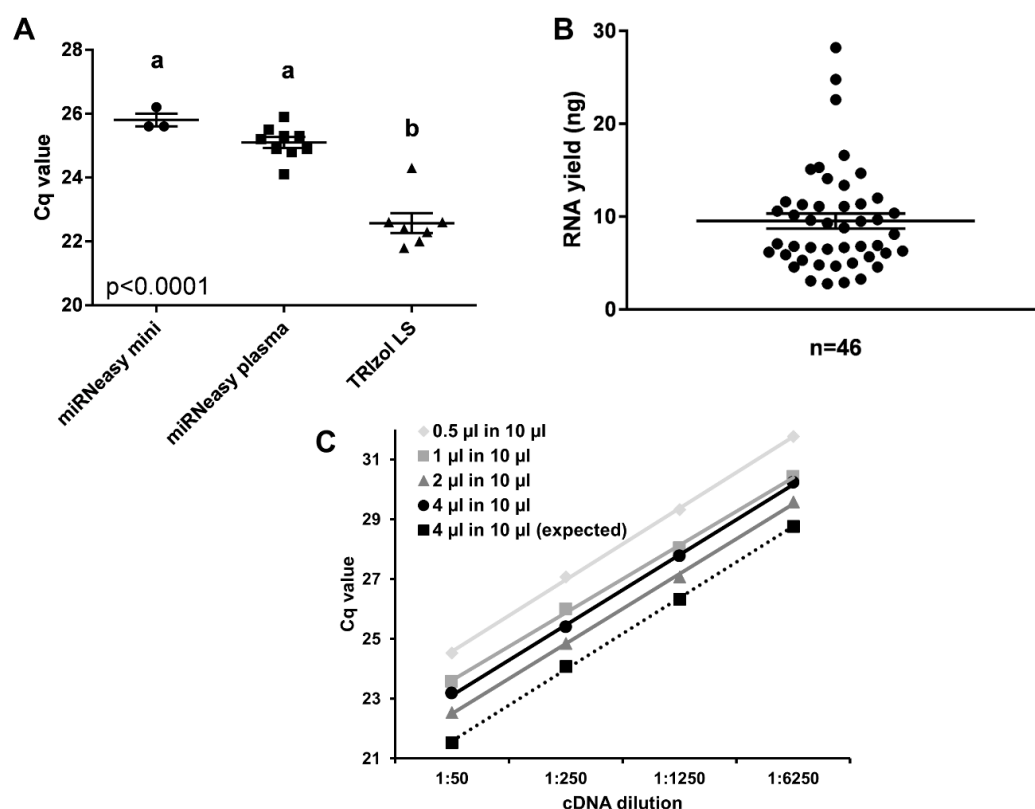


Figure 3-1. Optimisation for profiling miRNAs in bovine plasma.

A) RT-qPCR data plots (with mean $\pm$ SEM) showing abundance of the exogenous miRNA, cel-miR-39-3p, in plasma samples after RNA extraction using 3 different kits as indicated. B) RNA yield from 1.05 mL bovine plasma samples using TRIzol LS. C) Results of RT-qPCR quantification of miR-451 in plasma (mean $\pm$ SEM) using different RNA volumes for reverse-transcription; highest reaction efficiency was obtained using 2 μ L of RNA in a 10 μ L cDNA synthesis reaction.

The presence of high levels of enzyme inhibitors in plasma samples (for example, immunoglobulin G) can significantly reduce the efficiency of RT-qPCR (Al-Soud et al., 2000). To address this, I tested different input RNA volumes and determined that using 2 μ L of RNA extract in a 10 μ L cDNA synthesis reaction yielded the highest reaction efficiency (Figure 3-1C).

Another significant problem in plasma miRNA profiling is red blood cell contamination. As high levels of red blood cell-derived miRNAs such as miR-451 are associated with haemolysis (Kirschner et al., 2011; Pritchard et al., 2012), the Δ Cq between miR-451 and miR-23a has been proposed as a useful indicator of haemolysis (Kirschner, van Zandwijk, & Reid, 2013). I confirmed the validity of this approach for bovine samples by showing good correlation between the ratio of miR-

451 and miR-23a and optical densities at 414 nm (Figure 3-2, A-B). Based on this, I concluded that all samples used for miRNA profiling were within the normal range for non-haemolysed bovine plasma (Figure 3-2, C-D).

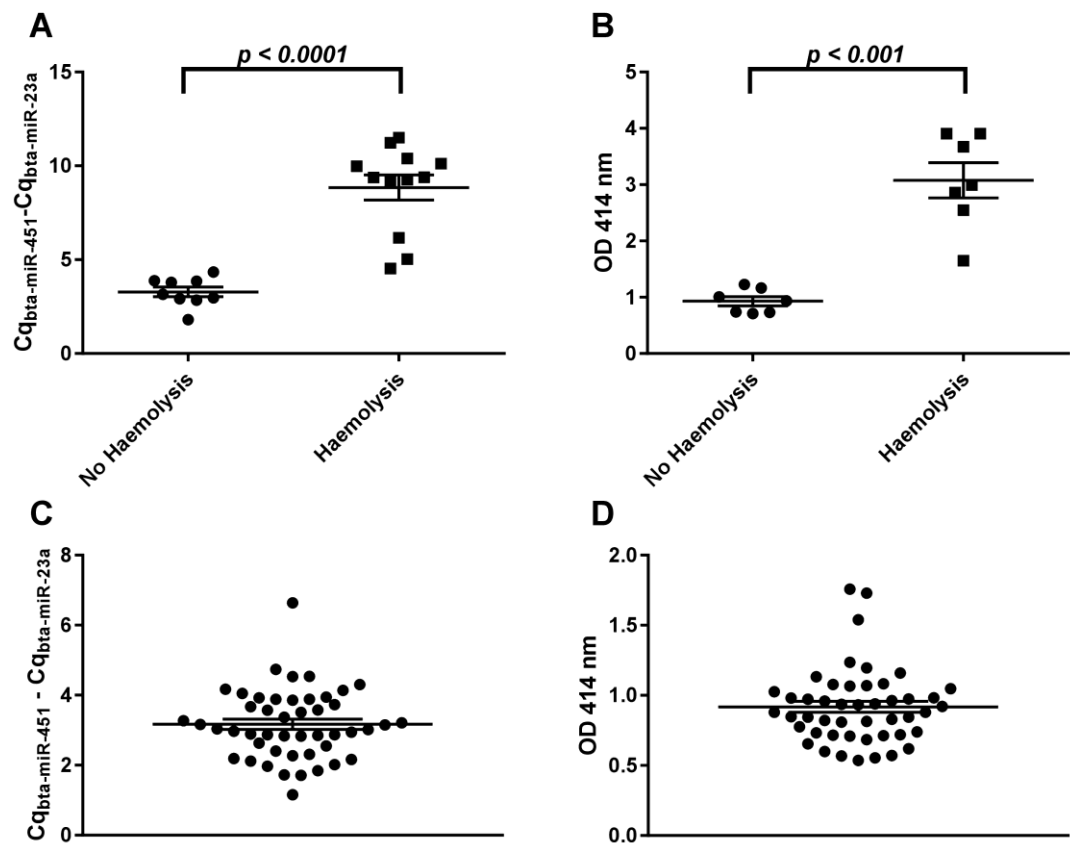


Figure 3-2. Determining haemolysis of bovine plasma samples.

Samples with or without visible haemolysis were analysed to determine A) the ΔCq between miR-451 and miR-23, and B) optical density at 414 nm (as a measure of oxyhaemoglobin levels). ΔCq (C) and optical density (D) values for all plasma samples ($n = 46$) used for small-RNA sequencing and PCR array profiling, showing the absence of detectable haemolysis. Mean $\pm$ SEM is shown.

3.5.2 Small-RNA sequencing of bovine plasma during pregnancy

I sequenced a total of 46 small-RNA libraries generated from plasma samples collected from 8 non-pregnant animals (on each of Days 0, 8 and 16 of the oestrous cycle), and 11 animals on each of Days 16 and 24 of pregnancy (encompassing the period for which a pregnancy biomarker would be desired (Balhara et al., 2013)).

I obtained a median of 9.2 million raw sequencing reads from each sample (Table 3-1). A length distribution plot of reads post-trimming showed a distinct peak at 20-23 nt indicating the majority of reads corresponded to mature miRNAs (Figure 3-3A). After removal of low-quality reads, a median of 4 million reads (43.5 % of total reads) from each sample were mapped to the bovine genome, 68 % of which (2.7 million) were identified as miRNAs (Table 3-1). The remaining mapped reads corresponded to non-coding regulatory and structural small RNAs (including Y-RNAs and spliceosomal RNAs), and fragments of larger RNA species such as mRNAs (Figure 3-3B). The vast majority of miRNA reads (99.8 %) corresponded to registered bovine sequences in miRBase; the remaining corresponded to human miRNA homologues (0.11 %) or to predicted novel miRNAs (0.06 %, Figure 3-3B). The percentage of miRNA reads obtained over the total sequencing reads (30.4 %) was higher than that reported from sequencing of bovine plasma in another study (5 %) but lower than obtained from human plasma using the same sequencing platform (57.7 %) (Spornraft et al., 2014; Williams et al., 2013).

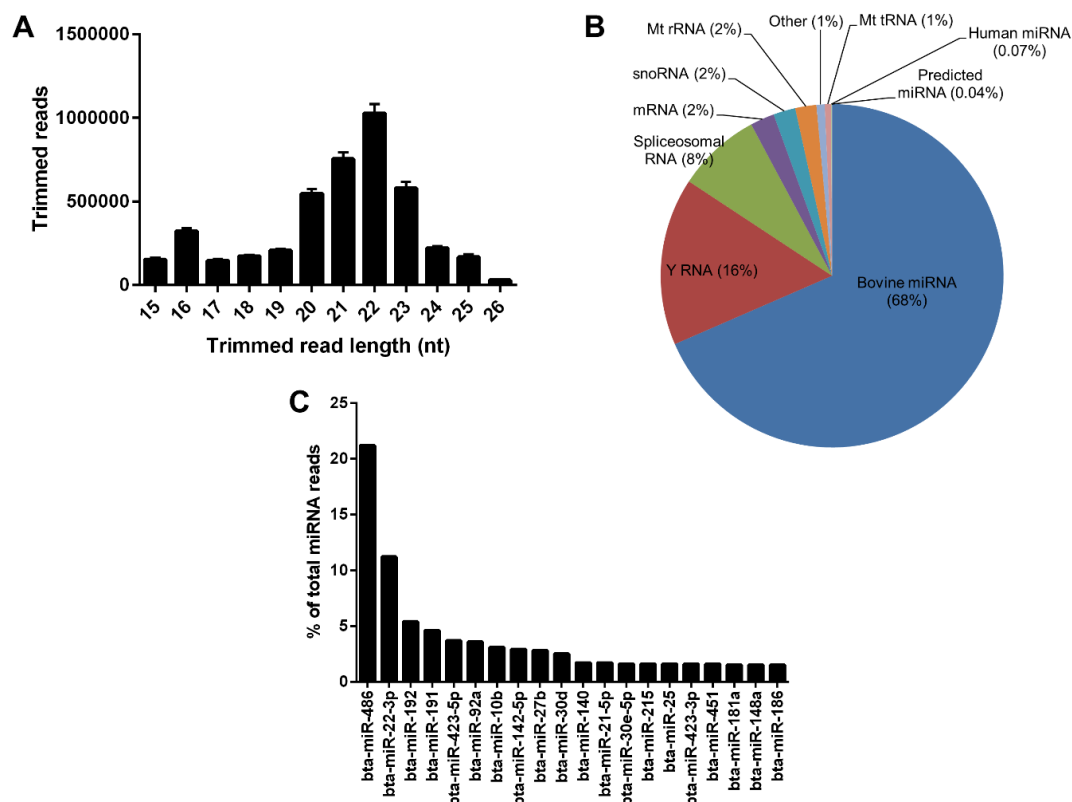


Figure 3-3. Small-RNA sequencing read count data during pregnancy.

A) Length distribution (mean $\pm$ SEM) of read counts (after trimming) from Illumina sequencing of bovine plasma samples. B) Relative abundance of different RNA species sequenced in bovine plasma. C) Top 20 miRNAs in bovine plasma detected by Illumina small-RNA sequencing; mean percentages of the total miRNA reads per million mapped (RPMs) are shown.

Table 3-1. Analysis endpoints from sequencing of plasma during pregnancy.

Median (in millions) and 25% - 75% percentile (in brackets) values obtained from a total of 46 plasma samples from non-pregnant heifers (NP; Days 0, 8 and 16 after oestrus combined; n = 24) and heifers on Days 16 (P16) and 24 (P24) of pregnancy (n = 11).

	NP	P16	P24	All
Total sequence reads	9.1 (8.8-9.6)	9.4 (8.7-11.6)	9.9 (8.8-10.7)	9.2 (8.7-10.5)
Reads with adapter	8.0 (7.7-8.9)	8.7 (7.7-10.6)	9.3 (8.0-9.8)	8.3 (7.7-9.6)
Reads which passed QC	4.5 (3.5-5.4)	4.0 (3.3-5.3)	4.0 (2.9-5.6)	4.2 (3.4-5.4)
Reads mapping to genome	4.3 (3.3-5.2)	3.7 (3.1-4.9)	3.6 (2.6-5.4)	4.0 (3.2-5.2)
Total miRNA reads	3.2 (2.2-3.8)	2.2 (2.1-3.0)	2.6 (1.5-3.5)	2.8 (2.1-3.5)

Overall, a total of 386 unique miRNAs across all individual samples (328 bovine, 37 human and 21 predicted novel) had more than 10 read counts. On average, the 10 most abundant miRNAs accounted for 61 % of the total miRNA reads in each sample (Figure 3-3C). Out of the most abundant miRNAs in bovine plasma, miR-486 and miR-92a are reportedly expressed primarily in erythrocytes, and miR-191 is expressed primarily in platelets (Ple et al., 2012; Pritchard et al., 2012). Almost half (40 %) of the 10 most abundant miRNAs were also identified as highly abundant in bovine plasma in another study whereas 20 % were very abundant in human plasma (Spornraft et al., 2015; Williams et al., 2013). Common miRNAs identified across studies include miR-486, miR-92a, miR-192 and miR-423-5p.

One sample from Day 16 of pregnancy was removed prior to differential expression analysis because it distinctly had very low total reads per million mapped (RPMs).

Different comparisons of miRNA expression data between non-pregnant (NP) and pregnant groups (P) were made involving 1) the average expression over Days 0, 8 and 16 of the oestrous cycle (NP; n = 8) for the non-pregnant group vs each of Day 16 and Day 24 of pregnancy (P16 and P24; n = 10 and 11, respectively) and 2) a direct comparison between Day 16 of the oestrous cycle (NP16) and Day 16 of pregnancy. PCA based on these comparisons revealed no clear separation according to pregnancy status (Figure 3-4A). Changes in the levels of 178 individual miRNAs which passed my quality filters (see Methods) were determined for each comparison (File 3-1). Differences in the expression of a limited number of individual miRNAs were detected although they were generally small (under 2.5-fold) and not significant after FDR adjustment (Figure 3-4B-D; Table 3-2). For one of the miRNAs, miR-133a, levels were up to 7.4-fold lower in Day 16 pregnant relative to non-pregnant heifers (FDR = 0.127) although the miRNA was not detectable by qPCR and thus those differences could not be validated further.

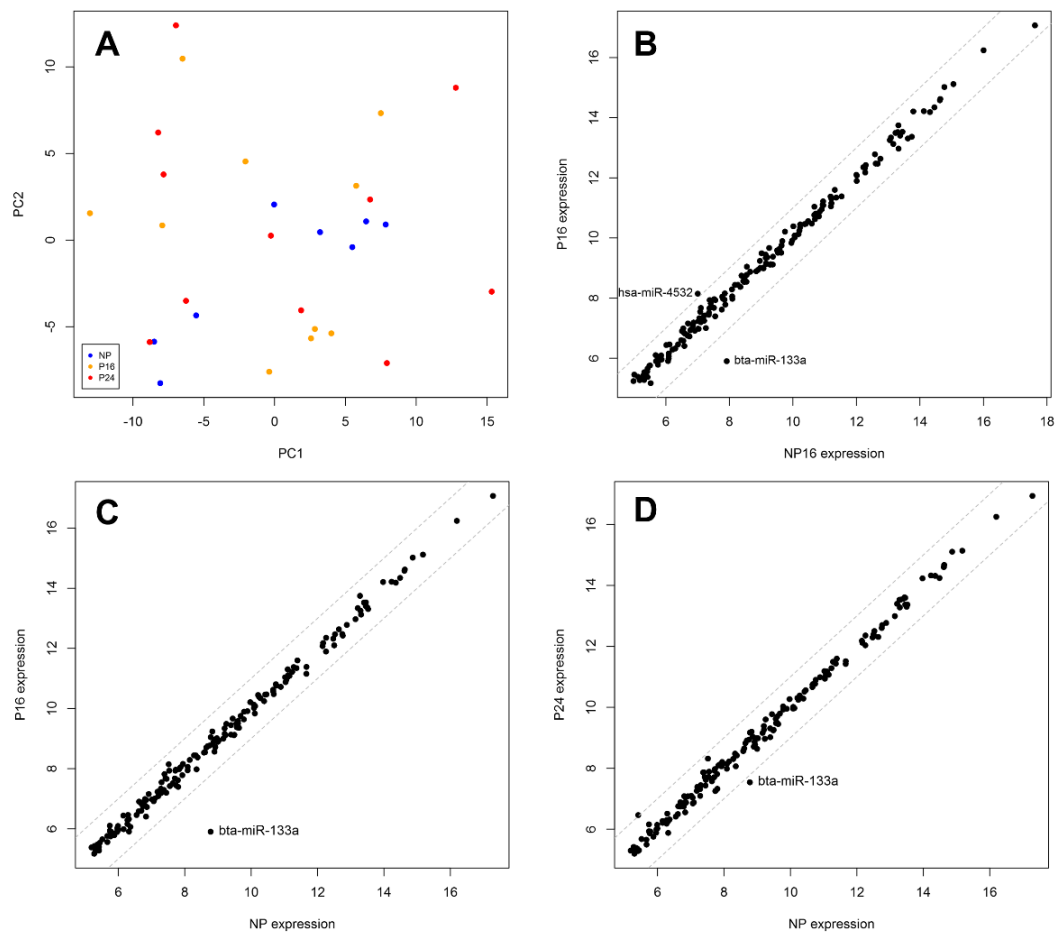


Figure 3-4. Differential expression analysis of sequencing data during pregnancy.

A) PCA plot using data from all pregnant (P16 and P24) and non-pregnant (NP) time-points obtained by Illumina small-RNA sequencing. Scatterplots for P16 vs NP16 (B) P16 vs NP (C) and P24 vs NP (D) comparisons using individual miRNA data are shown. The grey dotted lines indicate the 2-fold change threshold, and miRNAs which changed more than 2-fold between groups have been labelled. All data have been log₂ transformed.

Table 3-2. Changes in miRNA abundance during pregnancy using sequencing. List of plasma miRNAs with different abundance between pregnant and non-pregnant heifers (NP16 vs P16, NP vs P16 and NP vs P24 comparisons) based on RPMM values from Illumina sequencing. Fold change in pregnant relative to non-pregnant heifers are shown. FDR > 0.1 for all miRNAs.

	Comparison	Fold change	P value
bta-miR-101	NP16 vs P16	0.78	0.045
bta-miR-25	NP16 vs P16	0.81	0.014
bta-miR-19a	NP16 vs P16	1.22	0.029
bta-miR-130b	NP16 vs P16	1.33	0.019
bta-miR-328	NP16 vs P16	1.34	0.006
bta-miR-301b	NP16 vs P16	1.49	0.045
hsa-miR-4532	NP16 vs P16	2.21	0.006
bta-miR-133a	NP vs P16	0.14	0.001
bta-miR-193b	NP vs P16	0.74	0.021
bta-miR-365-3p	NP vs P16	0.75	0.026
bta-miR-2957	NP vs P16	0.77	0.048
bta-miR-152	NP vs P16	0.77	0.048
bta-miR-107	NP vs P16	0.82	0.036
bta-miR-103	NP vs P16	0.83	0.037
bta-miR-328	NP vs P16	1.22	0.003
bta-miR-133a	NP vs P24	0.42	0.028
bta-miR-125b	NP vs P24	0.71	0.024
bta-miR-99a-5p	NP vs P24	0.72	0.016
bta-miR-365-3p	NP vs P24	0.74	0.013
bta-miR-99b	NP vs P24	0.77	0.022
bta-miR-10b	NP vs P24	0.84	0.037

3.5.3 MicroRNA profiling during pregnancy using PCR arrays

To complement my sequencing analyses I used a commercial Custom PCR array platform to profile the expression of 308 unique bovine miRNAs in the same plasma samples. As it is not feasible to screen a large number of samples using PCR arrays, I analysed 3 sample pools (3-4 samples / pool) from each of Days 0, 8 and 16 from non-pregnant heifers and from Day 24 pregnant heifers (I reasoned I would more easily find differences in miRNA expression at Day 24 than at Day 16 of pregnancy).

I detected a total of 208 miRNAs in bovine plasma (based on mean Cq < 35 across all sample pools; Figure 3-5A), the most abundant of which (Figure 3-5B)

corresponded to miRNAs reportedly expressed at high levels in blood cells including erythrocytes (miR-451, miR-486, miR-16), leukocytes (miR-150, miR-27a, miR-23a) and thrombocytes (miR-223, miR-20a, miR-24), and which are putatively released into the plasma through apoptosis, lysis or active secretion (Haider et al., 2014; K. Wang et al., 2012; Willeit et al., 2013). The absence of detectable haemolysis in my samples (see above) indicates the detected blood cell miRNAs are predominantly the result of secretion / activation and not cell lysis.

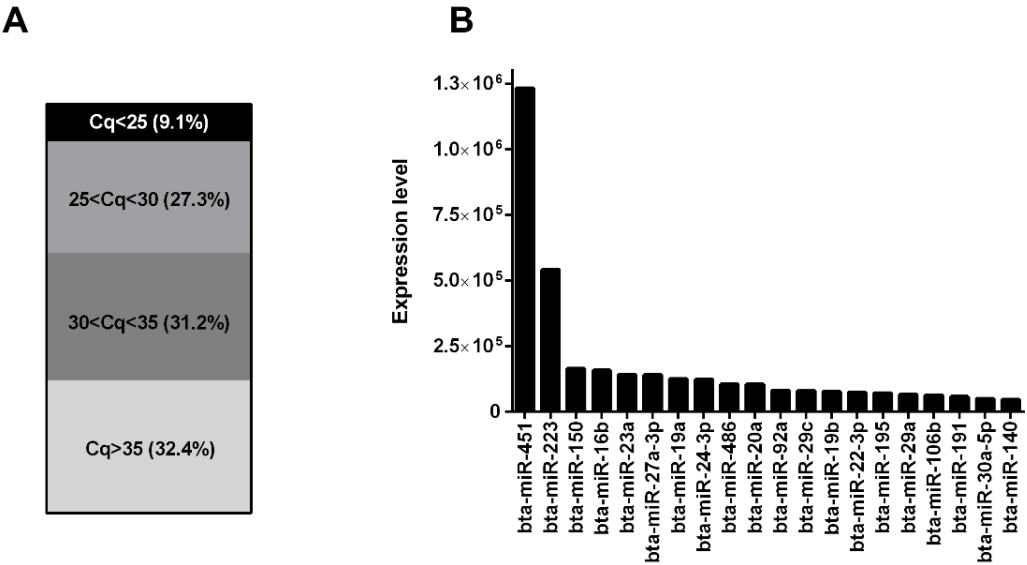


Figure 3-5. MiRNA abundance in bovine plasma pools determined by Qiagen PCR arrays.

A) Distribution of mean Cq values for 308 conserved miRNAs. B) Expression level of the 20 most abundant miRNAs in bovine plasma (calculated as $2^{(40-Ct)}$).

Comparing the 20 most abundant miRNAs in each of the PCR array and sequencing datasets, only 6 miRNAs (miR-486, miR-22-3p, miR-191, miR-92a, miR-140 and miR-451) were common. A very abundant miRNA in the sequencing dataset (miR-21-5p) could not be compared as it was not included in the PCR array. The overall poor agreement in abundant miRNAs identified by the two techniques can in part be explained by platform-specific biases such as those associated with sequencing adaptor ligation and differences in primer-specific PCR efficiency, which are known to affect the representation of miRNAs in a library therefore changing the perceived

order of abundance of the miRNAs within a sample (Fuchs et al., 2015; Sorefan et al., 2012; van Dijk et al., 2014).

As before, differential expression analyses for each miRNA was performed considering the average expression value over Days 0, 8 and 16 of the oestrous cycle for the non-pregnant group (NP). PCA of PCR array results showed a clear separation between NP and P24 groups (Figure 3-5A). Of the 176 unique miRNAs included in the analysis (after excluding low abundance miRNAs; see Methods), a total of 16 miRNAs were differentially expressed between the two groups (FDR < 0.1), 8 of which at ≥ 2 -fold (Figure 3-6BC; Table 3-3; File 3-2).

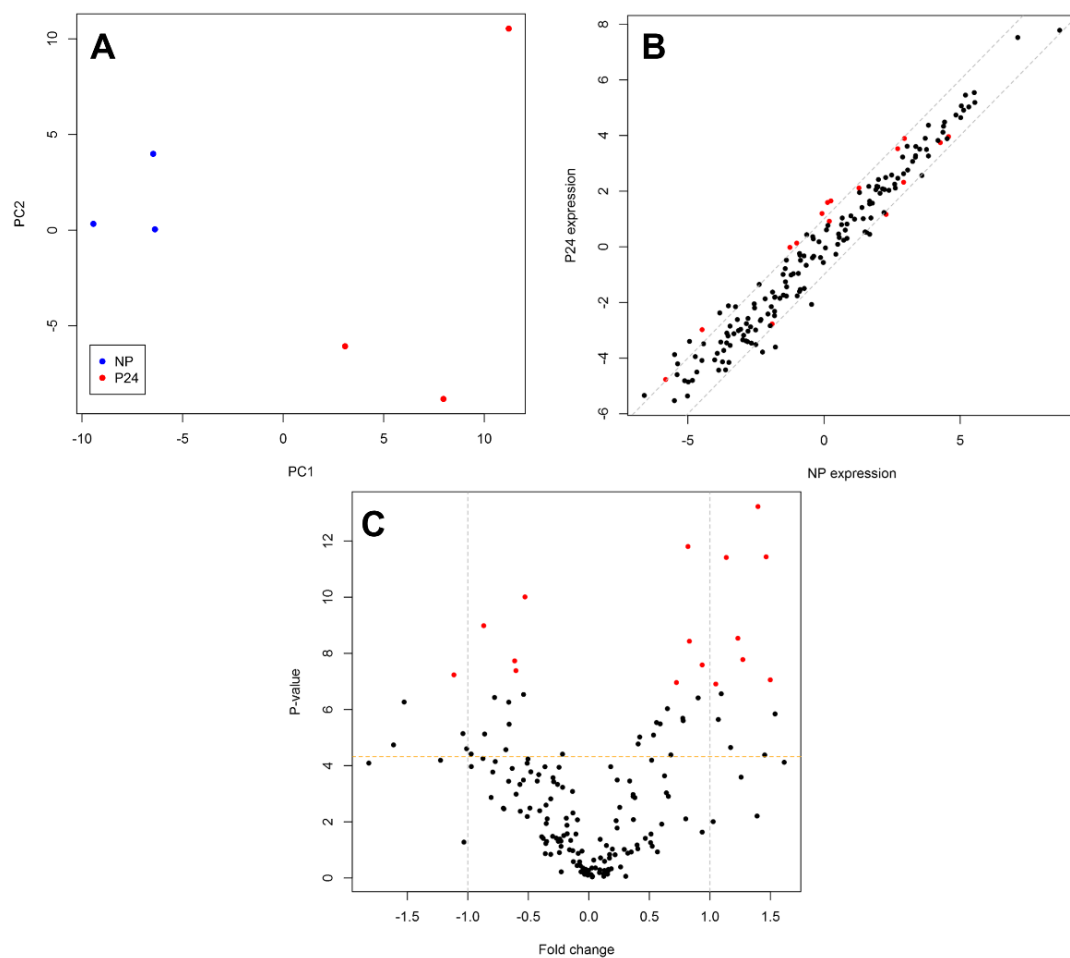


Figure 3-6. Differential expression analysis using PCR array data in pregnancy. A) PCA plot and B) scatterplot of individual miRNA data (NP vs P24). The grey dotted lines in B) indicate the 2-fold change threshold, and the highlighted data-points (in red) represent FDR < 0.1. The data have been log₂ transformed. C) Volcano plot for the same data shown in (B). The horizontal dotted orange line indicates the significance threshold (P = 0.05) and the vertical grey lines indicate the

2-fold change threshold. Data-points representing differences with FDR < 0.1 have been highlighted in red. The data have been log₂ transformed.

Table 3-3. Differences in miRNA abundance during pregnancy using PCR arrays.

List of differentially expressed miRNAs on Day 24 of pregnancy (NP vs P24; FDR < 0.1) based on Qiagen PCR array expression data. Fold changes were calculated as pregnant / non-pregnant.

	Fold change	P value	FDR
bta-miR-101	0.46	0.007	0.090
bta-miR-141	0.55	0.002	0.058
bta-miR-29c	0.65	0.005	0.083
bta-miR-339a	0.66	0.006	0.088
bta-miR-29a	0.69	0.001	0.034
bta-miR-103	1.65	0.008	0.092
bta-miR-26a	1.76	0.000	0.016
bta-miR-30b-5p	1.78	0.003	0.064
bta-miR-26b	1.91	0.005	0.083
bta-miR-631	2.07	0.008	0.092
bta-miR-374b	2.20	0.000	0.016
bta-miR-151-5p	2.35	0.003	0.064
bta-let-7d	2.42	0.005	0.083
bta-miR-30c	2.63	0.000	0.016
bta-let-7f	2.76	0.000	0.016
bta-miR-454	2.83	0.008	0.092

Generally, I observed poor overlap between differentially expressed gene lists from the two high-throughput datasets. Thus, only four of all miRNAs differentially expressed ($P < 0.05$) between pregnant (Day 24) and non-pregnant animals in the PCR array, i.e. miR-99b, miR-152, miR-101, miR-103, were also different ($P < 0.05$) between pregnant and non-pregnant groups (all comparisons) in the sequencing dataset. In some cases, the poor overlap was due to the miRNAs being under-represented in the sequencing libraries (e.g. miR-29c, miR-1249) resulting in low-confidence profiles. Other miRNAs such as miR-4532 were not included in the PCR arrays, therefore cross-platform validation was not possible. Platform-specific biases (indicated above) and the fact that pooled rather than individual samples were used in the PCR arrays may have also contributed to the relative lack of agreement between

sequencing and PCR arrays. Taken together, my results highlight the limitations of current circulating miRNA profiling technologies, especially when subtle differences in gene expression are investigated, such as here, and show the importance of cross-platform validation.

3.5.4 RT-qPCR validation of high-throughput data

From the results of PCR array and small-RNA sequencing I selected (based on both fold-change and P value) a total of 21 differences in miRNA abundance (involving 17 unique miRNAs) for validation by RT-qPCR (File 3-3). Since I did not want my selection of miRNA candidates to be constrained by the limited number of differences originally obtained after FDR adjustment ($\text{FDR} < 0.1$; Tables 3-2 and 3-3), in the validation analyses I included 13 differences which were significant ($P < 0.05$) only before FDR adjustment. Because Day 16 of pregnancy was not included in the PCR array analyses (from where a majority of differences for validation were obtained), for the sake of inclusiveness both P16 and P24 were included in all validation analyses.

I could robustly quantify 15 individual miRNAs by RT-qPCR representing a total of 17 differences in miRNA abundance between pregnant and non-pregnant groups. For 9 of these differences (Figure 3-7A) the results of RT-qPCR were consistent with those obtained by PCR array or sequencing although significance was only obtained for miR-26a; an increase in the levels of this miRNA was significant on Day 16 (NP vs P16, 1.7 fold, $P = 0.043$) but not on Day 24 (1.7 fold, $P = 0.208$ Figure 3-7B). A trend for an increase in miR-1249 levels on Day 16 of pregnancy was also identified but was not statistically significant (NP vs P16, $P = 0.081$; Figure 3-7B). To more robustly validate the changes in miR-26a during early pregnancy, particularly as they were relatively small, I analysed plasma samples from a different, larger group of heifers during early pregnancy (Figure 3-7C). In this group of animals, when compared to levels before insemination, plasma levels of miR-26a were higher on Day 24 of pregnancy (2.0-fold; $P = 0.027$). On average, miR-26a levels were also higher on Day 16 (1.7-fold) although this difference did not reach significance ($P = 0.118$). Thus, from analyses in two independent groups of animals I concluded that the levels of miR-26a increase during early pregnancy in heifers.

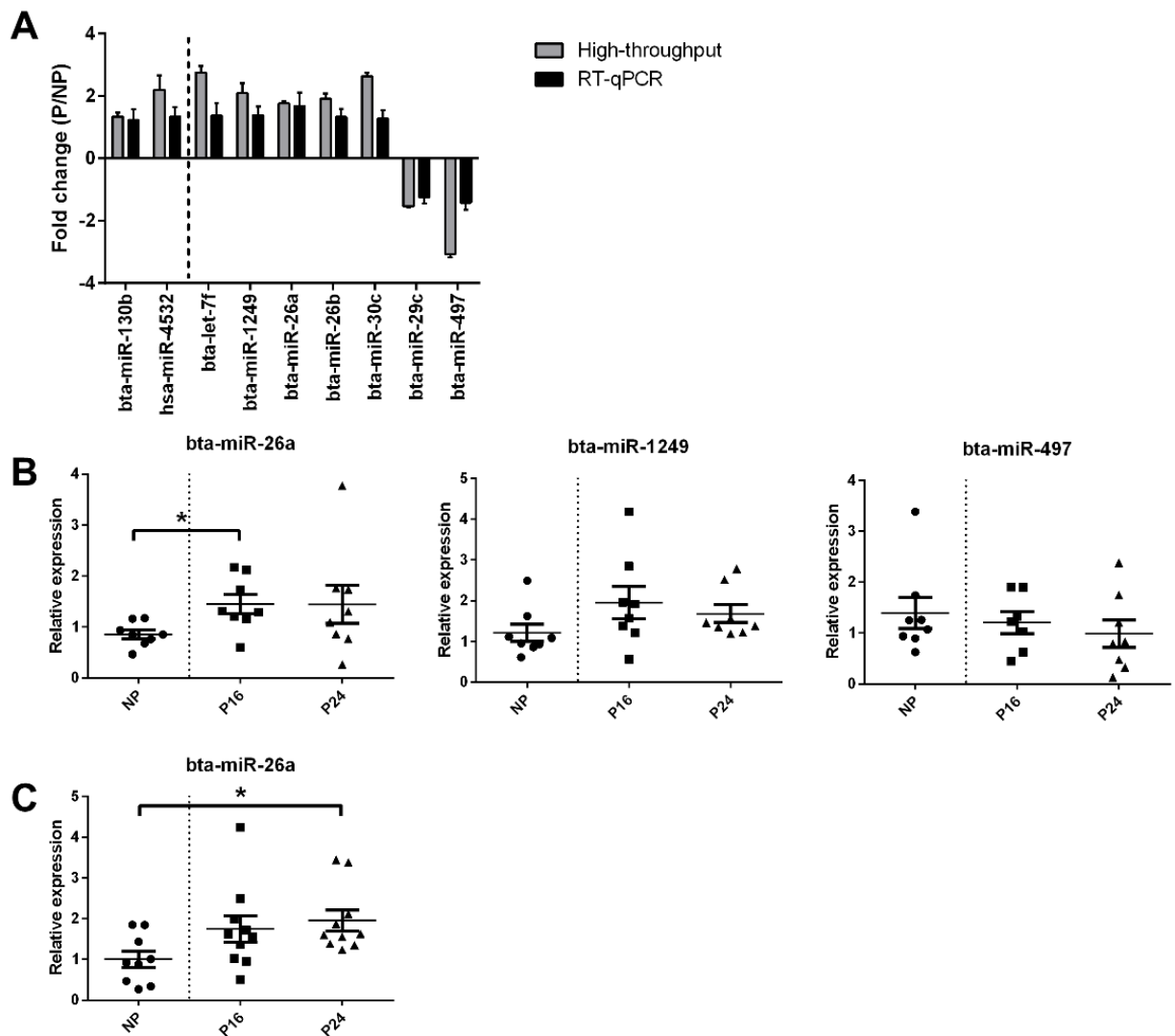


Figure 3-7. RT-qPCR validation of high-throughput results during pregnancy.

A) Comparative fold-changes (mean $\pm$ SEM) in miRNA abundance between pregnant and non-pregnant groups obtained by high-throughput analyses (PCR array or sequencing) and RT-qPCR. Results to the left and right of the dotted line correspond to NP16 vs P16 and NP vs P24 comparisons, respectively (File 3-3). B) RT-qPCR data plots (with mean $\pm$ SEM) for selected comparisons between pregnant and non-pregnant groups including the two differences in miRNA abundance that were significant (miR-26a, * indicates $P < 0.05$) or tended to be significant (miR-1249, NP vs P16, $P < 0.1$). C) RT-qPCR data plots (with mean $\pm$ SEM) obtained from an independent group of heifers and which confirms an increase in plasma miR-26a levels during early pregnancy. NP corresponds to plasma samples collected before insemination (Day 0) from the same animals.

Although expressed ubiquitously, distinctly high levels of miR-26a have been reported in the embryo, the ovary, and immune-related tissues and cells such as the thymus and B / T cells of different species (Coutinho et al., 2007; Huang et al., 2011;

Landgraf et al., 2007; Tripurani et al., 2010). Interestingly, miR-26a has been shown to directly down-regulate IFN- β , a major cytokine involved in innate and adaptive immune responses, suggesting a potential role for this miRNA as an immune suppressor during early pregnancy (Witwer, Sisk, Gama, & Clements, 2010). Additionally, recent studies in pig and goat have reported increasing levels of miR-26a in the conceptus and the ovary as early as Day 20 of pregnancy (Krawczynski, Najmula, Bauersachs, & Kaczmarek, 2014; X. D. Zhang et al., 2013). Also, high plasma miR-26a levels have been associated with pre-eclampsia in humans (L. Wu et al., 2012). These findings support the notion that miR-26a is involved in pregnancy, possibly by exerting immunomodulatory effects. Further studies should investigate the precise origin and function of this miRNA during pregnancy.

MiR-1249, identified by PCR array analyses as differentially expressed in pregnant animals, was severely under-represented in my sequencing library, with only 6.1 counts per million on average, which prevented any conclusions about its expression in bovine plasma. This may be the result of sequence- and secondary structure-dependent ligation biases during library preparation, a common issue with next-generation sequencing technology which affects all samples equally and thus is not considered to bias differential expression analyses (Fuchs et al., 2015; Sorefan et al., 2012; van Dijk et al., 2014). The case of miR-1249 is a good example of the need to use different quantification methods for cross-platform validation of results. The levels of miR-1249 in granulosa cells have been shown to vary during follicle development in bovine (Salilew-Wondim et al., 2014), and miR-1249 expression has been confirmed in bovine mammary glands, however little is known about its expression and functions in other tissues (R. Li et al., 2015).

The fact that results of RT-qPCR and high-throughput analyses agreed (i.e. differences between pregnant and non-pregnant groups showed the same trend) for only 9 of the 17 differences in miRNA abundance analysed may have been due in part to the intrinsically very low levels of RNA contained in plasma; this, together with the presence of several inhibitors of RT-qPCR enzymes in plasma make accurate quantification particularly challenging, especially when differences between the experimental groups being compared are small. Added to these are platform-

specific biases and intrinsic inter-animal variation. In the case of the PCR arrays, the use of pooled samples may also have contributed to disagreement in the results from different profiling platforms. A recent study reported a 54.6% agreement in human liver and brain tissue miRNA profiles across RT-qPCR, microarrays and small-RNA sequencing platforms (Mestdagh et al., 2014), which is comparable to the agreement reported in my study. This most likely reflects the limitations associated with quantification of plasma miRNAs discussed above. Finally, I only attempted to validate a fraction of the differences identified in the PCR array and sequencing experiments; further validation analyses may identify additional potential miRNA biomarkers of early pregnancy.

3.6 Conclusions

Through RNA sequencing and RT-qPCR profiling in cattle, I identified for the first time changes in the levels of miRNAs in plasma during early pregnancy. Specifically, I identified an increase (up to 2-fold) in the levels of miR-26a during Days 16 to 24 of pregnancy. These changes putatively reflect changes in miRNA expression in one or more body tissues and may play potentially important roles in the establishment of pregnancy. Overall, the differences in plasma miRNAs levels between pregnant and non-pregnant animals were small; larger differences in circulating miRNAs may occur at later stages of pregnancy. In addition, a limited ability to accurately quantify low abundance RNAs using available technologies may have prevented detecting larger differences in miRNA levels during early pregnancy. In summary, my results identify miR-26a as a novel candidate biomarker of early pregnancy in cattle.

4 Later Pregnancy Biomarkers

4.1 Chapter Introduction

In the following chapter I follow up on my findings from early pregnancy about differentially expressed miRNAs in plasma by identifying additional differences on Day 60 of pregnancy and retrospectively profiling miRNAs during early pregnancy. In addition, I investigate the tissue of origin of selected differentially expressed circulating miRNAs and I seek gender-specific differences in miRNA expression in my sequencing data from Days 16, 24 and 60 of pregnancy. The contents of this chapter are under review for publication to PLOS One with the title ‘Changes in circulating miRNAs levels can be identified as early as Day 8 of pregnancy in cattle’.

4.2 Abstract

Poor reproductive performance remains a major issue in the dairy industry, with extremely low calving rates (34 %) affecting milk production, profitability, and the economy in general. Early pregnancy detection can help identify non-pregnant animals within three weeks of insemination, minimising delays and shortening calving intervals. However current pregnancy detection methods are not accurate enough to support such reproductive management programs. MiRNAs have been proposed as non-invasive biomarkers of pregnancy and several reproductive diseases in humans. I previously identified miR-26a as a potential early pregnancy biomarker in cattle. In the current study, using miRNA sequencing, I identified and validated six differentially expressed miRNAs on Day 60 of pregnancy compared to Day 0 (non-pregnant). In a follow-up experiment I profiled selected miRNAs on Days 0, 8, 16 and 60 of pregnancy using RT-qPCR and I identified a significant increase in the levels of miR-26a (3.1-fold, $P = 0.046$) and the ratio between miR-26a and miR-205 (7.5-fold, $P < 0.001$) on Day 8 of pregnancy. RT-qPCR profiling of selected miRNAs across 14 bovine tissues revealed that miR-143 and miR-205 had markedly higher expression in tissues compared to blood cells, making them good candidate biomarkers for Day 60 of pregnancy.

4.3 Background

Based on UK government estimates for 2015 (DEFRA, (DEFRA, 2016)) the UK dairy herd comprises 1.8 million cattle which produce 14.3 billion litres of milk annually (7,944 litres per animal per annum). This generates approximately £3.7 billion per annum, which is of significant importance for the UK economy overall. However, for the past 50 years the dairy industry has suffered from reduced profitability, despite a continuous net increase in milk production (which has been estimated at 1 % per cow annually) (Dillon, Berry, Evans, Buckley, & Horan, 2006). Negatively correlated with milk production, fertility decreased during the same period, partially as a consequence of the introduction of North American Holstein-Friesian genes in European cattle populations (Dillon et al., 2006; Diskin et al., 2006). Calving rates as low as 34 % have been reported, with pregnancies being lost mainly during the first trimester in high-producing dairy cows (Diskin et al., 2015). A number of risk factors for poor reproductive performance have been identified, including immune, metabolic and endocrine parameters, with post-partum energy balance being of major importance (Aungier, Roche, Diskin, & Crowe, 2014). Recently, the inclusion of reproductive traits in genetic selection programs has begun in some countries, aiming to stop the decline in reproductive performance in dairy herds, however many issues still need to be addressed (Barbat et al., 2010; Diskin et al., 2015).

Key to the issue of low fertility is the inability to reliably detect early pregnancy in dairy cows, which prevents prompt re-insemination of animals failing to conceive at first service, in turn leading to extended calving intervals and suboptimal milk production (Lucy et al., 2004; NADIS, 2016). Current pregnancy detection methods, including trans-rectal ultrasound, palpation as well as biochemical methods such as P4 measurement in milk, are only accurate after the first three weeks of pregnancy, missing out the critical window of opportunity for re-insemination of cows after failed conception, thus being inadequate to support modern intensive management systems (Balhara et al., 2013).

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional gene regulators (reviewed in (Ul Hussain, 2012)). The intrinsically high stability of

miRNAs in bio-fluids together with, at least for some miRNAs (Z. Guo et al., 2014), their tissue-specific pattern of expression confers miRNAs with a significant potential as novel non-invasive biomarkers of disease (Casey, Kerin, Brown, & Sweeney, 2015; Mitchell et al., 2008; Williams et al., 2013). So far, a plethora of studies have shown the association between specific plasma and serum miRNA signatures and human cancer, heart disease and autoimmune diseases (reviewed here (Haider et al., 2014; He et al., 2015)), as well as, in a reproductive context, pregnancy progression and preeclampsia (Miura et al., 2015; Miura et al., 2010; Q. Wu et al., 2011).

Despite extensive evidence in humans, the potential utility of circulating miRNAs as tissue biomarkers in domestic animals has been largely overlooked, with a handful of recent studies identifying potential biomarkers of feed restriction in chickens and infection in pig and cow (Ahanda et al., 2014; Farrell et al., 2015; Hansen et al., 2016). I recently reported for the first time changes in circulating miRNA levels during early pregnancy in cattle (Chapter 3). Specifically, I identified a robust, significant increase in the plasma levels of miR-26a in dairy heifers during Days 16 and 24 of pregnancy (Ioannidis & Donadeu, 2016b); differences in miRNA levels between pregnant and non-pregnant heifers were in general very small, which may have been attributable to the very early stages of pregnancy being examined. The present report followed on that study by profiling circulating miRNAs at a later stage of pregnancy (Day 60), with the hypothesis that changes in the levels of selected miRNAs from that stage would be larger than those identified in earlier pregnancy, which may allow the retrospective identification of additional early pregnancy biomarkers.

4.4 Materials and Methods

4.4.1 Experimental animals and sample collection

Eleven Holstein-Friesian heifers were oestrus-synchronised and artificially inseminated at oestrus following previously described procedures (Ioannidis & Donadeu, 2016b). Blood samples were collected from the jugular vein in EDTA tubes (Becton Dickinson, USA) on Days 0, 8, 16 and 60 after insemination, as described in Chapter 2, using the ratio between miR-451 and miR-23a as an indicator

of haemolysis (Shah et al., 2016); most samples had a miR ratio < 5 , indicating no haemolysis was present (Figure 4-1).

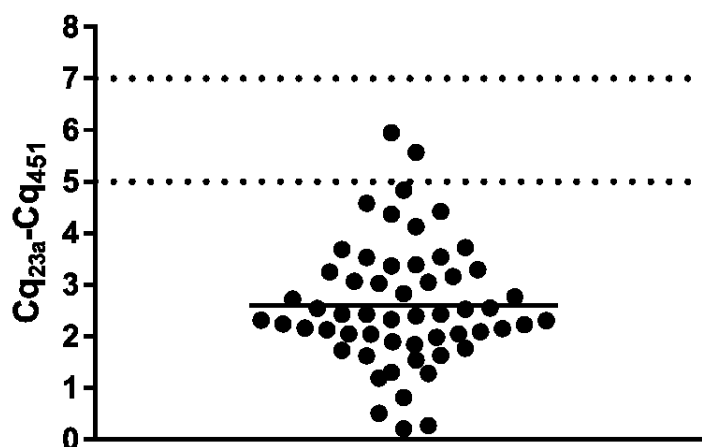


Figure 4-1. Haemolysis screen for plasma samples in this study.

MiR ratio between miR-451 and miR-23a for all of the plasma samples used in this study from Days 0, 8, 16 and 60 of pregnancy, used to detect haemolysis.

Blood cells were prepared from whole blood samples taken from healthy cycling heifers. Whole blood (100 μ L) was centrifuged at 1,900 $\times$ g for 10 min at 4 $^{\circ}$ C to separate blood cells from plasma. The resulting supernatant was discarded and the cell pellet was re-suspended to a volume of 250 μ L. The blood cell solution was then used (fresh) for RNA extraction as described in the section below.

Tissue samples were collected from 3-4 (typically non-pregnant) animals at a local abattoir and were placed in ice-cold PBS until arrival at the laboratory. Additional placental samples were collected from Langhill farm (term and D70 placenta).

Tissues were then dissected, labelled, weighed and snap-frozen using dry ice. All samples were stored at -80 $^{\circ}$ C until further use.

4.4.2 RNA extraction

RNA was extracted from 700 μ L of plasma using TRIzol LS (Life Technologies, USA) following the manufacturer's protocol. Following homogenisation with TRIzol LS, 3.5 μ L of exogenous cel-miR-39-3p (5.6×10^8 copies per sample; Qiagen, Germany) were spiked into the homogenate. Prior to RNA precipitation, 40 μ g of glycogen (Sigma-Aldrich, USA) were added to each sample to facilitate precipitation

of RNA in the presence of 1/10 per volume of 5 M ammonium acetate salt (Sigma-Aldrich) as recommended by the manufacturer. RNA pellets were re-suspended in 20 μ L of RNase-free water (Qiagen) and used immediately in downstream protocols or frozen at -80 °C until further use.

Blood cell suspensions (250 μ L) were homogenised using three volumes of TRIzol LS and extracted as per manufacturer instructions. RNA pellets were re-suspended in 10 μ L of RNase-free water and frozen at -80 °C until further use.

Bovine tissues (50 mg) were thawed in 1 mL of TRIzol LS and disrupted with Lysing Matrix D (MP Biomedicals, UK) and a FastPrep FP120 Tissue Disruptor (Thermo Electron, USA) using two consequent 30-second runs at a speed setting of 5. Following homogenisation, samples were centrifuged at 10,000 g for 5 min at 4 °C and the resulting supernatant was aspirated. The remaining steps for RNA extraction were carried out as described in the manufacturer's protocol and the resulting RNA pellets were re-suspended in 40 μ L of RNase free water. RNA content and quality in blood-cell and tissue samples were monitored using the Nanodrop ND-1000 Spectrophotometer (average RNA yield = 630 ng and A260/280 = 1.9, Thermo Fischer Scientific, USA) and *RnU6-2* expression levels.

4.4.3 Small-RNA sequencing analysis

Small-RNA sequencing was carried out as in Chapters 2 and 3, with the following additional details and modifications. Raw sequencing data (publicly available on the GEO database accession GSE83509) were analysed using the sRNAbench 1.0 tool (Barrett et al., 2013; Barturen et al., 2014). Briefly, the software was run in genome mode using default settings, with the bovine genome (bosTau4) and miRBase 21 (accessed on 30 April 2015) (Elsik et al., 2016; Griffiths-Jones et al., 2008) as reference in order to identify bovine miRNAs (bta-miR) and human miRNA homologues (hsa-miR). A single nt mismatch was allowed when mapping raw reads to known miRNA sequences and reads without sequencing adaptor, with undetermined bases and reads below 15 nt being automatically removed from the analysis. For additional details about the integrated analysis steps in sRNAbench please refer to the software manual.

Normalised reads (reads per million mapped, RPMM) obtained from sRNAbench were filtered before being passed to edgeR 3.10.2 for differential expression analysis in R language 3.2.1 (R Core Team, 2013; Robinson, McCarthy, & Smyth, 2010). Prior to analysis, miRNAs which were detected with less than 25 RPMM in more than 8 samples per experimental group were excluded to increase the confidence of the subsequent analysis and reduce technical noise. Differential expression analysis was performed using edgeR's paired mode on 197 miRNAs (File 4-1) using GLMfit. The statistical significance threshold was set to a false discovery rate (FDR) of 0.05.

For the additional foetal gender analysis we firstly re-filtered the sequencing dataset, including only 258 miRNAs which had RPMM > 25 in at least four animals within the same gender. The differential expression analysis carried out for the sequencing data used $\log_2(x+1)$ transformation of the data and the Shapiro-Wilk test to assess normality. T-tests were used to identify differences between groups for the majority of the miRNAs; in cases where non-parametric distributions were identified we used the Wilcoxon test as a non-parametric alternative, followed by an FDR correction (with the significance threshold set to FDR = 0.05, as above). All analyses of the sequencing data were carried out using R language 3.02 and R Studio 0.98 (R Core Team, 2013; RStudio Team, 2015).

4.4.4 RT-qPCR analysis

For plasma samples, cDNA was generated as described in Chapter 2. For blood cell and tissue samples, 500 ng of RNA were used in each 10 μ L reaction. All reverse transcription and qPCR reactions were prepared as in previous chapters. No-reverse transcriptase and no-template controls were used for each plate. Raw fluorescence data were collected using MxPro software (Agilent Technologies). The amplification efficiency ranged between 81 % and 115 %, with $R^2 > 0.91$. Expression levels were determined using freshly-made standard curves and data were analysed further using Microsoft Excel (Microsoft Corporation, USA). For plasma samples, expression levels were normalised to the mean expression of cel-miR-39-3p and miR-128 (miR-128 was the miRNA with the least variation across samples with coefficient of variation (CV) = 15 %). For blood cells and tissues, expression data were normalised to *RnU6-2*. Samples with distinctly low *RnU6-2* levels were excluded.

Statistical analyses were carried out using GraphPad Prism 7 (GraphPad Software, USA). Differences in miRNA expression between Day 0 and Day 60 were assessed using paired t-tests. For data involving more than two time points one-way rmANOVA was used to determine the effect of Day, followed by Dunnett's test to compare each time point with the Day 0 control. In all cases, normality was tested using the Shapiro-Wilk normality test, outliers were identified with the ROUT test and then removed, and data were $\log_2(x+1)$ transformed to meet the tests' normality criteria. Statistical significance thresholds for all tests were set to $P = 0.05$. For selected circulating miRNAs, normalised expression levels were correlated with plasma P4 levels using Pearson correlation in GraphPad Prism 7 (GraphPad Software), applying transformations as needed.

The methods followed for RT-qPCR analyses in the foetal gender analysis were the same as described above, with the difference that unpaired t-tests were used for differential expression analysis instead of the paired equivalent above.

4.4.5 Genomic cluster and pathway analyses

Information about the number of genomic clusters in cow, their location in the genome and the miRNAs belonging to each cluster was obtained from miRBase 21 (accessed 23/06/2016) (Griffiths-Jones et al., 2008). Bovine genomic clusters with inter-miRNA distance under 10,000 bases were numbered in order of decreasing size as shown on miRBase and the miRNA overlap with my sequencing gene list was identified manually.

Target and pathway analysis was carried out using DIANA miRPath 3.0 (Vlachos, Zagganas, et al., 2015). Experimentally validated miRNA targets were identified using DIANA-TarBase 7.0 and the Pathway Union option (an *a posteriori* method) was used for pathway analysis. The integrated Fischer's exact test followed by FDR adjustment were used for statistical analysis. Enriched pathways from the KEGG database were exported from the tool along with the associated FDR-corrected p-values. A significance threshold of $FDR = 0.05$ was used for the FDR-corrected p-values.

4.5 Results and Discussion

4.5.1 Small-RNA sequencing of bovine plasma

I sequenced a total of 22 plasma samples from Days 0 and 60 of pregnancy (11 cows on each day). On average, I generated 16.1 million raw reads per sample (Table 4-1). There was a distinct peak in read lengths between 20 – 23 nt, which matches the length of most mature miRNAs (Figure 4-2A). Out of the total raw reads, 5.6 million (34.7 %) corresponded to bovine and human mature miRNA sequences (Table 4-1, Figure 4-2B). I detected up to 389 unique miRNAs per sample when applying a miRNA detection limit of 10 reads. The 15 most abundant miRNAs in bovine plasma are listed in Table 4-2. Eleven of these miRNAs were also among the most abundant in my previous cattle study (Ioannidis & Donadeu, 2016b).

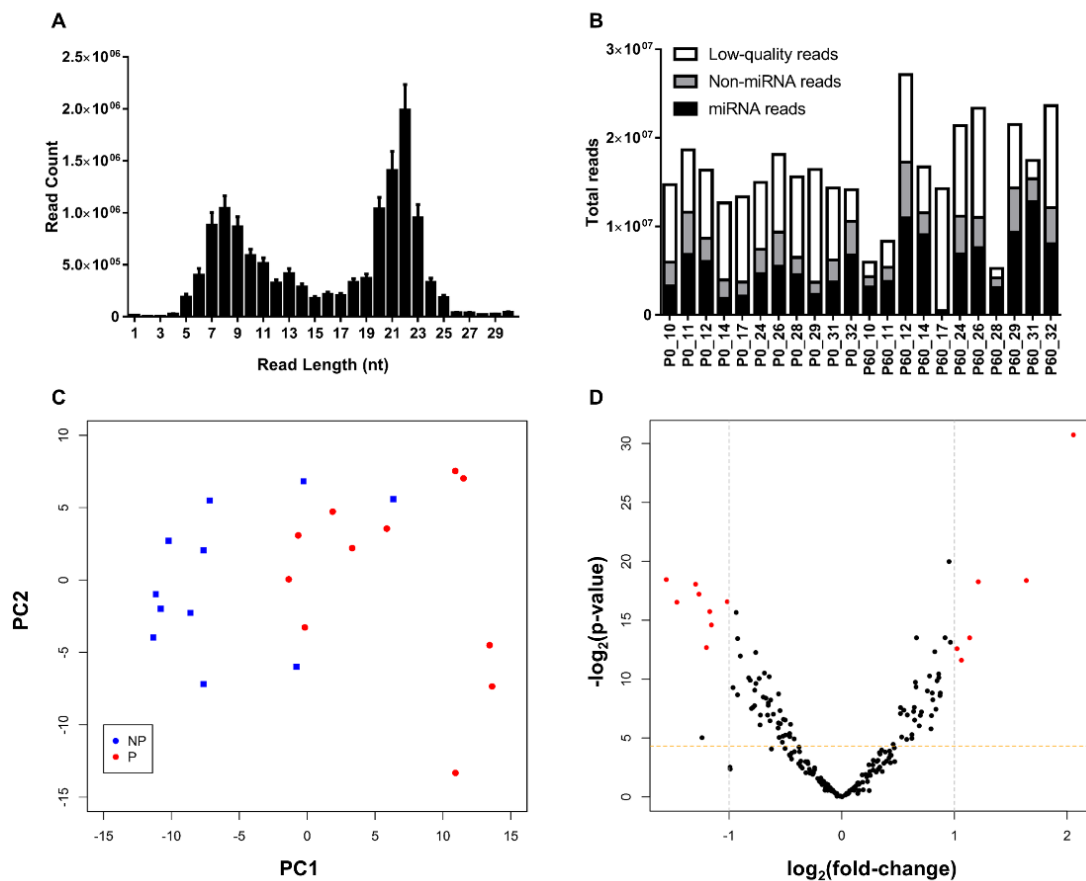


Figure 4-2. Small-RNA sequencing results from Day 60 of pregnancy.

(A) Read length distribution plot for all samples constructed using raw reads. Mean $\pm$ SEM. (B) Barplot showing total, mapped and miRNA reads for each plasma sample. Sample P60_17 generated fewer miRNA reads, however it was included because it clustered normally in subsequent PCA. (C) PCA plot showing the first two principle

components using transformed normalised data. (D) Volcano plot showing the significance of differences between samples from Days 0 and 60 of pregnancy, using transformed normalized data. MiRNAs with fold-change > 2 and FDR < 0.05 are highlighted in red.

Table 4-1. Sequencing analysis endpoints during later pregnancy.

Mean numbers of reads obtained from sequencing of plasma samples on Days 0 and 60 of pregnancy (n = 11 heifers per group) are shown, including reads which were excluded from further analyses.

	Day 0	Day 60	Mean
Raw reads	15,382,609	16,807,294	16,094,951
Reads without adapter (excluded)	120,529	143,187	131,858
Short reads < 15 nt (excluded)	8,185,927	6,897,753	7,541,840
Low quality reads (excluded)	23,372	26,179	24,775
Reads mapping to bovine genome	7,052,780	9,740,175	8,396,478
Bovine miRNA reads	4,322,232	6,814,346	5,568,289
Bovine miRNAs detected at > 10 reads (range)	268-337	193-353	N/A
Human homologous miRNA reads	7,469	5,451	6,460
Human miRNAs detected at > 10 reads (range)	18-32	5-36	N/A

Table 4-2. Most abundant miRNAs in bovine plasma during later pregnancy.

Numbers of miRNA reads in plasma samples from Days 0 and 60 of pregnancy (n = 11 heifers per group) identified by sequencing.

miRNA	Day 0	Day 60	Mean
bta-miR-486	163,304	187,148	175,226
bta-miR-22-3p	115,215	86,953	101,084
bta-miR-192	55,994	45,163	50,578
bta-miR-27b	50,069	39,846	44,957
bta-miR-143	16,617	67,181	41,899
bta-miR-10b	49,529	30,810	40,169
bta-miR-142-5p	31,254	40,063	35,659
bta-miR-191	32,881	33,804	33,343
bta-miR-423-5p	34,453	23,760	29,106
bta-miR-451	19,021	27,249	23,135
bta-miR-21-5p	21,387	24,164	22,775
bta-miR-101	17,776	25,853	21,814
bta-miR-92a	23,371	19,306	21,339

bta-miR-103	27,287	13,565	20,426
bta-miR-30d	17,498	18,882	18,190

From my comprehensive miRNA profiling dataset I sought to identify stable miRNAs which could be utilised for data normalisation in subsequent steps. The miRNA with the lowest variability across samples was miR-128 (CV = 15 %) followed by miRNAs 151-3p and 425-5p (Table 4-3). This result was confirmed by NormFinder, which listed miR-128 as the second least variable miRNA in the dataset after miR-181b, with stability values of 0.07 and 0.06, respectively. I chose miR-128 as an endogenous normaliser for subsequent RT-qPCR assays (see ‘Methods’) over miR-181b on the basis of its higher abundance.

Table 4-3. Most stable miRNAs in bovine plasma in later pregnancy.
The 15 most stable miRNAs in plasma across 22 samples from Days 0 and 60 of pregnancy identified using sequencing.

miRNA	RPMM	Coefficient of variation	NormFinder stability value
miR-128	2687	15%	0.07
miR-151-3p	6732	16%	0.12
miR-425-5p	1724	17%	0.07
miR-1307	266	20%	0.08
miR-181b	1295	21%	0.06
miR-148a	15092	21%	0.09
miR-130b	1085	21%	0.12
miR-148b	351	21%	0.13
miR-181a	13983	22%	0.11
miR-1842	113	23%	0.11
miR-6119-3p	40	23%	0.12
miR-92a	21339	24%	0.13
miR-30a-5p	6808	26%	0.12
miR-27b	44957	27%	0.13
miR-29c	733	27%	0.12
miR-151-5p	1667	27%	0.13
miR-326	174	30%	0.13
miR-194	442	33%	0.13
miR-224	86	37%	0.14

4.5.2 Differentially expressed microRNAs on Day 60

PCA revealed some separation of samples based on the day of pregnancy (Days 0 and 60) as shown in Figure 4-2C. Differential expression analysis identified 77 miRNAs which were differentially expressed on Day 60 compared to Day 0 with $FDR < 0.05$ (Figure 4-2D, Table 4-4; File 4-1). Fourteen miRNAs changed by more than two-fold between groups and the largest difference was observed for let-7c, which increased 4.5-fold on Day 60 of pregnancy.

Table 4-4. Top differentially expressed miRNAs between Day 0 and 60 of pregnancy.

The 15 miRNAs that had the highest fold-change in expression are shown. Values shown for Days 0 and 60 are mean RPMs. Note that miR-4532 is a novel human homologue (hsa-miR-4532, not annotated in miRBase 21 for cow).

miRNA	Day 0	Day 60	Fold-change	FDR
let-7c	273	932	4.53	0.000
miR-143	16617	67181	4.11	0.000
miR-16a	1965	4212	2.68	0.048
miR-155	250	477	2.32	0.000
miR-19a	298	569	2.28	0.001
miR-19b	1036	1913	2.13	0.003
let-7f	4265	6502	2.12	0.012
miR-32	387	792	2.01	0.002
miR-374b	71	112	2.00	0.007
miR-26a	9042	13806	1.95	0.006
miR-26b	2971	4506	1.94	0.011
miR-324	41	18	0.51	0.001
miR-665	103	45	0.51	0.000
miR-410	267	124	0.50	0.000
miR-328	143	54	0.49	0.000
miR-23a	7123	2795	0.49	0.000
miR-99b	1142	414	0.49	0.002
miR-424-3p	32	11	0.47	0.000
miR-205	1072	344	0.46	0.000
miR-4532	945	229	0.42	0.000

I next sought to determine which of the differentially expressed miRNAs were organised in genomic clusters. MiRBase lists 82 miRNA clusters in the bovine genome ranging in size from two to forty-seven miRNAs. Thirteen of these miRNA

clusters were represented in my list of differentially expressed miRNAs based on the presence of at least two miRNAs for any given cluster; these clusters contained a total of 31 out of the 77 miRNAs differentially expressed between Days 0 and 60. Notable clusters which were enriched for differentially expressed miRNAs included the C14MC cluster, represented by five miRNAs, and the miR-17-92 and miR-106b-25 clusters, represented by five and two miRNAs, respectively (Table 4-5). The C14MC cluster is known to be expressed in the placenta and is involved in foetal development; circulating levels of members of this cluster have been shown to increase during human pregnancy (Gu, Sun, Groome, & Wang, 2013; Labialle et al., 2014; Miura et al., 2010; D. M. Morales-Prieto et al., 2012). The miR-17-92 and miR-106b-25 clusters are paralogues, with a role in VEGF-mediated angiogenesis in endothelial cells through PTEN targeting and a suspected role in placental immune tolerance during the first trimester of pregnancy; both clusters are highly expressed in the human placenta (Chamorro-Jorganes et al., 2016; Gu et al., 2013; Thapa, Li, Jamieson, & Martinez-Maza, 2011; P. Xu, Zhao, Liu, Wang, Wang, Li, Zhu, Yao, Wang, et al., 2014). Finally, several members of the let-7 family were spread across multiple genomic clusters (Table 4-5). This miRNA family is differentially expressed in the human placenta during gestation, suggesting it may play a functional role in placental processes (Gu et al., 2013).

Table 4-5. Clusters enriched for differentially expressed miRNAs in pregnancy Day 60.

Bovine genomic clusters represented by at least two miRNAs from sequences that were differentially expressed in plasma on Day 60 of pregnancy compared to Day 0.

Cluster	Genomic Location	No. of miRNAs in cluster	Different miRNAs in cluster
miR-379-656	chr21:67561869-67604767	47	mir-380, mir-154c, mir-381, mir-369, mir-410
let-7a-3-3596	chr5:117119385-117120270	4	let-7a-3, let-7b
let-7a-1-7d	chr8:86884872-86887517	3	let-7a-1, let-7f-1
miR-100-let-7a-2	chr15:33353395-33347640	2	mir-100, let-7a-2
let-7-2-miR-98	chrX:96383532-96382746	2	let-7f-2, mir-98
miR-106b-25	chr25:36892046-36892532	3	mir-106b, mir-93
miR-17-92	chr12:66226554-66227332	6	mir-17, mir-18a, mir-19a, mir-20a, mir-19b
miR-199a-214	chr16:40491602-40485916	3	mir-199a-1, mir-214
miR-23a-24	chr7:12981970-12981713	3	mir-23a, mir-27a
miR-424-450b	chrX:18185439-18178985	7	mir-424, mir-450a-2, mir-450a-1, mir-450b
miR-452-3431	chrX:34665624-34662756	3	mir-452, mir-224, mir-3431

miR-493-136	chr21:67416605-67431509	7	mir-665, mir-136
miR-99b-125b	chr18:58014868-58015620	3	mir-99b, mir-125a

4.5.3 Validation of sequencing data using RT-qPCR

In an attempt to validate my sequencing results, I selected 13 miRNAs to be profiled in plasma using RT-qPCR. Eleven miRNAs were selected from the sequencing gene list (File 4-1) based on both relative abundance and fold-change between Days 0 and 60. I also included miR-30c and miR-152 as their expression tended to differ between the two days ($FDR < 0.1$) and moreover I previously found these miRNAs to be differentially expressed with $P < 0.05$ during early pregnancy in a previous experiment (Ioannidis & Donadeu, 2016b). RT-qPCR confirmed the significant differences in the expression of miR-7c, -7f, -101, -30c, -26a and -143 (Figure 4-3, Table 4-6), with an up to 6-fold increase on Day 60 for miR-26a and miR-143. Thus, six out of 13 differences in miRNA expression identified by sequencing were validated by RT-qPCR, comparable to the average between-platform concordance rate of 55 % reported by a recent study (Mestdagh et al., 2014).

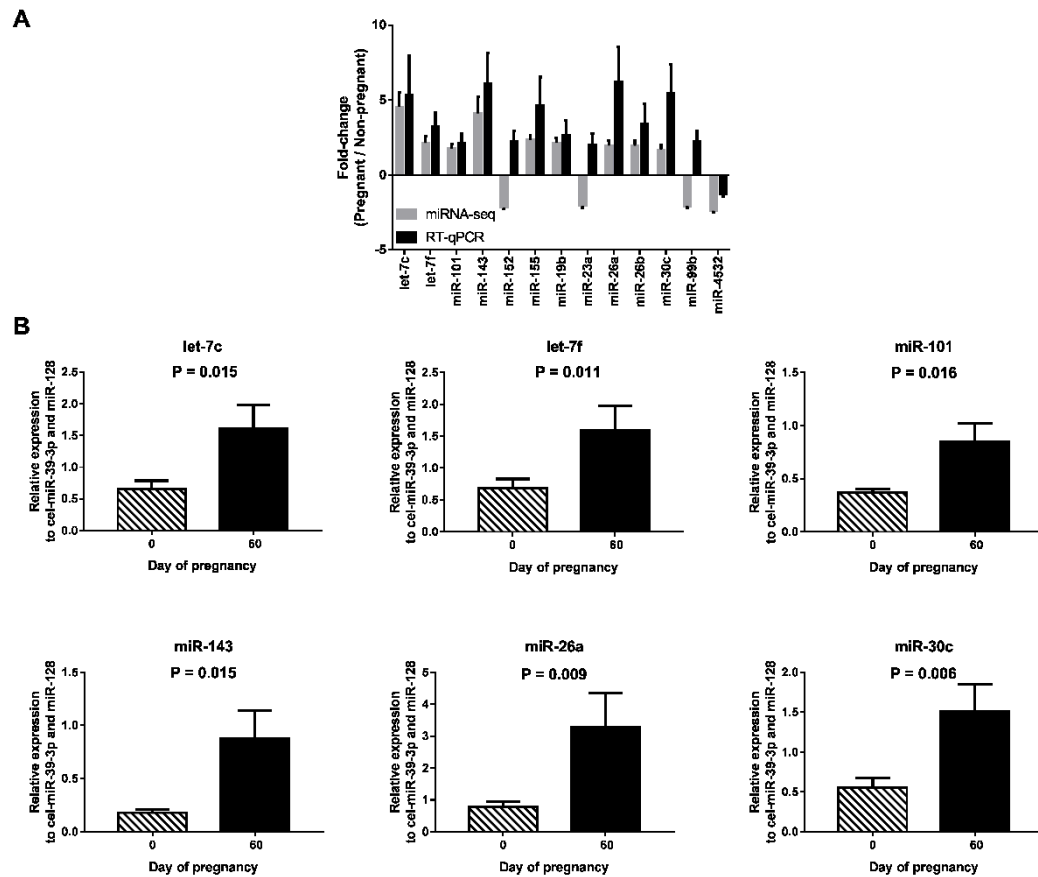


Figure 4-3. RT-qPCR validation of sequencing results on Day 60 of pregnancy.

(A) Fold-changes in plasma miRNA levels identified during pregnancy using sequencing and RT-qPCR. Mean $\pm$ SEM. (B) Relative expression of six miRNAs on Days 0 and 60 of pregnancy obtained by RT-qPCR. Mean $\pm$ SEM.

Table 4-6. Validation of sequencing results from pregnancy Day 60 using RT-qPCR.

Comparison of data for differentially expressed miRNAs between Days 0 and Day 60 of pregnancy obtained by sequencing and RT-qPCR.

	Next-generation sequencing			RT-qPCR		
miRNA	Fold-change	P-value	FDR	Fold-change	P-value	Concordance with sequencing results
bta-let-7c	4.53	0.000	0.000	5.30	0.015	✓
bta-let-7f	2.12	0.003	0.012	3.25	0.011	✓
bta-miR-101	1.76	0.003	0.014	2.12	0.016	✓
bta-miR-143	4.11	0.000	0.000	6.11	0.015	✓
bta-miR-155	2.32	0.000	0.000	4.60	0.053	
bta-miR-19b	2.13	0.000	0.003	2.63	0.929	
bta-miR-23a	0.49	0.000	0.000	2.00	0.312	
bta-miR-26a	1.95	0.001	0.006	6.22	0.009	✓
bta-miR-26b	1.94	0.002	0.011	3.38	0.054	
bta-miR-99b	0.49	0.000	0.002	2.20	0.687	
hsa-miR-4532	0.42	0.000	0.000	0.77	0.161	
bta-miR-30c	1.67	0.032	0.075	5.50	0.006	✓
bta-miR-152	0.46	0.031	0.073	2.21	0.869	

The expression levels of all six miRNAs were positively correlated with circulating P4 levels ($P < 0.01$, Pearson correlation > 0.5) during pregnancy. The highest correlations were observed for miR-30c ($r = 0.688$, $P = 0.001$), followed by miR-26a ($r = 0.654$, $P = 0.002$). Me and others previously identified significant correlations between miRNAs and steroid hormones in bovine follicular fluid (Donadeu, Sontakke, & Ioannidis, 2016) and there is evidence in support of functional inter-relationships between steroid hormones (including P4) and miRNAs (including let-7 and miR-26a) (Cochrane, Cittelly, & Richer, 2011). However, I did not find significant differences in the levels of circulating miR-26a, miR-205, miR-30c or the miR-26a/205 between the follicular and luteal phases of the oestrous cycle (low-high P4) in a separate study, which suggests the circulating levels of these miRNAs are not systemically dependent on P4 levels during the oestrous cycle (Ioannidis &

Donadeu, 2016a). Future work should further investigate the functional relationships between these circulating miRNAs and steroid hormones during bovine pregnancy.

4.5.4 Circulating microRNA levels throughout early pregnancy

Among the six miRNAs confirmed by RT-qPCR to be upregulated in plasma on Day 60 of pregnancy, miR-26a had already been shown to be increased in plasma as early as D16 (Chapter 3, (Ioannidis & Donadeu, 2016b)). I hypothesised that additional miRNAs may also increase at earlier stages of pregnancy (at or before D16), and that their identification may pave the way for developing novel early pregnancy diagnostics, possibly in the form of a miRNA panel. Accordingly, I profiled eight miRNA candidates from my sequencing data (let-7f, let-7c, miR-30c, miR-101, miR-26a, miR-143, miR-205 and miR-19a; File 4-1) by RT-qPCR on sequential plasma samples collected on Days 0, 8, 16 and 60 of pregnancy. For seven miRNAs (all except miR-19a) there was a significant effect of Day of pregnancy on miRNA expression levels (Figure 4-4). Inspection of individual miRNA profiles revealed that, on average, miR-101 and miR-30c gradually increased through Days 8, 16 and 60 although an increase relative to Day 0 was significant ($P < 0.05$) only at D60. In contrast, the mean levels of let-7c, let-7f and miR-143 remained unchanged until D16, increasingly significantly between 2.1- and 11.1-fold on Day 60. Interestingly, mean levels of miR-26a were distinctly increased as early as D8 (3.1-fold; $P = 0.046$) with no further elevation until up to D60 (Figure 4-4), suggesting a role for this miRNA very early during pregnancy. I also profiled miR-205 which, consistently with my sequencing data (Table 4-4), decreased during early pregnancy, although this was significant only on Day 60 (Figure 4-4). Because changes in miR-26a and miR-205 showed opposite trends starting very early during pregnancy I calculated the ratio between these two miRNAs to determine whether it may provide a potential biomarker of early pregnancy; indeed the miR-26a/205 ratio increased 7.5-fold between non-pregnant (Day 0) and Day 8 pregnant heifers which could be used in the future as a molecular indicator of very early pregnancy in cattle (Figure 4-4).

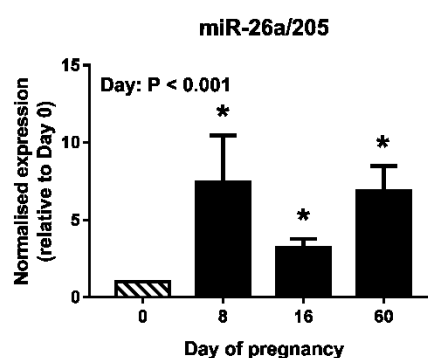
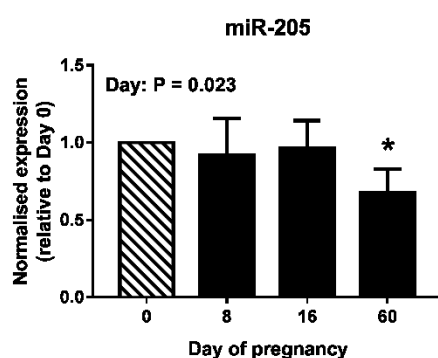
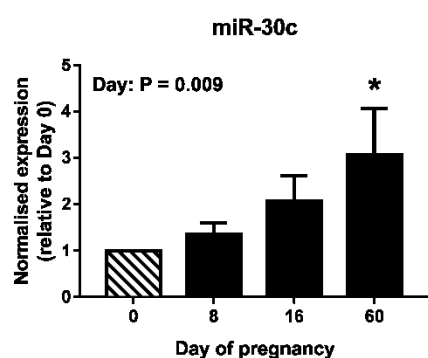
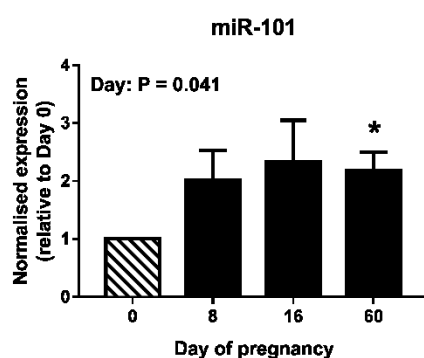
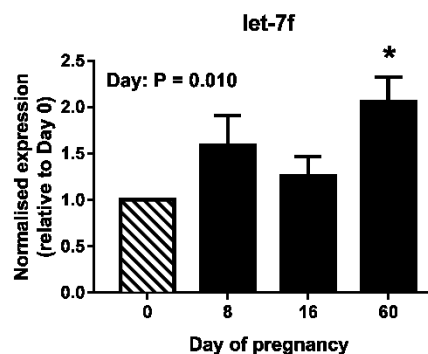
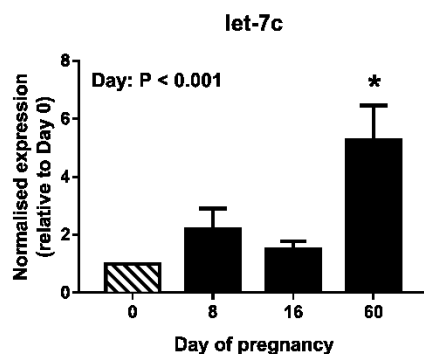
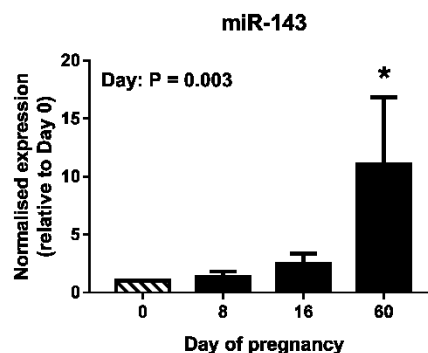
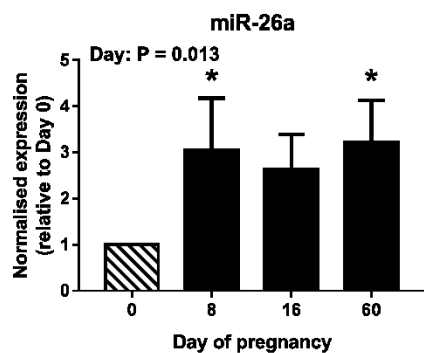


Figure 4-4. Timecourse experiment for pregnancy-associated miRNAs using RT-qPCR.

Relative expression of selected miRNAs in plasma during Days 0, 8, 16 and 60 of pregnancy relative to Day 0 (Mean $\pm$ SEM). Means with an asterisk are different ($P < 0.05$) from means on Day 0 (see Methods for more details).

It is possible that the differentially expressed miRNAs above cooperate during pregnancy. Four candidates, miR-143, miR-101, miR-30c and miR-26a, are involved in the regulation of metabolism according to previous studies, including insulin signalling, lipid metabolism and diabetes (Floris et al., 2015; Gil-Zamorano et al., 2014; Kilic et al., 2015). More specifically, there is evidence that miR-26a regulates insulin sensitivity in the liver (Fu et al., 2015). These miRNAs could putatively be involved in the metabolic changes that occur in the mother in the first trimester of pregnancy. In addition to metabolism, miR-26a and let-7f have been identified as regulators of immunity. Specifically, miR-26a regulates the levels of interferon-beta (INF- β) and interferon-gamma (IFN- γ) in macrophages and it has been shown also to regulate T-cell function via interleukin-6 (IL-6) (S. Liang, Wang, & Gou, 2015; Ni, Rajaram, Lafuse, Landes, & Schlesinger, 2014; Witwer et al., 2010). Another miRNA, let-7f, regulates interleukin-23 (IL-23) and interleukin-17 (IL-17) in T-cells (Z. Li, Wu, Brant, & Kwon, 2011). These miRNAs could cooperate to induce immune tolerance in the mother, which is required for the maintenance of pregnancy.

Finally, miR-101, miR-205 and miR-26a are known regulators of angiogenesis, which is another function that would be relevant in pregnancy because of placentation. Regulation of angiogenesis is achieved by miR-205 through targeting of transforming growth factor beta (TGF- β) (Tabruyn et al., 2013), while miR-26a has been shown to inhibit angiogenesis in endothelial cells by targeting similar to mothers against decapentaplegic 1 (SMAD1) (Icli et al., 2013) and miR-101 is an indirect regulator of vascular endothelial growth factor (VEGF) which drives angiogenesis (Smits et al., 2011; C. N. Zhang et al., 2010). Out of the miRNAs I profiled, miR-26a changed very early in pregnancy (Day 8) compared to the remaining miRNAs. This may indicate that this miRNA acts individually at the stage. A likely hypothesis for the function of miR-26a at such an early stage in pregnancy would be an immunomodulatory role, mediated by circulating blood cells. However it is also possible that this miRNA cooperates with other, unidentified

miRNAs which change by smaller increments to serve this or another role on Day 8 of pregnancy. Further work is required to investigate potential functional links between those miRNAs and the processes mentioned above during pregnancy.

4.5.5 Pathway analysis of pregnancy related microRNAs

In order to investigate the potential functions of the differentially expressed miRNAs in pregnancy I carried out pathway enrichment analysis using experimentally validated targets of the up-regulated and down-regulated miRNAs on Day 60 of pregnancy in two independent analyses. The analyses were carried out using the DIANA web-tool (Vlachos, Zagganas, et al., 2015) and I focused on non-neoplastic pathways for interpretation of the results. There was significant overlap between enriched pathways targeted by up-regulated and down-regulated miRNAs (File 4-2). Pathways included fatty acid metabolism, extracellular cell membrane (ECM)-receptor interaction, Hippo signalling and the thyroid hormone, p53 and cell cycle pathways. Up-regulated miRNAs in pregnancy also targeted the Foxhead O (FOXO) and TGF- β signalling pathways which are involved in apoptosis. It is difficult to elaborate on the potential functions of these miRNAs without first identifying their tissue(s) of origin during pregnancy, however this analysis suggests some miRNAs may be involved in metabolism, embryonic development and apoptosis.

4.5.6 Tissue expression of pregnancy-associated microRNAs

To investigate the origin of the miRNAs with differential expression levels in plasma, and at the same time gain insight on their potential role during early pregnancy, I decided to complement my analyses by performing expression profiling in a range of 14 tissues from cows, including blood cells. Previous studies have highlighted the importance for peripheral tissue-derived miRNAs not to be simultaneously expressed by blood cells if they are to be used as robust biomarkers (Pritchard et al., 2012); to this end, this analysis would highlight potential tissue-specific or tissue-enriched miRNAs (Pritchard et al., 2012).

Six of the seven miRNAs profiled (let-7c, let-7f, miR-101, miR-143, miR-30c and miR-26a) were expressed in a range of bovine tissues including the uterus and the placenta, with no particular tissue being clearly enriched for any particular miRNA

(Figure 4-5). The miRNA profiles obtained were consistent with those obtained using human tissues (Landgraf et al., 2007; Y. Liang et al., 2007). Notably, relative expression levels for most miRNAs were lowest in both early (day 70) and term placenta, indicating the developing conceptus is not a significant source of these miRNAs in plasma. In addition, relative expression profiles suggested that, for all miRNAs except miR-143 and miR-205, blood cells are a significant source of plasma miRNAs. Interestingly, miR-205 was clearly enriched in skin, with mean expression in that tissue being more than 8,000-fold higher than in any other tissue (Figure 4-5) and its expression was very low in blood cells, indicating that circulating levels of this miRNA are largely tissue-derived. In human and mouse, miR-205 is expressed in a range of tissues including the thymus, mammary gland, early embryo, digestive tissue and skin, making it difficult to hypothesise on possible functions (Abd El Naby et al., 2011; G. Liang et al., 2014; Y. Liang et al., 2007; M. Wang et al., 2012). However, a recent study in mouse found that miR-205 is downregulated in keratinocytes where it de-represses integrin subunit alpha 5 (ITGA5) and assists in wound healing (T. Wang et al., 2016). A possible hypothesis would be that miR-205 is involved in the development of the embryonic skin which would explain its downregulation on Day 60 of pregnancy, although it seems more likely that differential levels during angiogenesis (discussed previously) would be reflected in the maternal circulation. Further work is required to elucidate the role of miR-205 in pregnancy.

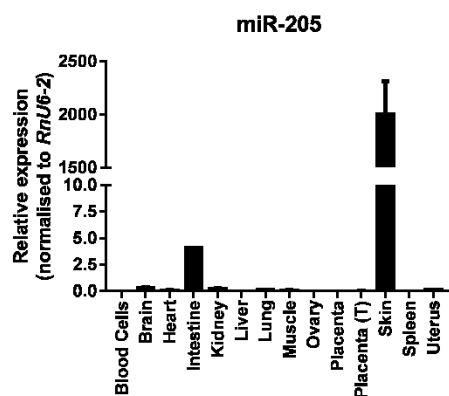
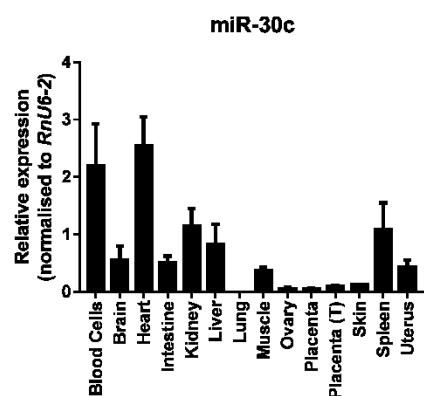
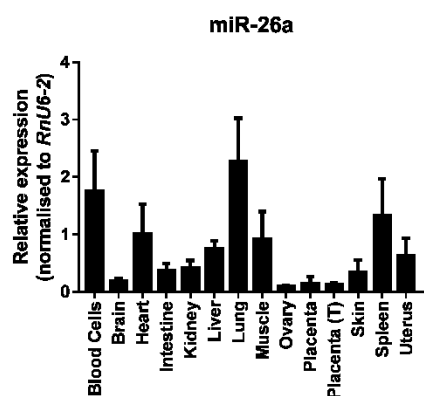
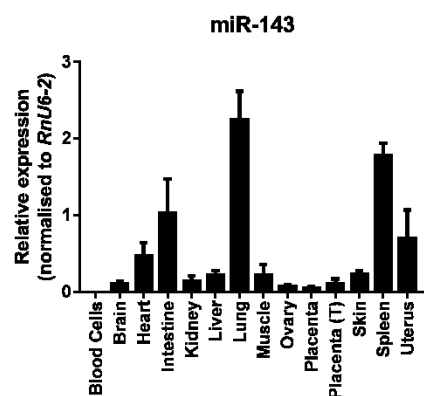
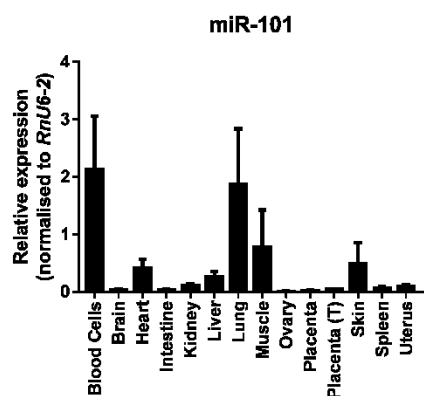
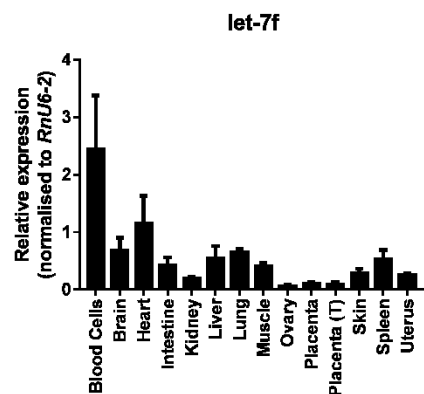
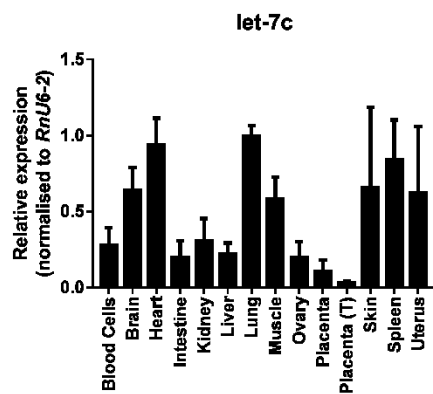


Figure 4-5. Expression of pregnancy-associated miRNAs in different tissues. Relative expression of pregnancy-associated miRNAs in 14 tissues from healthy cows (n = 3 animals per tissue). Placenta: Placenta from Day 70 of pregnancy; Placenta (T): Term placenta.

4.5.7 Foetal gender analysis using sequencing and RT-qPCR

As an additional set of analyses to complement my pregnancy analysis, I used sequencing miRNA expression data from Day 60 of pregnancy to identify differentially expressed miRNAs in plasma between foetal genders. Such miRNAs may provide non-invasive biomarkers of foetal gender. Out of the 11 pregnant animals in my study, six and five carried female and male foetuses, respectively. Differential expression analysis of 258 miRNAs identified no differentially expressed miRNAs on Day 60 of pregnancy after multiple testing correction (File 4-3). However four miRNAs (hsa-miR-6516-3p, hsa-miR-28-3p, hsa-miR-4508 and hsa-miR-5693, Figure 4-6) changed by more than two-fold between groups and had P-values lower than 0.05. Notably, miR-5693 was absent from the plasma of animals carrying male foetuses, but there is no literature suggesting possible functions of this miRNA (Figure 4-6). I also extended my analysis to earlier stages of pregnancy, looking for differences in circulating miRNA expression between male and female embryos on Days 16 and 24 of pregnancy (File 4-3) using sequencing data from my previous study (Ioannidis & Donadeu, 2016b). Only let-7a-3p changed significantly ($FDR < 0.01$) between groups, however it was only 1.3-fold higher in males and it was therefore excluded from further analyses.

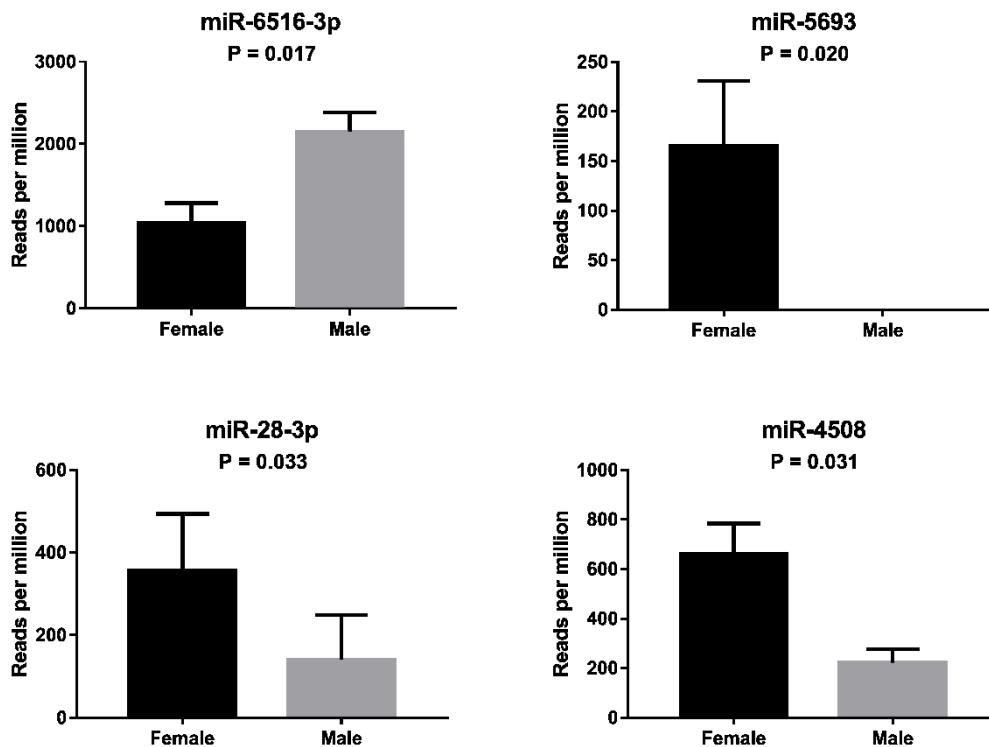


Figure 4-6. Foetal gender analysis candidates.

Sequencing expression profiles of four miRNAs which changed by more than two-fold between animals carrying foetuses of opposite sex on Day 60 of pregnancy. FDR > 0.1 for all miRNAs. Mean $\pm$ SEM; n = 6 female / 5 male.

In recent years two independent human studies on circulating miRNA gender differences have been published; unfortunately the agreement between those studies was limited to hsa-miR-28-3p which was identified as female-enriched (Ameling et al., 2015; Tonge & Gant, 2016). My sequencing data from Day 60 of pregnancy agree with this result. An additional 9 miRNAs, including miR-213, miR-17-3p, let-7a-5p, let-7f, let-7g and let-7i had common trends between my results and those of Tonge et al. (Tonge & Gant, 2016). Agreement with Ameling et al. (Ameling et al., 2015) was more limited, with only three additional agreeable miRNA profiles for miR-16b, miR-28-3p and miR-451.

Based on these findings I attempted to confirm the results for the miRNAs with the largest fold-changes (hsa-miR-6516-3p, hsa-miR-28-3p, hsa-miR-4508 and hsa-miR-5693) using RT-qPCR assays. Unfortunately, out of the four miRNAs above, only hsa-miR-28-3p was adequately quantifiable using RT-qPCR in a subset of 10 plasma

samples and there was no difference between animals carrying foetuses of different gender ($P > 0.1$, fold-change = 0.99). The low abundance of the differentially expressed miRNAs when using RT-qPCR was clearly a limiting factor in this analysis. Because of this and also due to the agreement between my sequencing data and previous studies in human, I cannot exclude the possibility that small gender-related differences exist during these stages in pregnancy.

4.6 Conclusions

I have identified a subset of miRNAs (miR-205, let-7f, let-7c, miR-30c, miR-101, miR-26a, miR-205 and miR-143) which are differentially expressed in the circulation on Day 60 of pregnancy in cattle compared to D0. Significantly, I have identified an increase in plasma levels of miR-26a as early as Day 8 of pregnancy, much earlier than previously reported for any miRNA during pregnancy in any species.

Importantly, miR-26a levels alone or in combination with miR-205 (miR-26a/miR-205 ratio), provides a novel and potentially valuable biomarker of very early pregnancy. The exact origin of the increased plasma miRNAs identified in this study is unknown, however I highlight miR-205 as potential skin-derived miRNA to follow up in future work.

5 Tissue Function Biomarkers

5.1 Chapter Introduction

Recent publications in the circulating miRNA biomarker field highlighted the need for identification of the origin of candidate biomarkers and suggested focusing on non-blood cell derived miRNAs. In this chapter I attempt to address these recommendations by distinguishing blood-cell miRNAs from tissue-derived miRNAs in the cow and profiling poorly-characterised bovine miRNAs across a range of bovine tissues, hoping to identify novel potential biomarkers of tissue function. The contents of this chapter will be submitted for publication with the title ‘Novel tissue-enriched miRNAs in the bovine circulation’.

5.2 Abstract

Circulating miRNAs have been proposed as biomarkers of several human diseases in human, however recent reports highlighted inconsistencies among biomarker studies. In the bovine context, limited resources are available for the identification of tissue-derived circulating miRNAs which carry the potential to provide robust biomarkers. In this study I attempted to identify novel tissue-derived circulating miRNAs which may be useful as biomarkers of tissue function in cow. Using sequencing, I identified 92 miRNAs with significantly higher expression in plasma compared to paired whole blood cells ($n = 4$). I then validated results for eight out of nine profiled candidates using RT-qPCR in six samples. These miRNAs are hypothesised to be tissue-derived and potentially tissue-specific. I then confirmed the tissue-specific expression of miR-122 (liver), miR-133a (muscle) and miR-215 (intestine) in cow, and I also established tissue-expression profiles for ten previously uncharacterised miRNAs in cow. I highlight circulating hsa-miR-802, a human homologue which was highly enriched in liver and is reported to regulate insulin sensitivity in humans and mice.

I propose that this novel circulating miRNA in cow may be useful as a tissue-specific biomarker of liver function, which could be further investigated in the context of post-partum negative energy balance in dairy cows. I also invite researchers to use my next-generation dataset as a resource to identify additional novel tissue-specific circulating miRNAs in cow.

5.3 Background

Since the discovery of *lin-4* in *Caenorhabditis elegans* in 1993, the microRNA (miRNA) field has expanded and matured (Casey et al., 2015; R. C. Lee, Feinbaum, & Ambros, 1993). It is now known that miRNAs are short, non-coding RNA molecules which are transcribed in cells and primarily fine-tune gene expression at the post-transcriptional level (Hammond, 2015). Following the discovery of their basic functional mechanism, the community worked towards uncovering the roles of miRNAs in mammalian biology. We have now established that miRNAs are involved in the regulation of virtually all biological processes, from embryonic tissue development (miR-134 in neurons, miR-122 in liver (Finch, Marquardt, Yeoh, & Callus, 2014; Motti, Bixby, & Lemmon, 2012)) to metabolism (miR-33 and miR-103 (Vienberg et al., 2016)) and reproduction in the adult (miR-202 (Sontakke et al., 2014)) and finally in disease (miR-21, miR-9 and the let-7/98 cluster in cancer (Farazi, Hoell, Morozov, & Tuschl, 2013)).

The discovery of cancer-related miRNAs in human in combination with the detection of miRNAs in biofluids in 2008 (Mitchell et al., 2008) sparked a wave of research into the potential use of extracellular miRNAs as biomarkers of disease. In recent years, circulating miRNAs have been proposed as potential diagnostic biomarkers of cancer (miR-21, miR-20, miR-221 (He et al., 2015; Schwarzenbach et al., 2014)), cardiovascular disease (miR-1, miR-133a (Creemers et al., 2012)), liver disease (miR-122 (Afonso, Rodrigues, Simao, & Castro, 2016)) and diabetes (miR-375 and miR-34 (Guay & Regazzi, 2013)) in humans. Progress in livestock miRNAs has been considerably slower, with a handful of studies linking circulating miRNA profiles with pregnancy (Ioannidis & Donadeu, 2016b), infection (Farrell et al., 2015; Hansen et al., 2016) and feed deprivation (Ahanda et al., 2014).

However the identification of novel non-invasive biomarkers of disease was followed by a number of cautionary studies highlighting contradictions in circulating miRNA research publications. A large number of candidate miRNA biomarkers of cancer were shown to be blood cell derived and were affected by blood cell numbers (Pritchard et al., 2012). This was supported by a second review of published studies in breast cancer which identified inconsistencies among studies and could not

reproduce published results (Leidner et al., 2013). In addition, a review of non-neoplastic miRNA biomarkers found that the majority of proposed candidates were non-specific and/or biologically implausible (Haider et al., 2014). These studies highlight the need for biomarker discovery studies to attempt to identify the origin of potential miRNA biomarkers and determine their tissue-enrichment in the tissue(s) of interest.

In recent years changes in miRNA levels have been identified in response to food deprivation, grazing, liver stress and infection in livestock species (Ahanda et al., 2014; Farrell et al., 2015; Fatima, Lynn, O'Boyle, Seoighe, & Morris, 2014; Hansen et al., 2016). These differential profiles could potentially provide useful biomarkers of pathophysiology in farm animals. To this end, but also as a resource for the livestock miRNA research community, I sought to identify potentially novel biomarkers of tissue function in cattle. I therefore hypothesised that previously uncharacterised, tissue-enriched miRNAs may be detectable in the circulation and in parallel absent from blood cells, which may be useful as novel biomarkers of tissue function in cattle. Through next-generation sequencing I identified several potentially tissue-enriched miRNAs and identified a previously-uncharacterised liver-specific miRNA in cow, miR-802, which may prove useful as a biomarker of metabolic stress.

5.4 Materials and Methods

5.4.1 Experimental animals and sample collection

Blood samples were collected from six healthy, open Holstein-Friesian cross cows at Langhill Farm of the University of Edinburgh. Tissue samples were collected from cows at a local abattoir with permission. All animal procedures were carried out under a UK Home Office license and with the relevant ethics approval (see Chapter 2).

Blood samples, blood cells and tissues were collected and processed as described in previous chapters (Chapter 2 and 4) with minor modifications. Following plasma preparation, plasma samples were filtered through 0.2 µm syringe filters (Sartorius, Germany) to ensure the removal of any residual contaminating cells and/or debris.

5.4.2 RNA extraction

RNA was extracted following the same procedures as in Chapter 4 for all samples (plasma, blood cells and tissues).

5.4.3 Small-RNA sequencing

Small-RNA sequencing was carried out as in Chapter 4 with minor modifications. Following initial data processing, read mapping and normalisation, miRNAs which were detected with less than 25 RPM in more than two samples per group were excluded to increase the confidence of the subsequent analysis and reduce technical noise. Differential expression analysis was performed using edgeR's paired mode on 212 miRNAs (File 5-1) using GLMfit. The statistical significance threshold was set to FDR = 0.05.

5.4.4 RT-qPCR

RT-qPCR analysis was as described in Chapter 4, with minor modification of the statistical analysis. The amplification efficiency of qPCR reactions ranged between 84.5 % and 117.4 %, with $R^2 > 0.86$. Expression levels were normalised to the mean expression of spiked-in cel-miR-39-3p for blood cells and plasma. For the tissue panel experiment, expression data were normalised to *RnU6-2*. Samples with low *RnU6-2* levels were excluded to ensure high quality of results.

Statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software, USA). Differences in miRNA expression between blood cells and plasma (six samples per group) were assessed using paired t-tests. In all cases normality was tested using the Shapiro-Wilk normality test, outliers were tested with the ROUT test before removal and data were $\log_2(x+1)$ transformed to meet the tests' normality criteria. Statistical significance thresholds for all tests were set to $P = 0.05$.

5.4.5 Genomic analyses

In order to identify miRNAs for profiling in my tissue panel (unique in cow or human homologues not previously identified in cow) I used Ensembl genome browser 85 (www.ensembl.org, (Aken et al., 2016)) NCBI (www.ncbi.nlm.nih.gov) and miRBase 21 (www.mirbase.org (Kozomara & Griffiths-Jones, 2011)) to look for miRNA annotations and their conservation in cow. I also used BLAST (accessed via

Ensembl at www.ensembl.org/Multi/Tools/Blast?db=core, (Camacho et al., 2009)) with default settings to identify the genomic location of selected miRNAs.

5.5 Results and Discussion

5.5.1 Small-RNA sequencing of blood cells and plasma

In order to identify circulating miRNAs which are more abundant in plasma compared to blood cells, I sequenced miRNAs in cells and plasma from four animals in a paired sample experimental design. The read length distributions (Figure 5-1A) show that both sample types are enriched for reads of 20-23 nt, which are characteristic of mature miRNAs. However, there is a much more distinct peak at this read length for blood cells compared to plasma samples, and the latter also generated a secondary peak for reads of shorter length (7-9 nt) which most likely consists of degradation products (Figure 5-1A). This difference between samples is likely the result of the lower RNA content of plasma and of the difficulty to visualise library bands clearly on a gel in the preparation process; this forces the inclusion of shorter sequences in the library when working with plasma samples.

On average, I obtained 14.9 million raw reads per sample (Table 5-1). After removing reads with low quality (reads without adapter, short reads and reads with multiple undetermined base calls), on average I mapped 13.3 million and 5.9 million reads to the bovine genome (see Methods) which correspond to 94.3 % and 38.1 % of the total reads for blood cells and plasma, respectively (Table 5-1, Figure 5-1B). The marked difference in the number of mapped reads is likely because of the low abundance of RNA in plasma samples compared to blood cells. On average, blood cell samples generated 12.3 million miRNAs reads and plasma samples generated 2.7 million reads, the majority of which were bovine miRNAs (Table 5-1, Figure 5-1B). I detected up to 315 unique miRNAs in plasma samples and 305 in blood cell samples and only 17 and 16 of these miRNAs were human homologues, respectively, when applying a detection threshold of 10 reads per sample (Table 5-1).

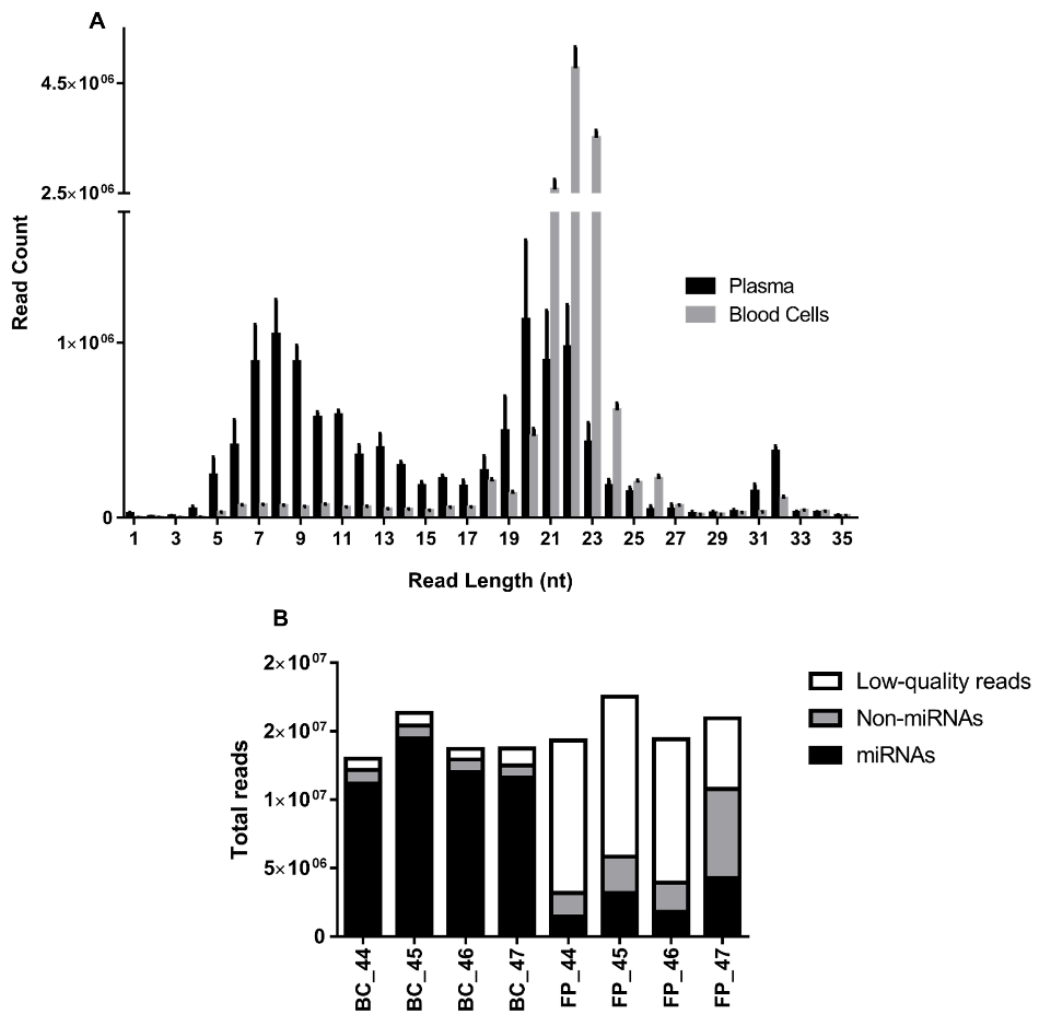


Figure 5-1. Small-RNA sequencing analysis parameters from cells and plasma. (A) Read length distribution plot for all samples constructed using raw reads. Mean $\pm$ SEM, four samples per group. (B) Barplot showing total, mapped and miRNA reads for blood cell samples (left, starting with 'BC') and plasma samples (right, starting with 'FP') in the sequencing experiment.

Table 5-1. Sequencing analysis endpoints from blood cells and plasma.

Mean values for each experimental group are shown, including reads which were excluded from further analyses. Detected miRNAs: the maximum number of miRNAs identified in a sample with more than 10 reads. Four samples in each group.

	Blood Cells	Plasma	Mean
Raw reads	14,194,750	15,553,150	14,873,950
Reads without adapter (excluded)	237,726	127,738	182,732
Short reads (excluded)	672,891	9,469,537	5,071,214
Low quality reads (excluded)	25,532	20,587	23,059
Mapped reads	13,258,602	5,935,288	9,596,945
Total miRNA reads	12,309,110	2,688,243	7,498,677
of which bovine	12,307,733	2,683,651	7,495,692
of which human	1,377	4,592	2,984
Detected miRNAs (> 10 reads)	305	315	N/A
of which bovine	289	298	N/A
of which human	16	17	N/A

I observed some differences in the most abundant miRNAs between blood cells and plasma as determined using sequencing (Table 5-2). Specifically, some of the most abundant miRNAs in plasma, such as miR-22-3p, miR-192, miR-27b and miR-423-5p were not among the most highly expressed miRNAs in blood cells (Table 5-2). Similarly, some of the highly-expressed miRNAs in blood cells in my dataset, which are known blood-cell derived miRNAs (Hunter et al., 2008; Pritchard et al., 2012), such as miR-451 and miR-26a, were not highly expressed in plasma in comparison. This result confirms that my differential centrifugation and subsequent filtration protocol effectively removed blood cells from plasma.

Table 5-2. Abundant miRNAs in blood cells and bovine plasma using sequencing.

The 15 most abundant miRNAs in blood cells and bovine plasma from four animals. MiRNAs are sorted by decreasing average abundance (RPMM), n = 4 samples per group.

Blood Cells		Plasma	
miRNA	RPMM	miRNA	RPMM
miR-486	324,749	miR-22-3p	126,167
miR-451	187,081	miR-486	125,775
miR-101	90,473	miR-192	101,853
miR-92a	44,722	miR-27b	43,371
miR-16b	33,980	miR-142-5p	39,727
miR-26a	32,849	miR-423-5p	35,409
miR-25	30,232	miR-191	32,448
miR-142-5p	20,925	miR-215	28,947
miR-191	19,656	miR-21-5p	25,484
miR-181a	15,753	miR-10b	25,277
miR-186	14,137	miR-92a	23,608
let-7f	12,830	miR-103	22,481
miR-30e-5p	11,955	miR-140	19,501
miR-144	9,847	miR-148a	19,108
miR-93	9,760	miR-30e-5p	19,058

5.5.2 Differentially expressed microRNAs in cells and plasma

In PCA of sequencing data for 212 miRNAs, blood cells could be clearly distinguished from plasma samples based on principal component 1 which accounted for 83.9 % of the variation in the data (Figure 5-2A). I assessed the statistical significance of the differences in miRNA expression between the two groups using EdgeR (see Methods), which identified 169 significant differences (FDR < 0.05, Figure 5-2B, File 5-1). Out of these, 131 miRNAs changed by more than two-fold between groups (within-animal), highlighting large differences in miRNA expression between blood cells and plasma (Figure 5-2B, File 5-1). Interestingly, miR-429 was moderately expressed in plasma (RPMM = 268) but remained undetected in blood cells, making it an ideal candidate for a potentially tissue-derived miRNA (File 5-1). Unfortunately I could not detect this miRNA in either sample group using RT-qPCR (see validation experiment). Overall, the observed fold-changes were very high, with

miRNAs being enriched by more than 50-fold in either group, although the largest fold-changes were observed for plasma-enriched miRNAs (Figure 5-2B, File 5-1).

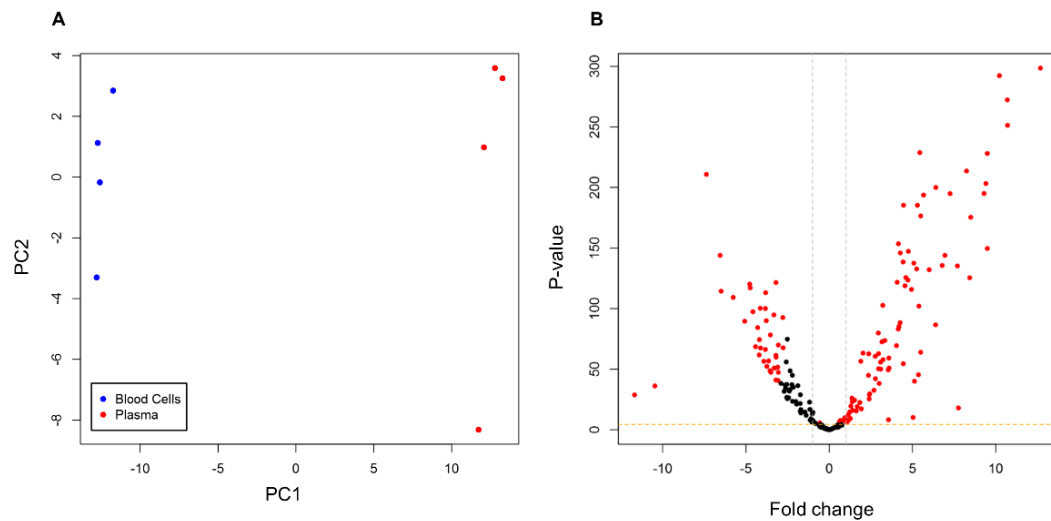


Figure 5-2. Small-RNA sequencing results from blood cells and plasma.

(A) PCA plot showing the first two principle components using transformed normalised data. (B) Volcano plot showing the significance of differences between blood cells and plasma, using transformed normalized data. MiRNAs with fold-change > 2 and FDR < 0.05 are highlighted in red. Gray dotted lines indicate two-fold differences. Yellow dotted line indicates P = 0.05, four samples have been used in each group.

Using this high-throughput approach I identified 92 miRNAs which are significantly enriched by more than two-fold in bovine plasma (Table 5-3, Figure 5-3A) and 39 miRNAs which are enriched in blood cells (Table 5-4, Figure 5-3B, File 5-1).

Among the enriched miRNAs in blood cells I identified many blood-cell derived miRNAs from human studies such as miR-144, miR-451, let-7f, miR-26a, miR-15b, miR-20a, miR-16a, miR-16b and miR-486 (Cheng et al., 2013; Haider et al., 2014; Hunter et al., 2008; Landgraf et al., 2007; K. Wang et al., 2012; Willeit et al., 2013). Also, there were several tissue-enriched miRNAs (identified in human studies) in my plasma-enriched miRNAs, such as miR-122 (liver), miR-133a (muscle), miR-127 (adrenal gland), miR-141 (adrenal gland and reproductive system) and miR-182 (thymus) (Z. Guo et al., 2014; Landgraf et al., 2007). The agreement of our results with existing reports from human partially validated my approach to identify novel tissue-enriched miRNAs in the bovine circulation.

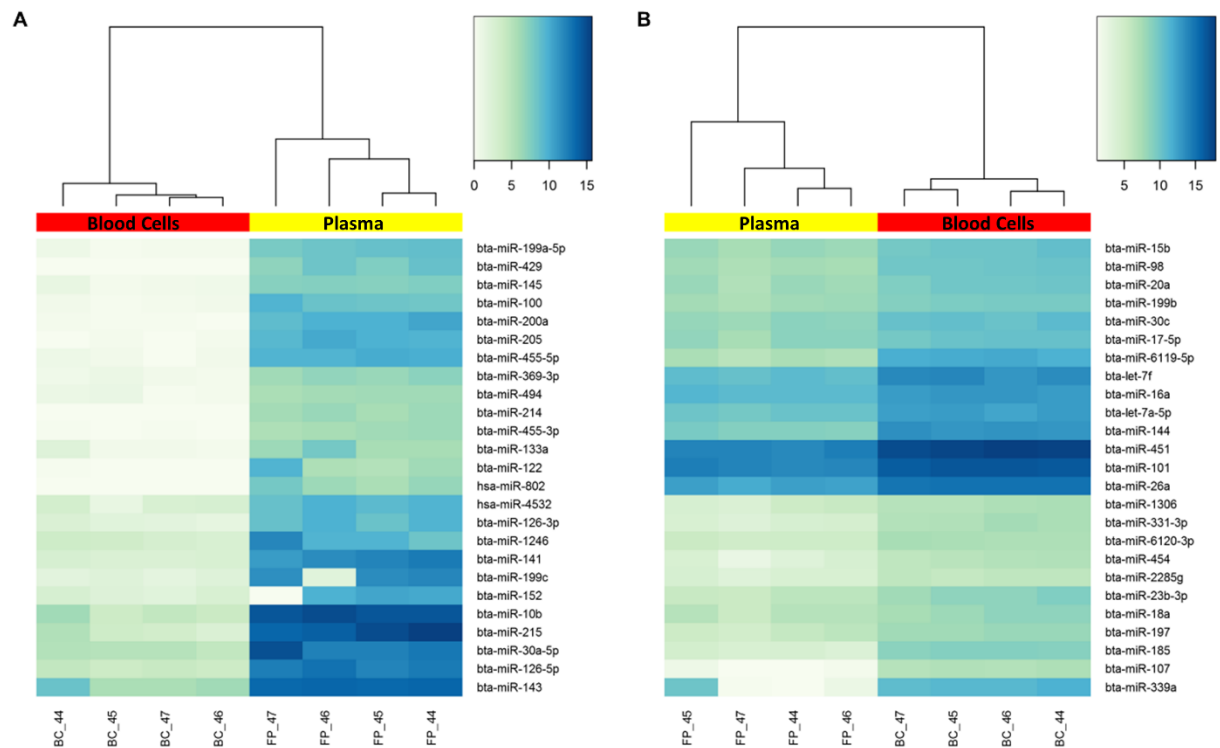


Figure 5-3. Differentially expressed miRNAs in cells or plasma using sequencing.

Heatmaps showing the 25 most up-regulated (A) and down-regulated (B) miRNAs in plasma (yellow bar) compared to blood cells (red bar). FDR < 0.05 for all miRNAs and four samples per group (n = 4). Samples have been ordered based on hierarchical clustering and the group with lowest mean expression is plotted on the left of each heatmap.

Table 5-3. The most up-regulated miRNAs in plasma using sequencing.

The 15 miRNAs that had the highest up-regulation in plasma compared to blood cells with FDR < 0.05, 4 samples per group. Fold-change: $\text{RPMM}_{\text{plasma}} / \text{RPMM}_{\text{cells}}$.

miRNA	Fold-change	P-value	FDR
miR-429	INF	0.000	0.000
miR-200a	6503.71	0.000	0.000
miR-205	5220.54	0.000	0.000
miR-215	2168.64	0.000	0.000
miR-455-5p	1707.35	0.000	0.000
miR-100	1498.54	0.000	0.000
miR-199a-5p	1359.19	0.000	0.000
miR-10b	1339.56	0.000	0.000
hsa-miR-802	1092.70	0.000	0.000
miR-199c	1041.52	0.000	0.000
miR-214	931.10	0.000	0.000
miR-141	699.78	0.000	0.000
miR-455-3p	635.12	0.000	0.000
miR-122	493.42	0.000	0.000
miR-126-5p	412.95	0.000	0.000

Table 5-4. The most up-regulated miRNAs in blood cells using sequencing.

The 15 miRNAs that had the highest up-regulation in blood cells compared to plasma with FDR < 0.05, four samples per group. Fold-change: $\text{RPMM}_{\text{cells}} / \text{RPMM}_{\text{plasma}}$.

miRNA	Fold-change	P-value	FDR
miR-107	54.19	0.000	0.000
miR-6119-5p	28.01	0.000	0.000
miR-185	27.94	0.000	0.000
miR-144	16.56	0.000	0.000
miR-451	10.31	0.000	0.000
miR-331-3p	8.55	0.000	0.000
miR-454	7.92	0.000	0.000
miR-339a	7.61	0.000	0.000
let-7f	7.16	0.000	0.000
miR-23b-3p	6.53	0.000	0.000
miR-26a	6.08	0.000	0.000
miR-98	5.72	0.000	0.000
let-7a-5p	5.60	0.000	0.000
miR-101	5.39	0.000	0.000
miR-197	5.36	0.000	0.000

5.5.3 Validation of sequencing results using RT-qPCR

I selected a total of 10 miRNAs which were enriched in plasma (miR-429, miR-233, miR-133a, miR-215, miR-375) or blood cells (let-7f, miR-144, miR-26a, miR-451, miR-6119-5p) according to my sequencing data for validation using RT-qPCR in an extended group of six animals. Unfortunately I could not quantify miR-429 accurately in blood cell and plasma samples using RT-qPCR, and therefore this miRNA was excluded from further analyses. However, I validated the significant differences identified in sequencing for eight of the remaining nine miRNAs using RT-qPCR, in some cases identifying larger differences compared to the sequencing result (Figure 5-4). I could not validate the significant enrichment of miR-375 in plasma compared to blood cells using RT-qPCR, although the direction of change was the same in both assays.

The validation rate of my high-throughput dataset was particularly high compared to previously reported miRNA validation results from the Donadeu laboratory and others (Ahanda et al., 2014; Ioannidis & Donadeu, 2016b; W. Long et al., 2014; Mestdagh et al., 2014). This is mainly due to the magnitude of the differences that I attempted to validate, which were higher than those seen in other miRNA studies and also allowed me to use lower biological replication compared to my previous studies (Ioannidis & Donadeu, 2016a, 2016b).

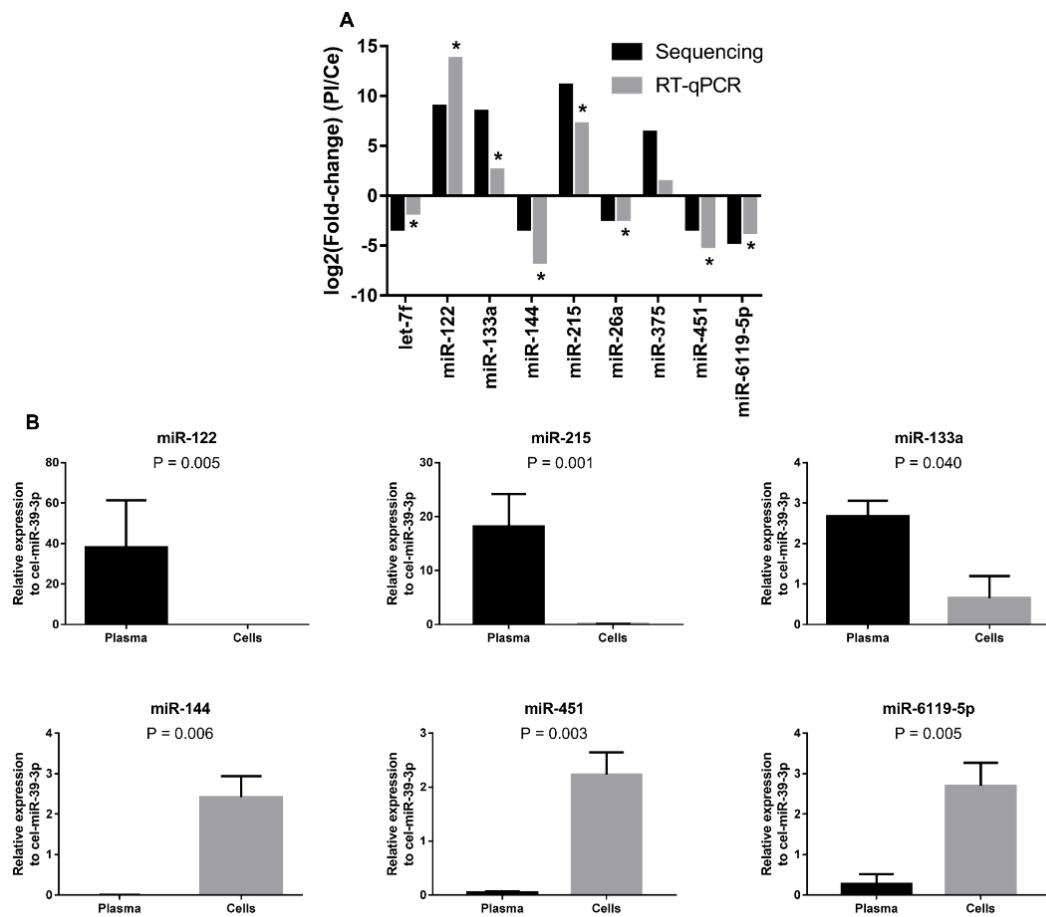


Figure 5-4. Validation of sequencing results in blood cells and plasma.

(A) Comparison of the fold-changes that were identified using sequencing and RT-qPCR in plasma. Asterisks indicate significant differences ($P < 0.05$) in the RT-qPCR dataset. Mean $\pm$ SEM, six samples per group. (B) Relative expression of six validated differentially expressed miRNAs between blood cells and plasma. Top row: up-regulated in plasma. Bottom row: down-regulated in plasma.

5.5.4 MicroRNA expression in a range of bovine tissues

In order to investigate the tissue specificity of miRNAs which were highly expressed in plasma compared to blood cells, I profiled selected miRNAs in 14 bovine tissues using RT-qPCR. I firstly profiled three miRNAs (miR-122, miR-133a and miR-215) which are well-characterised and tissue-enriched according to human studies. In accordance with human, miR-122, miR-133a and miR-215 were enriched in liver, muscle/heart and intestine, respectively in my bovine tissue set (Figure 5-5A) (Z. Guo et al., 2014; Landgraf et al., 2007). Some of these miRNAs were expressed at lower levels in other tissues however the expression in the enriched tissue remained

more than 85-fold higher compared to the mean expression in the remaining tissues I profiled (87.7-fold for miR-122, 253.8-fold for miR-133a and 150.2-fold for miR-215).

Previous studies have highlighted tissue-specific miRNAs in human (Landgraf et al., 2007; Y. Liang et al., 2007), however hypothetically there are additional tissue-enriched miRNAs in cow among the circulating miRNAs higher in plasma (Table 5-3) which have not been identified in human. I decided to pursue miRNAs that were either not present in human, or were newly discovered in this study in cow according to the list of bovine miRNAs on miRBase 21. Those miRNAs carry the highest potential to become novel tissue-specific miRNAs in cow which may also be useful as biomarkers of tissue function for their relevant tissue(s) of origin.

I identified 10 miRNAs which were either bovine miRNAs which are not present in humans (miR-1388-3p, miR-6529a, miR-1388-5p, miR-2376, miR-2285t and miR-3431, Figure 5-5B) or cow homologues of human miRNAs that have not been previously identified in cow (miR-802, miR-4532, miR-4792 and miR-210-3p, Figure 5-5C). Following profiling of these miRNAs in 14 bovine tissues using RT-qPCR, the majority (miR-2285t, miR-2376, miR-6529, miR-1388-5p, miR-1288-3p, miR-4532, miR-4792 and miR-210-3p, Figure 5-5B-C) proved to be expressed in most of the profiled tissues and therefore do not make good candidate tissue function biomarkers.

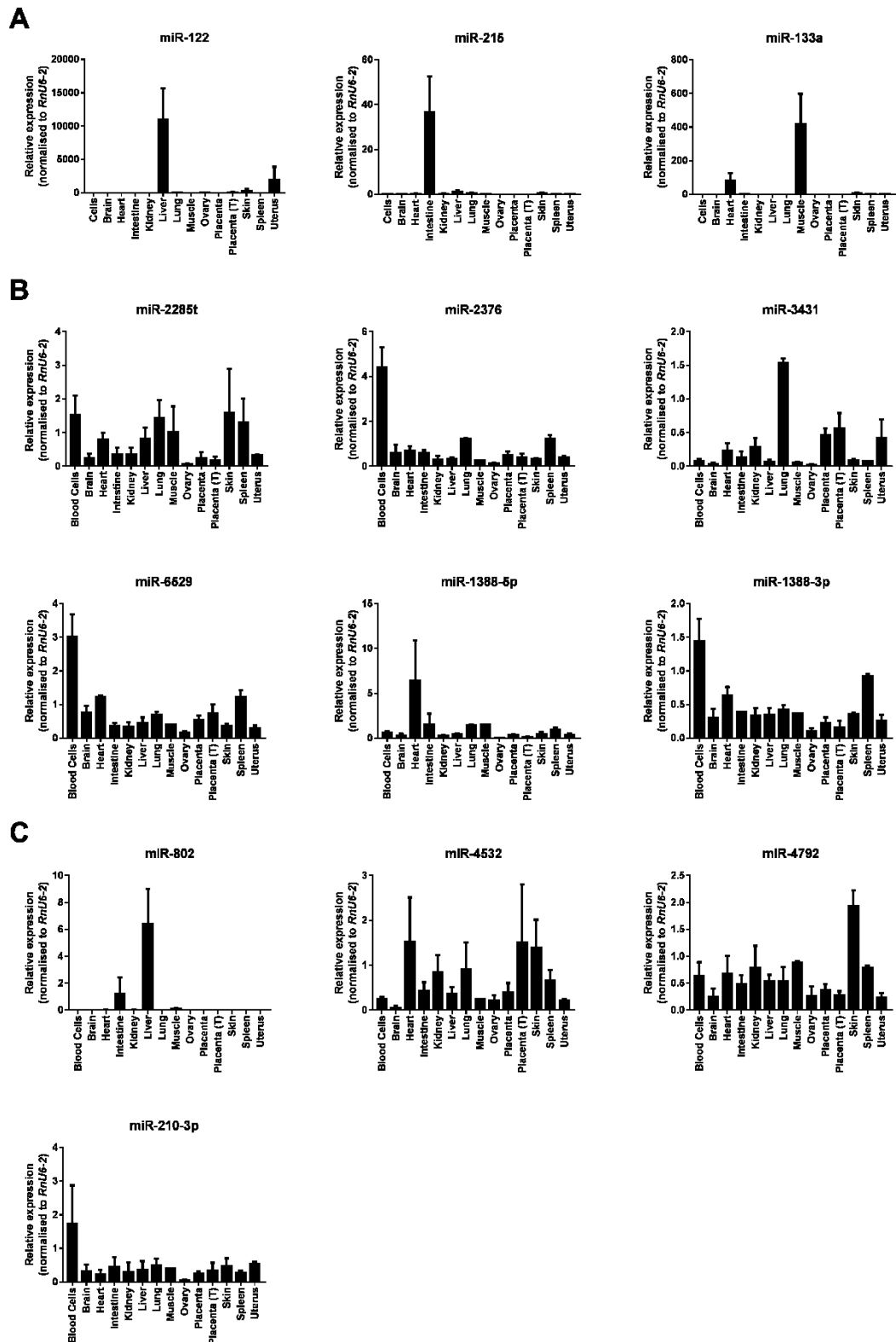


Figure 5-5. Tissue panel validation using RT-qPCR.

Relative expression of selected tissue-enriched miRNAs across 14 bovine tissues from healthy animals. A) Validation miRNAs with known tissue-enrichment in humans. B) Bovine miRNAs which are not present in humans. C) Human miRNA homologues in cow which have not been characterised Placenta: Placenta samples collected on Day 70 of pregnancy; Placenta (T): Placenta samples collected at birth (term). Mean $\pm$ SEM, 3 - 6 samples per tissue.

However, miR-3431 was highly expressed in lung compared to the remaining profiled tissues, followed by placenta (both at term and on Day 70) and uterus. The expression of miR-3431 in lung is 7.6-fold higher compared to the mean of the remaining tissues, which unfortunately is below the commonly accepted threshold for tissue-enrichment (Z. Guo et al., 2014). This miRNA is located on the X chromosome in cow (chrX: 34,662,660-34,662,756 [-]) but it has no identified homologues in other species according to the Ensembl genome browser 85 (UMD3.1, accessed 27/07/16). For confirmation I carried out a blast-like alignment tool (BLAT) search against the human genome (GRCh37, accessed 27/07/16) using the mature bta-miR-3431 sequence, which returned no hits. Unfortunately there is no literature on the role of this miRNA, however further work could focus on identifying potential functions.

Another interesting miRNA is hsa-miR-802, which was identified as a homolog from human and was very highly expressed in liver compared to the remaining tissues, showing an 86.7-fold enrichment (Figure 5-5C). This miRNA was also expressed in intestine although its expression level in this tissue was 5.2-fold lower than liver. This miRNA is not listed as a bovine miRNA on miRBase 21, however using BLAT (UMD3.1, accessed 27/07/16) I mapped the mature hsa-miR-802 sequence (as identified in my sequencing experiment) to bovine chromosome 1 (chr1: 149720302-149720392 [+]). Interestingly, this location in the bovine genome has been annotated as a novel miRNA with a secondary structure that matches hsa-miR-802, but the miRNA is absent from the cow on miRBase 21. In contrast to miR-3431 which appears to be unique to cow, miR-802 has homologues in many species including horse, rat, mouse and dog.

In mice, miR-802 has been linked to the regulation of metabolism. Specifically, miR-802 was identified as a candidate biomarker of type 2 diabetes along with miR-101

and miR-375 in obese mice (Higuchi et al., 2015). Furthermore, a second study showed that miR-802 is deregulated in the livers of both obese mice and humans, leading to insulin insensitivity via Hnf1b (Kornfeld et al., 2013). In my tissue expression data miR-802 was also expressed in the intestine, however lower than in liver. Interestingly a study identified miR-802 as a regulator of the angiotensin II type 1 receptor, ultimately affecting intestinal fluid, electrolyte balance and inflammation among other processes (Sansom et al., 2010). In the dairy cow, liver is a tissue of interest because of its involvement in post-partum negative energy balance and its contribution to low fertility which is a major problem in modern dairy herds (Fatima et al., 2014). I therefore propose that miR-802 may be useful as a non-invasive biomarker of metabolic stress and/or negative energy balance in the post-partum dairy cow.

5.6 Conclusions

I present a validated next-generation sequencing miRNA dataset identifying 92 miRNAs which are highly expressed in plasma compared to blood cells, which are believed to be tissue-derived. Using this resource I selected and profiled 10 plasma-enriched miRNAs in 14 bovine tissues, aiming to identify novel tissue-specific miRNAs which may be useful as biomarkers of tissue-function. Using this approach I identified the bovine specific miR-3431 as highly-expressed in lung compared to other tissues, although this miRNA showed a relatively low enrichment value. Interestingly, I also identified miR-802, a newly characterised miRNA in cow, as a liver-enriched miRNA which is involved in the regulation of insulin insensitivity in mice and humans and may be useful as a biomarker of post-partum negative energy balance in dairy cows. I invite researchers to investigate the tissue expression of additional miRNAs from my dataset which may provide novel biomarkers of tissue function in cow.

6 Discussion and Future Work

In this chapter I will expand on the discussion of the results that is included in the previous chapters and also discuss my findings as a whole. Although the aim of this discussion is to be generic and treat the work as a whole, some chapter-specific themes have been added after the publication of the original papers which make up my results chapters. In addition to this, I discuss future experiments which may shed light into areas that are still unclear, mainly associated with the origin and function of the circulating miRNAs identified in this work. Finally I attempt to briefly summarise my findings in the Conclusion.

6.1 Oestrus biomarkers

In Chapter 2 I describe the profiling of bovine circulating miRNAs during the oestrous cycle of heifers in order to identify differentially expressed miRNAs at oestrus compared to the luteal stages of the cycle. In this experiment I identified small differences in circulating miRNA levels at oestrus. An approach which would amplify the differences in circulating miRNAs that originate in the ovary would be to use superovulation. Hypothetically the presence of multiple follicles, and subsequently multiple CLs, would result in differences of higher magnitude compared to unstimulated animals.

Interestingly, a recent study in cow compared miRNA levels in the circulation of super-ovulated and unstimulated animals on Day 7 of the oestrous cycle and found lower expression of miR-145, miR-99a and miR-125b in the follicular fluid of stimulated animals compared to unstimulated animals. This downregulation persisted in plasma for miR-99a and miR-125b but not miR-145 (Noferesti et al., 2015). This suggests that the circulating levels of these miRNAs are contributed by the ovarian tissues. Analysis of circulating miRNAs on Days 0, 3 and 7 of the oestrous cycle in super-ovulated animals did not identify differences in the same miRNAs as in my experiment, putatively because different days of the cycle were sampled. A second study in humans found that super-ovulation disrupts the physiological expression of miR-200, affecting endometrial receptivity and decidualisation in early pregnancy. This suggests that endometrial and ovarian physiology may not be the same in

unstimulated and super-ovulated animals (Jimenez, Mainigi, Word, Kraus, & Mendelson, 2016). Future work could use super-ovulated animals on Days 0, 8 and 16 in order to identify circulating miRNAs that originate in the ovary and expand my findings.

In addition to the super-ovulation experiments outlined above, future work could also focus on clarifying if there is preferential secretion of miRNAs from the ovary into the circulation. We previously showed that miRNAs from the bovine ovarian follicle (miR-202 and miR-873) are released in the follicular fluid and are significantly correlated with E2 and aromatase levels across atretic and healthy follicles (Donadeu et al., 2016). However I could not quantify important ovarian miRNAs such as miR-202, miR-132 and miR-96 in bovine plasma at various stages of the oestrous cycle. Future work could focus on clarifying which ovarian miRNAs are secreted in the follicular fluid and why some appear to be also released in the circulation (e.g. miR-125b, miR-99a, miR-145 and let-7f) but others are not (e.g. miR-202).

In addition to identifying differentially expressed miRNAs I also carried out analyses to quantify isomiRs in the sequencing data. IsomiRs result from variations in miRNA processing, such as Drosha and Dicer cleavage, and from pre-miRNA editing events, and that they cooperate with canonical miRNAs to target common pathways (Cloonan et al., 2011; L. Guo & Chen, 2014). In total I identified 655 isomiRs corresponding to 134 canonical miRNAs. The most frequent single modifications occurred on the 3' end of the miRNA, followed by 5' end modifications and frame shifts (a shift of the miRNA sequence along the miRNA precursor template). This is in agreement with previous studies in mice and humans (Cloonan et al., 2011; Reid et al., 2008; H. Zhou et al., 2012) all of which observed higher frequencies of 3' end modifications compared to other modification types. Furthermore, the most abundant isomiRs were more abundant than their corresponding canonical miRNAs in my data and this confirms findings from previous studies in human and mouse (Cloonan et al., 2011; Morin et al., 2008). Perhaps a limiting factor in the progression of our understanding of isomiR biology has been the lack of validation of isomiR results using other methods, since RT-qPCR lacks the specificity required to study the individual isomiRs identified using sequencing (Schamberger & Orban, 2014).

Because of this difficulty, and also because evidence suggests that isomiRs serve similar functions to their corresponding ‘canonical’ miRNA, I did not pursue the analysis of differentially expressed isomiRs at oestrus (Cloonan et al., 2011).

Finally, perhaps unsurprisingly, the changes in plasma miRNA levels during the oestrous cycle were negatively correlated with progesterone concentration in this study. Considering that the expression profiles of these miRNAs show a decreasing trend during the luteal phase, it would be expected that some correlation with progesterone would be present at this stage (since progesterone increases over the same period). It is not clear if the differentially expressed miRNAs are functionally associated with progesterone and this is primarily because the source of these miRNAs is not known. The previously discussed study by Noferesti et al. showed that the miRNAs identified in this chapter were down-regulated in super-stimulated animals (experiencing higher progesterone) compared to controls, showing the same negative correlation with progesterone seen in my work (Noferesti et al., 2015). This suggests that these miRNAs are indeed associated with progesterone. Future work could focus on clarifying the relationship between the differentially expressed miRNAs identified in this chapter and progesterone.

6.2 Pregnancy biomarkers

In Chapters 3 and 4 I identified miRNAs which change in the circulation of pregnant compared to non-pregnant heifers. My initial hypothesis was that large differences in early pregnancy would be diagnostically useful in distinguishing non-pregnant from pregnant cows. MiR-26a was significantly increased as early as Day 8 of pregnancy, while miR-30c, miR-101, miR-143, let-7f, let-7c and miR-205 were significantly different on Day 60 of pregnancy. These miRNAs are likely to be biologically relevant in pregnancy, however the small magnitude of the changes I identified (perhaps with the exception of the miR-26a/miR-205 ratio which increased up to 7.5-fold on Day 8) makes their immediate application as biomarkers difficult. Currently there are also technical limitations in place, especially for small differences in miRNA levels and as quantification technologies improve it will be easier to develop miRNA based tools that will be useful for diagnostic purposes (perhaps in the form of a miRNA panel, as seen in diagnostic products for human cancer).

The origin and biological significance of the differences I identified in circulating miRNA levels during pregnancy are unclear. The ubiquitous expression of the majority of the differentially expressed miRNAs made it difficult to determine which tissue(s) contribute to the differential expression observed during pregnancy. A simple explanation of the differential expression of these miRNAs may be differences in plasma volume and blood cell counts which are part of the maternal adaptation to pregnancy. Specifically, plasma volume increases from the sixth week of pregnancy, while erythrocyte mass increases at a slower rate, resulting in low haematocrit (Chandra, Tripathi, Mishra, Amzarul, & Vaish, 2012). Neutrophil and monocyte counts increase, while lymphocyte counts decrease in the first trimester of pregnancy (Chandra et al., 2012). The magnitude of the dilution effect resulting from the increase in plasma volume is reportedly 14 % at the end of the first trimester of pregnancy (Thornburg, Bagby, & Giraud, 2015).

In Chapter 4 I identified differentially expressed miRNAs in plasma on Day 60 of pregnancy using sequencing. Interestingly, there was a noticeable enrichment for tissue-derived miRNAs (as identified in Chapter 5) among the miRNAs which were down-regulated on Day 60 of pregnancy, including miR-205, miR-4532 and miR-99b. A possible explanation for this enrichment could be that those miRNAs are affected by a blood volume dilution effect (discussed in the previous paragraph) at this stage. If this is true, then the miRNAs which were up-regulated on Day 60 of pregnancy are most likely white blood-cell derived, since the numbers of these cells increase during pregnancy. In retrospect, including blood cell counts as a co-variate in these experiments would have been informative; future studies could include this kind of analysis. MiR-205 is the only validated differentially expressed miRNA which was down-regulated in pregnancy. If a dilution effect tended to dilute circulating miRNA (resulting in apparent down-regulation in pregnancy) miR-205 would be the primary candidate to be affected by such an effect. However miR-205 has a known function as an inhibitor of angiogenesis via VEGF-A targeting, which may also explain the expression pattern of this miRNA. The ubiquitous expression of this miRNA based on published reports (thymus, mammary gland, early embryo, digestive tissue, skin) makes it difficult to hypothesise on possible functions and the

source of the difference observed during pregnancy (Abd El Naby et al., 2011; G. Liang et al., 2014; Y. Liang et al., 2007; M. Wang et al., 2012; T. Wang et al., 2016).

In addition to differences between pregnant and non-pregnant animals, in Chapter 4 I investigated the relationship between circulating miRNA levels and foetal gender at Days 16, 24 and Day 60 of pregnancy. Overall, there were not many statistically significant differences after multiple testing correction (only let-7a-3p) and only four miRNAs changed by more than two-fold between groups (hsa-miR-28-3p, hsa-miR-4508, hsa-miR-5693 and hsa-miR-6516-3p). In recent years two independent studies were published on circulating miRNA gender differences, however the agreement between the two studies was limited to hsa-miR-28-3p which was identified as female-enriched in human adults (Ameling et al., 2015; Tonge & Gant, 2016). My sequencing result for miR-28-3p is in agreement with these studies, even though validation of this result using RT-qPCR was not successful, partially because of the low abundance of this miRNA. The agreement of my sequencing results with these published reports supports the hypothesis that the small differences I identified in the circulation using sequencing are biologically relevant but lack the magnitude for accurate quantification and validation, similarly to my work on the oestrous cycle in Chapter 2.

In adults, sex-related differences in circulating miRNA expression have been attributed to differences in circulating cell numbers between males and females. Correction for differences in blood components between genders normalised the majority of the differentially expressed miRNAs, removing statistical significance (Ameling et al., 2015). However the cause of foetal sex-dependent differences in maternal circulation during pregnancy is not clear. They may potentially be the result of different sizes of female and male foetuses, resulting in variable miRNA expression in the maternal blood cells.

A possible criticism for the work presented in Chapter 4 is that Day 0 animals were used as controls for Day 60 pregnant animals. This was primarily done to minimise between-animal variation (which can be particularly high when working with large animals) since each animal can be used as its own control in a repeated-measure design. The comparisons between Day 60 and Day 0 carry the disadvantage that

miRNA expression is assessed across different hormonal backgrounds, that is, a high oestrogen background (Day 0) vs. as high progesterone background (Day 60). This makes it difficult to distinguish effects of pregnancy from effects of hormone background. For this reason, comparisons between days with similar hormone background (e.g. luteal phase non-pregnant vs pregnant animals) such as the comparison between Day 16 pregnant vs. non-pregnant animals might have been more suitable. Despite this, the differentially expressed miRNAs during pregnancy were not differentially expressed during the oestrous cycle in the data generated in Chapter 2, which suggests that the differential hormone levels between Day 0 and Day 60 have not affected the expression of these miRNAs. Further work such as the P4 experiments outlined in the following paragraph will clarify if the differences in miRNA expression during pregnancy are caused by the variable progesterone concentrations in pregnancy.

To elucidate the potential immune-regulatory function of my miRNA candidates, future work could investigate the effects of P4 and/or IFN- τ on blood cells and/or circulating leukocytes. IFN- τ is an embryo derived molecule, therefore larger embryos may contribute larger amounts of IFN compared to smaller embryos, affecting miRNA expression. A plethora of genes including ISG15 are induced in the maternal circulation during early pregnancy in response to IFN- τ (Han et al., 2006; Shirasuna et al., 2012). A recent study showed that in-vitro cultured bovine endometrial cells express miR-106 in response to IFN- τ , via the interferon response gene IRF-6 (Palma-Vera & Einspanier, 2016). It would be interesting to explore if this apparent effect of IFN- τ and its downstream factors on miRNAs extends to miR-26a in circulating blood cells. Additionally, since P4 tends to increase during pregnancy compared to non-pregnant animals, treatment with P4 would determine if these miRNAs are P4-inducible (Peters & Ball, 1995). These experiments will also potentially allow the identification of additional miRNA candidates and elucidate the function of these miRNAs in immune regulation during early pregnancy.

6.3 Tissue function biomarkers

In Chapter 5 I demonstrated the existence of uncharacterised organ-enriched miRNAs in cow which are easily quantifiable in the bovine circulation, such as miR-

802. Such miRNAs may become useful miRNA biomarker candidates of tissue function in cow. Conservation analysis is a simpler alternative approach which could drive the discovery of miRNAs in cow based on human miRNA expression data, however a cow-specific dataset such as the one I present in Chapter 5 also includes miRNAs which are unique to cow, which would not have been identified using human-derived data. This chapter also highlighted the importance of including tissue-enrichment analysis of potential biomarkers in the biomarker discovery process in the cow, as it has been suggested by studies in human (Pritchard et al., 2012; K. Wang et al., 2012). Studying the tissue enrichment of candidate biomarkers is important because it allows us to select markers with relevant expression to the condition of interest and helps identify blood-cell derived miRNAs which may not be ideal biomarkers in some cases due to variability in blood cell counts (Pritchard et al., 2012).

Plasma processing protocols in circulating miRNAs research are still being standardised and blood cell contamination is a significant factor that affects plasma miRNA levels (Cheng et al., 2013). In this context, I compared miRNA profiles from filtered and unfiltered plasma samples and found that filtered samples (Chapter 5) had similar profiles to unfiltered samples (Chapters 2, 3 and 4). Blood-cell derived miRNAs were almost identically distributed among the most abundant miRNAs in filtered and unfiltered plasma. The correlation between the two sample types was very high ($r = 0.88$, $P < 0.001$) and blood-cell derived miR-101, miR-451 and miR-486 were present in both 'Top 25' lists, while miR-16b and miR-26a ranked very closely in both samples. Previous reports showed that filtering effectively removed platelets and microvesicles from normal plasma (Cheng et al., 2013), however my data suggest that using a second, high speed centrifugation is sufficient to remove cell contamination from plasma samples when assessed on the miRNA level. This technical conclusion will help reduce the cost and sample processing times in future studies.

Future work stemming from the finding that miR-802 is a liver-enriched miRNA in cow could focus on its usefulness as a biomarker of metabolic stress and/or negative energy balance in post-partum dairy cows. Negative energy balance has been

identified as a major cause of low fertility in modern dairy herds (Diskin et al., 2015) and it is particularly important in the peripartum period due to increased metabolic demands induced by lactation. Recently, the liver-enriched miR-143 was found to be downregulated in the liver of cows which were under negative energy balance (Fatima et al., 2014). Future work could attempt to determine if the levels of miR-802 as well as miR-143 are differentially expressed in the circulation of post-partum dairy cows with differential energy balance, and also if these biomarkers have any predictive value, for example to identify stressed animals and allow for feed adjustment during pregnancy. It would also be interesting to compare these miRNAs with existing indicators of negative energy balance such as blood glucose, insulin and insulin-like growth factor 1 (IGF-1) (Butler, 2000).

6.4 General discussion

As mentioned in Chapter 1, during the course of my studies a tendency appeared in the published literature to begin using endogenous miRNA normalisers in the place of traditional spike-in exogenous normalisers for RT-qPCR. The importance of robust methodology was recently highlighted in a review of the field (Witwer & Halushka, 2016). In principle, endogenous normalisers correct for animal-derived variation in addition to technical variation, thereby offering a better normalisation approach. However identifying ideal normalisers for miRNA studies can be problematic and optimisation needs to be carried out for each individual study. Currently there appear to be mixed opinions on which normalisation method is best for plasma miRNA studies (Bae et al., 2015; D'Haene et al., 2012; Roberts et al., 2014; Sourvinou et al., 2013), and for this reason I opted for using both endogenous and exogenous normalisers in my later experiments and examining their effects on miRNA expression data. In both the pregnancy RT-qPCR experiments (Chapter 4) and the global biomarker RT-qPCR experiments (Chapter 5) I did not find significant effects of using different individual normalisers (cel-miR-39-3p, bta-miR-221, bta-miR-128) or combinations of endogenous and exogenous normalisers. However, some evidence suggests that spiked-in normalisers show variation that is unlike that of endogenous normalisers (Schlosser et al., 2015) and because of this I would conclude that normaliser optimisation for each individual study is vital at this point in the field.

Taken together, my results show that circulating miRNA profiling in the context of biomarker discovery is dependent on the technical ability of the profiling platforms used. The magnitude of change (fold-change) and inter-animal variation are key limiting factors for the success of a miRNA candidate. This is very obvious from the validation rates obtained from different experiments in this work; validating the larger differences (up to 6.2-fold) identified using high-throughput platforms on Day 60 of pregnancy was more successful (46 %) than validating smaller differences (up to 2.0-fold) in a comparable number of samples from early pregnancy (7 %). Despite this, I showed that miR-26a is consistently up-regulated in early pregnancy in two independent experiments, which improves confidence in this result and supports further work on this miRNA. In addition, my work has confirmed the need for standardisation of the methodology used and the use of multiple quantification platforms both in the discovery and validation stages of such studies. This will ensure studies are comparable and will also minimise the effect of quantification platform bias on biomarker discovery.

6.5 Conclusions

During this project I identified small differences in circulating miRNA expression levels during both early pregnancy and the oestrous cycle in the cow. For oestrus, the circulating levels are thought to reflect the expression of miRNAs in the ovary during this stage, while for pregnancy the origin of the changes is still unclear. Based on the existing literature the pregnancy-related miRNAs identified may be involved in angiogenesis, immune regulation and metabolism, while the identified oestrous cycle miRNAs are possibly associated with the follicular-to-luteal transition. In both cases, further experiments, which have been discussed above, will elucidate the origin of the differences and the function of the miRNAs which are involved in them. If the origin of the differentially expressed miRNAs at oestrus is confirmed to be the ovary, my data also suggest that miRNA release in the circulation is selective for ovary-derived miRNAs, since I could not quantify gonad specific miR-202 in the bovine circulation. This is another direction to be followed which may return some interesting findings about tissue-circulation crosstalk. Finally, I have identified novel miRNAs in the bovine circulation, some of which are tissue-enriched and may be useful as biomarkers of tissue function in the cow. An example of such a miRNA is

miR-802, which is conserved between cow and human and is liver-enriched, putatively being involved in controlling glucose metabolism. In one sentence, my work shows that circulating miRNAs are affected by the reproductive status of the cow and that the circulating miRNome includes novel cow miRNAs which may provide useful biomarkers of tissue function.

7 References

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Appendix I - MicroRNA indicators of follicular steroidogenesis

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Abstract

MiRNAs can provide useful biomarkers of tissue function. The present study aimed to determine in bovine follicles (n = 66, diameter: 4-22 mm) the relationship among several indices of steroidogenesis and the levels of 15 miRNAs previously identified to be associated with follicle development. Oestradiol levels, Oestradiol:Progesterone ratio (E:P) and CYP19A1 expression were strongly correlated to each other ($\rho > 0.8$) and to LHCGR expression ($\rho \geq 0.6$; $P < 0.01$). Levels of 9 different miRNAs in follicular wall were correlated ($P < 0.01$) with oestradiol, E:P and CYP19A1, with miR-873 showing the strongest correlation in each case ($\rho > 0.7$). Analyses of follicular fluid miRNAs identified miR-202 as correlated with oestradiol, E:P and CYP19A1 ($\rho > 0.5$; $P < 0.01$). We found that, when considering all follicle endpoints together, using a cut-off value of E:P = 1 overestimated the number of estrogen-inactive follicles, whereas using CYP19A1 as classifier provided a clearer separation of follicle samples based on estrogen activity, in agreement with E:P, LHCGR, miR-873 and miR-202. In conclusion, we identified follicular miR-873 and miR-202 as miRNAs which can be used as indicators of steroidogenic capacity in bovine. We show that these or other gene expression parameters, in addition or alternatively to E:P should be used to accurately classify follicles based on steroidogenic capacity.

Introduction

The development of a follicular wave in monovular species, including cattle, involves the selection of one (occasionally two or three) dominant follicle(s) simultaneously with the demise of all other (subordinate) follicles in the cohort [1]. A key feature of follicle selection is the upregulation of aromatase (CYP19A1) and the LH receptor (LHCGR) together with a sharp and distinct increase in the production of oestradiol by the dominant follicle, changes which are essential for follicular maturation and an eventual ovulatory LH surge. Dominant follicles developing in the presence of high progesterone levels during the luteal phase of the estrous cycle will fail to ovulate but will instead undergo atresia with a rapid decrease in the production of oestradiol, but not progesterone, and LH-binding capacity. Thus, differences in the relative levels of oestradiol and progesterone in follicular fluid provide an indication of steroidogenic capacity when reproductive status is unknown, e.g. in follicles sourced from abattoir ovaries, with oestrogen-producing (oestrogen-active) follicles typically being defined as having a ratio of oestradiol-to-progesterone (E:P) > 1 [2]. Although widely used, two limitations of the E:P ratio as a functional marker are 1) the inherent analytical variability between different steroid immunoassay platforms and 2) the use in some studies of different units for oestradiol and progesterone to calculate this ratio. These can lead to significant variability in the criteria used to identify estrogen-active follicles between studies. An additional limitation is that steroid immunoassays are not always available in-house requiring the use of relatively expensive commercial kits. Therefore, finding alternative biomarkers of follicle status would be of benefit.

MicroRNAs (miRNAs) are small RNAs that are ubiquitously involved in post-transcriptional control of gene expression. Different regulatory roles of miRNAs in the ovarian follicle have been proposed [3], including steroid production [4, 5]; in turn, steroidal hormones have been shown to actively regulate miRNA biogenesis [6]. Through microarray profiling in cattle we recently identified and characterised a specific subset of miRNAs which were differentially expressed during follicle development in association with changes in CYP19A1 expression [7]. We and others have also characterised miRNA populations in follicular fluid and suggested, at least for some miRNAs, an association between follicular cell and follicular fluid levels

[8-10]. These findings highlight the potential of using miRNA levels as a convenient indicator of follicle function. To test this, we determined the correlation among several established indices of steroidogenesis (including E:P ratio, expression of CYP19A1 and LHCGR) and the levels of 15 miRNAs of which the expression we previously showed to actively change during follicle development in bovine [7].

Materials and Methods

Collection and Processing of Bovine Tissues

Follicles from ovaries of cycling beef cattle obtained at an abattoir were collected as part of a separate study [7]. Individual follicles 4-22 mm in diameter were dissected out and the follicular fluid aspirated and centrifuged at 800 g for 10 minutes. The resulting supernatant was stored at -80 °C until further analysis and the cell pellet was combined with the follicular wall free of surrounding stroma and snap-frozen in liquid nitrogen until RNA extraction. Intra-follicular concentrations of oestradiol and progesterone were measured using competitive double antibody radioimmunoassay kits (Siemens Healthcare Diagnostics Inc., USA) following manufacturer's instructions. All assays were validated in our laboratory by showing parallelism between serial sample dilutions and the provided assay standard curve. Sensitivity of the assays was 0.56 ng/mL and 0.01 ng/mL, and the intra-assay CVs were 6 % and 4.3 % for oestradiol and progesterone, respectively.

RT-qPCR

Total RNA from snap-frozen bovine tissues or from follicular fluid (250 µL; pre-spiked with 5 fmol of synthetic cel-miR-39-3p) was extracted using the miRNeasy Mini kit (Qiagen, UK) and reverse-transcribed using the miScript II RT kit (Qiagen), as described [7]. QPCR for miRNA quantification was performed using the SensiFAST SYBR Lo-ROX Kit (Bioline Reagents Ltd, UK) and bovine-specific primers against CYP19A1 (CGCAAAGCCTTAGAGGATGA, ACCATGGTGATGTACTTTCC), LHCGR (GGACTCTAGCCCGTAGG, ACACATAACCACCATACCAAG) and *18S* (GCTGGCACCAGACTTG, GGGGAATCAGGGTTTCG). For miRNA quantification the miScript SYBR Green PCR kit and miScript Primer Assays (Qiagen) were used. All qPCRs were run on a MX3005P qPCR system (Stratagene, CA, USA) using a standard curve to calculate

copy numbers from Cq values [7]. Messenger RNA data were normalised using *18S* values within each sample and miRNA data were normalised using endogenous *RnU6-2* or, in the case of follicular fluid, spiked-in cel-miR-39-3p values.

Granulosa cell culture

Granulosa cells were obtained by dissecting out follicles (4-10 mm) from abattoir ovaries into hemispheres and gently scraping the follicle wall by blunt-ended forceps to release mural cells. Cells from several follicles were pooled and passed through a filter mesh (70 μ m, Fisher Scientific, UK) to separate out cumulus-oocyte-complexes. After washing with fresh McCoy's 5a medium, red Blood Cell lysis buffer (8.3 g/L NH₄Cl in 0.01 M Tris-HCl buffer pH = 7.5) was added to the cells followed by a further wash. Cells were placed in modified McCoy's 5a medium consisting of 0.02 M HEPES, 3 mM L-Glutamine, 0.1 % BSA, 1 % Pen-Strep, and AITS (androstenedione 100 nM, 10 ng/mL bovine insulin, 2.5 mg/L transferrin and 4 μ g/L sodium selenite) supplemented with 1 ng/mL of ovine follicle stimulating hormone (NHPP, USA), and cultured in 96-well plates (0.1 x 10⁶ per well; Nuclon, Nunc, Denmark) at 37 °C with 5 % CO₂ [7]. Twenty-four hours later, cells were transfected with LNA bta-miR-202 inhibitor (5 and 25 nM, Exiqon, Denmark) or with a scrambled oligonucleotide sequence (5 nM) using HiPerFect (Qiagen, UK) as per the manufacturer's instructions. Cells were harvested for RNA isolation and culture media was collected every 24 h for up to 4 days after transfection.

Statistical analyses

Dixon's outlier test was applied to both RT-qPCR and immunoassay data sets, and outlier values ($P < 0.01$) were excluded from subsequent analyses. Spearman's rank correlation test was used to determine the relationship between different follicular endpoints. Correlations were considered at $P < 0.01$. One-way or two-way ANOVA followed by Dunnett's test ($P < 0.05$) were used to analyse miRNA stability and cell culture data. All data were log-transformed prior to statistical analysis.

Results

Steroid and gene expression data from a total of 66 follicles (Figure 1) were used for analyses. Results of correlation analyses involving different follicular endpoints are shown in Table 1. Oestradiol, E:P ratio and CYP19A1 were strongly correlated

among them ($\rho > 0.8$), and each was correlated ($\rho \geq 0.6$) with LHCGR expression. A modest negative correlation with each of E:P ratio, CYP19A1 and LHCGR were found for progesterone levels.

Table 1. Spearman's correlation coefficient ($P < 0.01$) for different endpoints in bovine follicles (n = 66, 4-22 mm in diameter)

Endpoint	Diameter (mm)	Oestradiol (ng/ml)	Progesterone (ng/ml)	E:P	CYP19A1	LHCGR
Diameter (mm)	1.00	0.48		0.50	0.47	
Oestradiol (ng/ml)	0.48	1.00		0.97	0.83	0.60
Progesterone (ng/ml)			1.00	-0.44	-0.39	-0.42
E:P	0.50	0.97	-0.44	1.00	0.85	0.65
<i>CYP19A1</i>	0.47	0.83	-0.39	0.85	1.00	0.71
<i>LHCGR</i>		0.60	-0.42	0.65	0.71	1.00
miR-125b		-0.45	0.53	-0.51	-0.49	-0.41
miR-130a		-0.53	0.47	-0.57	-0.56	-0.36
miR-145		-0.38	0.55	-0.43	-0.44	-0.41
miR-150		-0.47	0.53	-0.54	-0.50	-0.39
miR-21		-0.44	0.57	-0.52	-0.55	-0.52
miR-378		-0.36	0.41	-0.42	-0.33	
miR-409a		-0.48	0.61	-0.57	-0.44	-0.42
miR-483						
miR-144	0.46					
miR-202	0.61	0.54		0.55	0.52	0.52
miR-210	0.50				0.34	
miR-31						
miR-451	0.58					
miR-652	0.67					
miR-873	0.50	0.70	-0.39	0.73	0.78	0.59
FF miR-130a						
FF miR-150						
FF miR-21						
FF miR-202		0.53		0.58	0.65	0.67
FF miR-873						

Gene expression measurements were done in whole follicle wall or, for the five miRNAs at the bottom, in follicular fluid (indicated by FF)

Coefficient values > 0.7 are shown in bold

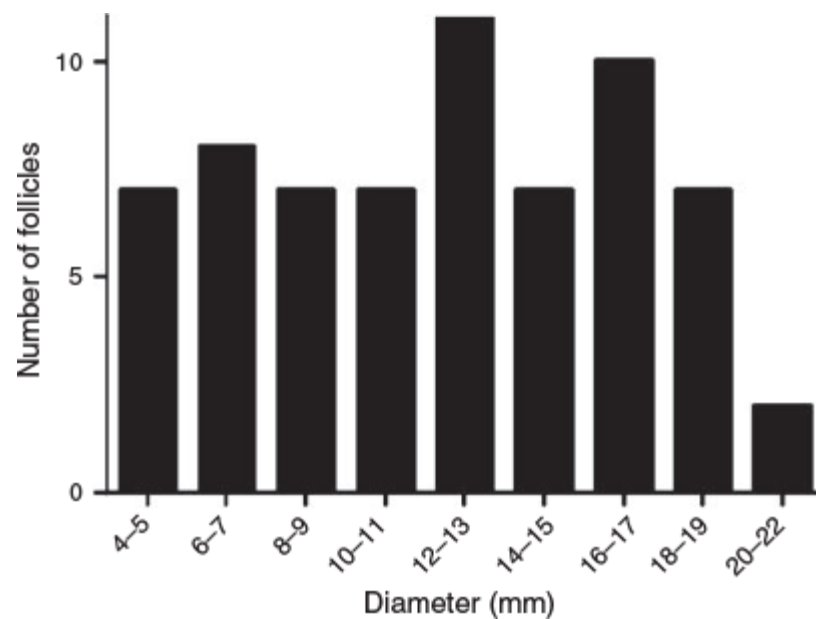


Figure 1. Histogram of the bovine follicle population (n = 66) used in this study.

MiRNAs chosen for analyses (Table 1) were those previously found to be associated with dominant-size follicles that expressed low CYP19A1 (miR-125b, miR-130a, miR-145, miR-150, miR-21, miR-378, miR-409a and miR-483) or high CYP19A1 (miR-144, miR-202, miR-210, miR-31, miR-451, miR-652 and miR-873). Follicular wall expression of all but miR-483 in the first category showed a negative but moderate correlation with each of oestradiol, E:P ratio, CYP19A1 and LHCGR, and a positive correlation with progesterone levels. Among miRNAs previously found to be associated with high CYP19A1 expression, miR-202 and, especially, miR-873 ($\rho=0.6$ to 0.8) were positively correlated with oestradiol, E:P ratio, CYP19A1 and LHCGR, whereas miR-210 was weakly correlated with CYP19A1 only.

Before examining correlations involving follicular fluid miRNAs, the stability of miRNAs in bovine follicular fluid samples was determined by RT-qPCR (Figure 2). This was done to establish optimal conditions for sample processing by determining whether incubation time and repeated freezing would have any effect on miRNA levels. Levels of 3 selected miRNAs (miR-125b, miR-145 and miR-21) were quantified after incubation of samples at room temperature for different periods and after several freeze-thaw cycles. Results showed a negative effect of incubation time, but not freeze-thaw cycle, on miRNA levels, with levels of miR-125b and miR-145 decreasing after 1 h incubation of follicular fluid at room temperature. MiRNAs

which levels in follicular wall displayed $\rho \geq 0.5$ with both E:P ratio and CYP19A1, namely miR-130a, miR-150, miR-21, miR-202 and miR-873, were then quantified in follicular fluid (available from a total of 38 samples) and their correlation coefficients were determined. MiR-202 was correlated ($\rho = 0.5$ to 0.7) with each of oestradiol, E:P ratio, CYP19A1 and LHCGR whereas none of the other 4 miRNAs was correlated with any endpoints.

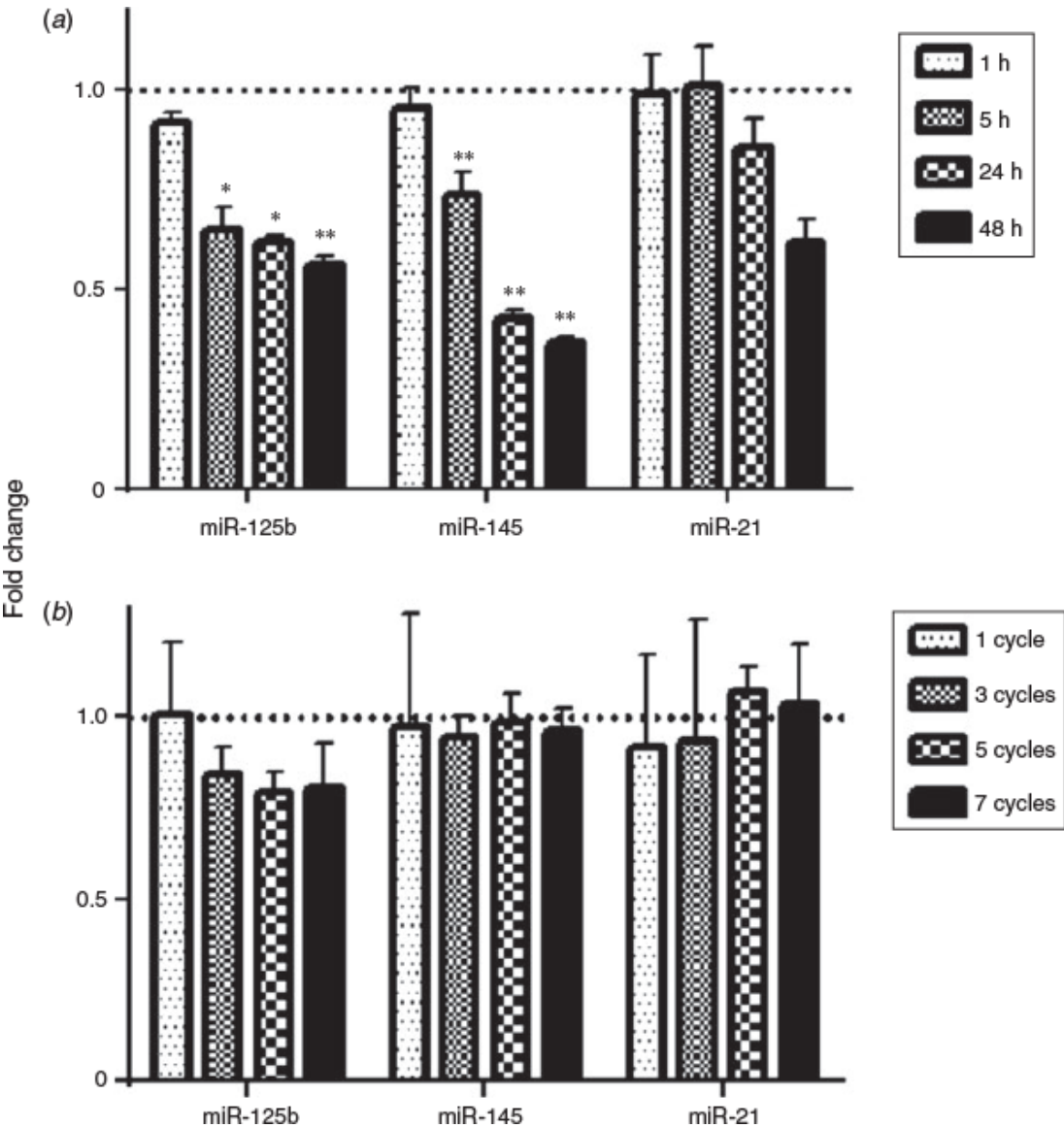


Figure 2. Changes (Mean $\pm$ SE) in levels of miRNAs in pools of bovine follicular fluid (n = 3) following incubation for different periods at room temperature (a) or after different freeze-thaw cycles (b).

Levels of each miRNA were normalised to spiked-in cel-miR-39-3p. Expression levels are shown as fold-change relative to baseline values before incubation or before freezing, respectively (represented by horizontal dotted lines). Mean differences from baseline are indicated by * ($P < 0.05$) or ** ($P < 0.01$).

We also wanted to determine the agreement among follicle sample groupings (oestrogen-active and -inactive) generated by different classifiers, namely, E:P ratio, CYP19A1, LHCGR, miR-873 and miR-202. Using a E:P = 1 threshold to separate follicle populations (Figure 3a) yielded a substantial overlap of oestrogen-active and -inactive populations based on individual sample values for CYP19A, LHCGR, miR-873 and miR-202 with, unexpectedly, some oestrogen-inactive follicles having higher values for these variables than all oestrogen-active follicles. Upon examining the distribution of CYP19A1 values alone, it was clear that, among all variables, this seemed to best separate follicle samples into two distinct populations. The boundary between these two populations was defined as the highest fold-change between two consecutive CYP19A1 expression values (Figure 3b). When simultaneously considering other endpoints, particularly LHCGR, miR-873 and follicular fluid miR-202, classification of samples based on CYP19A1 resulted in better defined follicle populations than using E:P = 1 as a classifier (Figure 3a), highlighting the potential of using these gene expression endpoints for experimental assessment of the steroidogenic capacity of bovine follicles.

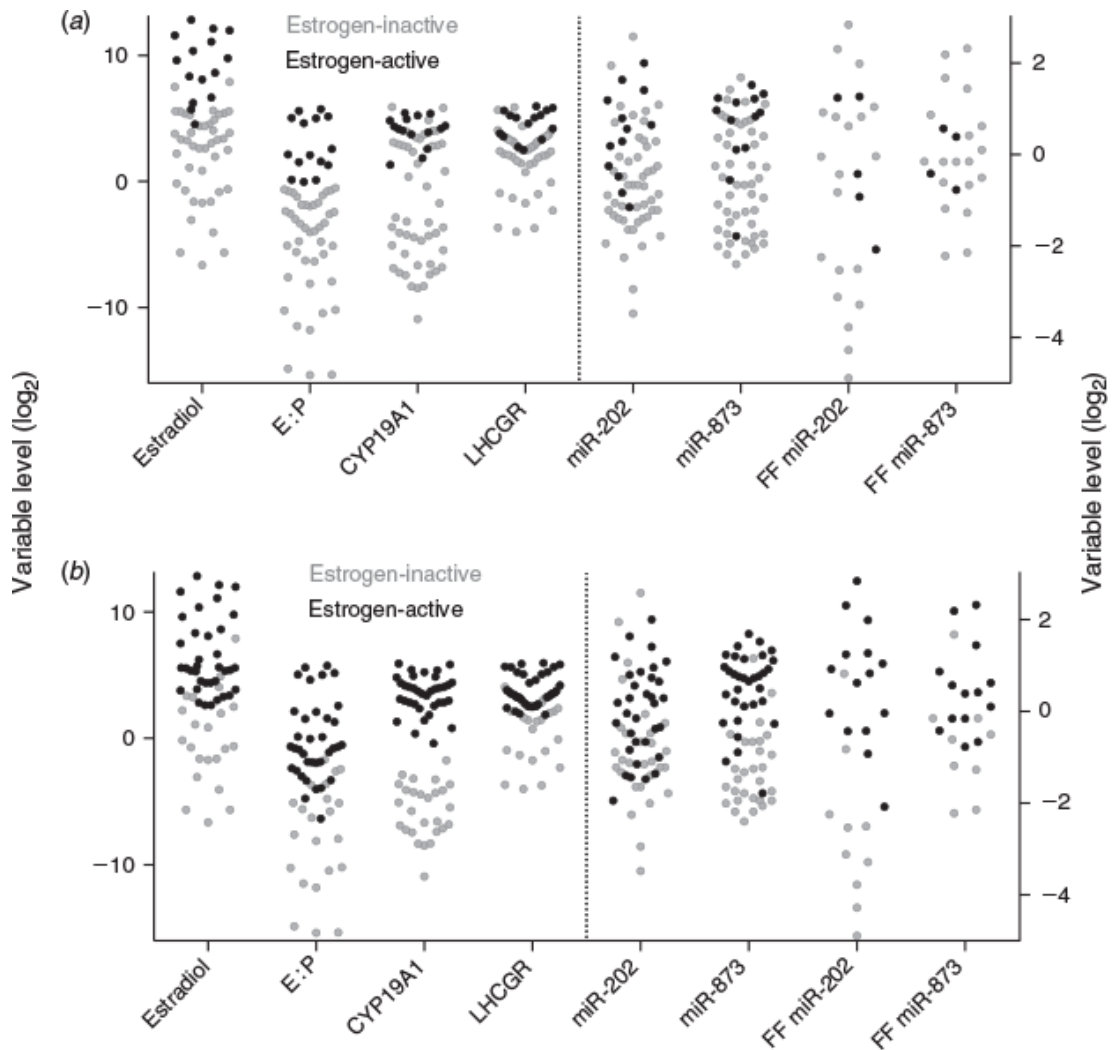


Figure 3. Distribution of values (log₂-transformed) for different endpoints in bovine follicular samples.

Follicles were classified as oestrogen-active and –inactive according to a) E:P ratio and b) *CYP19A1* levels. FF indicates miRNA values quantified in follicular fluid rather than in follicular wall.

Finally, we sought to investigate the biological implications of the observed temporal relationships between oestradiol and miRNA levels by examining the effects of miRNA inhibition on oestradiol production by bovine follicular cells in culture. We initially wished to examine miR-202 and miR-873, both of which are expressed predominantly in the granulosa cell compartment of bovine follicles. [7]. However, as reported in a previous study [7], the expression of miR-873 in follicular cells decreased very quickly upon culture, which prevented subsequent functional analyses of this miRNA. Thus, we focused our analyses on miR-202, a miRNA for which a temporal association with oestradiol was reported in embryonic chicken

gonads [11]. We transfected granulosa cells with different concentrations of LNA inhibitor and found that 5 nM of LNA reduced miR-202 to almost undetectable levels within 24 h (Figure 4a). The absence of miR-202, however, did not induce significant changes in the expression of CYP19A1 or the levels of oestradiol when measured for up to 4 days after cell transfection (Figure 4b, c).

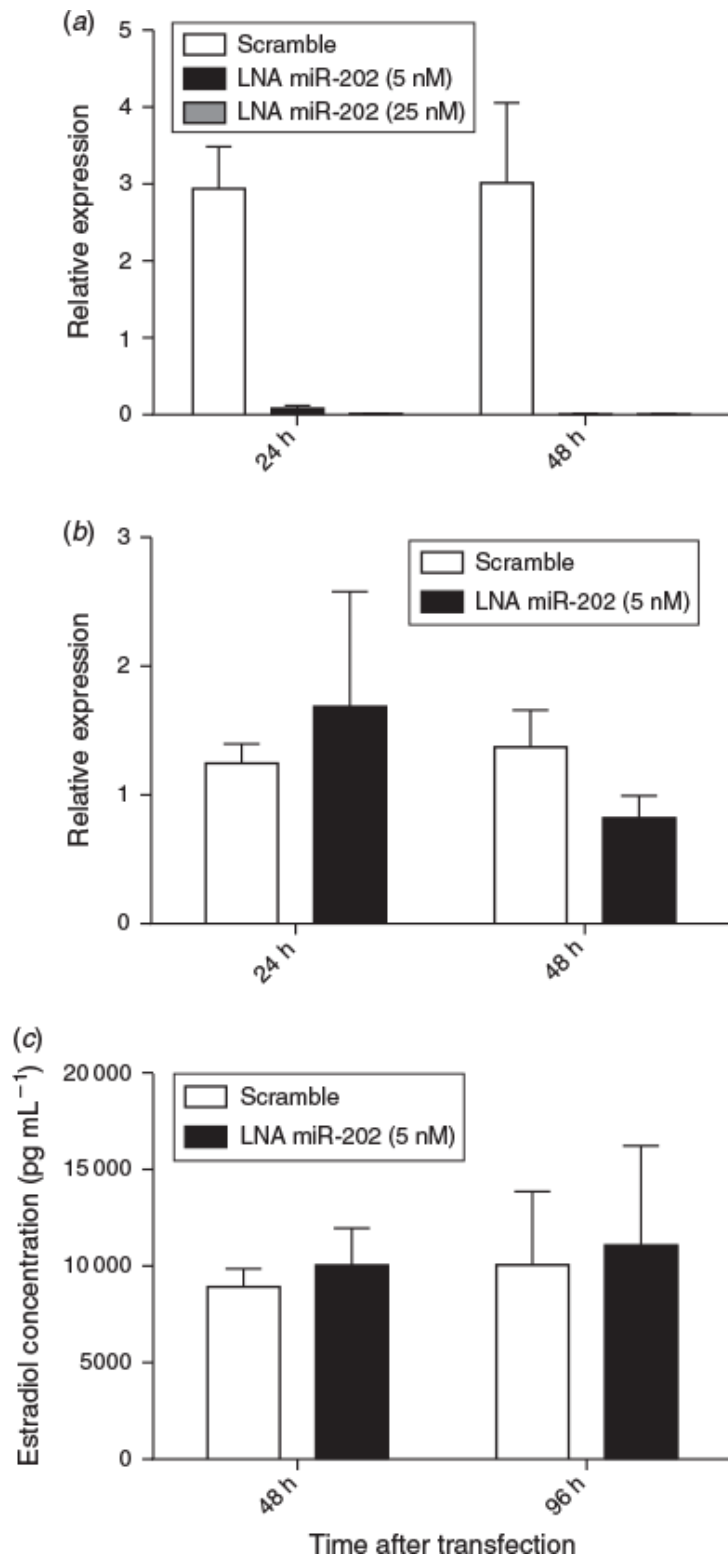


Figure 4. Mean ($\pm$ SE) levels of miR-202 (a) and *CYP19A1* (b) in cells (normalised to *RnU6-2* or *18S*, respectively), and oestradiol in culture media (c) after bovine granulosa cells were transfected with LNA miR-202 or scrambled oligonucleotide sequences.

There was a main effect of Treatment ($P < 0.0001$) for miR-202 (a).

Discussion

Although immunoassay determination of E:P ratio in follicular fluid has been used traditionally to determine the steroidogenic capacity and health status of follicles, qPCR analyses may provide a more convenient and cost-effective approach which can in addition be easily multiplexed to quantify several genes at the same time. To address this, in this study we determined, in a large population of bovine follicles representing a wide range of antral developmental stages (4-22 mm diameter range), the relationship among E:P ratio and the levels of CYP19A1, LHCGR and a total of 15 miRNAs which we identified in a previous study to be differentially expressed during follicle growth in cattle [7], with the aim of identifying novel miRNA biomarkers of follicular function.

Our finding of very strong correlations among E:P, CYP19A1 and LHCGR is consistent with a robust increase in both oestrogen-producing capacity and LHCGR expression in growing dominant follicles and a sharp decrease in both parameters during follicle atresia [12, 13]. Although most of the miRNAs analysed in follicular wall were correlated with several indices of steroidogenesis and LHCGR, a strong correlation ($p \geq 0.7$) with oestradiol, E:P and CYP19A1 was only found for miR-873. This is a mammalian-specific miRNA which we previously showed to be ubiquitously expressed in bovine tissues and to sharply increase in expression in oestrogen-producing > 8 mm follicles, mainly in mural granulosa cells [7]. Although there is little functional data on miR-873, this miRNA has been suggested to stimulate production of inflammatory cytokines [14], prevent macrophage apoptosis [15] and regulate tissue development by targeting the sonic hedgehog pathway [16], any of which effects could putatively result from the increase in miR-873 expression in dominant-size follicles. Interestingly, another study found miR-873 to have an inhibitory effect on oestrogen receptor transcriptional activity and cell growth by targeting CDK3 in breast cancer cells [17]. Notwithstanding the actual involvement, if any, of miR-873 in follicle development, the present results identify this miRNA as a novel marker of steroidogenically active follicles in cattle.

An ideal follicular biomarker should be measurable in follicular fluid. Follicular fluid levels of miR-202, a gonad-specific miRNA [7], but not the other miRNAs analysed, were correlated with oestrogen-production indices and LHCGR expression. A lack of correlation of follicular fluid miR-873 levels was unexpected given the high correlation between miR-873 levels in follicular wall and all three oestrogen-production indices ($\rho \geq 0.7$). The reason for this may have been the low abundance of miR-873 relative to other miRNAs analysed particularly in follicular fluid where mean Cq values for miR-202 and miR-873 were 25 and 34, respectively. In contrast, the absence of a correlation between miR-130a, miR-150 and miR-21 and any index of steroidogenesis or LHCGR cannot be explained by a low abundance of these miRNAs in follicular fluid. Unlike miR-202, these three miRNAs are associated with atretic follicles [7]. Although miRNAs are reportedly secreted by follicular cells, and levels in follicular cells and follicular fluid can be correlated [8, 10], whether miRNA secretion varies in relation to follicular status or with follicular cell of origin has not been determined. Indeed, the rate of secretion of miRNAs into follicular fluid or the stability of such miRNAs may differ between oestrogen-active and -inactive follicles. Likewise, miRNAs can be preferentially expressed in either granulosa or theca cells [7] which may influence the rate at which they gain access to the follicular fluid compartment. These are all important considerations that should be addressed in future studies.

Given that a relationship between oestradiol and miR-202 levels has already been reported in other species [11] and that miR-202 expression is readily maintained in cultured granulosa cells [7], we examined whether the relationships observed in bovine follicles may reflect a role of miR-202 in oestradiol production. Our results after inhibition of miRNA levels in bovine granulosa cells does not support such a role for miR-202, at least in the absence of downregulation of other miRNAs. Sexually dimorphic expression of miR-202 occurs during gonadal development in both chicken and mouse, with predominant expression of the miRNA in testis [11, 18]. Moreover, in the early chick embryo oestradiol can reportedly prevent miR-202 expression during gonadal masculinisation [11], an observation that is not consistent with the positive correlation between miR-202 and oestradiol levels in bovine follicles in our study. Aside from the studies above, very little information is

available on the involvement of miR-202 in non-transformed cells. Clearly, the involvement of miR-202, if any, in the adult gonad could be different from that reported in the embryo.

In summary, we identified two miRNAs, miR-873 and miR-202, which levels in follicular tissues could be used as indicators of steroidogenic capacity in bovine. We show that, in our follicular fluid sample population, using a threshold value of E:P = 1 does not effectively classify follicles into distinct oestrogen-active and -inactive populations based on other known indicators of steroidogenesis, namely CYP19A1 and LHCGR. We propose that expression analyses of one or several of CYP19A1, LHCGR and miR-873 in follicular wall and/or miR-202 in follicular fluid could be reliably used in combination or alternatively to E:P ratio to separate follicles based on their steroidogenic capacity. Future studies should elucidate the implications of these findings in terms of follicle health.

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Appendix II - A miRNA-target network putatively involved in follicular atresia

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Abstract

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

Introduction

The overwhelming majority of follicles recruited from the primordial pool during a female's reproductive life will undergo atresia before they can reach the ovulatory stage. Atresia is an active process involving not only cell death but also resorption of follicular tissue and its replacement by stromal and new follicular tissue; these processes involve infiltration by immune and other cells, very much resembling wound healing [1]. Among several key regulators of wound healing and tissue remodeling processes across body tissues are microRNAs (miRNAs) [2].

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

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

Materials and methods

Collection and processing of bovine tissues

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

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

RT-qPCR

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

Table 1. Primer sequences used in mRNA analyses

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

MiRNA target identification

Identification of putative miRNA targets was done using miRTarBase 6.0 and TargetScan 7.0 to select targets experimentally validated in human and/or rodents (by reporter assay, western blot and/or RT-qPCR, as detailed in <http://mirtarbase.mbc.nctu.edu.tw/>) and computationally predicted targets within the bovine genome (<http://www.targetscan.org>), respectively. For convenience, each identified miRNA-target interaction was classified as high-, medium- or low-

confidence based on whether it was present in both miRTarBase and TargetScan, miRTarBase only or TargetScan only.

Statistical analyses

ROUT outlier test was applied to data sets, and outlier values ($P < 0.01$) were excluded from subsequent analyses. Gene expression data were assessed for normality using the D'Agostino & Pearson normality test and were log-transformed prior to statistical analysis where necessary. Two-way ANOVA followed by unpaired t-tests to identify differences in gene expression between healthy and atretic follicles within each cell type were used. Significance was considered at $P < 0.05$ whereas differences with $P < 0.1$ were considered to approach significance. Nomenclature according to miRBase release 21 is used throughout the manuscript. All miRNAs referred to are bovine (bta-) except otherwise specified.

Results

MicroRNA expression analyses in follicular tissues

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

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

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

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

**FDR; Benjamini and Hochberg adjustment

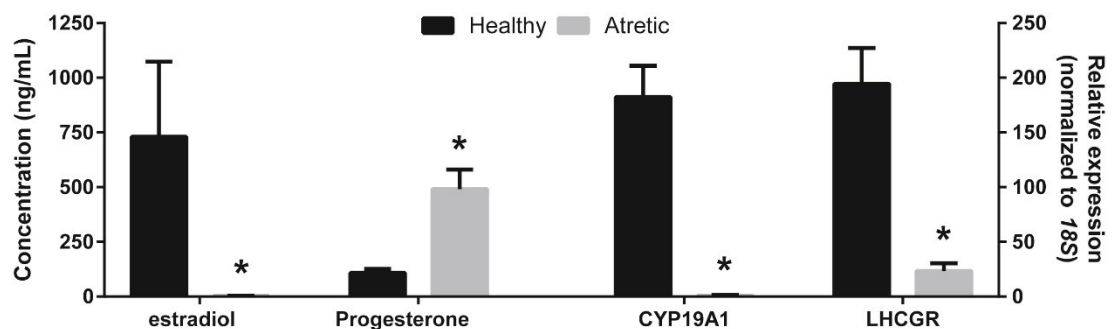


Figure 1. Mean ($\pm$ SE) concentrations of Oestradiol, Progesterone, and transcript levels of *CYP19A1* and *LHCGR* in bovine follicles (9-17 mm) classified as healthy (n = 26) or atretic (n = 15).

Differences between group means are indicated by asterisks ($P \leq 0.05$). Data adapted from Sontakke et al. 2014.

To gain insight on the involvement of these miRNAs in follicle development we quantified their relative expression in granulosa and theca cell compartments. This showed that all miRNAs except for miR-21-3p and miR-378 were expressed in greater abundance (between 2-fold and 25-fold) in theca than in granulosa cells (cell type, $P < 0.05$; Figure 2). Moreover, an effect of Follicle Status ($P < 0.05$), owing to

overall higher expression levels in atretic follicles, was detected for all miRNAs except miR-21-5p and miR-378; for miR-378, an interaction approached significance ($P = 0.07$), reflecting higher expression levels in granulosa but not theca cells from atretic follicles.

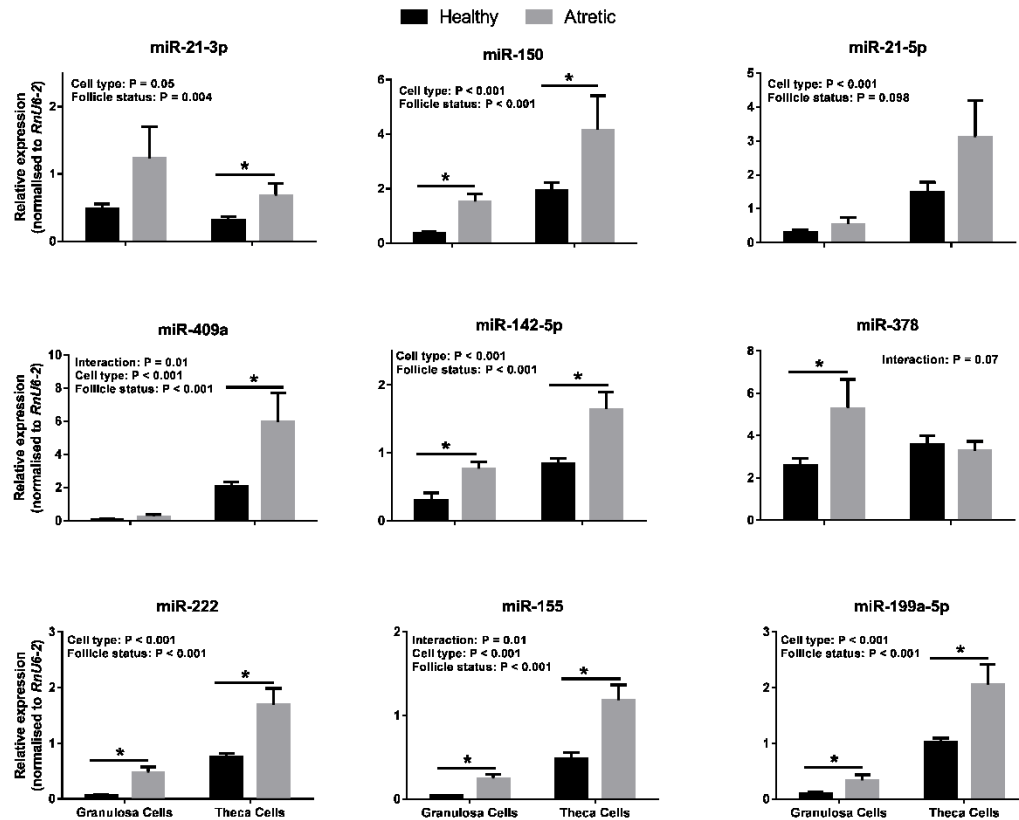


Figure 2. Relative miRNA levels (Mean $\pm$ SE; normalised to levels of *RnU6-2*) in granulosa and theca cells from bovine healthy (n = 9) and atretic (n = 6) follicles. Differences between group means within each Cell type are indicated by an asterisk ($P < 0.05$).

Analyses of microRNA targets

To identify putative targets of the 9 miRNAs in cattle we used miRTarBase, a database containing experimentally validated targets of human, mouse and rat miRNAs, and we selected 36 genes that were simultaneous targets of ≥ 2 of those miRNAs. To increase confidence in our target selection, we then searched all 36 genes in TargetScan and selected those, 16 in total, which bovine homologues were computationally predicted targets of one or more of the corresponding bovine miRNA sequences. Finally, we assessed the validity of these predictions by

analysing by RT-qPCR the expression in follicular cells of 8 of the 16 genes identified (Table 3 and Figure 3; an additional two targets, E2F2 and SIRT1, were also selected but could not be detected in follicular cells by RT-qPCR). For completeness, our RT-qPCR analyses also included one gene (MYD88) which interactions with two miRNAs were experimentally validated but not computationally predicted in bovine (Table 3 and Figure 3), and 2 genes, IGF1 and PAPP, which were computationally predicted bovine targets of 2 miRNAs (miR-222 and miR-378) and 3 miRNAs (miR-142-5p, miR-150 and miR-378), respectively, but none of which were experimentally validated in any species, i.e., they were present in TargetScan but not in miRTarBase (Table 3 and Figure 3).

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

	miR-142-5p	miR-150	miR-155	miR-199a-5p	miR-21-5p	miR-222	miR-378
<i>ETS1</i>							
<i>JAG1</i>							
<i>RECK</i>							
<i>TIMP3</i>							
<i>HIF1A</i>							
<i>IGF1R</i>							
<i>MSH2</i>							
<i>VEGFA</i>							
<i>MYD88</i>							
<i>IGF1</i>							
<i>PAPP</i>							

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

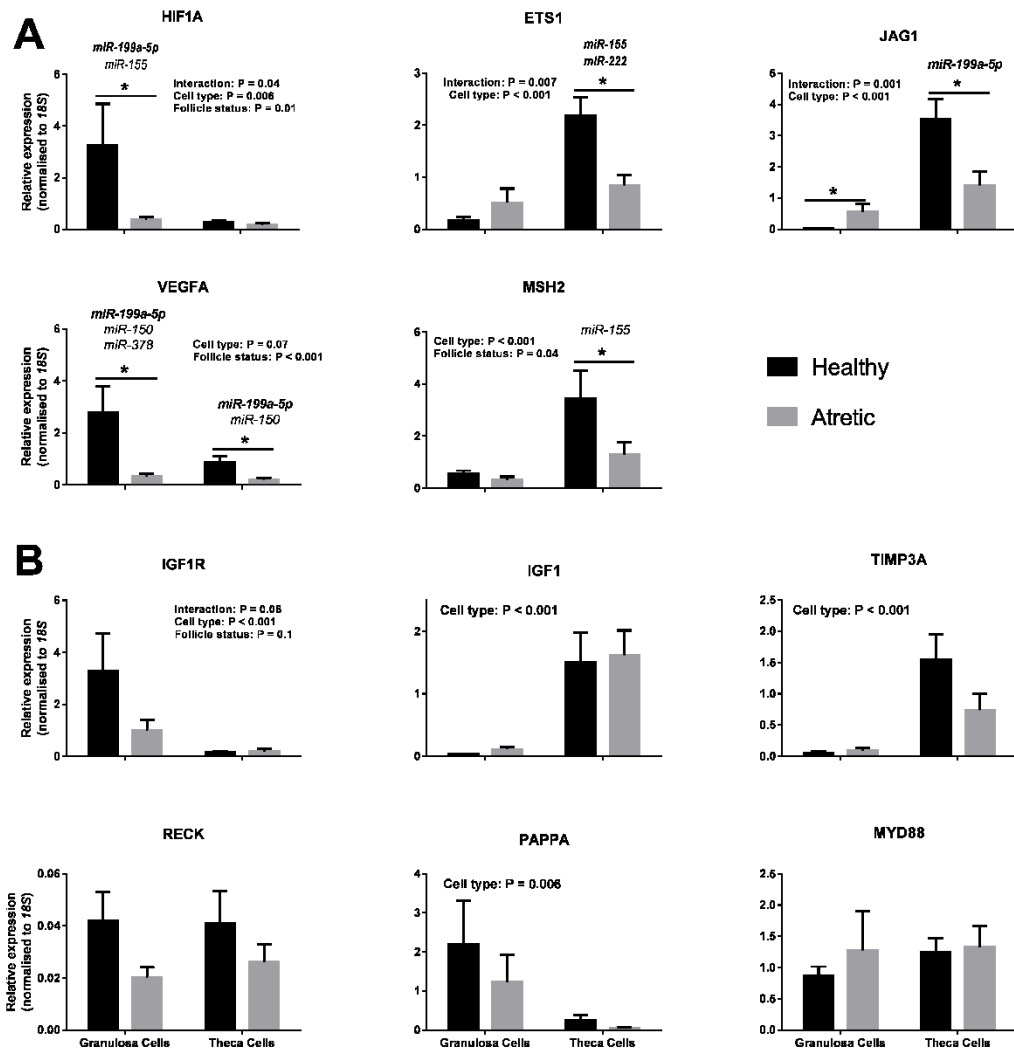


Figure 3. Relative mRNA levels (Mean $\pm$ SE; normalised to levels of *18S*) in granulosa and theca cells from bovine healthy ($n = 9$) and atretic ($n = 6$) follicles. Genes confirmed as miRNA targets by PCR are shown in A), other genes are shown in B). Putative targeting miRNAs are shown on top of the corresponding graph bars in A); all indicated miRNA-target interaction were obtained from miRTarBase (database of experimentally validated miRNA-target interactions in human and/or rodents), and interactions that were in addition computationally predicted in cattle (obtained from TargetScan) are indicated by miRNAs in bold (e.g., miR-199a-5p-*HIF1A*). Differences between group means within each Cell type are indicated by an asterisk ($P < 0.05$).

As shown in Figure 3, out of the remaining miRNA targets, 8 were enriched (Cell type, $P < 0.05$) in either granulosa cells (*HIF1A*, *IGF1R* and *PAPP*) or theca cells (*ETS1*, *JAG1*, *MSH2*, *IGF1* and *TIMP3A*), and 5 targets were differentially expressed according to follicle status, in all cases involving a reduction in atretic follicle cells,

as indicated by a significant effect of Follicle Status or a Follicle Status x Cell Type interaction (*HIF1A*, *ETSI*, *JAG1*, *VEGFA* and *MSH2*).

Validation of microRNA-target interactions

Comparing miRNA and mRNA expression profiles (Figures 2 and 3) allowed for the testing, based on a negative association between the expression of a miRNA and that of its predicted target(s) within a follicular cell type, of a total of 12 high-confidence miRNA-target interactions (i.e. obtained from both miRTarBase and TargetScan; indicated by dark grey in Table 3), 8 medium-confidence interactions (obtained from miRTarBase but not present in TargetScan; indicated by light grey in Table 3) and 5 low-confidence interactions (identified in TargetScan but not present in miRTarBase; indicated by an “X” in Table 3).

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

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

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

Discussion

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

Our miRNA-target pair analyses provides novel insight on the molecular regulation of follicular atresia by identifying specific miRNA networks putatively involved within different follicular compartments (summarised in Figure 4). To identify high-confidence bovine miRNA targets we selected genes that both 1) had already been experimentally validated for 2 or more miRNAs in different cellular contexts in humans and/or rodents (as bovine-specific information is not available) and 2) contained predicted miRNA target sites in the bovine homolog 3' UTR. In choosing

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

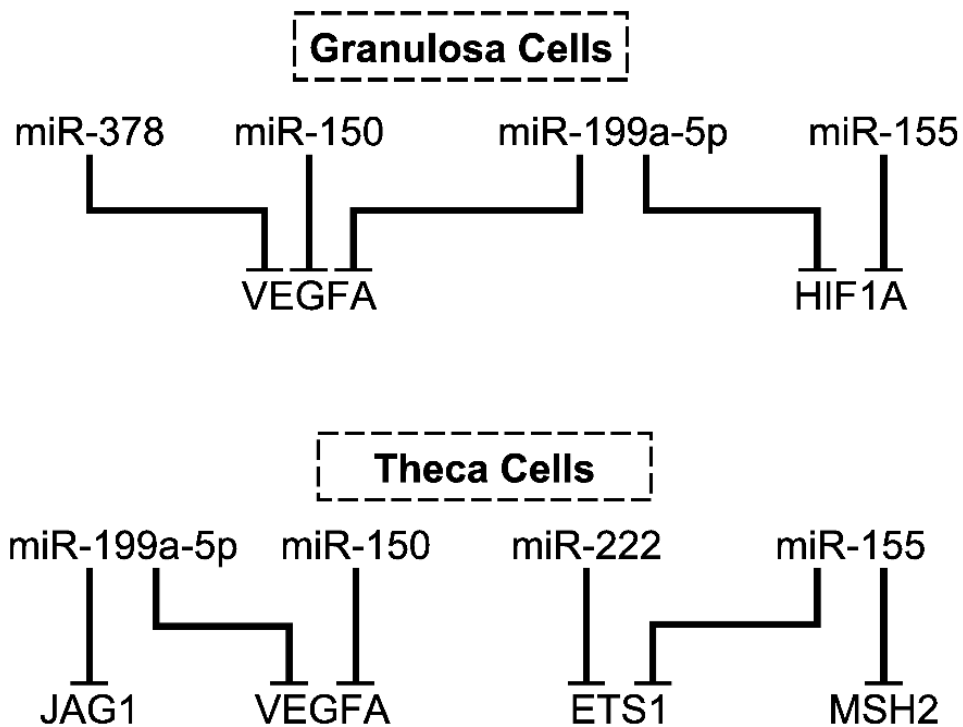


Figure 4. Schematic summary of putative miRNA-target interactions in granulosa and theca cells during follicular atresia identified in this study.

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

one miRNA, miR-199a-5p, simultaneously targeting both genes, as reported in other cell types [33].

Two miRNA targets identified in theca cells and which, albeit much less characterised, may also be involved in angiogenesis, are the transcription factor, ETS1, and the NOTCH1 ligand, JAG1. ETS1 is a proto-oncogene highly expressed in immune and vascular cells. In the ovary, it has been shown to be expressed in theca and granulosa cells and its expression to dynamically change during the oestrous cycle [34, 35]. An involvement of ETS1 in regulating RGS2 expression during ovulation has been shown [36]. Moreover, its role in promoting angiogenesis or as a pro-apoptotic factor in different cell types [37] could account for the changes in expression during follicle atresia. Likewise, apart from its reported role in promoting early follicle development, little information is available on the role of JAG1 in the ovary. An involvement of JAG1 in follicular angiogenesis is suggested by its expression in endothelial and other vascular mural cells in mouse ovaries [38]. The reason for the opposite trends in *JAG1* expression in granulosa and theca cells of healthy and atretic follicles in our study is unknown, warranting further study of the functions of this gene in the adult ovary.

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

Finally, because of the heterogeneous nature of follicular tissue, particular theca, it is not possible to determine, from our data, the specific cell types involved in the identified miRNA-target interactions. For example, although most of the miRNAs analysed in this study are known not to be cell-specific, miR-150 and miR-155 are highly expressed and primarily regulate hematopoietic and vascular cells. Nonetheless, paracrine regulation of gene expression by miRNAs has also been

described whereby miRNAs are produced by one cell type and then secreted to regulate gene expression of a different cell type within a tissue [40], adding further complexity to the role of miRNA-target interactions during follicular atresia.

In summary, by establishing the expression patterns of miRNAs and their putative targets in granulosa and theca cells of healthy and atretic follicles in cattle we have identified a network of miRNAs including miR-199a-5p, miR-155, miR-222, miR-150 and miR-378 which we propose are involved in follicle atresia through combined targeting of genes involved in cell survival, proliferation and differentiation; namely *HIF1A* and *VEGFA* in granulosa cells, and *MSH2*, *ETSI*, *JAG1* and *VEGFA* in theca cells. Although the identified miRNA-target interactions should be confirmed by gene targeting or other molecular approaches in future studies, our results will provide fertile ground for further hypothesis-testing towards a better understanding of the molecular mechanisms involved in follicle atresia.

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Appendix III - Additional files (CD-ROM)

File 2-1. Oestrous cycle small-RNA sequencing dataset. Read counts and differential expression analysis for bovine and human-homologue miRNAs in bovine plasma. FDR > 0.1 for all statistical results.

File 2-2. Oestrous cycle PCR array dataset. Cq values and differential expression analysis results for bovine miRNAs in plasma measured by Qiagen PCR arrays. FDR > 0.1 for all statistical results.

File 2-3. Oestrous cycle target and pathway analysis data. KEGG pathways enriched among experimentally validated targets of let-7f, miR-125b, miR-99a-5p and miR-145 using miRPath 3.0 and TarBase 7.0.

File 2-4. Oestrous cycle isomiR dataset. A list of isomiRs in bovine plasma identified using sequencing.

File 3-1. Early pregnancy small-RNA sequencing data. Normalised expression data (reads per million mapped) and differential expression analysis results for bovine plasma miRNAs, generated using small-RNA sequencing.

File 3-2. Early pregnancy PCR array data. Normalised expression data and differential expression analysis results for bovine plasma miRNAs, generated using PCR arrays.

File 3-3. Early pregnancy differences in miRNA levels validated by RT-qPCR. List of differences ($P < 0.05$) in miRNA abundance obtained by PCR array or sequencing (NP16 vs P16, NP vs P16 and NP vs P24) that were selected for validation by RT-qPCR. Fold changes were calculated as pregnant / non-pregnant.

File 4-1. Day 60 sequencing miRNA profiles. Normalised expression for all of the miRNAs which were used in differential expression analysis

File 4-2. Day 60 pathway analysis. Pathways which are targeted by up-regulated and down-regulated miRNAs on Day 60 of pregnancy using sequencing data, based on experimentally validated miRNA targets.

File 4-3. Foetal gender analysis. Results of the differential expression analysis using sequencing data to identify differentially expressed miRNAs between animals carrying foetuses of different gender on Day 60 of pregnancy.

File 5-1. Tissue biomarkers sequencing miRNA analysis. Normalised miRNA expression levels in bovine blood cells and plasma and the results of the differential expression analysis between the two groups.