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The role of microRNAs in the pre-implantation mammalian embryo

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This thesis is submitted to the University of Nottingham in partial fulfillment of the requirements for the degree of the Doctor of Philosophy

April 2021

DECLARATION

I hereby declare that this thesis is my own work and that it has not been submitted anywhere for any other degree or award. The work presented herein is my own work and where other sources of information have been used, they have been duly acknowledged.

Klaountia Dimitra Makri

ACKNOWLEDGEMENTS

It is a pleasure to thank those who made the completion of this degree possible. I wish to thank, first and foremost, my primary supervisor Dr. Walid Maalouf for trusting in me and giving me the opportunity to continue my studies. I am grateful for his advice, guidance, and support during the past years.

I would like to make a special reference to Dr Adam Watkins and Dr Juan Hernandez Medrano whose encouragement, continuous support, and expertise were invaluable for the completion of my studies. I am also thankful to Prof. Kevin Sinclair for his insightful feedback that helped me complete this thesis. Equally I would like to thank the entire team of the CHO&G department for welcoming me and making me feel a part of the team since the beginning.

Special thanks to my colleagues and dear friends Nader Eid, Afsaneh Khoshkerdar, Adwoa Dadson, and Sutham Suthaporn who provided stimulating discussions as well as happy distractions during the last few years. Your moral support and friendly advice has made the completion of this degree easier and I am grateful for the unforgettable memories we have shared.

Getting through my PhD required more than academic guidance and I would like to thank my parents Vera and Loukas for their immense support during this time. I am extremely grateful to my sister Vaso, my partner Thomas, and my friend Ioanna. Without their understanding and encouragement in the past few years, it would be impossible for me to complete my studies. Some special words of gratitude go to my closest friends in the UK and my newest friends in my new home in Germany who have been there for me on a daily basis and were a major source of support throughout this period.

ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNA molecules that exert regulatory actions in gene expression acting mainly via RNA silencing and post-transcriptional changes. Their actions are involved in important cell processes such as stress signalling, cell cycle progression, differentiation, and cell death. Mammalian gametes and pre-implantation embryos selectively express miRNAs that control oocyte maturation, spermatogenesis, fertilisation, embryonic division and growth, development and differentiation, and implantation.

Blastocyst-stage embryos release miRNAs in the surrounding micro-environment and these can be quantified *in vitro*. The levels of specific cell-free miRNAs are linked to embryo aneuploidy and implantation outcomes in human assisted reproduction cycles. However, there are no reports about the value of miRNAs as biomarkers of embryonic quality and their use for embryo (de-)selection purposes. Importantly, basic science evidence about the roles of secreted miRNAs at the peri-implantation period is lacking. The main objective of this thesis is to investigate the biological reason that embryos differentially release specific miRNAs and whether miRNAs have effects on reproduction with specific focus on embryo-maternal communication at the pre-implantation period.

This was addressed firstly by analysing miRNAs in spent media samples from blastocysts and correlating their levels with embryo quality markers (apoptosis and morphokinetics). It was found that highly apoptotic mouse embryos release more miR-294 in spent media. Further research showed that it is the process of apoptosis which triggers the release of this miRNA. This finding was reproduced in bovine embryos where the homologue miR-371 was also linked to extensive blastomere apoptosis. Lastly, human endometrial cells were transfected with the miR-371 mimic

and were analysed for transcriptomic changes using microarray technology. Global genome changes were caused by this miRNA which impaired processes related to implantation.

The original findings presented here suggest that miRNA analysis of individual spent media samples using current technology could be a valuable method for assessment of embryo quality. Potentially, miRNA analysis could be integrated in the future in multi-OMICS platforms for increasing the effectiveness of embryo (de-)selection for clinical purposes. The findings that apoptosis triggers the release of specific miRNAs from pre-implantation embryos which then cause major gene expression changes influencing implantation provide novel insights on the roles of miRNAs in reproduction. Ultimately, this thesis concludes that embryo-secreted miRNAs have key-roles in the communication between the embryo and the maternal cells and act as molecular cues to promote or impair implantation. Future research is advised to elucidate the miRNA molecular network at the embryo-maternal interface.

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ORIGINAL PAPERS

MAKRI, D., EFSTATHIOU, P., MICHAILIDOU, E. & MAALOUF, W. E. 2020. Apoptosis triggers the release of microRNA miR-294 in spent culture media of blastocysts. *Journal of Assisted Reproduction and Genetics,* 37**,** 1685-1694. This publication comprises results obtained in Chapters 3 and 4.

NATIONAL AND INTERNATIONAL CONFERENCES

MAKRI, D., CASTELLANOS-URIBE, M., MAY, S., MAALOUF, W. E. 2021. MiR-371a disrupts the stroma-epithelium communication in human endometrium. This presentation contains results obtained from Chapter 6 and was presented as an oral presentation at the SRF PhD Prize Session, Fertility 2021 Annual Meeting, Online, 6-10 January, 2021. Abstract SRF.4.

MAKRI, D., MAALOUF, W. E. 2020. Increased DNA fragmentation in blastocysts triggers the release of miR-294 in spent culture medium. *Human Reproduction,* 35, Supp. 1, i204. This work was presented by poster at the European Society of Human Reproduction and Embryology 2020 Annual Meeting, Virtual Meeting, 5-8 July, 2020. Abstract P-142.

MAKRI, D., EFSTATHIOU, P., MICHAILIDOU, E. & MAALOUF, W. E. 2020. Embryo-secreted microRNA miR-294 is correlated to DNA fragmentation at the blastocyst stage. This work was presented by poster at the Fertility 2020 Conference, Edinburgh, 9-11 January, 2020. Abstract P-123.

MAKRI, D., MAALOUF, W. E. 2019. MicroRNA miR-294 in spent culture medium is correlated to embryo apoptosis and can serve as a biomarker for non-invasive embryo assessment. *Human Reproduction,* 34, Supp. 1, i206. This work was presented by poster at the European Society of Human Reproduction and Embryology 2019 Annual Meeting, Vienna, 23-26 June, 2019. Abstract P-133.

LIST OF ABBREVIATIONS

aCGH array comparative genomic hybridisation

ART assisted reproduction technology

CL corpus luteum

COC cumulus oocyte complex

DSC decidualised stromal cells

ECM extracellular matrix

EEC endometrial epithelial cells

EPI epiblast

FF follicular fluid

GC granulosa cells

GO gene ontology

GSEA gene set enrichment analysis

GV geminal vesicle

HA hyaluronic acid

hCG human chorionic gonadotropin

HPG hypothalamic-pituitary-gonadal

ICM inner cell mass

ICSI intracytoplasmic sperm injection

IVF *in vitro* fertilisation

KEGG Kyoto Encyclopaedia of Genes and Genomes

LH luteinising hormon

MI/II metaphase I/II

MP mammalian phenotype

MRE miRNA response element

NES normalised enrichment score

NGS next generation sequencing

PCOS polycystic ovarian syndrome

PE primitive endoderm

PGC primordial germ cells

PGD pre-implantation genetic diagnosis

PGS pre-implantation genetic screening

PGT-A pre-implantation genetic testing for aneuploidy

PLCζ phospholipase C zeta

PN pronuclear

POF premature ovarian failure

SCM spent culture media

SSC spermatogonial stem cells

TCN total cell number

TE trophectoderm

TUNEL TdT-mediated dUTP-X nick end labelling method

WOI window of implantation

XCI X chromosome inactivation

ZGA zygote genome activation

ZP zona pellucida

1. CHAPTER ONE: LITERATURE REVIEW

1.1 Assisted Reproduction Technologies

In a biological sense reproduction ensures the self-perpetuation of the species and is a mechanism of life to move forward. In humans creating a family is much more than conserving our species – reproduction fulfils emotional, behavioural, and social needs. Unfortunately, 15% or reproductive-aged couples worldwide, translating to 48 million couples or 186 million individuals, are affected nowadays by infertility according to the World Health Organization (WHO). Infertility is defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse and may occur due to male factors, female factors, a combination of both, environmental and lifestyle factors, or may be unexplained (WHO, 2021). Assisted reproduction technology (ART) treatment is often the only solution for affected people and the European Society of Human Reproduction and Embryology (ESHRE) estimates that around 2.4 million ART cycles are carried out worldwide each year, with about 500.000 babies born per year (ESHRE, 2020).

Since the first baby was born in 1978 through *in vitro* fertilisation (IVF), major steps have been made to improve medical treatment, optimise techniques, and develop assessment methods that overall aim to increase the success of IVF. ART treatments vary from simple interventions like intrauterine insemination (IUI) and fertility drug administration to conventional IVF and intracytoplasmic sperm injection (ICSI) and more complex interventions like surgery of the reproductive system, fertility preservation, surrogacy, embryo testing, and add-on treatments. Sadly despite all the advancements in the field the success rates remain relatively low, with recent figures from the Human Fertilisation and Embryology Authority (HFEA) showing an average

of 23% in live birth rates per embryo transferred that falls dramatically to below than 5% as the maternal age increases to 43 and above (HFEA, 2020). Achieving a live birth from the first IVF cycle is very important firstly because of the high costs that are covered by national or private healthcare providers or by the patients themselves. Even more importantly, patients undergo considerable physiological and psychological stress throughout IVF and thus it is crucial to increase the chances of a live birth the earliest possible. Therefore, identifying the factors affecting IVF success and adjusting the clinical treatment to overcome these are major topics of study and discussion in the assisted reproduction field.

1.1.1 Factors affecting IVF success

Reproductive system pathologies, diseases, advanced maternal age, obesity, smoking, and stress are all factors that can have negative effects on IVF treatment (Bashiri et al., 2018). Many of these affect the gamete quality which ultimately results in a developmentally compromised embryo that fails to develop, implant, or grow further into a healthy foetus. Indeed, oocytes from patients of advanced age have more frequently chromosomic errors or have spindle abnormalities that lead to high aneuploidy incidence in the embryo (Cimadomo et al., 2018). Moreover, poor sperm quality is associated with aneuploidy, mosaicism, and abnormal morphokinetic development of embryos (Kahraman et al., 2020) which possibly explains the lower pregnancy rates in these patients (Chapuis et al., 2017). Even when embryo quality is excellent, uterine pathologies as well as hormonal and metabolic disorders can have adverse effects on the implantation process, inflammatory response, placental growth, and overall the maintenance of pregnancy (Vannuccini et al., 2016).

Technical factors can also impair IVF success mainly because of the effects they have on oocyte and embryo quality. Conventional IVF treatment comprises four main steps: the collection of oocytes from the ovaries and collection and processing of sperm, the fertilisation *in vitro* of the oocytes, the culture of the embryos, and the transfer of the embryo(s) into the uterus. Initially ovarian stimulation drugs are administered to the patient to allow the retrieval of multiple mature oocytes that can be used for fertilisation. Undoubtedly the regimen used for ovarian stimulation impacts IVF outcomes primarily because the oocyte quality is strongly influenced by different stimulation drugs (Bosch et al., 2016).

Moreover, fertilisation and *in vitro* culture methods become more and more refined in the latest decade in order to mimic as close as possible the conditions *in vivo*. At the very early stages embryos depend on the ingredients of the culture media (amino acids, chelators etc.) and are susceptible to environmental changes, namely temperature, pH, and atmospheric conditions (CO_2, O_2). It is therefore not surprising that extended culture in *in vitro* conditions impairs clinical outcomes. Additionally, the culture medium type impacts on live birth rates, meaning that not all commercially available media are equally efficient for IVF treatment (Castillo et al., 2020). Lastly, the timing of embryo transfer, the type of catheter used, and the uterine location where the embryo is placed are all critical factors of implantation success at the last step of IVF treatment (Schoolcraft, 2016). Naturally, specialised training and advanced skills are required for all the laboratory procedures to ensure fast, safe, and efficient performance.

Apart from the technical difficulties to mimic *in vivo* conditions, one of the problems embryologists face is choosing which embryos have the best chances of implantation and should be therefore transferred. This has been made even more

challenging since the HFEA started to actively monitor and regulate embryo transfers in the UK (HFEA, 2017) in an effort to eliminate the adverse effects of multiple gestations (Sazonova et al., 2013). As predicted by Gardner and colleagues (Gardner et al., 2000), single transfers indeed lower multiple gestation rates and the associated risks without compromising pregnancy rates (Hodes-Wertz et al., 2013, Grady et al., 2012). Consequently, the need for assessment systems that can effectively distinguish the best embryo for transfer became even more apparent.

1.2 Embryo assessment

1.2.1 Pre-implantation embryo development

The events and mechanisms occurring at the early stages of mammalian embryo development are briefly described to introduce the topic of embryo assessment. Initially the fertilised egg undergoes a series of division cycles producing increasing numbers of cells known as blastomeres. Protein synthesis in the zygote relies on the maternal mRNA cargo. During the initial cleavage cycles the embryo transcribes its own mRNA machinery upon the process of zygotic genome activation (ZGA). In humans, the initial divisions produce an embryo with 16 blastomeres that exhibit augmented intercellular adhesion known as compaction. At this stage the blastomeres slowly acquire polarity and as the embryo grows, two populations of cells are created: outside, polar cells and inside, non-polar cells. This polarity is maintained through the next division cycle which produces 32 blastomeres. From the 32-cell stage onward, the two cell populations have distinct developmental fates with the outside cells contributing to the trophectoderm (TE) lineage and the inside cells contributing to the inner cell mass (ICM), the group of cells that will further diverge into epiblast (EPI) and primitive endoderm (PE) lineages by the late blastocyst stage. The next stage is

the formation of a fluid-filled cavity known as blastocoel which marks the early blastocyst stage. During this stage, the embryo actively pumps water in the inside compartment through the actions of membrane transporters and the TE forms tight junctions that create a seal which prevents water leakage. As the pressure from the blastocoele increases and cell divisions take place, the embryo diameter grows larger until the surrounding zona pellucida (ZP) breaks and the fully-grown blastocyst hatches in preparation for implantation into the endometrium (Cockburn and Rossant, 2010).

The mouse embryo and other animal models are often used in experiments to elucidate the mechanisms controlling these early stages of life due to the many morphological similarities with the human embryo. However, caution must be taken before any conclusions are made for human embryology because of important differences between species. For instance, the ZGA occurs in different stages between human, murine, and bovine and compaction is also initiated later in the human embryo. Additionally, the molecular mechanisms guiding the cell fate are mostly studied in mouse embryos with obvious differences in the transcription factors involved in human embryo development. In Figure 1.1 the embryonic stages are illustrated in two species, human and mouse, to allow for comparisons.

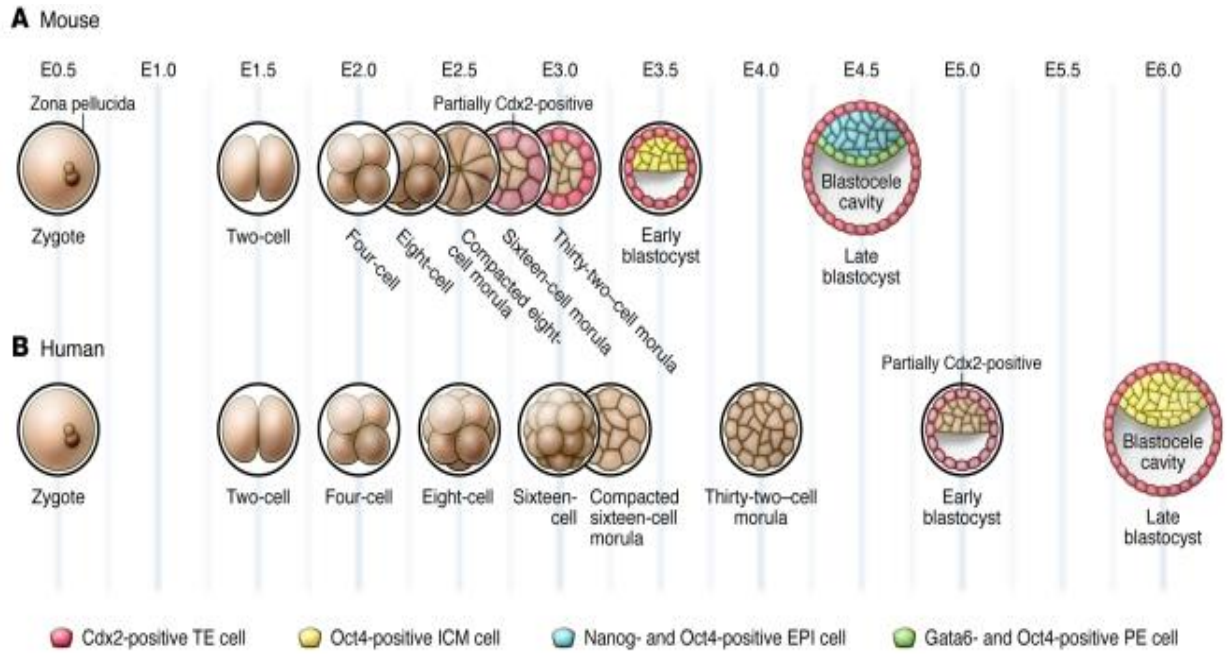

Figure 1.1 Mouse and human pre-implantation embryo development. (A) In the mouse embryo compaction starts at the 8-cell stage. Subsequent divisions produce two cell populations occupying the inside and outside regions of the embryo. At the 32-cell stage, the outside cells increase their expression of Cdx2 whereas Oct4 is limited in the inside cells The blastocoele starts forming at this stage. By the late blastocyst stage, the inside cells differentiate into two cell types with different gene expression profiles (Nanog/Gata6). **(B)** Compaction happens at a later stage in the human embryo while the mutually exclusive CDX2/OCT4 expression profiles are not established until the late blastocyst stage. The transcription factors guiding the second lineage decision are unclear in human embryos. E0.5-6: Day of embryonic development (Cockburn and Rossant, 2010).

1.2.2 Embryo morphology assessment

The most widely used approach to assess the quality of pre-implantation embryos is the observation of an embryo through a microscope and the subsequent categorisation of the embryo in quality grades (A, B, or C) based on specific morphological markers. In the early pronuclear oocyte morphological scoring is based on the location of the maternal and paternal pronuclei and the orientation and polarisation of the enclosed nucleolar precursor bodies. The embryos are then further cultured and morphological assessment is typically carried out before the embryo transfer process. Therefore morphological assessment differs depending on the day of transfer (usually cleavage-stage or blastocyst). For cleavage-stage embryos the cell

number, percentage of fragmentation, symmetry of blastomeres, multinucleation, and vacuole presence are considered when scoring the embryos (Balaban et al., 2011). However, morphological scoring of the early embryo is a poor indicator of later development because it is not possible to predict whether embryos will continue to arrest, grow, or form excellent quality blastocysts (Graham et al., 2000). Therefore blastocysts transfers are preferred so that only embryos which have already reached the final stages of pre-implantation development and are developmentally competent are transferred. In Figure 1.2, the features that are assessed in traditional morphological assessment of pronuclear stage oocytes, cleavage-stage embryos and blastocysts are described.

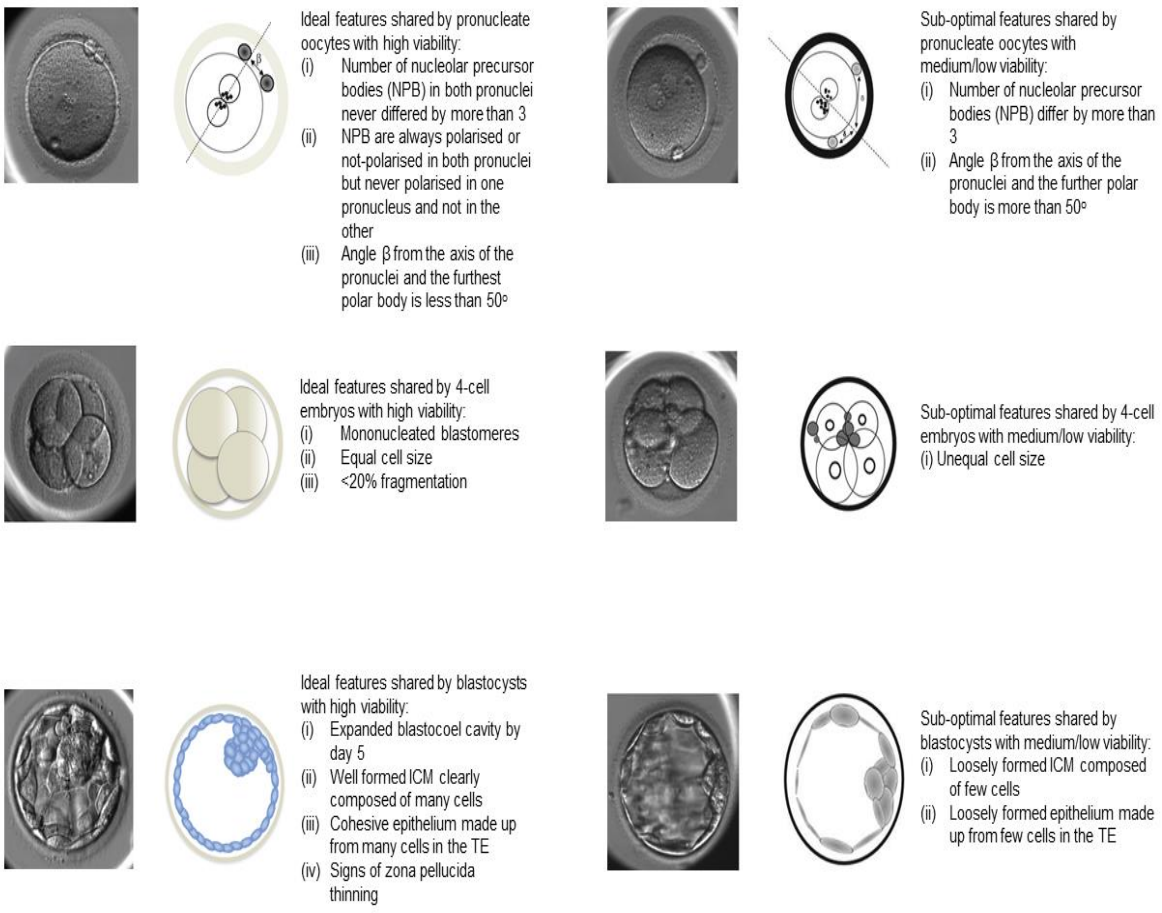

Figure 1.2 Key morphological features of human embryos with high (left) and medium to low (right) viability. ICM: inner cell mass; TE: trophectoderm (Gardner and Balaban, 2016).

Historically Gardner and Schoolcraft (1999) introduced a blastocyst scoring system which described the blastocyst quality in terms of expansion degree, cell number and cohesiveness of the ICM and TE populations (Gardner and Schoolcraft, 1999). This system set the basis for minor changes and additions in later years which included phenomena such as vacuolation and hatching patterns (Hardarson et al., 2012, Balaban et al., 2011). Although blastocyst scoring can indicate the risk of aneuploidy in human embryos (Figueira et al., 2012), studies fail to show an association of blastocyst grade with clinical outcomes in IVF cycles (Capalbo et al., 2014, Van Den Abbeel et al., 2013). The weakest points of morphological evaluation is that firstly the observation is static, i.e at a specific timepoint, and thus dynamic phenomena cannot be observed and secondly that it is highly subjective and depends on the experience and training of an embryologist.

1.2.3 Morphokinetics

Nowadays, high-tech incubation systems with integrated cameras are used in clinics worldwide for thorough observation of embryo development. Time-lapse technology generates detailed information regarding morphology and kinetics throughout the whole pre-implantation development and aids the observation of dynamic events, defined as morphokinetic variables. For instance, embryologists can now annotate the time of pronuclear fading at the zygote stage, durations and synchronicities of division cycles, abnormal cleavage patterns such as trichotomous mitosis and reverse cleavage, and blastocoel collapsing patterns at the blastocyst stage (Ciray et al., 2014) (Figure 1.3). Perhaps the strongest advantage of time-lapse technology is the ability to save all records and annotate at a later time with more than one observer present or even apply artificial intelligence (AI) software to automatically score and rank embryos for transfer. This reduces the subjectivity of

traditional morphological assessment and refines the selection process. Furthermore, time-lapse incubation has the added benefit of a closed, undisturbed culture system which means that the environmental conditions are stable throughout the culture period and fluctuations that jeopardise embryo quality are eliminated.

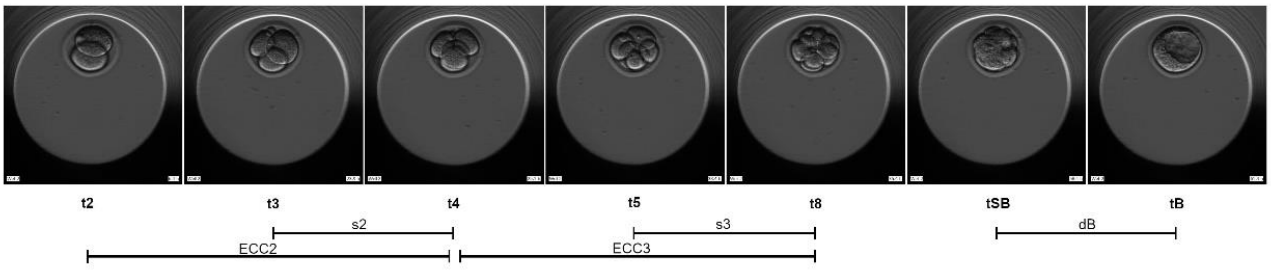

Figure 1.3 Time-lapse snapshots of mouse embryo development. The exact timings of 2-cell, 3-cell, 4-cell, 5-cell, and 8-cell divisions are depicted and the durations and synchronicities of the second (ECC2 and s2 respectively) and third cell cycles (ECC3 and s3 respectively) and duration of blastulation (dB) are calculated using the exact timings.

In regards to its diagnostic value, specific morphokinetic variables have been linked to embryo quality and clinical outcomes. For example, the exact times of the 2- , 3-, 4-, and 5-cell divisions and morula formation predict blastocyst formation and quality (Cruz et al., 2012, Motato et al., 2016, Milewski et al., 2015) and the durations between the 2- with the 3-cell stage, 3- with the 4-cell stage, and 5- with the 8-cell stage predict blastocyst formation and implantation (Motato et al., 2016, Milewski et al., 2015). Moreover, the time of compaction, initiation of blastulation, and full blastocyst formation are associated with the risk of aneuploidy in embryo selection cycles (Campbell et al., 2013). The timing of the 5-cell stage and expansion stages significantly reflect on the implantation potential of embryos (Meseguer et al., 2011, Motato et al., 2016) and time of morula formation is correlated with live birth rates after euploid blastocyst transfers (Rienzi et al., 2019). Additionally, duration of blastulation can be predictive of ongoing pregnancy rates (Mumusoglu et al., 2017).

Although in the beginning time-lapse technology was expected to become a valuable tool for selecting embryos in a non-invasive manner (Kirkegaard et al., 2012), there is now an ongoing debate about whether this add-on treatment is indeed improving clinical outcomes (Harper et al., 2017). For instance, whereas pregnancy rates do not improve with the use of time-lapse (Goodman et al., 2016), a minimum of 19% increase in the incidence of live birth has been found when morphokinetic data are used to select embryos (Fishel et al., 2017). It is evident that unless there is a consensus as to which morphokinetic markers are the most valuable, and further studies control for patient characteristics, treatments, and time-lapse systems used, direct comparisons between findings and formulation of conclusions are impossible. Indeed, most clinics develop in-house selection algorithms which are only beneficial for on-site applications (Barrie et al., 2017).

Apart from these inconsistencies, perhaps one of the weakest points of morphological and morphokinetic evaluation is the inability to accurately detect and identify aneuploidies, now and in the future; them being a major reason of low success in IVF cycles. Indeed, it is impossible to assess the type of chromosomal error and whether an embryo carries certain genetic loci that cause a diseased phenotype by sole observation of the embryo's phenotype. To identify genomic abnormalities one needs to analyse the genetic material itself. Therefore, more accurate and reproducible methods are needed for selecting embryos for transfer.

1.2.4 Biomolecular methods

Biomolecular techniques were employed for embryo assessment purposes because of the high accuracy, sensitivity, reproducibility, and objectivity that these methods offer. For those techniques, embryonic material is obtained through embryo biopsy, either cleavage-stage or blastocyst, and the cells are then processed to amplify RNA or DNA. From there on, monogenic mutations, aneuploidy and chromosomal abnormalities, SNP genotyping, and whole genome sequencing can be carried out depending on the technology used. Conventionally, pre-implantation genetic diagnosis (PGD) refers to the identification of monogenic mutations whereas PGT-A refers to pre-implantation genetic testing for aneuploidies (formerly pre-implantation genetic screening, PGS). Initially, polymerase chain reaction (PCR) technology was used for identifying monogenic mutations however technological advancements in array comparative genomic hybridisation (aCGH) allowed DNA copy number profiling and SNP genotyping simultaneously. Lately, improvement of next generation sequencing (NGS) platforms allowed whole genome sequencing from a single cell with high accuracy thereby providing information of the whole genome that can be used to identify gene mutations and assess the chromosomal status (Vermeesch et al., 2016).

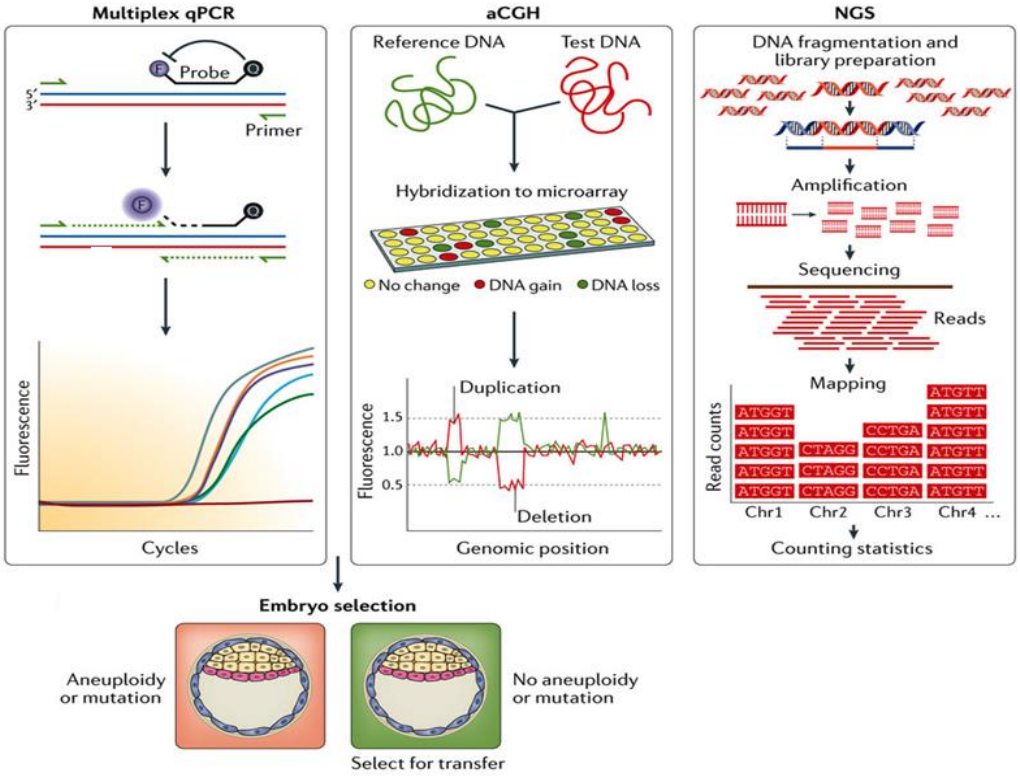

Figure 1.4 The available methods for diagnostic services. Multiplex quantitative PCR (qPCR) for mutation detection and/or genotyping, array comparative genomic hybridisation (aCGH) for aneuploidy screening and/or diagnosis, and next-generation sequencing (NGS) for aneuploidy screening. Embryos are prioritised for transfer based on the genomic (PGD) and chromosomal status (PGT-A). F:fluorophore; Q:quencher. Figure adapted from Vermeesch *et al*. (2016).

Although during the last decade these methods are more and more refined, there are also some inevitable disadvantages. For instance, the embryologists need to be specially trained to perform biopsies which also increases the treatment costs for patients, the embryo is even minorly damaged and is also exposed to laboratory conditions throughout the biopsy (Cimadomo et al., 2016), and the small number of biopsied cells is often not adequate to assess the degree of mosaicism in the whole embryo and thereby accurately assess the chromosomal status (Ou et al., 2020). In an effort to overcome these problems, nowadays the focus of clinical embryology is to use available technology for assessing embryos in a non-invasive manner.

Currently, there is active research on the use of spent culture media for analysis of embryo-secreted/released molecules. The development of -OMICS platforms supports this research and nowadays it is possible to analyse spent culture medium from individually-cultured embryos for genomics, proteomics, metabolomics, and transcriptomics. One of the hot topics for research with immediate clinical applications is the detection of DNA in embryo culture media. Specifically, cell-free embryonic DNA is present in culture media and NGS can detect this DNA for PGT-A purposes. This method is intensively studied to establish the concordance rate between cell-free DNA and embryonic cells and if this method is superior for assessing the degree of mosaicism compared to embryo biopsies (Rubio et al., 2019). Moreover, clinical proteomics and metabolomics are candidate emerging technologies with possible applications in embryo assessment. In proteomics, protein profiling in spent culture medium identifies biomarkers that are associated with embryo viability and even implantation potential of an embryo. In metabolomics, the molecular metabolites in spent medium are used as biomarkers to determine the degree of oxidative stress and the embryonic potential and viability (Egea et al., 2014). Furthermore, transcriptomic profiling of small RNA molecules is a newly-introduced approach for non-invasive embryo quality assessment. In this approach, microRNAs (miRNAs) are candidate molecules for research because of their numerous biological functions and involvement in epigenetic regulation and gene silencing.

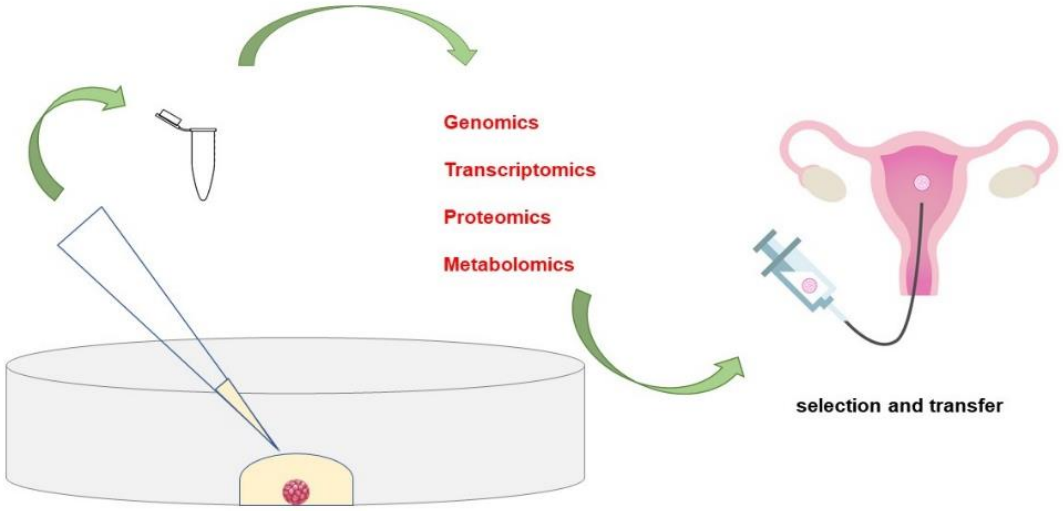

Figure 1.5 Spent culture media analysis for non-invasive embryo assessment. The media from single-cultured blastocysts is collected and analysed with respective platforms for genomics, transcriptomics, proteomics, and metabolomics analyses. The molecular profiles are analysed and used to select the embryo with the highest probabilities for implantation and pregnancy. This embryo is then transferred to the uterus.

The present thesis studies the roles of cell-free miRNAs in embryo development and reproduction in order to contribute to the limited knowledge in this topic. The literature review continues with describing the biology of miRNA molecules and how these regulate mammalian reproduction. Chapter 1 ends with the hypothesis and objectives of the thesis. In Chapter 2 the general methodology and techniques used throughout are described in detail. Moreover, Chapters 3-6 include the original work and experiments carried out for the purposes of this thesis. Lastly, a general discussion with conclusions are given in Chapter 7.

1.3 microRNAs

MicroRNAs (miRNAs) are small (~22 nucleotides), single-stranded, non-coding and highly conserved RNAs whose genes either express a single miRNA or are found in transcriptional miRNA clusters. miRNAs act as guide molecules for RNA silencing by direct translational repression, mRNA destabilisation, or a combination of the two (O'Brien et al., 2018). To date it is estimated that approximately 2.300 mature miRNA forms exist in human cells (Alles et al., 2019). Their actions are involved in numerous cell processes including stress signalling, cell cycle progression, development, differentiation, and apoptosis (Mendell and Olson, 2012). It is therefore not surprising that specific miRNAs have been linked with the onset of diseases such as cancer, cardiovascular disorders, and neurodegenerative diseases (Kumar et al., Wijnhoven et al., 2007, Condorelli et al., 2014).

The biogenesis of miRNAs is under tight temporal and spatial control and the involved pathways have been reviewed extensively in the past (Ha and Kim, 2014b, Winter et al., 2009). In brief, miRNA genes are transcribed by RNA polymerase II which produces a long primary transcript (pri-miRNA) with a local hairpin structure within which the mature miRNA sequence is found. The primary miRNA then undergoes several steps of maturation starting with processing of the molecule by the "Microprocessor complex" formed by the nuclear RNase III Drosha and essential co-factor DGCR8. This complex precisely recognises and cleaves the pri-miRNA stem-loop releasing a hairpin-shaped RNA molecule (~65 nucleotides) known as precursor miRNA (pre-miRNA). Then the pre-miRNA is transported from the nucleus to the cytoplasm via the action of Exportin-5 in complex with Ran-GTP. The RNase III Dicer completes the maturation process by cleaving the terminal loop to generate the mature duplex miRNA molecule which is ~22 nucleotides long. miRNAs are then loaded onto

an Argonaute (AGO 1-4) protein forming a complex known as the miRNA-induced silencing complex (miRISC). The directionality of the miRNA strand determines the name of the mature miRNA. The 5p strand derives from the 5´ end of the pre-miRNA hairpin whereas the 3p comes from the 3´ end; however the selection of the strand is based partly on the thermodynamic stability of the 5´ end with the less stable strand being preferentially loaded into AGO (guide strand). The miRNA then guides the RISC complex to complementary sequences on the target mRNA where it exerts its actions on miRNA-mediated silencing. The unloaded strand (passenger strand) will be unwound from the guide strand and the strands that contain no mismatches are cleaved by AGO2 and degraded by cellular machinery. Alternatively, miRNA duplexes with central mismatches or non-AGO2 loaded miRNA are passively unwound and degraded (Ha and Kim, 2014a). The Drosha-Dicer pathway is the most-described miRNA biogenesis pathway yet; however non-canonical mechanisms of miRNA processing are also emerging.

In general, the non-canonical miRNA biogenesis can be grouped into Drosha/DGCR8-independent and Dicer-independent pathways (Figure 1.6). In the mirtron pathway short introns are processed by spliceosomes and debranching enzymes in the nucleus to produce miRNA hairpins that are exported by Exportin-5 in the cytoplasm where they are cleaved by Dicer. Thus this pathway replaces the microprocessor processing with splicing activity and then merges with the canonical miRNA pathway. Another example is the 7-methylguanosine (m^7G)-capped pre-miRNA. These small RNAs are exported to the cytoplasm through Exportin-1 and are cleaved by Dicer following the canonical pathway. Dicer-independent miRNAs are processed by Drosha/DGCR8 from endogenous short hairpin (shRNA) transcript,

transferred in the cytoplasm via Exportin-5 and require AGO2 to slice their strand ends and complete their maturation (O'Brien et al., 2018).

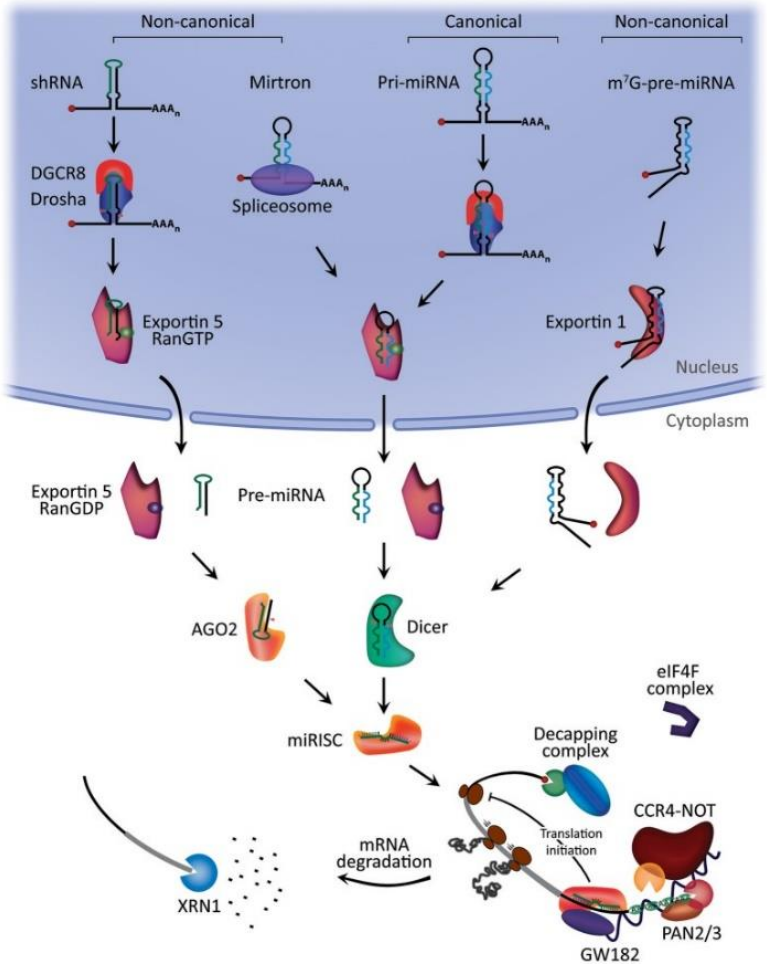

Figure 1.6 miRNA biogenesis pathways. Canonical biogenesis begins with the generation of the pri-miRNA transcript in the nucleus which is processed by the Drosha/DGCR8 complex to produce the precursor-miRNA (pre-miRNA). The pre-miRNA is exported to the cytoplasm via Exportin-5/RanGTP action and is processed by Dicer to produce the mature miRNA duplex. Either miRNA strand is then loaded into the Argonaute (AGO) family of proteins forming the miRNA-induced silencing complex (miRISC). In the Drosha/DGCR8-independent non-canonical pathways, mirtrons are processed by spliceosomes and exported by Exportin-5 whereas 7-methylguanosine (m^7G)-capped pre-miRNAs are transferred via Exportin-1 in the cytoplasm where they are cleaved by Dicer activity and follow the canonical pathway. In the Dicer-independent biogenesis, small hairpin RNAs (shRNA) are initially cleaved by the microprocessor complex and are exported via Exportin-5/RanGTP where they are further processed via AGO2-dependent cleavage. All pathways lead to a functional miRISC complex which ultimately binds to target mRNA sequences possibly by interacting with the EIF4F complex. Next, GW182 proteins bind to AGO and recruit the poly(A)-deadenylases PAN2/3 and CCR4-NOT. The former initiate deadenylation of the mRNA and CCR4-NOT removes the m^7G cap on target mRNA (decapping). The mRNA may then undergo 5´-3´ degradation through exoribonuclease XRN1 (O'Brien et al., 2018).

Even when cells utilise other mechanisms to produce functional miRNAs, it is important to note that Dicer is almost always indispensable in the production of miRNAs and without it the vast majority of the miRNA cargo in a cell is lost. On the other hand, Drosha and Dgcr8 are only needed to process miRNAs in the canonical pathway so their deletion causes complete loss of canonical miRNAs but preserves non-canonical miRNA biogenesis. Therefore, the understanding of miRNA biogenesis and the actions of key-factors in each pathway is necessary when knockout experiments are designed to study specific miRNA roles. For instance, deletion of *Dicer* will completely deplete cells from miRNAs whereas deletion of *Drosha/Dgcr8* will only affect the miRNAs produced by the canonical pathway. Deletion of *Exportin-5* will affect the transport of miRNAs in the cytoplasmic region for final miRNA maturation and deletion of *Ago* genes will affect the loading of miRNAs in RISC complexes which will block the transportation of miRNAs to their targets.

After they are synthesised, miRNAs bind to specific sequences at the 3´ UTR of their target mRNA to induce translational repression and mRNA deadenylation and decapping (Ipsaro and Tor, 2015). miRNAs also bind to 5´ UTR and coding regions and have silencing effects on gene expression (Forman et al., 2008), whereas interactions with promoter regions has been reported to induce transcription (Zhang et al., 2018).

MicroRNA-mediated gene silencing starts with the miRISC specifically targeting complementary sequences on mRNA called miRNA response element (MREs). This interaction enables the AGO protein to act as a mediator of RNA interference and is followed by the recruitment of the GW182 family of proteins which provide the scaffolding needed to recruit other effector proteins such as the poly(A)-deadenylase complexes PAN2/3 and CCR4-NOT (Behm-Ansmant et al., 2006, Jonas

et al., 2015, Christie et al., 2013). These proteins initiate poly(A)-deadenylation after which decapping takes place through the actions of decapping protein 2 (DCP2) and associated proteins (Behm-Ansmant et al., 2006), followed by 5´-3´ mRNA degradation by exoribonuclease 1 (XRN1) (Braun et al., 2012).

Translational activation through miRNAs is less common and is activated under specific conditions. The mechanisms are not clear yet, however miRNAs are able to mediate activation of translation which involves association with AU-rich elements at 3´ UTR through AGO2 and FXR1 actions or binding to the 5´ UTR of mRNAs encoding ribosomal proteins (Vasudevan et al, 2007). Lastly, miRNAs also mediate transcriptional and post-transcriptional gene regulation within the nucleus. This is achieved through the actions of Importin-8 or Exportin-1 which allows the human AGO2 to shuttle between the nucleus and cytoplasm (Nishi et al., 2013). When the miRISC is transferred inside the nucleus it can regulate both transcriptional rates and post-transcriptional levels of mRNA and associate with euchromatin at gene loci with active transcription (Cernilogar et al., 2011, O'Brien et al., 2018).

Intracellular miRNAs are important regulators of many biological processes. Interestingly, miRNAs circulate extracellularly and cell-free miRNAs are found in abundance in body fluids including serum and amniotic fluid (Cortez et al., 2011). miRNAs circulate using miRNA transporters or by binding to other molecules (Figure 1.7). Often miRNAs are encapsulated in vesicular transporters, namely exosomes and microvesicles, which are secreted by cells and fuse with plasma membranes releasing the miRNA cargo in other cells, serving thus as mediators of cell-to-cell communication. Another miRNA carrier is the apoptotic body which is formed at the final stages of cellular apoptosis and can have post-apoptotic effects in other living cells. The main difference between exosome packaging and binding to apoptotic

bodies is that the latter is a random process and independent of cell types. Some proteins such as NPM1, HDL, and Ago2 are also used as miRNA carriers by cells. miRNA-protein complexes circulate in body fluids and enable inter-cellular communication via miRNAs. Exosome release by cells is an ATP-dependent process, as it includes membrane fusion and breakage, and similarly formation of apoptotic bodies as well as protein secretion are also energy-demanding processes. A small fraction of miRNAs could also be released passively by leakage from damaged cells; however, it is generally thought that miRNA release/secretion is an active, selective process carried out by cells for specific biological functions (Xu et al., 2013).

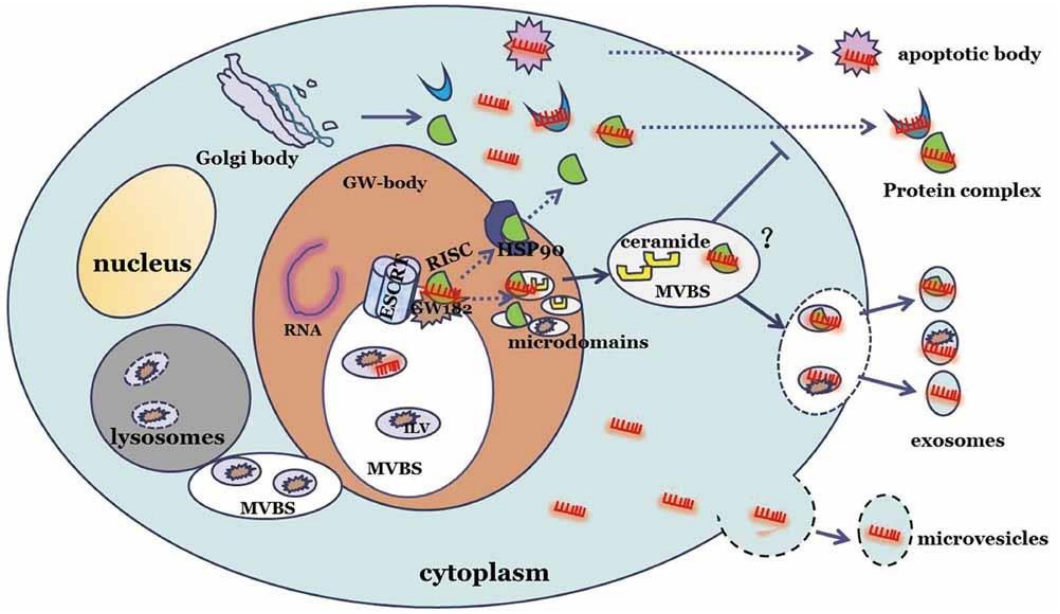

Figure 1.7 miRNA transporters. In the GW body, which is formed by accumulated GW182 in the Processing Body (P-body), the GW182 dissociates the RISC on the membranes of multivesicular bodies (MVBs) by endosomal sorting complex required for transport (ESCRT) and the RISC is sorted into intralumenal vesicles (ILVs) for degradation in the lysosomes. An alternative pathway for sorting miRNA cargos into ILVs is the selection of raft-based microdomains in the MVBs and exosome release (MVBs-exosomes pathway). Ceramide accumulation in these microdomains promotes exosome secretion. miRNA transport via exosomes is achieved through fusion between MVB and plasma membranes or through budding of plasma membranes of microvesicles. Binding to proteins or apoptotic bodies are also mechanisms of miRNA transport in the extracellular environment (Xu et al., 2013).

Circulating miRNAs are highly stable in body fluids and experiments show that these molecules do not degrade as easily as other RNAs withstanding prolonged exposure in room temperature, repeated freeze-thaw cycles (Mitchell et al., 2008, Gilad et al., 2008), and pH changes (from pH=1 to pH=13) (Xi et al., 2008). Endogenous miRNAs are also resistant to RNase digestion in contrast to other cell-free RNAs that are destroyed from extracellular RNases (Mitchell et al., 2008, Gilad et al., 2008). However, synthetic miRNAs are easily degraded by RNase activity which suggests that endogenous miRNAs are protected from degradation probably by vesicles or proteins with which they bind (Mitchell et al., 2008). Indeed, miRNAs rapidly destabilise and degrade after treatment with proteinase K at 55^{o}C (Arroyo et al., 2011) and addition of membrane permeabilising factors (Triton X-100), meaning thus that miRNA stability is achieved by encapsulation of miRNAs in membrane vesicles and/or protection by RNA-binding proteins.

Overall the available information on miRNA roles and actions highlights the competence of these molecules as gene regulators and their potential as mediators of cellular processes. Considering the multiple roles of miRNAs in normal cellular functions it is not surprising that a number of research work has been carried out in female and male reproduction and lately in embryo development.

1.3.1 miRNAs in male reproduction

MiRNAs are expressed in male reproductive tissues and have essential roles in male fertility (Reza et al., 2019). Aberrant miRNA expression in male tissues can cause structural changes in male gonads, affect spermatogenesis, increase germ cell apoptosis, and impair the production of spermatozoa and their maturation. Complete miRNA absence caused by deletion of *Dicer* in germ cells of mutant mice results in reduced testis size, significant reduction in testis weight by 55%, and smaller diameter of seminiferous tubules. Cellular associations within the seminiferous tubules are also disturbed, including disbalanced proportions of different cell populations, presence of leptotene spermatocytes, absence of elongated spermatids and presence of round spermatids. Additionally, meiotic progression is severely affected by global miRNA deletion and cell apoptosis is 8-fold higher in mutant testes compared to controls. Moreover, acrosome fragmentation, abnormal head shape and chromatin condensation, and abnormal mitochondria shape are prominent morphological defects in round and elongated spermatids of mutant mice. All these changes cumulatively result in the absence of functional spermatozoa and complete infertility in *Dicer* knockout males (Romero et al., 2011).

The roles of specific miRNAs have also been studied in relation to male reproduction. Starting from germ cell commitment and differentiation to spermatogonia, the pluripotency-related miR-290/295 cluster members are downregulated whereas the pro-proliferation and pro-differentiation miR-136, -743a, -483 are upregulated in spermatogonia compared to primordial germ cells (PGC) and gonocytes (McIver et al., 2012). MiR-143 is transiently increasingly expressed from PGC to spermatogonia cells and spermatozoa which suggests that this miRNA is likely necessary for the differentiation of PGC to spermatozoa (García-López et al., 2015).

Moreover, miR-21, and miR-221/222 are expressed in spermatogonial stem cells (SSC) with actions on stemness, proliferation, and survival which overall promote the maintenance of the SSC population reserve (Niu et al., 2011, Yang et al., 2013a). Additionally, miRNAs miR-20 and miR-106a trigger post-translational molecular mechanisms which promote renewal of SSCs (He et al., 2013) while miR-17-92 and miR-106b-25 cluster members cooperatively regulate spermatogonial differentiation and development (Tong et al., 2012). It is therefore evident that miRNAs form complex regulatory networks controlling the very early stages of male reproduction.

Moreover, miRNAs are involved in the regulation of the subsequent stages of spermatogenesis – from the differentiation and mitotic proliferation of spermatogonia to the meiotic divisions of spermatocytes and later on the process of spermiogenesis which involves the maturation of round spermatids to elongated spermatids (He et al., 2009). More specifically, miR-146 is a key miRNA for spermatogonial differentiation and is highly expressed in undifferentiated spermatogonia compared to differentiated, by 180-fold difference. MiR-146 actions are exerted through direct binding on the retinoic acid mediator *Med1* and overexpression of this miRNA modulates the effect of retinoic acid on spermatogonial differentiation (Huszar and Payne, 2013). Let-7 miRNA family members also seem essential for differentiation of spermatogonia and expression of these miRNAs is decreased upon differentiation to primary spermatocytes. These miRNAs act through targeting *IGF1* and regulating Erk1/2 and PI3K signalling pathways and overall block the IGF1-induced spermatogenesis (Shen et al., 2014). On the other hand, miR-383 expression is restricted to spermatogonia and is necessary for the production of spermatocytes. MiR-383 regulates the expression of proliferation factors (cyclin dependent kinases CCND1, CDK2 and p21 inhibitor) and overall controls the process of mitotic divisions to spermatocytes (Lian

et al., 2010). Furthermore, miR-34c promotes the meiotic divisions of spermatocytes by inhibiting *Nanos2* and simultaneously upregulating the *Stra8* retinoic acid mediator (Yu et al., 2014). Additionally, the stage-specific meiotic miRNAs miR-221, -203, and -34b target *c-Kit*, *Rbm44*, and *Cdk6* genes respectively and coordinate this way the meiotic progression of spermatocytes to produce haploid gametes (Smorag et al., 2012).

MiRNA expression is also necessary for the structural and molecular transition of spermatocytes to elongated spermatids during spermiogenesis. Indeed, miRNA-dependent regulation of *Tdrd6* is crucial for the formation of chromatin bodies in round spermatids (Vasileva et al., 2009). Additionally, the testis-specific miR-469 plays an important role in chromatin remodelling and elongation of spermatids via repression of *Tnp2* and *Prm2*. Spermatids fail to elongate in absence of this miRNA which severely impacts on male fertility (de Mateo and Sassone-Corsi, 2014). Lastly, the sperm miRNA signature is substantially modified during epididymal maturation compared to other spermatozoal stages (Nixon et al., 2015) and differential expression is also found among the segments of the epididymis (caput, corpus, cauda) and compared to ejaculated sperm in different species, including human (Belleannée et al., 2012) and mouse (Nixon et al., 2015). This suggests that epididymal miRNAs are involved and necessary for the final stages of in sperm maturation contributing to sperm motility and the content of seminal fluid. It is evident from the above that abnormal miRNA expression can impair any of these processes, separately and cumulatively, leading to male-factor infertility.

Lastly, cellular apoptosis during spermatogenesis appears to be, at least partly, under miRNA regulation. Notably, spermatocyte apoptosis is a necessary process in order to establish the precise homeostasis of cell types in the testis needed for

spermatogenesis. In mice, the miR-449 cluster and miR-34b/c are expressed in high levels upon meiotic initiation in the testis and act redundantly to suppress the activity of E2F1. This suppression has protective effects that prevent meiotic male germ cells from undergoing apoptosis, which is crucial to overcome at this stage as the mitotic to meiotic progression requires double strand breaks for DNA recombination (Bao et al., 2012). In sheep with impaired spermatogenesis, miR-144 is highly expressed and inhibits the expression of apoptosis-related genes *FASL*, *CAS3*, and *TP53*, suggesting that controlled apoptosis is crucial for normal spermatogenesis in the adult testis (Guan et al., 2015). In pigs, miR-34 and miR-16 are upregulated during germ cell development and negatively regulate the cell cycle progression (Luo et al., 2015) and miR-16 in the mouse testis specifically targets and downregulates the *Ccnd1* gene which suppresses cell growth and promotes cell apoptosis (Li et al., 2016b). Furthermore, miR-17-92 has pro-survival actions in mouse testicular cells as deletion of this miRNA upregulates pro-apoptotic proteins (Bim, Stat3, c-Kit, Socs3) and leads to increased germ cell apoptosis, testicular atrophy, empty seminiferous tubules, and depressed sperm production (Xie et al., 2016).

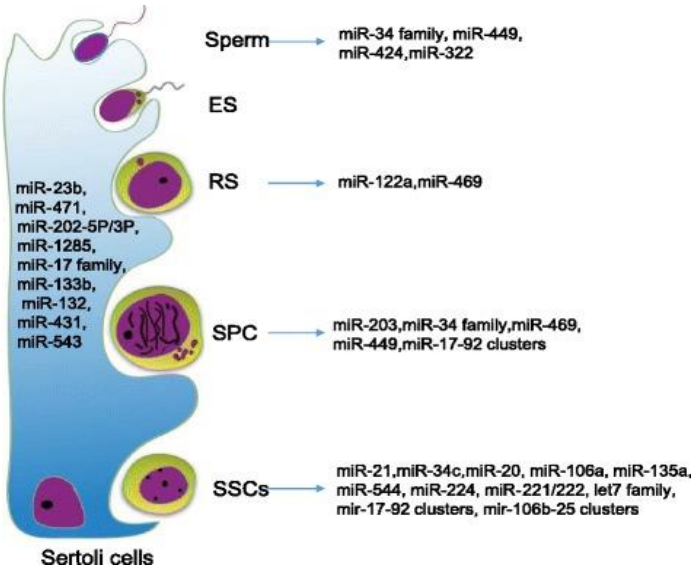

Figure 1.8 miRNA regulations of spermatogenesis. miRNAs involved in each stage of spermatogenesis are given for Sertoli cells, SSCs: spermatogonial stem cells, SPC: spermatocytes, RS: round spermatids, ES: elongated spermatids, and sperm (Chen et al., 2017).

Many studies have investigated whether miRNA profiling in ejaculated semen is predictive of male infertility, embryo quality, and even clinical outcomes. In specific, miRNAs let-7a, miR-7-1-3p, -131, -200a, and -429 are significantly upregulated whereas miR-15b, -34b, and -122 are upregulated in infertile males compared to normozoospermic men. These profiles are consistent in spermatozoa and seminal fluid samples and are significantly correlated with sperm concentration (Mokánszki et al., 2019). Moreover, miR-449 and miR-15b are decreased while miR-141 and miR-200a are increased in semen samples of oligozoospermic males (McCubbin et al., 2017), but miR-122 is decreased which contradicts the findings of Mokánszki and colleagues (2019). Additionally, miR-371a-3p levels in ejaculate are positively correlated with sperm concentration and total sperm count (Radtke et al., 2019). Furthermore, the promoters of miR-34 family members (miR-34a,b,c) are hypermethylated in sperm samples of infertile men which leads to the under-expression of these miRNAs with possible impacts on spermatogenesis (Momeni et al., 2020). A recent study identified miRNA pairs as potential biomarkers for classification in male infertility groups. The best biomarker potential was found for pairs miR-942-5p/miR-1208 (asthenozoospermia), miR-265-5p/miR-328-3p (teratozoospermia), miR-139-5p/miR-1206a (oligozoospermia), and miR-34b-3p/miR-93-3p (unexplained male infertility) (Corral-Vazquez et al., 2019).

Regarding embryo parameters, miR-191-5p in sperm samples is an indicator of fertilisation rate, blastocyst formation, and embryo quality and as such is a potential non-invasive marker for improving success rates in IVF cycles (Xu et al., 2020). Lastly, miR-34c expression in spermatozoa is linked to higher rates of good-quality embryos, implantation, pregnangy, and live birth rates in ICSI cycles, which suggests that this miRNA can be used as an indicator for clinical otucomes (Cui et al., 2015).

1.3.2 miRNAs in female reproduction

Similar to male reproduction, miRNAs are expressed in female reproductive tissues and play essential roles throughout folliculogenesis and oogenesis (Reza et al., 2019) (Figure 1.9). Dicer knockout experiments offer insights about the broad regulatory roles of miRNAs in female reproduction. Dicer1 is abundant in ovarian tissues of mice and is expressed throughout the different developmental stages in oocytes and supporting cells – from oocytes of primordial follicles to oocytes of the follicles from primary to antral stage, in granulosa cells, and notably in the corpus luteum and stromal cells (Lei et al., 2010).

Unsurprisingly, inactivation of *Dicer1* in ovaries inevitably affects the normal functions of ovaries with adverse effects on oogenesis. Firstly, deletion of *Dicer1* leads to morphological alterations as shown by *Dicer1* knockouts which have more tertiary follicles, more degenerate follicles, presence of cysts in the centre of the ovaries, and abnormal vascularisation and cytoplasmic size of the corpus luteum (Lei et al., 2010). Additionally, follicular development is altered with knockout ovaries showing an increased primordial follicle pool endowment, enhanced early follicle recruitment, increased number of tertiary follicles, but lower numbers of antral follicles which suggests increased atresia before ovulation (Leit et al., 2010). Moreover, gene expression is altered in *Dicer1* knockout ovaries in different reproductive stages (day 4, day 8, 8 weeks), with significant dysregulation of *Bcl2*, *Bax*, *Nobox*, and *Inhba* in day 4 ovaries, downregulation of *Zp2* and *Bmp15* in day 8 ovaries, and downregulation of *Inhba* (activin/inhibin assembly), *Cyp11a1*, *Cyp17a1*, and *Cyp19a1* (hormone synthesis), *Casp3*, *Ccnd2*, and *Cdkn1b* (granulosa cell development), *Zp2*, *Zp3*, *Gdf9*, and *Bmp15* (oocyte-specific) in 8 week ovaries (Lei et al., 2010). Taken together, these findings suggest that miRNAs are essential for maintaining the morphological

structures and molecular profiles of the ovarian cell populations which cooperate for the production of the maternal gamete.

Specific miRNAs regulating the different stages of folliculogenesis have been identified by a number of studies. During the initiation of primordial follicle development in mice, 24 miRNAs are differentially expressed in ovaries of 3- and 5-day-old mice and these miRNAs are involved in at least 77 miRNA-mRNA regulatory networks which modulate growth, development, and maintenance of primordial follicles. One of the networks is the TGF-beta signalling pathway in which miR-145 has important regulatory actions. MiR-145 is abundant in ovaries in which follicle development has been initiated and specifically targets and downregulates *Tgfbr2* while activating Smad signalling. These actions are essential for controlling early folliculogenesis as loss of miR-145 over-activates primordial follicles (decreased proportion and number of primordial follicles and increased number of growing follicles), and affects the oocyte size (increased diameter in primordial follicles, smaller in growing follicles) (Yang et al., 2013b). In addition, miR-224 directly targets *Smad4* in mouse ovaries and promotes early folliculogenesis through enhanced granulosa cells (GCs) proliferation in cooperation with *Tgfb1* (Yao et al., 2010).

Moreover, miRNAs have actions in later stages including selection of dominant follicles, formation of germinal vesicle (GV) oocytes, oocyte maturation, ovulatory follicles, and development of metaphase II (MII) oocytes. In bovine, there are distinct miRNA profiles between granulosa cells of subordinate and dominant follicles in the early luteal phase, with at least 16 miRNAs differentially expressed at day 3 of the oestrous cycle and 108 miRNAs at day 7. Temporal miRNA expression dynamics is ,also observed in dominant follicles from day 3 compared to those from day 7, with miRNAs involved in important processes of follicular development such as Wnt, TGF-

beta, GnRH signalling pathways and meiosis. This shows that miRNA expression patterns are likely associated with follicular recruitment, selection, and dominance during the early luteal phase (Salilew-Wondim et al., 2014).

Additionally, miR-424 and miR-10b are abundantly expressed in GV oocytes compared to ovarian somatic cells in bovine and these patterns persist to the MII oocyte and early stage embryos (16-cell) before declining in further development, meaning that these miRNAs are possibly essential for the formation of GV oocytes, progression to the MII stage, with possible involvement in maternal transcript turnover in early embryo development (zygote genome activation occurs at 8-cell stage in bovine embryos) (Tripurani et al., 2010).

Furthermore, bovine immature oocytes preferentially express 31 miRNAs compared to matured oocytes, and inversely 28 miRNAs are distinctly expressed in mature oocytes compared to immature (Tesfaye et al., 2009). miRNAs miR-205, -150, -122, -96, -146a, -146b-5p dramatically decrease as the oocyte matures (0 to 22 h maturation) and their levels are maintained up to the 8-cell embryo stage before further reduction following a typical bovine maternal transcript profile (Abd El Naby et al., 2013). In pigs, miR-27b-3p has negative effects in oocyte maturation (GV to MII) through regulation of *PPARγ* which is essential for fatty acid metabolism (Song et al., 2016) and miR-574 supresses maturation through direct downregulation of *hyaluronan synthase 2* (*HAS2*) (Pan et al., 2018). In contrast, miR-205 mediates the expansion of cumulus-oocyte complexes (COCs) through *Ptx3* targeting, promoting this way maturation (Li et al., 2016a). Large bovine follicles highly express miR-144, -202, -451, -652, and -873 compared to small follicles and these miRNAs regulate multiple signalling pathways involved in follicular cell proliferation, steroidogenesis, luteinisation, and oocyte maturation (Sontakke et al., 2014). Moreover, miR-21 is

essential in mouse ovaries where it is highly expressed in granulosa cells transitioning to luteal cells and exerts its pro-maturation actions through inhibition of cellular apoptosis (Carletti et al., 2010). Lastly, human MII oocytes express higher levels of 4 miRNAs and lower levels of 11 miRNAs compared to GV oocytes, with miR-15a and miR-20a gradually decreasing through transition of GV to the MI and MII stages which suggests that dynamic temporal expression is essential for the successive stages of oocyte maturation (Xu et al., 2011).

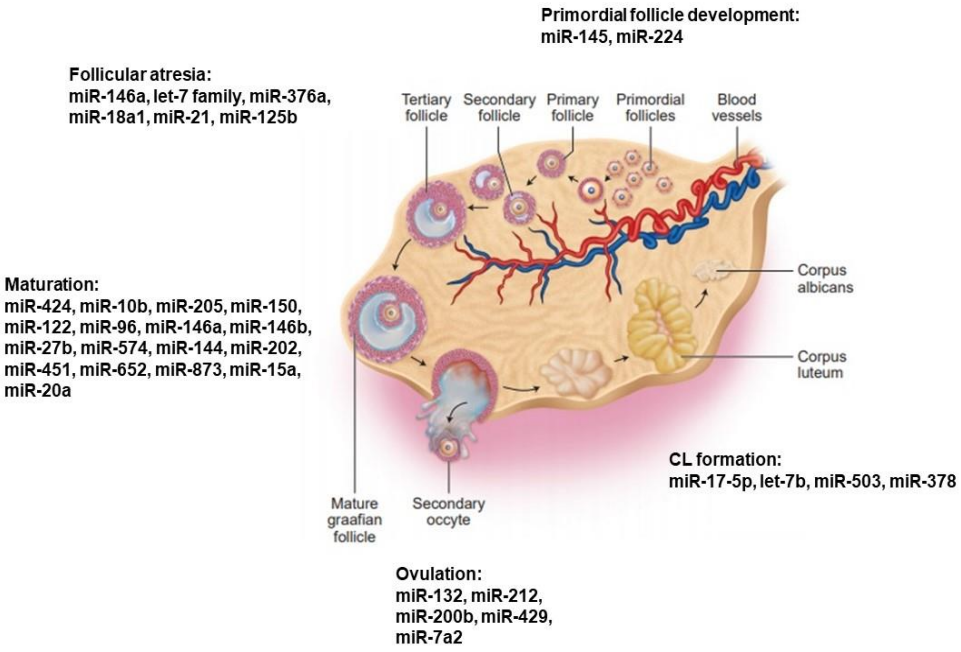

Figure 1.9 miRNA regulation of oogenesis. miRNAs are listed according to their involvement in each stage. The miRNA roles are discussed within the text using literature around mammalian oogenesis in general. Therefore, their actions may differ between species. CL: corpus luteum.

Interestingly, hormone-miRNA interactions in ovarian cells and the pituitary gland control the ovulation process. More specifically, LH/hCG triggers the differential expression of 13 miRNAs in peri-ovulatory granulosa cells, with miR-132 and miR-212 amongst the highly upregulated miRNAs causing elevated transcription of the pro-ovulation gene *Ctnp1* (Fiedler et al., 2008). MiR-200b and miR-429 are essential for female fertility and are expressed in the pituitary gland where they suppress *Zeb1*. Deletion of these miRNAs leads to lowered serum LH concentration, impaired LH surge, and ovulation failure in mice (Hasuwa et al., 2013). Moreover, miR-7a2 is essential for pituitary development and hypothalamic-pituitary-gonadal (HPG) function in adulthood with genetic deletion of this miRNA resulting in low levels of gonadotropic and sex hormones, small ovaries, anovulation, and infertility. Mir-7a2 is expressed in the pituitary where it suppresses *Glg1* and *Ptgfrn* (FSG and LH inhibitor) and BMP4 signalling, regulating this way sexual maturation and reproductive function (Ahmed et al., 2017).

MiRNAs have pivotal regulatory roles during apoptosis of granulosa cells and follicular atresia. Programmed cell death and the consequent follicular atresia is a normal process that regulates the number of follicles in the developing pool. In humans, granulosa cell apoptosis is promoted by miR-146a which directly targets the interleukin-1-receptor-associated kinase, *IRAK1*, and tumour necrosis factor receptor-associated factor 6, *TRAF6*, and indirectly affects factors of the caspase cascade family (Chen et al., 2015). Moreover, the let-7 family controls follicular atresia in pigs through MAPK, TGFβ and p53 pathways, with significant reduction of let-7a, -7b, -7c, -7i and increase of let-7g in atretic follicles compared to healthy ones (Cao et al., 2015). Moreover, miR-26b promotes granulosa cell apoptosis in porcine ovaries through direct targeting of *ATM* (Lin et al., 2012). In mice, miR-18a1 regulates ovarian

follicle development and inhibits GC proliferation by supressing *Acvr2a* and activin signalling (Zhang et al., 2013a). Additionally, miR-21 blocks apoptosis in granulosa cells, prevents follicular atresia, and regulates follicle survival (Carletti et al., 2010) and miR-125b suppresses pro-apoptotic genes of the *Bcl2* family in GCs and promotes follicular survival (Sen et al., 2014). Furthermore, miR-376a in ovaries regulates follicle assembly, by modulation of the proliferation factor *Pcna*, and is correlated with increased number of primordial follicles and reduced apoptosis in oocytes (Zhang et al., 2014). Finally, in bovine, miRNAs miR-21-5p, -21-3p, -222, -155, -199a-5p are upregulated in atretic follicles compared to healthy follicles meaning that these miRNAs possibly form a regulatory network which promotes atresia (Donadeu et al., 2017).

Moreover, the formation and normal function of the corpus luteum (CL) appears to be regulated by miRNAs. Firstly, *Dicer1* knockouts display impaired growth of capillary vessels in the ovary which results in CL insufficiency. More specifically, the lack of miRNAs miR-17-5p and let-7b leads to dysregulation of the anti-angiogenic factor tissue inhibitor of metalloproteinase 1 (*Timp1*) which results in impaired angiogenesis (Otsuka et al., 2008). The follicular-luteal transition and CL regression are also under miRNA regulation. In ruminants, 17 miRNAs are differentially expressed during luteal transition and these miRNAs regulate at least 29 gene targets specifically involved in follicular differentiation (McBride et al., 2012). Specifically, miR-503 is a key-factor during luteinisation as this miRNA targets, amongst others, hormone receptors in the ovary (*Fshr*, *Lhr*, *Ar*, *E2r*, *Actr*, *Lhr)*, cyclin and caspase genes that altogether are directly involved in CL formation and maintenance (Lei et al., 2010). Reduction of this miRNA in pre-ovulatory follicles marks the onset of luteinisation and its expression is restored in subsequent CL development (Lei et al.,

2010, McBride et al., 2012). Furthermore, 7 miRNAs are preferentially expressed in non-regressed CL and 6 miRNAs are highly expressed in regressed CL in bovine. Of these, miR-378 is upregulated by 8.54-fold in non-regressed CL and this miRNA plays an anti-apoptotic role in luteal cells by targeting *interferon gamma receptor 1* (*IFNGR1*) and suppressing its expression (Ma et al., 2011). Considering the above, miRNAs are indispensable molecules for the CL formation, maintenance, and regression in female reproduction.

Due to their numerous actions in female reproduction, the value of miRNAs as biomarkers is under research. Currently, miRNA profiling in cumulus cells and follicular fluid (FF) are promising non-invasive approaches for assessing the oocyte quality, embryo developmental potential, and even predicting the reproduction outcome in assisted reproduction cycles. Unfortunately, preliminary evidence shows that miRNA expression profiles in cumulus cells of oocytes that formed blastocysts and non-cleaved oocytes were not significantly different in the bovine species (Uhde et al., 2017).

However, follicular fluid is rich in miRNAs and cell-free miRNAs may have roles in follicle maturation (Santonocito et al., 2014, Sang et al., 2013, Da Silveira et al., 2015). In bovine, differences in 25 miRNAs in exosomes and 30 miRNAs in non-exosomal fraction are found between good oocyte quality and poor oocyte quality (distinguished by BCB staining) FF samples (Sohel et al., 2013). Regarding circulating miRNAs in human FF, miR-92a and miR-130b are over-expressed in samples from oocytes that fail to fertilise, miR-888 is over-expressed and miR-214, miR-454 are under-expressed in samples that result in impaired Day 3 embryo quality compared to top quality embryos (Martinez et al., 2018). In addition, FF samples from oocytes that develop into blastocysts have significantly lower levels of miR-663b compared to FF

samples from oocytes that do not produce viable blastocysts in women undergoing IVF (Fu et al., 2018). Moreover, FF let-7b predicts the development of expanded blastocysts with 70% sensitivity and 64.3% specificity and miR-29a is promising as a predictor of clinical pregnancy with a sensitivity of 83.3% and specificity of 53.5% in a cohort of women with normal ovarian reserve and polycystic ovarian syndrome (PCOS) (Scalici et al., 2016).

MiRNA differences are also found in relation to reproductive diseases in women. FF from PCOS patients differentially express 29 miRNAs compared to the healthy control group (Butler et al., 2019) and miR-451 is significantly lower in FF of women with endometriosis which impairs the developmental potential of embryos deriving from this group (Xiong et al., 2019). Although the evidence so far is limited, studies on miRNAs as non-invasive biomarkers of female fertility are currently ongoing and new evidence is expected to shed more light on the miRNA-mediated regulation of human reproduction.

1.3.3 miRNAs in early embryonic development

Since miRNAs are by nature regulatory molecules that control thousands of genes which in turn create complex molecular networks, it is only logical that miRNAs actions are pivotal in early embryonic development. During the initial stages of development tightly-controlled cellular processes support the normal growth of an embryo – this is the result of cooperative action of epigenetic reprogramming, DNA synthesis, cellular proliferation, division, cell differentiation, programmed cell death (just to name a few) – and miRNAs are key-factors mediating these pathways. Initially miRNAs are transferred to the zygote upon fusion of the parental gametes and

fertilisation, hence the miRNA cargo in the zygote is of parental inheritance until the zygote genome activation (ZGA) takes place to transcribe the RNA machinery.

Sperm-borne miRNAs are crucial for the early stages of embryo development and there is evidence to support that paternal transfer of miRNAs at the time of fertilisation is required for normal embryo developmental potential. For instance, sperm from *Drosha* and *Dicer* knockout male mice is able to fertilise wild-type eggs, but the embryos created have significantly impaired developmental potential in terms of disrupted maternal transcript turnover and failure of zygotic gene activation. Interestingly, when wild-type sperm is injected to these embryos, the deleterious knockout effects are reversed (Yuan et al., 2016).

Altered expression of a single paternal miRNA can also affect normal embryo development. Indeed, paternal miR-34c is necessary for the zygotic division and absence of this miRNA supresses the first cleavage division possibly via inhibition of DNA synthesis and modulation of the *Bcl-2* pro-apoptotic factor (Liu et al., 2012). Moreover, sperm-borne miR-216b is associated with low fertility in bovine, specifically affecting cell proliferation via *K-RAS* targeting which affects the first cleavage rate, division number, and blastocyst cell number in the resulting embryos (Alves et al., 2019). In mice it is estimated that around 20% of miRNAs found in the nuclear and peri-nuclear compartments of spermatozoa are introduced into the oocyte upon fertilisation (Amanai et al., 2006). It is therefore evident that the spermatozoon does not only transfer the DNA, acrosome enzymes, and the oocyte activation factor PLCζ, but is also contributing to the RNA content in the early embryo which is essential for the initial stages of development.

Maternal contribution of miRNAs is necessary for normal embryonic growth. Even though the embryo transcribes its own miRNAs upon zygote genome activation, oocyte miRNAs are vitally important for the early stages of development and their loss or malfunction has consequences in further embryonic development. miRNA expression patterns in mature oocytes are similar in zygotes which is a first indication that the miRNAs detected in zygotes are maternally inherited and not *de novo* transcribed. miRNA levels then dramatically drop through global active degradation (~60%) before the embryo initiates the transcription of embryonic miRNAs (Tang et al., 2007). To investigate the effects of maternal miRNA absence, specific deletion of *Dicer* is used to block miRNA cleavage and deactivate the mature miRNA cargo in growing oocytes. Interestingly, deletion of this gene in *Dicer* mutant oocytes results in gene dysregulation (specifically upregulation) of more than 1/3 of genes expressed in control oocytes which means that miRNAs control gene expression of an astonishingly large number of genes. Naturally embryo growth is impaired in mutant mice, with zygotes failing to progress through the first cell division mainly due to anomalies in spindle formation (Tang et al., 2007).

Unlike the highly differentiated gametes, the early embryo is able to differentiate into any cell type. The totipotency properties are gained by extensive molecular reprogramming which starts with degradation of the maternal transcriptome (loss of oocyte identity) and is followed by reprogramming of gene expression (through alterations in epigenetic marks) during the ZGA (2-4-cell stage in mouse, 4-8-cell stage in human, 8-16-cell stage in bovine) (Svoboda et al., 2015). miRNAs likely play some roles in ZGA, as the maternal miRNA cargo is present up to this event with the most abundant family being the let-7, followed by miR-30 and miR-16. Maternal miRNA levels rapidly decline as the embryo approaches the two-cell stage (mouse

species), and the zygote transcribes its own miRNAs with the miR-290 family being the earliest *de novo* synthesised. There are also maternal-zygotic miRNAs present in oocytes and embryos, like the miR-17-92 cluster, miR-103, miR-342, and miR-200 family with a transiently lower expression at the two-cell stage (Svoboda et al., 2015).

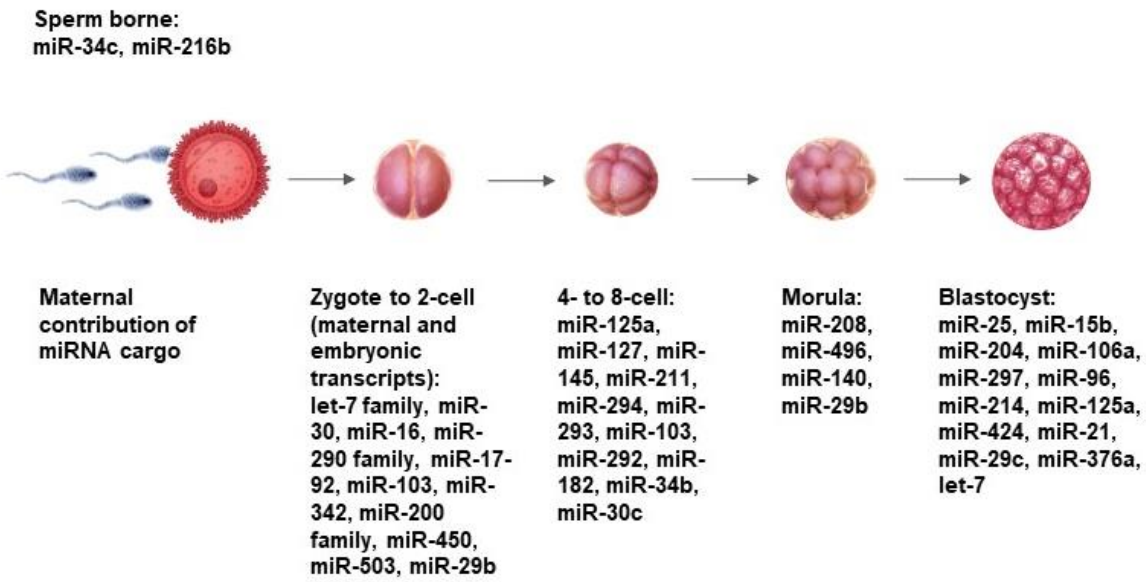

Figure 1.10 miRNAs involved in embryonic development. The miRNAs are listed according to their expression profiles and actions in each stage using available literature around mammalian embryogenesis. However, the exact roles may differ between different species.

Upon development of mouse embryos, there is a shift in the presence of small RNA molecules from predominantly siRNAs and piRNAs at the zygote to predominantly miRNAs by the blastocyst stage (Ohnishi et al., 2010). This is further supported by the lowered expression of genes involved in the canonical biogenesis of miRNAs at the zygote stage. In specific, the genes for *Dgcr8* and *Drosha* splicing enzymes, the *Dicer* processing protein, *Exportin5*, and the *Ago-1, -2, -3, -4, -5* loading proteins are all downregulated in zygotes. The expression of *Ago-1 -3 -4* increases again in the 2-cell embryo and of *Ago-2* in 4- and 8-cell embryos (García-López and

del Mazo, 2012). Thus the embryos have reduced capacity to process miRNAs at the very early stages of embryogenesis. However, miRNA duplexes or bound-to-target forms are protected by degradation and potentially act as a reservoir of mature miRNAs for functional use in cells without the need for canonical processing (García-López and del Mazo, 2012).

In early zygotes and 2-cell stage mouse embryos miRNAs are also protected from massive degradation through a modification at the miRNA strand (3´ mono- and oligo-adenylation), and this modification is removed after the 2-cell stage to enable the embryo to recruit active miRNAs and regulate genes important in embryonic development (Yang et al., 2016). In support of the above, *Dgcr8* oocyte and zygotic knockouts do not show impaired pre-implantation development, with normal allocation patterns of inner cell mass and trophectoderm populations. The transcriptomic profiles between wild type and *Dgcr8* null oocytes are identical, meaning that miRNAs biogenesis is not essential during oocyte maturation and early pre-implantation development and that the inherited stock of miRNAs is sufficient to carry out gene regulation in these stages whereas other small RNAs likely have more prominent roles than miRNAs in early embryogenesis (Suh et al., 2010).

Knockout studies shed some light on the importance of miRNAs in later stages of embryonic development. Deletion of *Dicer1* is embryonically lethal, with the number of *Dicer1*-null embryos being ~50% lower than expected. At Day 7.5 of development these embryos are depleted of stem cells, and they do not express neither the primitive streak marker *T brachyury* or stem cell marker *Oct4* (Bernstein et al., 2003). Moreover, *Dgcr8* knockouts arrest early by Day 6.5, and their stem cells fail to silence self-renewal upon differentiation. Upon chemical induction of differentiation (with retinoic acid) the stem cells abnormally continue to express pluripotency

markers *Oct4*, *Rex1*, *Sox2*, and *Nanog* which suggests that miRNA functions are necessary for lineage commitment at the blastocyst stage (Wang et al., 2007).

MiRNA are expressed in a temporal fashion in early embryogenesis, with miR-125a expression at the 4-cell stage, miR-127 and miR-145 predominantly at the 8-cell stage, miR-208 and miR-496 at the morula stage, and miR-25 at the blastocyst stage in bovine (Tesfaye et al., 2009). In mice, miR-450 and miR-503 are abundant in 2-cell stage embryos, miR-211, -294, -293, -103 in 4-cell, miR-292, -182, -34b, -30c in 8-cell, miR-140 in morula, and miR-15b, -204, -106a in blastocysts (Viswanathan et al., 2009). It is likely that although early embryos do not synthesise their own miRNAs, specific stored miRNAs are activated and have roles in different stages of development, possibly involved in proliferation, DNA synthesis, cell communication, and differentiation.

Unfortunately, there is limited evidence on the exact roles of miRNAs in embryogenesis. MiR-29b facilitates the establishment of gene imprinting at the ZGA by downregulation of *Dnmt3a/4b* – the suppression of methyltransferases at this stage safeguards proper DNA demethylation (Zhang et al., 2015). Moreover, miR-29b is involved in pluripotency after the morula stage by promoting the expression of *Klf4* and *Nanog* pluripotency markers (Wang et al., 2016). The miR-290/302 cluster facilitates the transition from naive pluripotency to primed pluripotency – a prerequisite for lineage commitment in embryos (Gu et al., 2016). Additionally, 9 miRNAs (miR-297, -96, -214, -125a, -424, -21, -29c, -376a, and let-7) are candidate miRNAs involved in trophectoderm specification and maintenance of pluripotency in mouse embryos (Viswanathan et al., 2009).

1.3.4 miRNAs in spent culture medium

MiRNAs regulate the expression of thousands of genes, circulate in biological fluids and travel in distant organs to exert their regulatory actions, serving thus as secretory molecules (Cortez et al., 2011). miRNAs are protected by degradation because they are either enclosed in exosomes or bound in proteins, lipids, or apoptotic bodies (Xu et al., 2013) and thus they are resistant to repetitive freeze/thaw procedures, can be stored for prolonged times in the freezer without degrading, and are also stable after pH changes (Xi et al., 2008). Due to these natural characteristics of miRNAs, scientists of the reproduction field suggested that these molecules may serve as non-invasive biomarkers for embryo selection purposes and prediction of clinical outcomes in IVF cycles. It was therefore hypothesised that if miRNAs could be isolated and detected in spent culture media (SCM) of embryos and specific miRNAs correlated with embryo quality and/or clinical outcomes, then miRNA analysis of SCM could be a valuable method for increasing IVF success.

The first results linking miRNAs with ART outcomes come from a study conducted by McCallie *et al.* (2010) that found six miRNAs (let-7a, miR-19a, -19b, -24, -92, -93) significantly under-expressed in blastocysts produced by infertile patients (PCOS or known male factor infertility) compared to fertile donor controls. The predicted gene targets of these miRNAs were involved in several processes of pre-implantation development such as transcription, cell growth, proliferation, and differentiation. Further analysis of three selected targets (*ARIH2*, *KHSRP*, *NFAT5*) showed that their expression was indeed altered and consistent with the aberrant miRNA expression profiles. Importantly, this study was the first to show that morphologically similar embryos expressed aberrant miRNA levels relating to infertility. The authors further suggested that quality might be impaired in the embryos

produced by infertile patients as a result of abnormal miRNA expression (McCallie et al., 2010).

In a later study, 754 miRNAs were assayed in human donated blastocysts and the association between their expression with the chromosomal status (euploidy) of the embryos was examined (Rosenbluth et al., 2013). It was found that 39 miRNAs were differentially expressed between euploid and aneuploid embryos, with four of them (miR-141, -27b,, -339-3p, -345) demonstrating an >8-fold difference in expression in euploid embryos. Gene target scan analysis showed that these genes were all critical for embryo development and differentiation, being involved in cell signalling pathways, adhesion, and proliferation. The results of this study highlighted the potential use of miRNA analysis for PGT-A assessment in IVF cycles. Additionally, it was the first study to report differential expression according to the sex of the human embryo, meaning that when miRNA profiling is carried out in embryos the result may vary due to the sex (Rosenbluth et al., 2013).

So the above studies formed a strong basis for further research on miRNA roles in assisted reproduction. The next step was to prove that mammalian embryos actually release miRNAs in the surrounding micro-environment, which is necessary for using miRNA profiling as non-invasive assessment method. Initially Kropp and colleagues (2014) examined the presence of 5 miRNAs (miR-25, -302c, -196a1, -181a2, -370) in the medium of *in vitro*-cultured bovine embryos. These miRNAs were specifically chosen because of their roles on mammalian embryogenesis. Culture media samples were collected from individually-cultured embryos and pooled together depending on whether the embryo formed a blastocyst or degenerated. The SCM samples and the groups of embryos (blastocysts vs degenerate embryos) were then analysed for the presence of miRNAs. Furthermore, SCM samples from human embryos were pooled

and analysed for the same miRNAs. The results showed that degenerate embryos (those that failed to reach blastocyst stage) expressed (intracellularly) significantly higher levels of all miRNAs except miR-370. The SCM analysis found that at least one of the miRNAs, miR-25, is released by both bovine and human embryos and thus can be quantified in spent medium. Based on these findings the authors further implied that quantification of specific miRNAs secreted in embryo culture media could be used for non-invasive embryo quality assessment (Kropp et al., 2014). However, this study also pinpointed two main problems of miRNA analysis, namely contamination of SCM samples (from serum additives and proteins) and the value of single sample analysis as pools of samples might not reflect the status of each individual embryo.

In a parallel study, Rosenbluth *et al.* (2014) extended their research to associate miRNA secretion from human embryos with their chromosomal status (euploidy) and clinical outcomes. Donated PN frozen embryos were cultured to the blastocyst stage and SCM from Days 4 and 5 were assayed for a panel of 754 miRNAs. The chromosomal status was assessed by analysis of Day 5/6 trophectoderm biopsies. It was found that miR-372 and miR-191 are released by human embryos and that SCM from aneuploid embryos has significantly higher levels of miR-191. Moreover, further experiments were carried out to associate the two miRNAs with clinical outcomes. Spent media from Days 4 and 5 were analysed and it was found that both miRNAs are present in higher levels in SCM of Day 5 embryos that failed to lead to a live birth. Based on these results it was therefore suggested that secreted miRNAs have potential value as biomarkers of embryo chromosomal status and clinical outcomes (Rosenbluth et al., 2014).

Later studies focused on the roles of embryo-released miRNAs in implantation as it is the first interaction of the embryo with the maternal body that occurs immediately after the end of the pre-implantation embryo development period and hence any biological effect of secreted miRNAs would be observed at this stage. A study by Cuman and colleagues (2015) specifically focused on the relation of miRNAs secreted by human blastocysts with their implantation potential, defined as the number of pregnancies that were carried to term. The analysis was carried out in pooled spent frozen media samples from Days 3-5 of culture. Their findings showed that from the 47 miRNAs present in SCM, 19 were exclusively secreted by the group of blastocysts that implanted whereas 22 were secreted only by the non-implanted group. Of these, miR-661 was the most differentially expressed miRNA and further experiments in individual SCM samples verified that non-implanted embryos intensively secrete this miRNA whereas in the implanted group this miRNA is undetected (Cuman et al., 2015).

MiRNA secretion and implantation were also linked by another study in human blastocysts that found that miR-20a and miR-30c are differentially secreted in SCM from euploid implanted and non-implanted embryos (Capalbo et al., 2016). The implanted group secreted significantly higher levels of the two miRNAs and their predicted gene targets were found to be involved in implantation-related pathways by *in silico* analysis. In addition, TE analysis showed that >96% of the secreted miRNAs were present in TE cells suggesting overall that the blastocyst stage embryo secreted factors to the environment for its interaction with the uterine wall and its subsequent implantation. Interestingly, culture media from the cleavage and morula stages were also analysed and showed similar results with the blank controls suggesting thus that only blastocyst media is useful for miRNA profiling, especially for individual sample

analysis (Capalbo et al., 2016). This finding is in agreement with the suggestion that embryos produce their miRNA machinery after ZGA for cellular functions and they become secretory at the later stages (blastocyst) where they package and release miRNAs for inter-cellular communication with the endometrium which ultimately regulates implantation (Liang et al., 2017). The relevant miRNA studies published so far are discussed throughout this thesis and are presented in summary in Table 1.1. Further thorough details for each study can be found in Appendix A.

Table 1.1 Summary of studies in relation to miRNAs in spent culture media of embryos as non-invasive biomarkers. The studies are listed chronologically**.**

Reference	Species	Primary findings
Kropp *et al.* (2014)	bovine, human	miRNAs are released by human and bovine embryos
Rosenbluth *et al.* (2014)	human	miRNAs in SCM are candidate biomarkers of aneuploidy and clinical outcomes
Kropp and Khatib (2015)	bovine	miRNAs in SCM are candidate biomarkers of embryo quality
Cuman *et al.* (2015)	human	miRNAs in SCM are potential predictors of implantation outcome
Capalbo *et al* (2016)	human	miRNAs in SCM derive from the TE and may be predictive of implantation potential
Borges *et al.* (2016)	human	miRNAs in SCM can predict implantation failure
Lin *et al.* (2019)	bovine	miRNAs in SCM can be used as biomarkers of embryo developmental potential

1.4 Rationale and hypothesis

Refined methods for assessing the embryonic quality prior to transfer are needed in order to improve IVF success rates worldwide. Choosing the embryo with the higher potential to implant and develop further into a healthy foetus will benefit first and foremost the thousands of patients undergoing IVF each year, financially, physically, and psychologically, as well as national and private healthcare systems that cover to costs of IVF. Ideally an embryo assessment method should be quick, easy, safe, sensitive, accurate, reproducible, and cost-effective. Collection of the medium in which an embryo has developed until the latest pre-implantation stages is safe for the embryo (non-invasive) and easy for the embryologist (no training required, no special equipment). Further analysis with established biomolecular methods fulfill the requirements for sensitivity, accuracy, reproducibility, and cost-effectiveness.

MicroRNAs are key-factors of gene regulation in mammals, they circulate in body fluids and exert secretory functions (-cortez et al., 2011), and they are resistant to environmental changes (Mitchell et al., 2008, Xi et al., 2008). As such, miRNAs are candidate molecules for non-invasive techniques in research and clinical applications. In the last decade, analysis of spent culture medium for the presence of miRNAs has emerged as a method for determining the quality of an embryo and predicting clinical outcomes (Appendix A). As described in detail in this Chapter, miRNAs form complex molecular networks and pathways that have pivotal regulatory roles in the specialisation and maturation of mammalian gametes, fertilisation, and embryonic growth. However, there is a lack of evidence regarding specific miRNA roles in embryo development and there is no deep understanding how miRNAs are released by embryos and what is the biological role of miRNAs at the peri-implantation period.

The hypothesis in the present thesis is that mammalian embryos release specific miRNAs that are indicative of their developmental potential and quality. For this, specific miRNAs were investigated in relation to two processes that are essential for normal embryonic growth: developmental dynamics/kinetics and apoptosis. These served as measurable outcomes that directly reflect on the potential of an embryo to implant and establish a healthy pregnancy. It was moreover suggested that miRNA release is triggered by certain processes/cues in a selective manner rather than this being a passive and random process. Lastly, it was further hypothesised that embryo-released miRNAs have secretory functions and cause changes in endometrial cells acting as mediators of the embryo-maternal communication.

1.5 Aims and objectives

Initially, the aim was to verify that current molecular methods can be employed to detect miRNAs in minimum volume of medium from single-cultured embryos. The relevant miRNA studies have pooled media samples and grouped them according to different criteria (Kropp et al., 2014, Rosenbluth et al., 2014, Capalbo et al., 2016), which introduces bias and increases the risk of false positive/negative findings. Moreover, the use of miRNAs for non-invasive purposes is only clinically relevant when it applies to selection of a single embryo for transfer, meaning that this method needs to be refined for SCM deriving from single-cultured embryos. Therefore, the use of qPCR technology was firstly attempted to establish a detection protocol for the purposes of single embryo assessment. qPCR method was readily available at the time and it is a known technology that is highly sensitive and accurate and has been used for pre-implantation genetic testing of embryos.

After this was achieved, the aim was then to link specific miRNAs with the developmental potential of embryos, measured quantitively as duration of stages and events using time-lapse monitoring, and the DNA quality, expressed as apoptotic index at the blastocyst stage. The morphokinetic properties and apoptotic index served thus as measurable outcomes of embryonic quality which directly reflect on the potential of embryos to implant and establish a healthy pregnancy. The present study therefore explored the potential value of miRNA analysis on assessing the quality of embryos, even in cases where this is not phenotypically obvious. The only publication regarding miRNAs in SCM and embryo quality grouped the embryos in those that reached blastocyst stage and those that degenerated (Kropp et al., 2014), which however renders miRNA analysis redundant since degenerate embryos are easily detected through the microscope and will of course be excluded for transfer. The established protocol and the first study along with the findings are described in detail in Chapter 3.

Where there was an association between apoptosis and miRNA in SCM, the mechanism involved was further explored. Usually, miRNA studies transfect miRNA mimics in embryos (Kropp and Khatib, 2015, Lin et al., 2019) or use *in silico* target prediction tools to explain their findings (Rosenbluth et al., 2014, Capalbo et al., 2016). It is a common misconception to assume that miRNA levels in spent medium reflect on the intra-cellular content, i.e. the more miRNA you have in SCM the more you have inside the blastocyst cells. However, no study has proven yet that extra-cellular miRNA levels are in concordance with the intra-cellular content. It is important to establish the direction of association between miRNA release and embryonic quality in order to understand whether miRNAs have functions as secretory molecules at the window of implantation or they are randomly released and serve no

other actions outside the embryonic cells. In Chapter 4, the direction of association was investigated firstly by measuring intra- and extra-cellular miRNA levels in individual embryos and running correlation tests and secondly by artificially causing apoptosis at the blastocyst stage and then measuring SMC miRNA levels to identify any differences. Hence the mechanism of release of specific miRNAs was further investigated to understand whether miRNAs are passively and randomly extruded by cells or there is an active cellular process involved to miRNA packaging and release which suggests a secretory function for these molecules.

The experiments in Chapters 3 and 4 were carried out using the mouse as a model organism to study mammalian pre-implantation embryo development. The mouse embryo was initially chosen due to the fact that pre-implantation development has many shared events between humans and mice, the genomes between the species are ~85% identical, and the mouse has been used extensively in embryo studies in the past with detailed information available in the existing literature. Additionally, mouse embryos are ideal to study morphokinetic events due to their cytoplasmic appearance which is almost transparent making thus observations and annotations quite easy. However, some miRNAs that were studied in these chapters were mouse-specific and exist in homologue conserved clusters in other mammalian species. Because this thesis involves basic scientific research that ultimately aims to form a strong basis for applications in humans, it was suggested that the preliminary findings be reproduced in another mammalian species with the exact same miRNA sequence as the human. Hence in Chapter 5 bovine embryos were cultured and the levels of specific miRNAs were measured in SCM in relation to apoptosis. This study confirmed the roles of specific miRNAs in relation to embryonic quality and as such it opened the way towards the final experiments.

Finally, effects mediated by miRNAs in endometrial cells were examined to elucidate the biological reason behind embryo-secreted miRNAs at the peri-implantation period. miRNAs control cell processes in embryonic and uterine cells (Liang et al., 2017), are actively released (Kropp et al., 2014, Vilella et al., 2015), and internalised by blastocysts and endometrial cells (Vilella et al., 2015, Burns et al., 2016), meaning that these molecules likely act as mediators of the embryo-maternal interactome at the window of implantation. Possibly circulating miRNAs function as regulators of implantation by triggering cell signalling cascades and controlling the expression of adhesion molecules, inflammatory proteins, and growth factors. However, to date there is no published work about miRNA gene targets in endometrial cells using large-scale platforms. Therefore, in Chapter 6 the effects of a specific miRNA on human endometrial cells were studied using transfections in cell cultures and microarray gene profiling. This study concluded the research presented in the present thesis and provides a holistic picture of the roles of specific miRNAs in mammalian reproduction.

2. CHAPTER TWO: METHODOLOGY

2.1 Mouse embryo culture

Frozen 1-cell stage mouse embryos (B6C3F-1 × B6D2F-1 strain) were thawed in Hepes-buffered medium (Sigma-Aldrich, UK) following the manufacturer's instructions (Embryotech, USA). The embryos were individually placed in micro-wells of EmbryoSlides™ (Vitrolife, Sweden) previously prepared with 25 µL of Embryomax KSOM medium overlaid with 1.4 ml of Embryomax light mineral oil (Merck, UK) and equilibrated at 37^{o}C in 5% CO_2. The embryos were cultured in the EmbryoScope™ time-lapse incubator (Vitrolife, Sweden) at 37^{o}C and 5% CO_2 until they reached the full blastocyst stage (~80 h post-thaw).

2.2 Triple blastocyst staining (FITC/TxRed/DAPI)

All staining steps are performed in glass plates coated with Sigmacote (Sigma-Aldrich, USA). The blastocysts were separately placed in 4% PFA for 12 min at room temperature for fixation. After washing in 2% BSA/PBS, the TritonX100 reagent (Sigma-Aldrich, USA) was used at a 0.1% concentration for permeabilising the membranes. The embryos were washed and placed in drops containing the primary antibody H2AK119Ub (1:200) (Cell Signaling Technology, USA) overnight under mineral oil at 4^{o}C. The next day, the blastocysts were incubated with the Anti-Rabbit IgG secondary antibody (1:300) (Alexa Fluor 488 FITC-conjugated) under mineral oil at room temperature for 1 h. Next, they were washed twice in 2% BSA/PBS and stained for apoptosis using the TdT-mediated dUTP-X nick end labelling method (TUNEL) with the *In Situ* Cell Death Detection Kit with TMR red following the manufacturer's instructions (Sigma-Aldrich, USA). Briefly, each embryo was placed in a mixture of the Enzyme and Label solutions in a 1 in 10 dilution respectively and

the dishes were incubated for 45 min at 37^{o}C. Lastly, the embryos were washed and loaded with the Vectashield Mounting Medium containing DAPI (Vector Laboratories, USA) in 10-well slides (ThermoFisher, UK) for nuclear staining.

2.3 Fluorescence imaging

The embryos were visualised using a Nikon eclipse Ti 90x microscope with an adjusted Hamamatsu digital camera (C4742-80-12AG) and the appropriate filters corresponding to the maxima excitation and emission wavelengths of the dyes (490/525nm Alexa Fluor 488, 596/615nm TxRed, 350/470nm DAPI). The Volocity 3D imaging software (PerkinElmer Inc., USA) was used to capture images and annotate the total cell number (TCN), apoptotic extent, and identify the sex of embryos.

2.4 Time-lapse annotations

Each embryo was captured with the in-built camera every 15 min at 9 focal planes. The EmbryoViewer software (Vitrolife, Sweden) was used to process the data. The following morphokinetic events were annotated following previously proposed guidelines (Ciray et al., 2014): pronuclei fading (tPNf), first cleavage (t2), division to the 3-cell (t3), 4-cell (t4), 5-cell (t5), and 8-cell (t8) stages, start of blastulation (tSB), and full blastocyst (tB) stages (Table 2.1). Using this information, the durations of the second (ECC2 or t4-t2) and third cell cycle (ECC3 or t8-t4) and the synchronicity of the second (s2 or t4-t3) and third cell cycle (s3 or t8-t5), and duration of blastulation (dB) were calculated. Additionally, the ratios of the cleavage synchronicities from 2 to 4 cells (CS2-4), 2 to 8 cells (CS2-8), and 4 to 8 cells (CS4-8) were calculated as described previously (Cetinkaya et al., 2015) (Table 2.2).

Table 2.1 Exact timings, developmental stage, and criteria used to annotate the time-lapse parameters of the embryos.

	Cell stage	**Criteria**
tPNf	Pronuclear fading	First frame in which both pronuclei disappear
t2, t3, t4, t5, t8	2-cell, 3-cell, 4-cell, 5-cell, 8-cell	First frame in which the 2, 3, 4, 5, or 8 cells are clearly separated by membranes accordingly
tSB	Small blastocoel	First sign of blastocoel formation
tB	Blastocyst	Last frame before the embryo starts pushing against the ZP

Table 2.2 Calculations of developmental events using the exact timings described above.

Relative timings	**Morphodynamic event**	**Calculation**
ECC2	Duration of second cell cycle	t4-t2
ECC3	Duration of third cell cycle	t8-t4
S2	Synchronization of cell divisions	t4-t3
S3	Synchronization of cleavage pattern	t8-t5
CS2-4	2- to 4- cell stage cleavage synchronicity	(t4-t3) / (t4-t2)
CS2-8	2- to 8- cell stage cleavage synchronicity	((t3-t2) + (t5-t4)) / (t8-t2)
CS4-8	4- to 8- cell stage cleavage synchronicity	(t8-t5) / (t8-t4)
dB	Duration of blastulation	tB-tSB

2.5 miRNA extraction

2.5.1 miRNA extraction from spent culture media

When culture was terminated, 20 µL of spent media were collected from each embryo, immediately snap-frozen in LN_2, and stored at -80^{o}C. The miRNeasy Serum/Plasma Kit by Qiagen (USA) was used for miRNA extraction. The manufacturer's instructions were followed with small alterations to the protocol aiming to increase the miRNA concentration (Figure 2.1). The 20 µL samples were first diluted 1 in 2 with RNase-free water, 200 µL of the QIAzol Lysis Reagent were added, and the homogenate was incubated at room temperature (15-25^{o}C) for 5 min. Morover, 3.5 µL of the spike-in control (cel-miR-39-3p, custom-made from Invitrogen) concentrated at 10 pmol/µL and 40 µL of choloroform were added and the mixture was shaken vigorously and incubated at room temperature for 2-3 min. The tubes were centrifuged for 15 min at 12,000 x g at 4^{o}C, 120 µL of the upper aqueous phase were transferred to a new collection tube, and 180 µL of 100% ethanol were added. The samples were pipetted into RNeasy MinElute spin columns and centrifuged at ≥8,000 x g for 15 s, 700 µL of Buffer RWT were then added to the spin column and centrifuged for 15 s at ≥8,000 x g. After this, 500 µL Buffer RPE were pipetted and the samples were centrifuged for 15 s at ≥8,000 x g. Lastly, 500 µL of 80% ethanol were added and centrifuged for 2 min at ≥8,000 x g., and the column was then centrifuged at full speed for 5 min to dry the membrane. The spin column was placed in a new collection tube, 10 µL of RNase-free water were added, and the tube was centrifuged for 1 min at full speed to elute the RNA. The samples were stored at -80^{o}C until further processing or immediately processed for building cDNA templates.

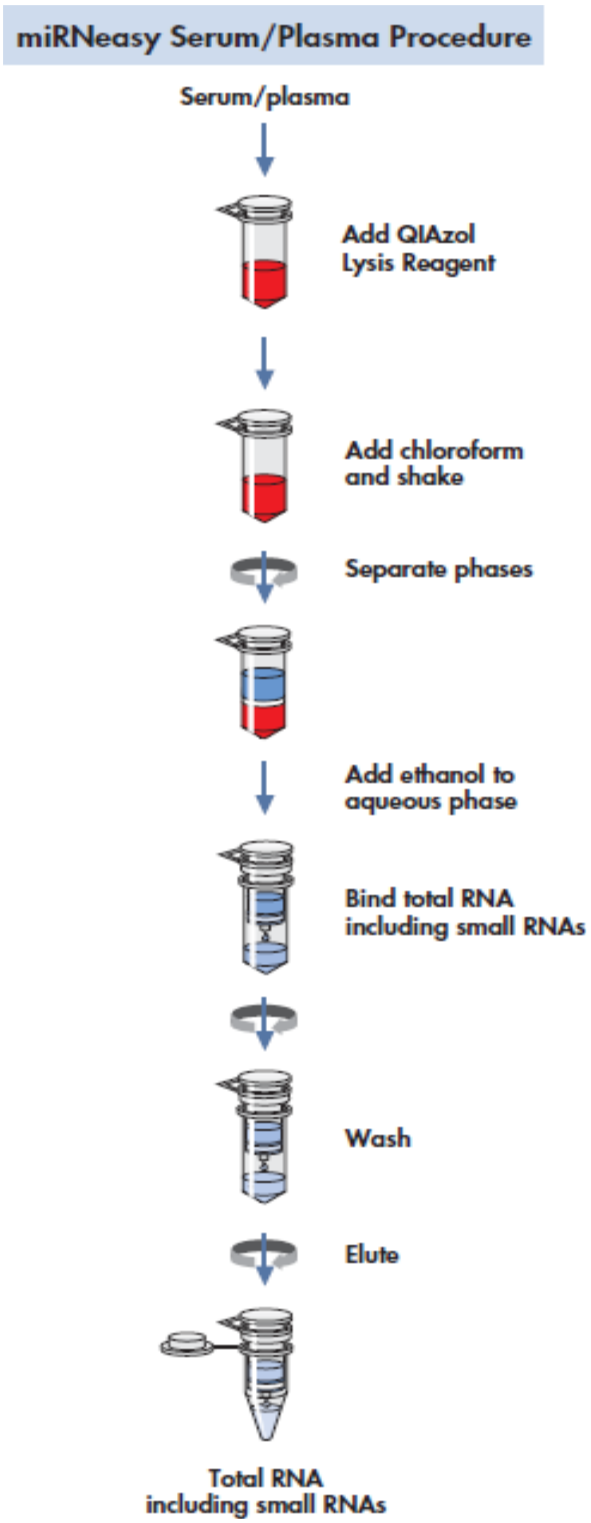

Figure 2.1 miRNA extraction from culture medium procedure using the miRNeasy Serum/Plasma Kit (Qiagen, USA).

2.5.2 Total RNA (miRNA + RNA) extraction from blastocysts

Tubes containing a single blastocyst in 10 µL of RNase-free water were immediately immersed in LN_2 and stored at -80^{o}C until extraction. Total RNA was extracted using the miRNeasy Micro Kit (Qiagen, USA) (Figure 2.2). Firstly, 700 µL of the QIAzol Lysis Reagent were added and the cells were homogenised by vortexing for 1 min. The homogenate was incubated at room temperature for 5 min, 3.5 µL of the spike-in control (cel-miR-39-3p, custom-made from Invitrogen) concentrated at 10 pmol/µL and 140 µL of chloroform were added. The samples were shaken vigorously for 15 s, incubated at room temperature for 2-3 min, and centrifuged for 15 min at 12,000 x g at 4^{o}C. Moreover, 350 µL of the upper aqueous phase were collected into a new tube and 525 µL of 100% ethanol were added. The samples were pipetted into RNeasy MinElute spin columns, centrifuged at at ≥8,000 x g for 15 s at room temperature, 700 µL of Buffer RWT were then added to the spin column and centrifuged for 15 s at ≥8,000 x g. After this, 500 µL Buffer RPE were pipetted and the samples were centrifuged for 15 s at ≥8,000 x g. Lastly, 500 µL of 80% ethanol were added and centrifuged for 2 min at ≥8,000 x g., and the column was then centrifuged at full speed for 5 min to dry the membrane. The spin column was placed in a new collection tube, 14 µL of RNase-free water were added, and the tube was centrifuged for 1 min at full speed to elute the RNA. The samples were stored at -80^{o}C until further processing or immediately processed for building cDNA templates.

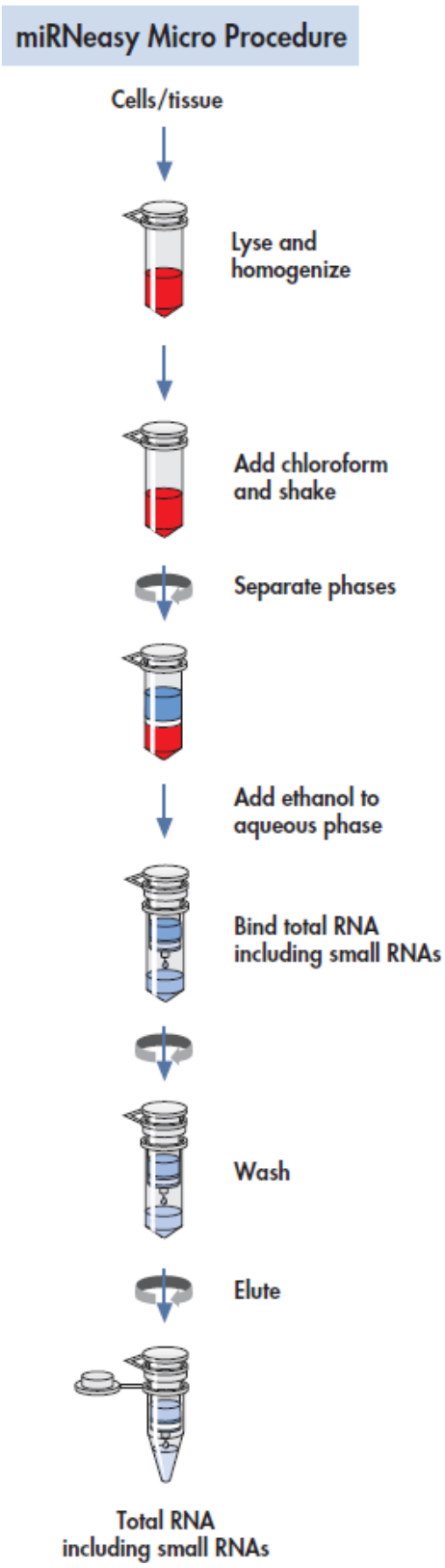

Figure 2.2 Total RNA extraction from blastocysts procedure using the miRNeasy Micro Kit (Qiagen, USA).

2.6 RNA extraction from cells

The RNease Mini Kit (Qiagen, USA) was used for RNA extraction from endometrial stromal cells (Figure 2.3). Firstly, cells were collected in 600 µL RLT buffer, 1% β-mercaptoethanol, 1 volume of 70% ethanol was added to the lysate, and the samples were loaded onto RNeasy Mini spin clomuns and centrifuged for 15 s at ≥8000 x g. Then, 700 µL of Buffer RW1 were added and the columns were centrifuged for 15 s at ≥8000 x g. The columns were then washed with 500 µL Buffer RPE followed by centrifugation at ≥8000 x g for 15 min. This step was repeated with extended centrifugation to 2 min. The spin columns were then centrifuged at full speed for 1 min to dry the membrane, placed in new collection tubed, and eluted with 20 µL of RNase-free water by centrifugated for 1 min at ≥8000 x g. The RNA samples were stored at -80^{o}C until further processing.

2.6.1 RNA clean-up by ethanol precipitation

This protocol was carried out for the preparation of RNA samples for microarray analysis due to the high guanidine salts: RNA concentration ratio which interferes with the amplification steps of the microarray protocol. Briefly RNA clean-up was carried out using ethanol precipitation, where: RNA sample, 1 µl Glycogen, 1/10th of sample volume of 3 M sodium acetate (pH 5.2), and 2 sample volumes of 100% ethanol were mixed well and kept at -80^{o}C overnight. The tubes were centrifuged for 15 min, 12.000 x g at 4^{o}C and the supernatant was discarded. In each sample, 500 µl of cold 70% ethanol were added and the tubes were centrifuged for 5 min, 12.000 x g at 4^{o}C – this step was carried out twice. Lastly, the pellet was air-dried for ~5 min and the pellet was resuspended in appropriate volume of RNase-free H_2O (~20 µl/sample) to achieve >50 ng/µl RNA concentration. The samples were stored at -80^{o}C until further processing.

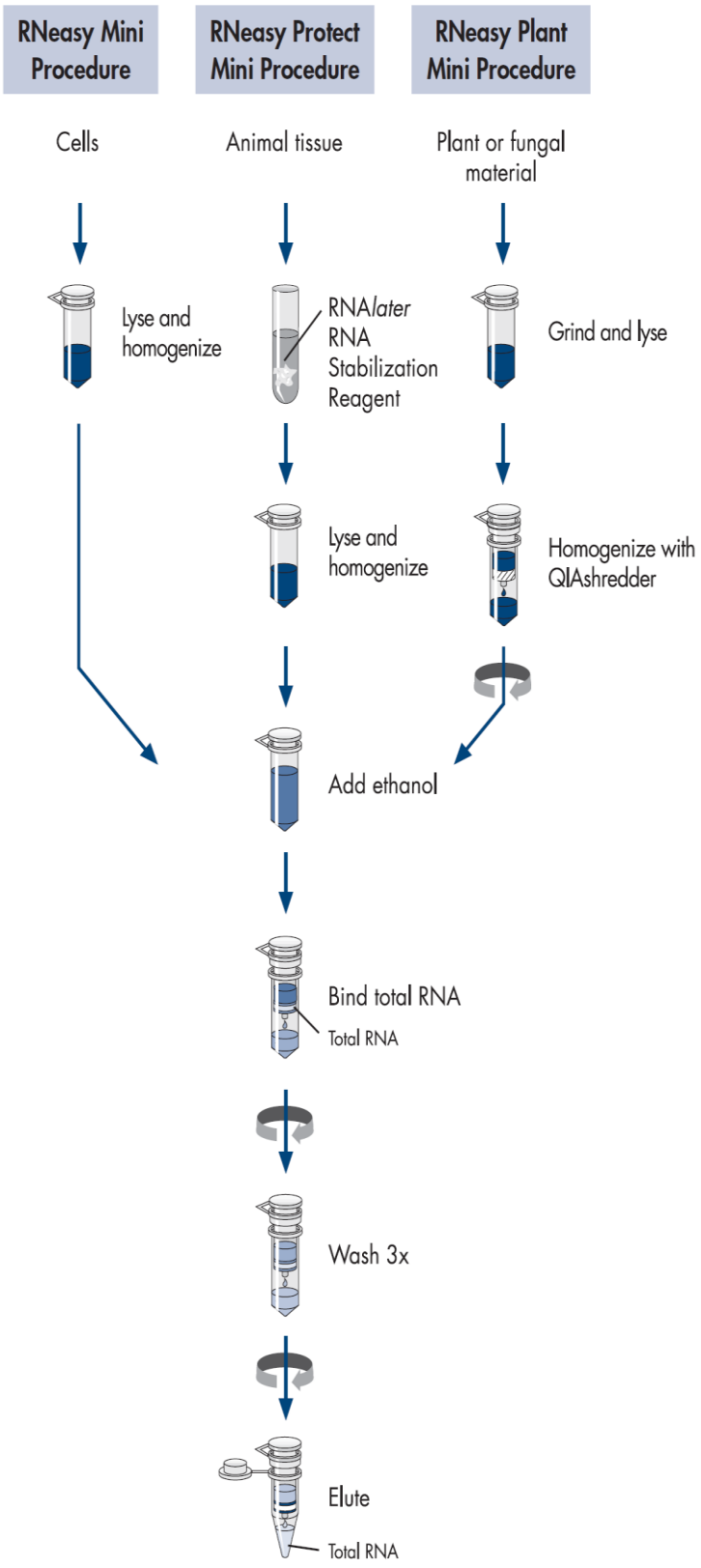

Figure 2.3 Total RNA extraction from cells procedure using the RNeasy Mini Kit (Qiagen, USA).

2.7 cDNA synthesis from miRNAs

The TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, UK) was used for building cDNA libraries from miRNAs. Firstly, the Poly(A) Reaction Mix was prepared using the volumes depicted in Table 2.3 for the appropriate number of reactions (samples). For each reaction, 1.3 μL of Poly(A) Reaction Mix were mixed with 3.7 μL of miRNA eluate. The tubes were placed into the Techne Touchgene Gradient Thermal Cycler, model FTGRAD2D (Techne, USA), and incubated following the conditions described in Table 2.4.

Table 2.3 Reagents and quantities needed for the poly(A) reaction.

Component	Volume (μL) x 1 reaction
10X Poly(A) Buffer	0.5
ATP	0.5
Poly(A) Enzyme	0.3
Total Poly(A) Reaction Mix	1.3

Table 2.4 Cycling conditions for the polyadenylation reaction.

Step	Temperature	Time
Polyadenylation	37ºC	45 min
Stop reaction	65ºC	10 min
Hold	4ºC	Hold

The adaptor ligation reaction was then carried out by preparing sufficient Ligation Reaction Mix for the required number of reactions according to Table 2.5. For each reaction, 10 μL of the mix were added to the 5 μL of the polyadenylated miRNA product. The tubes were then incubated following the conditions described in **Error! Reference source not found.**.

Table 2.5 Reagents and quantities needed for the ligation reaction.

Component	Volume (μL) x 1 reaction
5X DNA Ligase Buffer	3
50% PEG 8000	4.5
25X Ligation Adaptor	0.6
RNA Ligase	1.5
RNase-free water	0.4
Total Ligation Reaction Mix	10

Table 2.6 Cycling conditions for the ligation reaction.

Step	Temperature	Time
Ligation	16ºC	60 min
Hold	4ºC	Hold

Moreover, the reverse transcription reaction was carried out by preparing the RT Reaction Mix for the required number of reactions according to Table 2.7. In each tube containing the adaptor ligation reaction product, 15 μL of the RT mix were added and the tubes were incubated following the settings of Table 2.8.

Table 2.7 Reagents and quantities for the reverse transcription reaction.

Component	Volume (μL) x 1 reaction
5X RT Buffer	6
dNTP Mix (25mM each)	1.2
20X Universal RT Primer	1.5
10X RT Enzyme Mix	3
RNase-free water	3.3
Total RT Reaction Mix	15

Table 2.8 Cycling conditions for the reverse transcription reaction.

Step	Temperature	Time
Reverse transcription	42ºC	15 min
Stop reaction	85ºC	5 min
Hold	4ºC	Hold

The miRNA amplification reaction was carried out by preparing miR-Amp Reaction Mix for the required number of reactions (Table 2.9). For each sample, 45 µL of miR-Amp were mixed with 5 µL of the reverse transcription product. The tubes were incubated in the thermal cycler following the cycling conditions described in Table 2.10.

Table 2.9 Reagents and quantities for the miR-Amp reaction.

Component	Volume (µL) x 1 reaction
2X miR-Amp Master Mix	25
20X miR-Amp Primer Mix	2.5
RNase-free water	17.5
Total miR-Amp Reaction Mix	45

Table 2.10 Cycling conditions for the miRNA amplification reaction.

Step	Temperature	Time	Cycles
Enzyme activation	95ºC	5 min	1
Denature	95ºC	3 s	14
Anneal/Extend	60ºC	30 s	
Stop reaction	99ºC	10 min	1
Hold	4ºC	Hold	1

2.8 cDNA synthesis from RNA

The High Capacity RNA-to-cDNA kit (Applied Biosystems, UK) was used for cDNA synthesis from RNA. The reverse transcription mix was prepared using the reagents and quantities described in Table 2.11. A minor modification to the manufacturer's protocol was carried out for blastocyst samples due to the low RNA concentration. While 5 μL of RNA product were used for endometrial cellls, 9 μL were added for each blastocyst sample. The difference was substituted with nuclease-free water making up to 20 μL of total mix. Lastly, cDNA libraries were built following the cycling conditions of Table 2.12.

Table 2.11 Reagents and quantities for cDNA library building from RNA.

Component	Volume (μL)
2X RT Buffer Mix	10
20X RT Enzyme Mix	1
RNA sample	9[a] or 5[b]
Nuclease-free H_2O	1[a] or 4[b]
Total RT mix	20

volumes used for [a] blastocysts (Chapter 4) and [b] for endometrial cells (Chapter 6)

Table 2.12 Cycling conditions for cDNA library building from RNA.

Setting	Step 1	Step 2	Step 3
Temperature	37ºC	95ºC	4ºC
Time	60 min	5 min	∞

2.9 PCR for miRNA amplification

The TaqMan® Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems, UK) and TaqMan® Advanced miRNA Assays (20X) (assay information in Appendix B) were used for miRNA amplification. Firstly, the PCR Reaction Mix was prepared using the reagents and quantities described in Table 2.13. For each reaction, 2.5 μL of the cDNA product were added to 7.5 μL of the mix in each well of a 96-well PCR plate. The plates were run in the 7500 Fast Real-Time PCR System (Applied Biosystems, UK) setting the cycling conditions of Table 2.14.

Table 2.13 Reagents and quantities for the PCR for miRNA quantification.

Component	Volume (μL) x 1 reaction
TaqMan® Universal PCR Master Mix	5
TaqMan® Advanced miRNA Assay (20X)	0.5
RNase-free water	2
Total PCR Reaction Mix	7.5

Table 2.14 Cycling conditions for the PCR for miRNA quantification.

Step	Temperature	Time	Cycles
Enzyme activation	95ºC	20 s	1
Denature	95ºC	3 s	40
Anneal/Extend	60ºC	30 s	

2.10 PCR for RNA amplification

The TaqMan® Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems, UK) and the corresponding TaqMan® Gene Expression Assays (20X) were used for RNA amplification. Firstly, 1:100 dilutions of the cDNA templates were prepared for the samples derived from endometrial cells. The samples coming from blastocysts were not diluted due to the low RNA concentration. The manufacturer's protocol was generally followed, with small modifications depending on whether simplex or duplex reactions were carried out (Table 2.15). For simplex reactions, 2 μL of the cDNA product were added to 3 μL of the PCR Reaction mix in each well of a 96-well PCR plate. For duplex reactions, 2 μL of the cDNA product were added to 8 μL of the PCR Reaction mix, making a total volume of 10 μL per well. The plates were run in the 7500 Fast Real-Time PCR System (Applied Biosystems, UK) setting the cycling conditions of Table 2.16.

Table 2.15 Reagents and quantities for the PCR for RNA quantification.

Component	Volume (μL) x 1 reaction
TaqMan® Universal PCR Master Mix	2.5[a] or 5[b]
TaqMan® Gene expression Assay (20X)	0.25 assay 1 + 0.25 assay 2 (duplex)[a] or 0.5[b]
Nuclease-free H_2O	0[a] or 2.5[b]
Total PCR Reaction mix	3[a] or 8[b]

[a]volumes used for blastocyst samples in duplex PCR reactions (Chapter 4)

[b]volumes used for endometrial cells with single plex PCR (Chapter 6)

Table 2.16 Cycling conditions for the PCR for RNA quantification.

Stage	Temperature	Time
Hold	50°C	2:00
Hold	95°C	0:20
Cycle (40 cycles)	95°C	0:03
	60°C	0:30

2.11 Analysis of PCR data

All data were analysed with the 7500 Software V2.3 (Applied Biosystems, UK) using the Ct values as read-outs. miRNAs let-7b and cel-miR-39 spike-in were used for normalising the Ct values in the experiments were miRNAs were quantified. During the optimisation phase of miRNA analysis, let-7b showed consistent, stable expression in all samples and was chosen as a normaliser. The spike-in miR-39 is recommended by the manufacturer as it is species-specific (*C.elegans*) and is therefore not expected to amplify in samples from other species. Moreover, *Rpl5* was chosen for normalising the RNA expression in mouse blastocyst samples whereas *18S* was used for normalising the Ct values in samples from human endometrial cells. The Ct-value of 35 was used as cut-off to capture all the potentially valid signals in the miRNA experiments. The amplification plots were visually examined, the baseline and threshold values adjusted where necessary (threshold~0.1), the Ct values were viewed and the ΔCt and ΔΔCt values were calculated based on the recommendations by Schmittgen and Livak (2008).

3. CHAPTER THREE: MIRNAS IN SPENT MOUSE CULTURE MEDIUM AS BIOMARKERS OF DEVELOPMENT AND APOPTOSIS

3.1 Introduction

MicroRNAs are important regulators of cell processes such as stress signalling, cell cycle progression, development, differentiation, and apoptosis (Mendell and Olson, 2012). During the differentiation of mammalian gametes and the subsequent embryo development miRNAs are dynamically expressed in a tightly controlled and time-dependent fashion (Hossain et al., 2012, Tang et al., 2007, Du et al., 2014, Qi et al., 2013). As described in detail in Chapter 1, miRNAs control normal growth and development of the preimplantation embryo. Embryos also release miRNAs in the surrounding micro-environment at the peri-implantation period. Therefore, analysis of spent embryo culture medium for miRNAs is a promising method for assessing the quality embryos in a non-invasive manner.

To date there are only two published studies that have investigated the association between embryonic quality and miRNA presence in medium. Initially Kropp and Khatib (2015) identified and characterised miRNAs in SCM of bovine embryos with differing developmental competence. In this experiment the embryos were cultured in groups to the morula stage at which point they were placed in individual culture up to the blastocyst stage. The SCM were then collected and pooled according to the developmental status in SCM from degenerate embryos and SCM from blastocysts accordingly. Small RNA sequencing technology was used to analyse the samples. It was found that degenerate embryos released significantly higher levels of 11 miRNAs compared to blastocysts. Based on these findings the authors suggested

that miRNAs in SCM indeed have potential as non-invasive biomarkers of quality (Kropp and Khatib, 2015). Nevertheless, this study only provided preliminary evidence and had two significant limitations that need to be addressed in the context of non-invasive embryo assessment. Firstly, the SCM samples were pooled in groups and then analysed which increases the probability of false positive/negative findings as it is not known whether all embryos release miRNAs and at which levels. Secondly in the clinical application degenerate embryos can easily be detected morphologically and would never be transferred to the uterus. Thus, there is no need for molecular analysis to distinguish between degenerate and normal-looking embryos. As with all molecular assessment methods the aim is to distinguish embryos of similar morphology and select the one with the best developmental potential for transfer.

Moreover, a very recent study in bovine embryos questioned whether miRNAs in SCM can be used as biomarkers of developmental competence (Lin et al., 2019). For this embryos were cultured individually to the blastocyst stage and they were grouped according to the timing of the first cleavage (“slow”, “intermediate”, “fast”) and the blastocyst formation competence (degenerate, D and blastocysts, B). The SCM were pooled for each group and miRNAs were sequenced. It was found that 2 miRNAs, namely miR-30c and miR-10b, were highly secreted by “slow” embryos compared to “intermediate”, miR-45 was higher in the degenerate group whereas miR-113 and miR-139 were higher in the blastocyst group. In their bioinformatics analysis the differentially expressed miRNAs affected pathways related to embryo development and apoptosis amongst others (Lin et al., 2019). Although this study provided evidence that possibly the miRNA levels in SCM reflect on the developmental competence of embryos, there were also some weak points that call for caution when interpreting the results. The SCM samples were once again pooled to

achieve the concentration needed for sequencing which increases the risk of false findings. Even with pooled samples the miRNA was very low for an accurate analysis and some groups were consequently excluded.

3.2 Rationale and hypothesis

In this preliminary study the initial hypothesis was that miRNAs can be detected in spent culture media deriving from single-cultured embryos using available technology. It was further hypothesised that embryos release differential levels of miRNAs relating to embryo quality which was assessed through the DNA fragmentation index and morphokinetic timings. These were chosen as primary outcomes since both DNA quality of the blastomeres and the morphokinetic characteristics of embryos reflect on the potential of embryos to implant, grow further, and establish a healthy pregnancy. Overall, the hypothesis of this first experiment was that miRNA analysis of spent culture medium from single-cultured blastocysts can be used as a non-invasive method to identify embryos of different quality and potential. If this proves to be true then it could form a strong basis for future research and clinical applications in human assisted reproduction technologies.

3.3 Aims and objectives

Although miRNAs are intensively studied lately, there is still limited evidence regarding the involvement of specific miRNAs on key-processes controlling embryo pre-implantation development. Considering the lack of information, the aim of the study presented in this chapter was to examine whether embryo-released miRNAs reflect on two processes that are essential for embryonic growth: developmental dynamics/kinetics and apoptosis. More specifically, it was examined whether certain developmental events occurring at the pre-implantation stages are linked to the active

release of specific miRNAs. The mouse embryo was used as a model organism in the context of this study.

In this study, the levels of miRNAs in SCM of mouse blastocysts were measured and analysed in relation to morphokinetic annotations and extent of apoptosis. For this, miR-24, miR-124, miR-291a, and miR-294 were studied as candidate biomarkers because they have key actions in proliferation, differentiation, and cell death. For instance, miR-24 inhibits proliferation through direct targeting of cell cycle genes *E2F2* and *MYC* (Lal et al., 2009) and is additionally involved in apoptosis (Wang et al., 2014, Wang and Qian, 2014). Moreover, miR-124 targets CDK6, an important protein for G1/S transition (Lujambio et al., 2007) and induces apoptosis (Yuan et al., 2017). MiR-290 family members are amongst the earliest miRNAs expressed *de-novo* in embryonic development in mice. MicroRNAs miR-291a and miR-294 promote cell divisions by translational regulation of the G1/S factor *Cdkn1a*. These miRNAs promote cell survival by targeting apoptotic mediators *Casp2* and *Ei24* (Zheng et al., 2011, Li et al., 2017). Finally, all four miRNAs act on pluripotency factors such as *Nanog* and *Oct4* regulating this way cellular differentiation, a process that is crucial for normal developmental progress (Marson et al., 2008, Furuta et al., 2010, Lee et al., 2016).

For the purposes of this study it was essential to have a detailed picture on the association of the miRNAs with development and apoptosis. Therefore, individual samples of SCM deriving from single-cultured embryos were analysed - a technical approach which has not been reported previously. miRNA data were then associated with morphokinetic information and apoptotic percentage for each embryo individually. Morphokinetics were assessed using time-lapse incubation technology because it allows to continuously monitor the embryos and annotate dynamic events,

cleavage patterns, and timings of events and extract detailed descriptive information about the development of each embryo individually. Embryo quality was assessed by using specific apoptosis detection staining and calculating the percentage of apoptotic cells at the blastocyst stage.

3.4 Methodology

3.4.1 Embryo culture, morphokinetic annotations, and blastocyst scoring

Preliminary experiments were firstly carried out to establish the methodology and design the experimental procedure. After this, 1-cell stage mouse embryos (*N=70*) (B6C3F-1 x B6D2F-1) were thawed and cultured until Day 5 of development (80 h post-thaw) in EmbryoScope in 2 consecutive repetitions of this experiment following the protocols described in Section 2.1. Morphokinetic annotations were carried out according to the criteria provided in Section 2.4 and the data were processed to generate the absolute and relative timings to the various developmental stages using the time of pronuclear fading (tPNf) to normalise the values (time O). Moreover, fragmentation percentage at the 4- and 8-cell stages, multinucleation at the middle of t3-t2 and t5-t4 intervals, and blastomere size at the 2-, 4-, 8-cell stages were noted as morphological markers of embryo quality. Lastly, the blastocysts were graded at the specific timeframe of 70.0-70.3 h (post-thaw) based on the quality of the trophectoderm and inner cell mass (Table 3.1) as good, fair, or poor morphology (Table 3.2) (Heitmann et al., 2013).

Table 3.1 ICM and TE characterisation criteria used to score the blastocysts.

Grade	ICM	TE
A	Prominent, easily discernible, with many cells that are compacted and tightly adhered together	Many cells forming a cohesive epithelium
B	Easily discernible, with many cells that are loosely grouped together	Few cells forming a loose epithelium
C	Difficult to discern, with few cells	Very few cells

Table 3.2 Simplified SART system used to categorise the blastocysts in three morphology groups.

SART grade	Embryo grade (ICM/TE)
Good	AA, AB
Fair	BA, BB, BC
Poor	CB, CC

3.4.2 Blastocyst staining

To exclude sex-related differences in miRNA expression as reported for other species (Rosenbluth et al., 2013, Gross et al., 2017b) sexing of embryos was carried out. Sex determination was based on the molecular phenomenon of X inactivation which happens at the blastocyst stage in female mouse embryos. Histone 2a ubiquitin is enriched in the inactive X chromosome (Xi) in females, therefore by immuno-targeting this protein a Xi patch appears in cells of female embryos. Furthermore, the embryos were stained for apoptosis using the TdT-mediated dUTP-X nick end labelling method (TUNEL) - a well-established method for apoptosis detection in mammalian embryos, including murine (Abdelhafez et al., 2011), bovine (Vichera et al., 2014), porcine (Chen et al., 2018), and human embryos (Li et al., 2012). Thus, the

blastocysts were separately stained following the triple staining protocol described in section 2.2. The embryos were visualised with fluorescent microscopy (2.3), the sex was identified and the apoptotic extent at the blastocyst stage (% apoptotic cells / total cell count) was assessed for each embryo.

3.4.3 miRNA quantification

Spent culture media were collected from each well of the EmbryoSlides where embryos were cultured along with blank media controls from wells not exposed to embryo culture. The samples were placed in PCR 0.2 ml microtubes which were immediately immersed in LN_2 and stored in -80^{o}C until further processing. miRNAs were extracted from each sample according to the protocol provided in Section 2.5.1. The products were either stored for a short period of time in -20^{o}C or immediately processed further. cDNA libraries were built (2.7) and PCR was carried out (2.9) to amplify the miRNA signals and quantify the expression. The probes used in this study are listed in the table below (Table 3.3). The data were analysed with the 7500 Software V2.3 (Applied Biosystems, UK) using the Ct values as read-outs (2.11). The let-7b-3p miRNA was used for normalising the Ct values. The Ct-value of 35 was used as cut-off to capture all the potentially valid signals.

Table 3.3 miRNA assays used for amplifying each miRNA. The target miRNA and the mature sequence are displayed.

TaqMan miRNA probes			
Target	**Assay name**	**MiRBase accession number**	**Mature miRNA sequence (5'-3')**
miR-291a	mmu-mir-291a-3p	MIMAT0000368	AAAGUGCUUCCACUUUGUGUGC
miR-294	mmu-mir-294-3p	MIMAT0000372	AAAGUGCUUCCCUUUUGUGUGU
miR-24	hsa-miR-24-3p	MIMAT0000080	UGGCUCAGUUCAGCAGGAACAG
miR-124	mmu-mir-124-3p	MIMAT0000134	UAAGGCACGCGGUGAAUGCC
let-7b	hsa-let-7b-3p	MIMAT0004482	CUAUACAACCUACUGCCUUCCC

3.4.4 Statistical analysis

The Statistics Clinic at the School of Medicine (UoN) was consulted for the processing of the data produced throughout the experiments. The SPSS Statistics 26 software (IBM, USA) was used for data analysis. Data distributions were checked using the Shapiro-Wilk test for normality and visual examination of the histograms. Values greater than twice the standard deviation for each parameter were considered outliers and excluded from further analysis. The Kruskal-Wallis test was performed to examine the distribution of apoptosis across the good, fair, and poor morphology groups. The $2^{-\Delta Ct}$ values were used for all statistical tests using the miRNA data (Schmittgen and Livak, 2008). Log transformations were carried out where necessary to achieve normal distribution of the values. Independent samples t-tests were carried out to examine the expression of miRNAs between the sex groups. Spearman's and Pearson's correlation coefficients were calculated for examining potential correlations between morphokinetics and apoptosis with miRNA levels in SCM. A *p* value of <0.05 was considered significant for all tests. Graphs were generated using GraphPad PRISM 8.

3.5 Results

3.5.1 Embryo development

From the 70 mouse embryos thawed, 59 (84%) cleaved, formed morulae, and reached the blastocyst stage by day 4 of culture. After normalisation of the annotation data with the tPNf, the average time ± standard deviation (in hours) for each developmental stage were calculated and are presented in Table 3.4 and Table 3.5.

Table 3.4 Average value and standard deviation for the time in hours to reach each developmental stage.

Absolute timing	Average (h)	Standard deviation (h)
t2	1.7	0.4
t3	22.4	1.2
t4	23.1	1.4
t5	33.1	1.8
t8	34.6	1.9
tB	59.6	2.8
tSB	62.8	3

Table 3.5 Average value and standard deviation for the interval in hours to complete each dynamic event.

Relative timing	Average (h)	Standard deviation (h)
ECC2	21.4	1.3
ECC3	11.5	1.2
S2	0.7	0.6
S3	1.5	0.8
CS2-4	0.03	0.03
CS2-8	0.9	0.03
CS4-8	0.1	0.06
dB	3.2	1.8

3.5.2 miRNA expression in spent culture medium

The SCM from 59 blastocysts were analysed for the presence and levels of the miRNAs. All samples amplified for let-7b that was used for normalising the Ct values for the rest of the miRNAs. For miR-24, 17 samples (29%) showed amplification below the cut-off Ct value and accordingly 36 samples (61%) were positive for miR-124, 20 (34%) for miR-291a, and 43 (73%) for miR-294. For miR-24 one sample was excluded as an outlier, for miR-124 3 samples were outliers, for miR-291a 1 sample, and for miR-294 1 sample was an outlier. The remaining samples for each miRNA were either negative or amplified beyond 35 cycles thus being treated as negative samples. Blank samples, namely water and culture media not exposed to embryo culture, were also run in parallel and showed no amplification for the miRNAs. The average and standard error of mean of the Ct and ΔCt values are shown in Table 3.6.

Table 3.6 Average Ct and ΔCt values with standard error of mean for each miRNA in spent media.

miRNA	Ct	ΔCt
miR-24	33.3 (± 0.5)	28 (± 2)
miR-124	32.7 (± 0.3)	27.8 (± 1.5)
miR-291a	31 (± 0.7)	25.7 (± 3.5)
miR-294	27.8 (± 0.4)	22.9 (± 2.7)
let-7b	5 (± 0.8)	

3.5.3 miRNAs and embryo sex

A total of 49 blastocysts were successfully stained for sex assessment. Embryos in which the FITC dye was highly concentrated in a genomic region in each cell, appearing like a green dot, were easily observed and classified as female whereas in embryos where the FITC dye was diffused in each cell were classified as male (Figure 3.1). Sample size differed between sex groups with 30 male versus 19 female mouse embryos. The mean miR-24 ΔCt was 28.1 ± 0.7 (SEM) for male embryos and 27.3 ± 1 for female embryos. MiR-124 was 27.8 ± 0.4 and 28 ± 0.4, miR-291a was 26.9 ± 0.9 and 23.5 ± 1.4, and miR-294 was 23 ± 0.6 and 22.7 ± 0.6 for male and female blastocysts respectively (Figure 3.2). Mean comparisons showed that miRNA levels in SCM were similar between male and female embryos ($p>0.05$). Therefore, further correlation tests were not adjusted for sex-related bias.

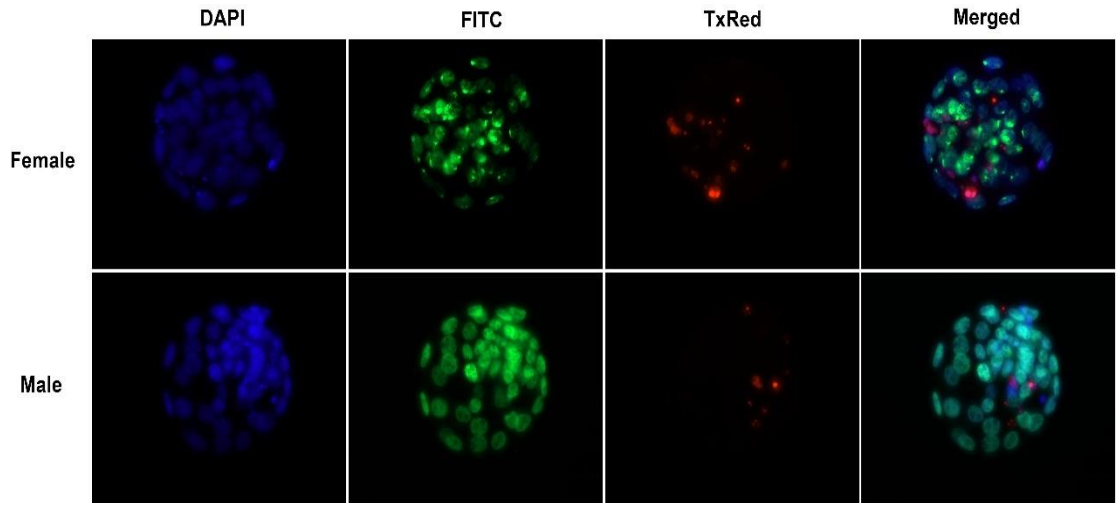

Figure 3.1 Mouse blastocysts x40 magnification after triple staining. The DAPI nuclear dye was used to count the TCN and the TxRed dye specifically binds to broken DNA, indicating this way cell death. The apoptotic extent was calculated by dividing the apoptotic cells with the TCN (%). Gender determination was based on the molecular phenomenon of X inactivation. Histone 2a ubiquitin is enriched in the Xi in females, therefore by immuno-targeting this protein an Xi patch appears in cells of female embryos. In the first row a female embryo is displayed with the characteristic FITC nuclear patch whereas in the male embryo (lower row) the dye is diffused since Histone 2a ubiquitin is not concentrated in a genomic region**.**

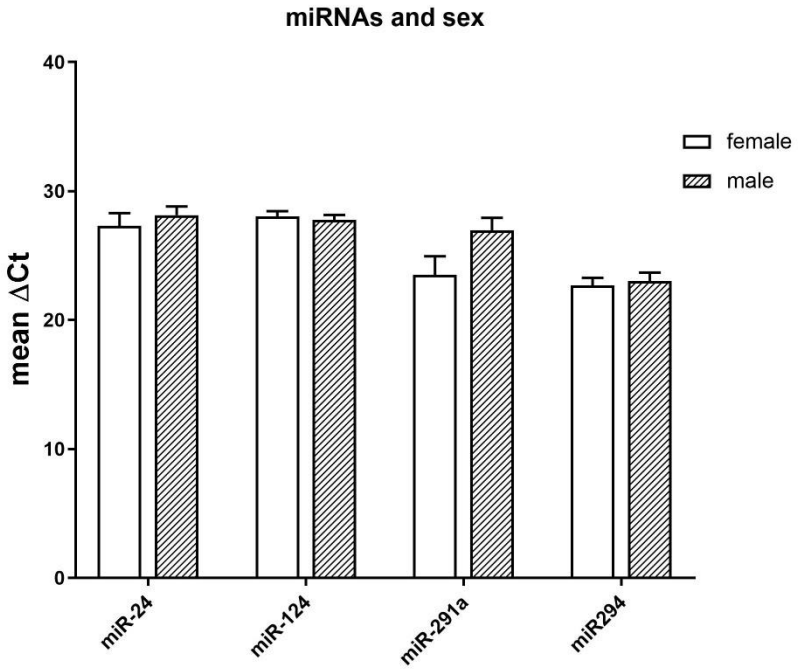

Figure 3.2 Mean ΔCt for each miRNA in male and female embryos. T-bars depict the standard deviation for each variable.

3.5.4 miRNAs and development

Data from 59 mouse embryos were analysed to examine the relationship between pre-implantation development and miRNA levels in SCM. Thus, for each embryo miRNA levels were compared with absolute timings (t2, t3, t4, t5, t8, tSB, tB - normalised with tPNf) and relative timings of ECC2, ECC3, s2, s3, dB, CS2-8, CS4-8, and CS2-4. The miRNA levels were not significantly associated with the duration of distinct developmental events for most of the dataset. However, a significant correlation was found between miR-124 and the duration of blastulation dB (*N=36, p<0.001*) and further analysis revealed that the direction of the association is negative (*r=-0.528*) (Figure 3.3). Post-hoc power calculation with GPower using the sample size and correlation value found that the power of this finding reached 0.57 (Faul et al., 2009).

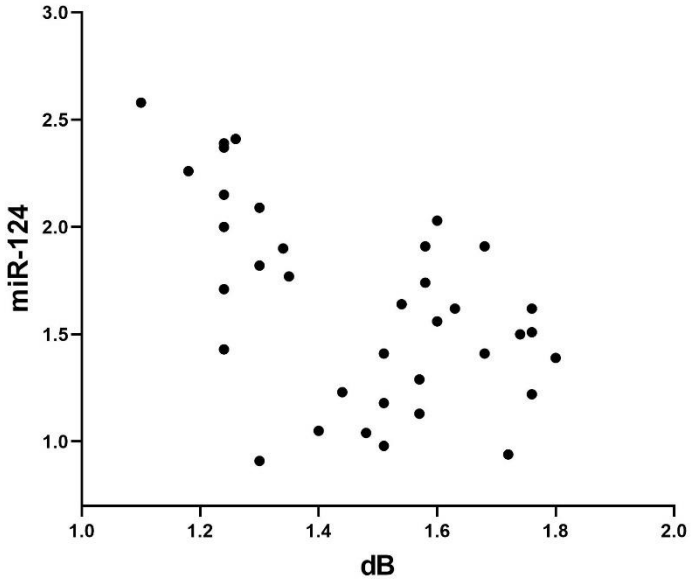

Figure 3.3 Scatter plot of miR-124 in relation to duration of blastulation (dB). The variables are transformed using log10 (x*10) to achieve a Gaussian distribution. The transformed values are depicted in the x and y axis. MiR-124 and dB are significantly correlated (*N=36, p<0.001)* in a negative relationship (*r=-0.528*).

3.5.5 Morphology, miRNAs and apoptosis

No signs of fragmentation and multinucleation were observed in the 59 embryos while they were cultured in time-lapse. Blastomere size was uneven for 2 embryos at the 2-cell stage, 4 embryos at the 4-cell stage, and 5 embryos at the 8-cell stage. Cell lysis was observed in 5 embryos that developed into blastocysts. After assessing the morphology of 59 blastocysts, 4 were categorised as good, 42 as fair, and 6 as poor, whereas 7 embryos had not reached the full blastocyst stage at the 70.0-73.0 interval and were not classified. Moreover, 47 blastocysts were stained for apoptosis and the average apoptotic percentage was 12% ranging from 0% up to 56% (Figure 3.4). Analysis of variance tests showed that the average extent of apoptosis was similar across the three morphology groups (good=21 ± 6%, *N=4*; fair=15 ± 1%, *N=37*; poor=9 ± 1%, *N=6*) (*p>0.05*). The 5 blastocysts with signs of cell lysis did not show different expression of miRNAs in the respective spent media samples compared to

the rest of the population (*p>0.05*). The SCM levels of miRNAs -24, -124, and -291a were not associated with apoptosis (*p>0.05*). However, miR-294 was significantly correlated with the extent of apoptosis (*N=36, p<0.001)* with Pearson's correlation coefficient revealing a positive relationship between these variables (*r=0.560*) (Figure 3.5). The power of the study was found 0.96 in the post-hoc analysis with GPower (Faul et al., 2009).

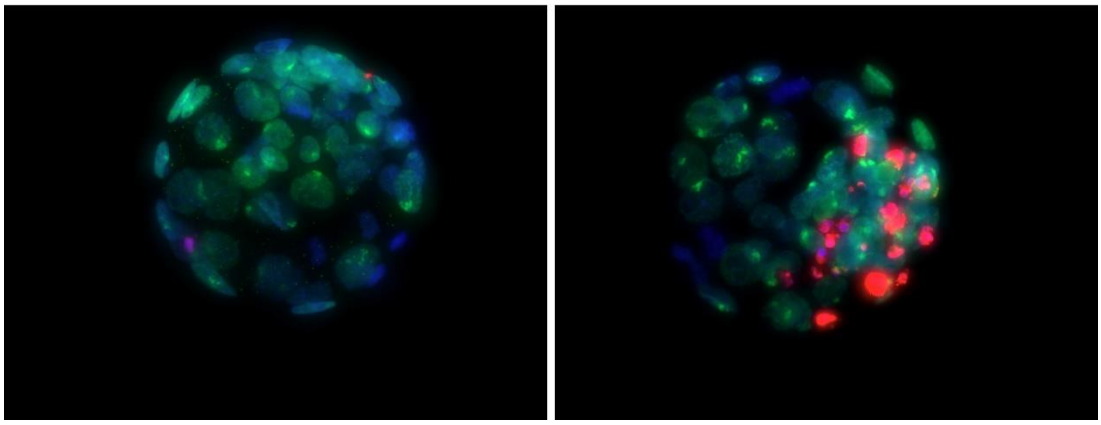

Figure 3.4 Mouse blastocysts with different apoptotic indexes (x40 magnification). The Tx-Red dye specifically binds to broken DNA, indicating this way cell death. On the blastocyst on the left the dye is barely detectable whereas on the blastocyst on the left the dye marks almost half of the embryo and is localised exclusively in the embryonic part that contains the ICM.

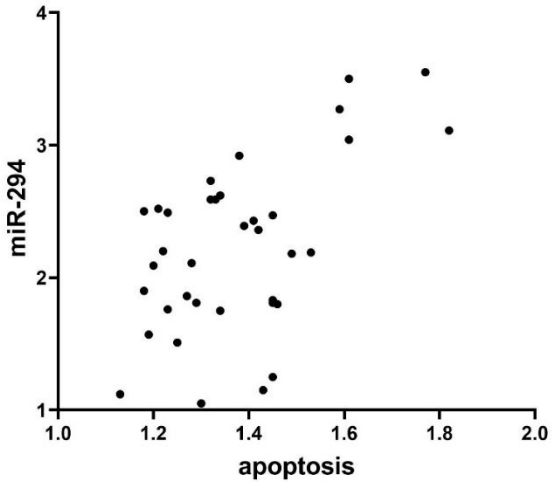

Figure 3.5 Scatter plot of miR-294 in relation to apoptosis. The variables are transformed using log10 (x+10) to achieve a Gaussian distribution. The transformed values are used as inputs for the x and y axis. MiR-294 and apoptosis are significantly correlated (*N=36, p<0.001)* in a positive relationship (*r=0.560*).

3.6 Discussion

The present experiment shows that miRNA quantification in SCM from single-culture embryos is possible and provides preliminary evidence linking specific miRNAs with morphokinetics and apoptosis. The main findings are that miR-124 is inversely associated with the duration of blastulation and miR-294 is directly correlated with the extent of apoptosis in mouse embryos.

3.6.1 Mouse blastocysts release miR-24, miR-124, and the miR-290 cluster in spent culture media

It was hypothesised that miR-24, miR-124, and the miR-290 cluster would be found in SCM of embryos since these miRNAs are expressed in embryonic cells (Landgraf et al., 2007, Tang et al., 2007, Rosenbluth et al., 2013, Kropp and Khatib, 2015), control processes involved in embryonic development such as cell cycle progression and differentiation (Grandjean et al., 2009, Zhao et al., 2015, Yuan et al., 2017), and are found in serum meaning that they are released by cells (Rotkrua et al., 2013, Meng et al., 2014, Santangelo et al., 2017).

In contrast to other studies that pool SCM samples using different grouping criteria (Rosenbluth et al., 2014, Cuman et al., 2015, Kropp and Khatib, 2015, Gross et al., 2017b), in the present study its was shown that it is possible to isolate and quantify miRNAs from single-cultured embryos. The significance of single sample analysis is highlighted by the finding that not all embryos release miRNAs in detectable levels, therefore by grouping samples together false positive/negative findings are very likely. Indeed the amplification rate ranged from 29% for miR-24 up to 73% for miR-294, even when the reference miRNA let-7b was detected.

As expected, the presence of the selected miRNAs was verified in the culture media of mouse embryos. This finding agrees with studies showing that mammalian blastocyst-stage embryos release microRNAs in their environment. This is known for two species –bovine blastocysts (Kropp et al., 2014, Gross et al., 2017b) and human embryos (Rosenbluth et al., 2014, Cuman et al., 2015, Capalbo et al., 2016). To date there are no publications showing similar findings for the mouse embryo. Additionally, from the selected panel miR-24 has been found in bovine SCM (Kropp and Khatib, 2015) whereas there are no reports for miR-124 and the miR-290 cluster. Taken together, these findings show that miRNA analysis is possible for single samples and hence could be further explored as a non-invasive embryo assessment method.

3.6.2 miR-24, miR-124, miR-291a, and miR-294 levels do not differ between male and female mouse blastocysts

In mammals there is a surprisingly high density of miRNA sequences on the X chromosome and an almost complete absence of miRNA genes in Y chromosome. This feature is highly conserved in mammals including humans and it is also observed in lower-level organisms like *D.melanogaster* and *C.elegans* (Ghorai and Ghosh, 2014). It is estimated that approximately 10% of miRNA genes are located on the X chromosome which according to the miRBase database (www.mirbase.org) corresponds to 118 miRNAs of which 62 are classified with high annotation confidence (Di Palo et al., 2020). This sex-specific genomic feature equips females with a larger miRNA machinery compared to males and possibly contributes to gender-biased functions in organisms. Thus miRNA studies in mammalian species, and especially those with human implications, need to take into consideration the sex as a potentially confounding factor.

Information on differential miRNA expression in embryos come mainly from two studies. Initially, Rosenbluth and colleagues (2013) identified 20 miRNAs that differed significantly between female and male human embryos (Rosenbluth et al., 2013). This study analysed biopsy material to quantify the intra-cellular expression of 754 miRNAs and found that 20 miRNAs are differentially expressed according to embryo sex. Of these, 14 miRNAs were more highly expressed in male embryos and 6 miRNAs were highly expressed in female embryos (Rosenbluth et al., 2013). Interestingly from the 20 differentially-expressed miRNAs only 2 are located in X chromosome according to miRBase which begs the question: why is there sexual dimorphism in expression of miRNAs located in chromosomes other than the X? The answer to this remains unclear, however it could be that since miRNAs are relatively newly-discovered molecules the genomic context needs to be further investigated and validated. In addition, it is highly likely that biological differences in epigenetic patterns and endogenous compounds (hormones, cytokines) are responsible since they affect the regulation of miRNA expression (Gulyaeva and Kushlinskiy, 2016).

Moreover, a study in bovine embryos analysed the expression of 68 miRNAs in media collected from female and male blastocysts (Gross et al., 2017b). It was found that at least 8 miRNAs were released in significantly higher levels by female blastocysts compared to males. Of these, miR-122, miR-22, and miR-320a were further validated and supplemented in bovine endometrial epithelial cells to investigate the potential roles of these miRNAs in communication with the maternal environment. These miRNAs were uptaken by the endometrium where they caused transcriptomic changes in implantation-related genes. Based on these findings, the authors therefore suggested that there are dimorphic differences in miRNA secretion by blastocysts and these can have further effects in the blastocyst-endometrium

crosstalk (Gross et al., 2017b). It must be highlighted that although these findings show different expression patterns, none of the 8 differentially-released miRNAs are located in X chromosome. Once again it could be that biological differences that influence miRNA transcription and/or secretion are responsible for the observed differences.

Considering both studies described above it was therefore decided to carry out embryo sexing in this experiment to exclude any bias relating to sex, even though none of the studied miRNAs are located on X. More specifically, miR-24 is located in chromosome 12, miR-124 in chromosome 2 and the miR-290 cluster in chromosome 7 – all with high annotation confidence according to miRBase. Indeed, in the present study these miRNAs were released in similar levels by female and male mouse blastocysts and thus sex was not considered as a confounding factor in the following statistical analyses.

3.6.3 Longer blastulation is negatively associated with miR-24 levels in spent media

Due to the involvement of all chosen miRNAs with cell proliferation and growth it was expected that the variable expression of these miRNAs in SCM would associate with differences in the timings of morphokinetic events. Development was assessed by measuring absolute and relative timings of specific kinetic and dynamic events that are characteristic in mammalian preimplantation embryo development. For instance, blastomere division cycles and blastocoel formation are events that occur during the development of bovine, murine, and human embryos amongst other species. In turn morphodynamic events are informative regarding the developmental potential of each embryo. Indeed in human embryos specific timings reflect on the ability for blastocyst

formation, implantation, and post-implantation potential of embryos (Meseguer et al., 2011, Dal Canto et al., 2012, Cetinkaya et al., 2015, Motato et al., 2016).

The main finding comes from the comparison of miR-124 and dB where lower miR-124 levels were linked to longer duration of blastulation. This association could be a result of miR-124 regulatory actions in cell proliferation and differentiation that occur at the dB interval. For instance, during blastulation the first lineage decision separates the cells in the TE and ICM populations and by the late blastocyst the ICM differentiates further into the epiblast and primitive endoderm (Zernicka-Goetz et al., 2009). Interestingly one of the targets of miR-124 is SMYD3, a methyltransferase that regulates the transcription of lineage-specific factors that are essential for progression to the latest pre-implantation stages (Furuta et al., 2010, Zeng et al., 2012). Because of the involvement of this miRNA in cell differentiation it could be that variable miR-124 levels reflect on the ability of embryos to complete blastulation. It must be noted however that miR-124 has more than 1.500 gene targets (www.targetscan.org) and therefore alternative pathways cannot be ruled out and further work is needed to decipher those exact mechanisms.

When the present experiment was designed and carried out there were no publications regarding the association of miRNAs in SCM with embryo development. Recently some interesting findings were published in this topic. More specifically, Lin *et al.* (2019) carried out an experiment whereby bovine embryos were cultured individually to the blastocyst stage and were grouped according to the first cleavage pattern in “fast”, “intermediate”, and “slow” embryos (Appendix A). The respective SCM samples were pooled and analysed with sequencing for the identification of marker miRNAs in each developmental group. Although there were technical difficulties with the “fast” group, it was found that miR-30c and miR-10b were highly

released by “slow” embryos compared to “intermediate” and this difference was statistically significant. Bioinformatics analysis revealed that these miRNAs affected pathways relevant to embryo development and signalling and thus the authors further suggested that these miRNAs are candidate biomarkers of impaired developmental potential (Lin et al., 2019). Despite the key differences of this study with the present experiment (pooled samples, no timelapse, bovine vs mouse), the general findings are in agreement and further strengthen the suggestion that embryos with different developmental potential release miRNAs differentially.

Regardless of the biological mechanism behind miR-124 release and blastulation, the duration of this stage could be a sign of poor developmental potential. Indeed, there is evidence to suggest that developmentally compromised embryos require longer time to complete blastulation. More specifically, a study in human IVF where single euploid blastocysts were transferred showed that shorter blastulation is associated with increased ongoing pregnancy rates (Mumusoglu et al., 2017). Although not clear, it seems that the completion of this stage within a specific timeframe reflects on the potential of embryos to establish a healthy pregnancy. In the present study faster blastulating embryos transport excessive amounts of miR-124 to the surrounding micro-environment compared to slower embryos. Since faster dB is a sign of superior quality, higher miR-124 levels in SCM could be measured to identify embryos with favourable developmental potential.

Nevertheless, it would be advised that this finding is studied further and reproduced in other experiments before investigating its importance. What became apparent in the statistical analysis and can be visualised in Figure 3.5 is that the distribution of values between 1.3 and 1.5 of dB does not follow the pattern of the negative correlation, ie there is a gap of expected miR-124 values in this timeframe.

In specific, embryos that completed blastulation within this interval showed a high dispersion of miR-124 in SCM. The problem is particularly evident for embryos with dB values around 1.4 where, according to the correlation test, the expected miR-124 values would range between 1.3 and 1.7; however these levels were not detected. Evidently there are few datapoints in this specific dB timeframe (only 2) and thus a larger sample size could clarify this discrepancy of values. Although the sample size in the present study was calculated a priori, 39% of the original population did not express miR-124 in SCM which significantly lowered the sample size for the correlation test. Possibly miRNA information from more embryos with dB in this interval would prove or disprove the correlation, which would make this finding more reliable. Indeed a post-hoc calculation of statistical power showed that these findings achieved a 0.57 power (Faul et al., 2009) - which was considerably lower from the minimal expected value of 0.8 – meaning that this association must be interpreted with caution.

3.6.4 Extended blastomere apoptosis is positively correlated with miR-294 release

Since miR-24, miR-124, and the miR-290-295 cluster are microRNAs that are expressed in mouse preimplantation development and they all have roles in DNA repair mechanisms (Tulay and Sengupta, 2016), it was hypothesised that the release of these miRNAs within culture media would reflect on the apoptotic status of the embryos. Perhaps the most interesting finding of the present experiment is that miR-294 in SCM is strongly associated with apoptosis at the blastocyst stage.

Although the mechanism behind this association needs to be further studied it is possible that the actions of miR-294 on apoptosis regulation led to the observed difference. Target prediction analysis was carried out to identify potential regulatory effects of miR-294 on apoptosis-related factors (www.targetscan.org). Interestingly, miR-294 is involved in the post-transcriptional regulation of the apoptosis facilitator *Bcl2l11*– a regulatory mechanism which could possibly explain the association. Additionally, this miRNA regulates the expression of cyclin-dependent kinases *Cdkn1a* and *Cdk2* genes that are key-mediators of DNA damage recognition in the p53 signalling pathway which initiates apoptosis in cells. However, it is likely that the association of miR-294 and apoptosis is not that simplistic and straightforward as this miRNA targets more than 700 genes and therefore complex regulatory networks activated through miR-294 could also lead to increased apoptosis in blastomeres.

Because miR-294 and apoptotic extent are directly associated, it could be that higher miRNA levels lead to increased DNA damage in embryonic blastomeres. This possibility was recently described for another miRNA in the bovine species. More specifically, Lin and colleagues (2019) firstly identified that miR-30c was found in

elevated levels in SCM of developmentally compromised embryos. The miR-30c mimic was then supplemented in embryo culture and it was found that the up-take of miR-30c by embryos significantly increases apoptosis at the blastocyst stage by 8%. It was further shown that miR-30c downregulates a gene of the DNA damage response pathway (*CDK12*) which according to the authors was the reason behind the impaired blastocyst quality and extended apoptosis (Lin et al., 2019). Although the findings of Lin *et al.* (2019) are in line with these of the present experiment, the pathways involved in release of miRNAs by pre-implantation embryos are not yet understood and thus the mechanism of miR-294 release and its association with apoptosis inside the embryonic cells needs to be further explored.

Regardless of this, it must be mentioned that apoptosis is a crucial "rescue" mechanism for the normal development of preimplantation embryos as it corrects cell abnormalities that occur. Nevertheless, extended apoptosis has negative effects on embryo survival which reflects on implantation and pregnancy rates (Ebner et al., 2001). The extent of apoptosis in embryos cannot always be assessed morphologically and to further support this blastocyst scoring was carried out and compared with the number of apoptotic cells in each embryo. Additionally, other quality markers such as multinucleation at the t3-t2 and t5-t4 intervals, fragmentation at the 4- and 8-cell stages, and blastomere symmetry at the 2-, 4-, and 8-cell stages were annotated. No associations between these quality markers and apoptosis were found meaning that sole morphological assessment has limited value on assessing cellular quality. In contrast, this study provides preliminary evidence that miRNA analysis of SCM can be used to distinguish highly apoptotic from lower apoptosis embryos.

3.7 Limitations

The findings of the present experiment are limited to mouse embryos and caution must be taken for any assumptions for the human or other mammalian species. Although miRNAs are highly conserved molecules across evolution, genomic differences are found between species. For instance, the miR-290 cluster is mouse-specific and therefore any follow-up experiments in higher species should be carried out with the homologue miR-371-373 cluster. Additionally, the embryos used in this experiment were frozen at the 1-cell stage and subsequently thawed and cultured. The freezing-thawing procedure might result in altered miRNA profiles compared to fresh embryos due to higher cellular stress and damage from cryopreservation. Moreover, the oocytes were fertilised with traditional IVF so discrepancies might be found if similar experiments are carried out in ICSI-fertilised embryos due to the zona pellucida opening in these embryos through which molecules can be passively transported to the extra-cellular environment.

Another limitation was the small panel of miRNAs studied in this experiment. Although a total of 14 miRNAs were chosen as candidate molecules (Appendix B), preliminary experiments found that either some miRNAs were present in the culture medium (possibly from serum albumin contamination) or they were completely absent in spent culture media samples (Appendix C). Additionally, because the aim was to analyse individual SCM samples there was a very small concentration of total miRNAs recovered from each embryo (< 10 ng/μL) which is inevitably limiting for the number of reactions/targets. Due to these reasons only four miRNAs (miR-24, miR-124, miR-291a, miR-294) were carefully chosen for this study. However, because of the numerous biological roles of miRNAs it is expected that other miRNAs in spent media are also associated with embryo development and quality.

Lastly as described previously for miR-124 and blastulation, the sample size was suboptimal for the correlation test and the power of the study was below the aimed value which make these results weak and possibly unreliable. Additionally the amplification rate varied for all miRNAs and it reached a maximum value of 73% for miR-294; however, 100% amplification was not achieved in the present study. This is a considerable limitation that might have hindered any significant findings and that lowers the effectiveness of miRNA analysis as a non-invasive method for embryo assessment.

3.8 Conclusions

In summary, miRNAs are present in SCM and are detectable in minimum volume. Extra-cellular miRNAs reflect on pre-implantation development as it has been found for miR-124 and the developmental competence of embryos to complete blastulation. Additionally, embryo quality reflects on miRNA release since miR-294 in SCM is directly linked to the apoptotic percentage in mouse blastocysts.

Overall, this preliminary evidence shows that embryo developmental potential and DNA quality are associated with the extra-cellular levels of miRNAs. Since early morphokinetics and apoptotic extent at the blastocyst stage have effects on implantation and post-implantation outcomes, specific miRNAs could be quantified and serve as markers of embryonic potential.

Nevertheless, the nature of regulation and mechanisms involved need to be further explored to understand the biological role behind the release of miRNAs by early embryos. Additionally, it is necessary to carry out similar work in other mammalian species to understand the degree of conservation of those mechanisms. These are the research questions that are investigated further in the following chapters.

4. CHAPTER FOUR: MIR-294 MECHANISM OF RELEASE BY MOUSE BLASTOCYSTS

4.1 Introduction

Based on the finding that highly apoptotic embryos intensively release miR-294 it was decided to extend the research and study the nature of this association and the mechanism involved. Two possibilities exist in regards to the association between apoptosis and high extra-cellular levels of miR-294. On the one hand it is possible that miR-294 is the factor causing cell death in blastomeres and its presence in SCM can either be a result of passive release, for example due to membrane damage. On the other hand it could be that increased apoptosis is the triggering factor of intense release of this miRNA, meaning that poor quality embryos "intentionally" transcribe, package, and release miR-294 in the surrounding micro-environment. In this case miR-294 could serve secretory functions as a signalling molecule for communication with other cell types.

If miR-294 is the factor causing apoptosis then it is expected that the expression of this miRNA is higher inside the blastocyst cells. There it could up- or down-regulate genes involved in cell death pathways which would ultimately lead to the observed effect. As discussed in the previous chapter, miR-294 has hundreds of targets including genes of the Bcl-2 apoptotic pathway. Dysregulation of such genes could disrupt molecular networks with downstream effects on DNA fragmentation and cellular apoptosis. The abnormally high DNA fragmentation would quickly result in degradation of membranes and organelles and ultimately cell death. Molecules including small nucleic acids like miRNAs could then "leak" from the damaged membranes out to the surrounding micro-environment.

If apoptosis in blastomeres (regardless of the causal factor) triggers the expression and/or exocytosis of miR-294 then the secreted profile will not necessarily reflect the miRNA composition of the cells and moreover the gene targets of miR-294 will also not be dysregulated. In this case it is the cellular stress that activates miRNA secretion mechanisms which results in higher miRNA levels in the medium. Although such mechanism has not been described in embryos yet it is worth mentioning that stress signalling and miRNA release have been studied extensively in association with human diseases. It is now known that in body fluids there are distinct miRNA signature profiles relating to several types of cancer and also cardiovascular diseases (Mendell and Olson, 2012). miRNA export from cancerous cells is selective and regulated and the circulating miRNAs are protected from enzyme degradation due to their carriers (see Chapter 1). Specific miRNAs are then enclosed by other cells and exert their regulatory actions on mRNA targets. Hence these molecules are part of the inter-cellular communication network which is related to the onset and progress of various diseases (Boon and Vickers, 2013). Although the existing evidence favours the second possibility behind the miR-294 mechanism of release in this chapter, since there are no studies yet investigating the biological mechanisms behind embryo-released miRNAs these possibilities were further explored.

Initially, a review of the relevant literature was carried out to establish the experimental procedures. What was common in miRNA studies was the use miRNA mimics to cause the downregulation of *in silico* predicted targets in order to explain their findings. For example in the bovine study by Kropp and Khatib (2015) it was reported that degenerate embryos released higher levels of miR-24-3p compared to fully-formed blastocysts (amongst 11 other miRNAs). The miR-24 mimic was then supplemented in the culture medium of morulae and the embryos were further grown

to the blastocyst stage. Up-take of the mimic was verified in the grown blastocysts and it was found that addition of the mimic caused 27.3% decline in blastocyst formation. Further analysis found that one of the targets *CDKN1b* was significantly repressed by 33% in transfected embryos. This gene has important roles in cell cycle progression and thus affects cell proliferation and growth. Based on the above, the authors suggested that high expression of miR-24 possibly dysregulated *CDKN1b* and this in turn impaired the developmental potential of embryos and led to their degeneration (Kropp and Khatib, 2015).

Moreover, in the study by Lin *et al*. (2019) it was found that bovine embryos that completed the first cleavage later released more miR-30c in the culture medium. More specifically, "slow" embryos had significantly higher levels of miR-30c compared to "intermediate" embryos and hence this miRNA was further studied in relation to embryo development. The miR-30c mimic was supplemented to embryo culture and the embryos were cultured to the blastocyst stage. MiR-30c was up-taken by embryos and it caused higher apoptotic extent by 8% at the blastocyst stage. Additionally, this miRNA downregulated *CDK12*, a kinase which alters DNA damage response pathways. Based on these findings the authors suggested that elevated miR-30c possibly disrupts signalling and apoptosis pathways which impair the developmental potential and quality of embryos (Lin et al., 2019).

Therefore in both studies the embryos were transfected with the respective miRNAs and then the expression of gene targets was analysed. However, neither of these studies showed that in fact the levels of miRNAs inside the blastomeres are in concordance with the levels outside of them in the surrounding micro-environment. It is a common misconception to assume that miRNA levels in spent culture medium reflect on the intra-cellular content, i.e. the more miRNA you have in SCM the more

you have inside the blastocyst cells. If this was the case, using mimics is of course an acceptable approach, however no study has proven this yet. The next questions were therefore: Do embryos that release higher levels of a specific miRNA in SCM have increased levels of this miRNA inside their cells? Does the intra-cellular miRNA trigger apoptotic pathways? Or do highly apoptotic embryos actively release more miRNA extra-cellularly, which could then serve another function such as cell signalling?

4.2 Rationale and hypothesis

The hypothesis of the present experiment was that when apoptosis in early embryonic stages occurs, it triggers molecular pathways associated with miR-294 transcription, packaging, and release which results in higher levels of this miRNA in the extra-cellular environment. This hypothesis is based on previous knowledge that miRNAs are actively released by cells rather than non-selective passive release. Importantly, no lysis was observed in the preliminary experiments which further strengthens the suggestion that this is not a process of passive transport.

4.3 Aims and objectives

The aim of this experiment was to study the direction of the association between the extended apoptosis and the high levels of miR-294 in SCM of blastocysts. For this it was firstly investigated whether the intra-cellular levels of miR-294 correspond with the extra-cellular levels. In case there was a concordance between them it is likely this miRNA that causes apoptosis; thus miR-294 mimic transfection could be used to further study the molecular mechanisms involved. In the same context the expression of key-apoptotic targets predicted *in silico* was also analysed in relation to miR-294 SCM levels.

Furthermore, another experiment was carried out where DNA damage was artificially induced in mouse embryos and the SCM was collected at the end of culture and analysed for miR-294 levels. If cellular apoptosis triggers miR-294 release, then higher levels of this miRNA are expected in the study group compared with control embryos. In this case it would mean that apoptosis precedes miR-294 exocytosis, and it is in fact the process of apoptosis that triggers the packaging and release of miR-294 outside the embryonic cells.

4.4 Methodology

4.4.1 Intra-cellular miR-294 levels

Mouse embryos (B6C3F-1 x B6D2F-1) were thawed (*N=54*) and cultured in individual drops of pre-equilibrated Embryomax KSOM medium overlaid with EmbryoMax light mineral oil (Merck, UK) (Section 2.1). The embryos were cultured to the blastocyst stage at 37^{o}C in 5% CO_2. Spent media samples and blastocysts were then collected and stored in individual microtubes at -80^{o}C until further processing. miRNA extraction, amplification, and quantification were carried out in the SCM samples according to the protocols described in Sections 2.5.1, 2.7, and 2.9. The Ct values were normalised with the cel-miR-39-3p spike-in control recommended by the manufacturer (Qiagen, USA).

According to the miR-294 values the embryos were divided in 4 groups: null, low, medium, and high release groups. Embryos from each group were then individually processed for miRNA and total RNA extraction following the respective protocol (2.5.2). cDNA templates were built (2.7, 2.8) and the expression patterns of miR-294, *Bcl2* and *Apaf-1* were analysed using TaqMan™ assays and the PCR conditions described respectively in 2.9 and 2.10. Duplex reactions were carried out

using the *Rpl5* gene to normalise the Ct values. The cut-off Ct value of 35 was used to filter out fluorescence artefacts.

4.4.2 DNA damage induction

Preliminary experiments were carried out to optimise the conditions that would cause the level of DNA breakage for the purposes of this study. Mouse 1-cell stage embryos (B6C3F-1 x B6D2F-1) were thawed and cultured to the early blastocyst stage (~70 h post-thaw). The embryos were exposed to UV radiation by placing them on the benchtop UV Transilluminator (UVP, USA) for 5 s (wavelength 302 nm). A control group with no UV treatment was also cultured in parallel. The embryos were cultured for 16 more hours (in total 86 h post-thaw) to allow for transcriptional and translational responses to DNA damage. SCM samples were then collected and analysed for miR-294 expression. All blastocysts were collected and stained with TUNEL (2.2) to assess DNA breakage.

4.4.3 Statistical analysis

The SPSS Statistics 26 software (IBM, USA) was used for data analysis. Data distributions were checked using the Shapiro-Wilk test for normality and visual examination of the histograms. The ΔCt values were used for all statistical tests using the miRNA and RNA data. Log transformations were carried out where necessary to achieve normal distribution of the values. ANOVA and Tukey's post-hoc comparisons were performed to detect significant differences in miRNA and RNA expression between the null, low, medium, and high miRNA release groups in Experiment A. A t-test comparison was carried out to detect significance in miRNA levels between the UV-treated and control groups for Experiment B. A p value of <0.05 was considered significant for all tests. Graphs were generated using GraphPad PRISM 8.

4.5 Results

From the 54 SCM samples, 13 were null for miR-294 (76% amplification rate) and the average Ct from the rest of the samples was 27.9 ± 2.2 (ΔCt 8.9 ± 2.1). By calculating the Q1 and Q3 quartiles of the population, the blastocyst samples were categorised in null (Ct > 35, zero expression), low (ΔCt 11.6 ± 0.8), medium (ΔCt 9.1 ± 0.6), and high (ΔCt 6 ± 0.9) miR-294 groups, *N=10* for each group. The average intraceullular ΔCt was 3.2 for null, 3.1 for low, 2.7 for medium, and 4.8 for high release groups. After logarithmic normalisation, ANOVA detected significant difference in expression between the groups (*p<0.001*). Post-hoc comparisons showed that it was the high group that expressed significantly less miR-294 compared to the null (*p<0.01,* -3.1 FC), low (*p<0.001,* -3.1 FC), and medium (*p<0.001,* -4 FC) groups (Figure 4.1).

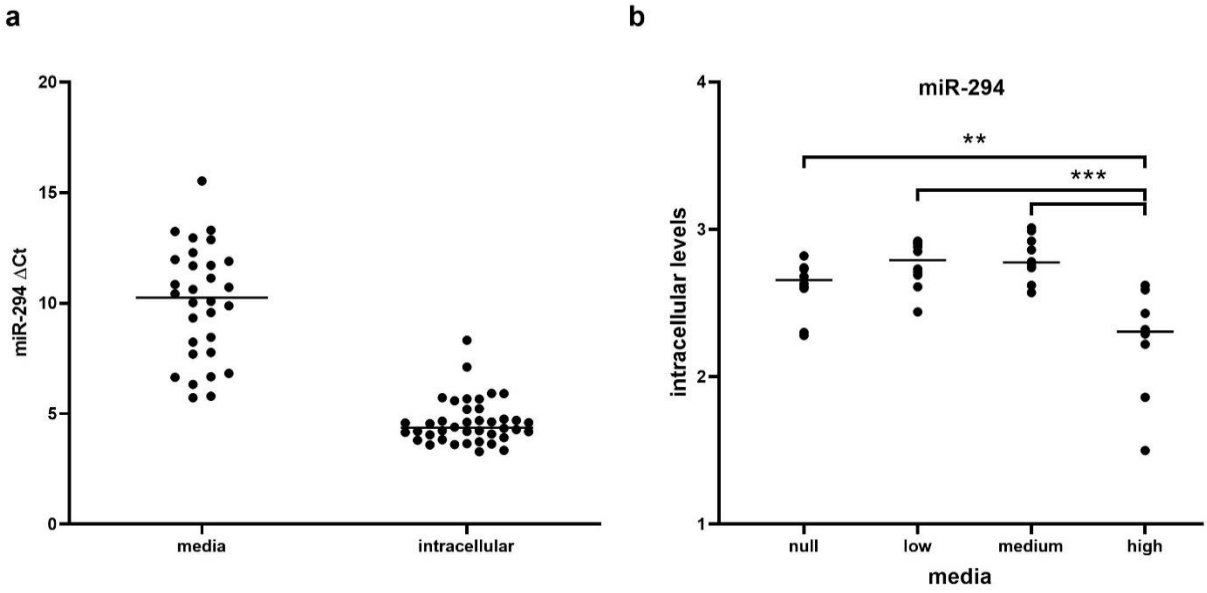

Figure 4.1 miR-294 expression in spent culture media samples and in blastocyst cells. Corresponding samples for SCM and cells are shown here for each embryo. Thus SCM samples are in total 30 (10 had zero expression of miR-294) and blastocyst samples are in total 40. The average miR-294 ΔCt was 10.0 for media samples and 4.6 for blastocyst cells. b) Comparison of miR-294 levels (log10 (x +10) values) between null, low, medium, and high miR-294/SCM groups. The boxplots contain the middle 50% of the values (Q3-Q1) and the median (horizontal line) and the whiskers depict the maximum and minimum values for each group. Significant differences in expression between the high and the null, low, medium groups are marked with asterisks.

Furthermore, *Bcl2* expression was null for all 40 blastocyst samples. However, *Apaf-1* was expressed in embryonic cells with an average Ct of 33.4 ± 0.6 (ΔCt 5.3 ± 0.6) in the null group, 33.7 ± 0.7 (ΔCt 5.4 ± 0.6) in the low group, 32.7 ± 0.7 (ΔCt 5.4 ± 0.5) in the medium, and 33.4 ± 0.7 (ΔCt 4.9 ± 0.4) in the high group. ANOVA testing showed that the expression of *Apaf-1* was similar between the groups (*p>0.05*) (Figure 4.2).

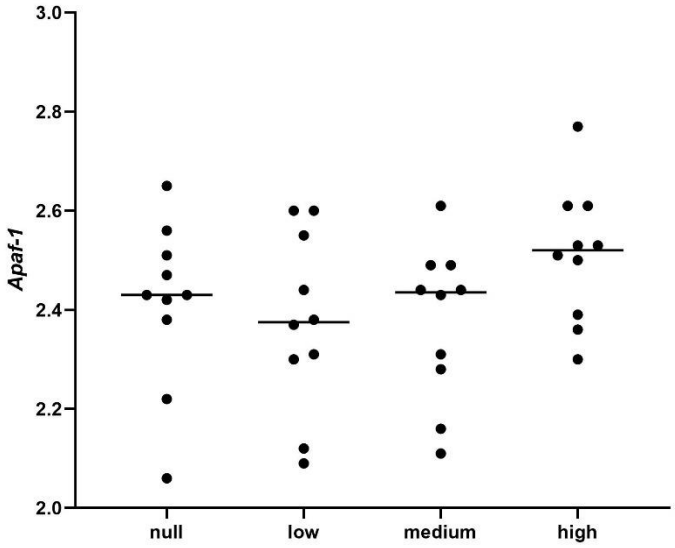

Figure 4.2 Comparison of intra-cellular *Apaf-1* expression between the null, low, medium, and high miR-294 release groups. The y axis contains the normalised Ct values for Apaf (log10 (Ct) values). Each group consists of *N=10* samples. The boxplots contain the middle 50% of the values (Q3-Q1) and the median (horizontal line) and the whiskers depict the maximum and minimum values for each group. Embryos from the 4 groups expressed similar levels of *Apaf-1* (*p>0.05*).

For the UV experiment, a total of 15 blastocysts were subjected to UV radiation (302 nm) for 5 s and 13 blastocysts were used as control of the study. Using TUNEL staining, the direct effects of UV on DNA damage was verified, with the UV group showing higher extent of apoptosis and reduced cell proliferation compared to the control group (Figure 4.3).

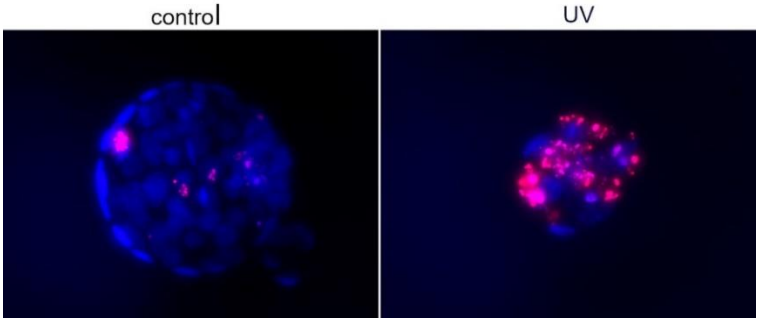

Figure 4.3 Induced DNA breakage using UV radiation. On the left image, a control blastocyst with few apoptotic cells and on the right image a UV-treated embryo with reduced TCN and many apoptotic cells. Embryos from both groups were cultured in parallel and for the same period.

The average miR-294 Ct in the SCM of the UV group was 25.1 ± 1.1 (ΔCt 6.3 ± 1) and in the control group 27.1 ± 1.5 (ΔCt 8.3 ± 1.5). The results from the t-test showed that the blastocysts with UV-induced DNA damage released significantly more miR-294 in the media ($p<0.01$) with a fold-change of 4 (Figure 4.4).

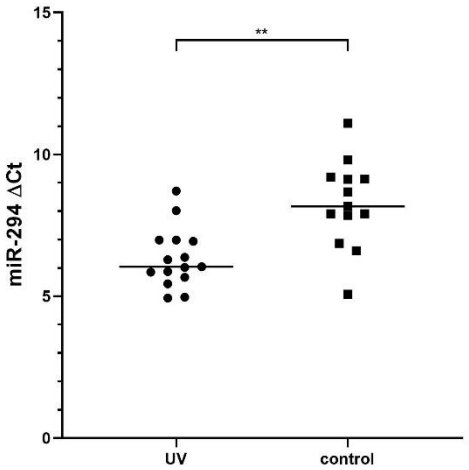

Figure 4.4 Average miR-294 ΔCt between the UV-treated and control embryos. The embryos treated with 5 s of UV (*N=15*) released significantly more miR-294 in the SCM compared to the control group (*N=13*) (*p<0.01*). T-bars show the standard deviation of the values.

4.6 Discussion

In agreement with the hypothesis, taken together the findings of this study indicate that embryonic apoptosis activates mechanisms of miR-294 release which results in higher levels of this miRNA in spent culture medium. Notably to date there is no published evidence regarding the mechanisms controlling miRNA release by embryos.

4.6.1 Extracellular miR-294 levels are negatively associated with the intracellular content

Unsurprisingly, the average miRNA levels were generally higher in blastocyst samples compared to culture media samples. However, when the embryos were divided in groups according to their SCM miRNA profiles it was found that embryos releasing more miR-294 in SCM had significantly less miR-294 in their cells compared to the null, low, and medium release groups. Additionally, the expression of *Bcl2* (a target of miR-294) was analysed in the same samples but was undetectable. Because of this, the apoptotic regulator *Apaf-1* which is involved in the same pathway as *Bcl2* was chosen as an alternative apoptotic marker. *Apaf-1* acts as a key-activator at the last regulatory level of the *Bcl2* apoptotic pathway through direct activation of caspases which then degrade cellular components (Bratton and Salvesen, 2010). *Apaf-1* was expressed in detectable levels in all the blastocysts, however its expression was similar across the groups. Considering these, no further conclusions could be made regarding the specific apoptotic pathway. Nevertheless, these results clearly suggest that miRNA levels in SCM, at least for the specific miRNA, are not positively correlated with cellular content. Therefore the first possible mechanism that this miRNA targets apoptotic genes causing cell death is not supported by these findings.

Only one embryo study has analysed the intracellular content for specific miRNAs and found corresponding intra- and extra- cellular profiles for miR-30c, miR-10b, miR-45, miR-113, and miR-139 in bovine blastocysts (Lin et al., 2019). This study is described in more detail in Chapter 3; however, in the context of the present experiment it is worth mentioning once more that this study grouped embryos and hence analysed miRNA profiles in pooled samples. In chapter 3 it was shown that there are individual embryos that do not release some miRNAs, i.e. expression rate is not 100% in a random study population. Therefore, by pooling samples it is probable to find false positive/negative associations. It is highly likely that the disagreement of the present results with those from the study by Lin and colleagues (2019) are due to the different study designs - individual versus pooled samples analysis.

Notably a study in stem cells showed that miRNAs packaging and release in microvesicles is a selective organised process rather than a random event (Collino et al., 2010). In this study miRNA profiles were compared between cell content and microvesicle cargo in human bone marrow stem cells and liver stem cells. Interestingly, miRNA expression profiles in microvesicles were shared with the patterns in their cells of origin for the majority of the miRNAs. Additionally, selected miRNAs were only detectable in circulating microvesicles and were absent within the cells (Collino et al., 2010). In the present experiment miR-294 expression in spent medium was negatively associated with cellular content. This finding in agreement with those of Collino and colleagues (2010) support the theory that miRNAs are specifically accumulated, packaged, and secreted by cells rather than them being leaked from damaged cells.

4.6.2 Induced cellular apoptosis triggers the release of miR-294 by mouse blastocysts

To study whether in fact apoptosis triggers miRNA release, UV radiation was used to artificially cause DNA damage in blastocysts. A similar methodology has been reported previously for UV-induced apoptosis in sea urchin embryos (Lesser et al., 2003). The embryos were subjected to UV radiation specifically when they reached the early blastocyst stage for two reasons. Firstly, it was important not to compromise embryo survival and blastocyst formation because it is mainly the trophectoderm population that actively releases miRNAs in the extra-cellular environment (Capalbo et al., 2016). Additionally, by UV-treating the embryos at the final stages of development, DNA repair mechanisms which are activated in the cells had minimum time to reverse the effect (Jaroudi and Sengupta, 2007). On the other hand, it was crucial for this study to maintain embryo viability for enough time to allow for transcriptional responses to DNA damage and, more specifically, cause enhanced transcription, packaging, and release of miR-294. In parallel, it was important to cause high damage in order to observe the maximum effect of the treatment. After some optimisation steps, it was found that UV radiation for 5 s at 302 nm caused direct DNA breakage in the blastocysts without instantly killing them. The increase in DNA breaks and cell apoptosis was verified using TUNEL staining and miR-294 in SCM was measured. In agreement with the hypothesis, the UV-treated embryos released significantly more miR-294 in the media compared to the control group.

Although the exact process that is triggered by DNA damage to release miRNAs is unknown, there is some evidence on the regulation of miRNA expression in DNA damage response. For example, DNA damage can regulate miRNA expression at the transcriptional level by the p53 damage-induced transcription factor that enhances the

transcription of miR-34. This miRNA then targets and downregulates cell cycle genes which leads to cell cycle arrest (He et al., 2007). Additionally, post-transcriptional processing of miRNAs is also regulated in the DNA damage response. Post-transcriptional upregulation of miRNAs including miR-16-1, miR-143, and miR-145 following DNA damage changes the expression of miRNA targets and decreases the rate of cell proliferation (Wan et al., 2011). Lastly, UV damage promotes miRNA expression which are then localised in stress granules and mediate gene silencing. This process is believed to be an integral part of the DNA damage response in cells (Pothof et al., 2009).

It is not believed that increased miRNA levels in media result from membrane fragmentation or cell lysis but rather it is a result of active release. In Chapters 3 and 4 cell lysis was recorded and no association was found with miRNA levels. Increasing evidence shows that miRNA release is regulated, rather than a result of cell injury, and these molecules have hormone-like properties causing autocrine, paracrine, and endocrine responses. Although the secretion mechanisms are less clear, intra-cellular stress and other pathways possibly induce miRNA release (O'Brien et al., 2018). Indeed, Ca^{2+} stimulates the release of miRNAs from neuroendocrine cells which then participate in cell-to-cell communication and function as neuromodulators (Gümürdü et al., 2017). Additionally, in prostate cancer cells a stress-induced protein complex (including Ago-1 and Ago-2) transports miRNAs to the nucleus to change gene expression in response to cellular stress (Castanotto et al., 2018). Considering the above and the findings presented so far, it appears that cells respond to stress through transporting miRNAs between cellular compartments and outside to the extra-cellular environment where these molecules exert functions in inter-cellular communication.

4.7 Limitations

The findings are limited to mouse blastocysts and the specific miRNA. Further validation is needed for assumptions for other species due to sequence differences in miRNAs between species. Specifically miR-294 belongs to the miR-290 cluster which is mouse-specific whereas the homologue cluster in human and bovine is the miR-370 cluster. Moreover, although it was aimed to establish the molecular mechanism behind miR-294 release unfortunately no conclusions could be made in regards to this. The evidence presented in this chapter provide information about the direction of association between apoptosis and miR-294 secretion by embryos without however establishing the molecular pathways that are involved. Possibly experiments on miRNA transporting proteins could elucidate the exact mechanism that is activated by apoptosis.

4.8 Conclusions

In summary apoptosis triggers the mechanism of miR-294 release by mouse blastocysts in the surrounding micro-environment. This conclusion is based on three findings presented so far in the thesis. Firstly, no lysis or signs of membrane damage were observed in embryos that released high miR-294 levels. Secondly, the spent media miR-294 levels were inversely associated with cellular content which eliminates the possibility that intra-cellular miR-294 targets apoptotic genes and promotes DNA fragmentation. Finally, induced apoptosis with UV radiation led to elevated levels of miR-294 in the spent media which proves that the direction of association is that apoptosis is the causal factor of high miR-294 release by embryos. Possibly this miRNA could be secreted for inter-cellular communication purposes which will be explored in a later chapter.

5. CHAPTER FIVE: EMBRYO-RELEASED MIRNAS IN RELATION TO BLASTOMERE APOPTOSIS IN BOVINE EMBRYOS

5.1 Introduction

The experiments presented so far showed that DNA damage in embryonic blastomeres increases the release of miR-294 by blastocysts in the extra-cellular environment. At the time of writing (January 2021) there are no publications regarding apoptosis-triggered mechanisms of miRNA release in pre-implantation embryos. Although these findings are original, one of the limitations throughout the experiments is that mouse embryos were studied as model animal organisms for implications in human embryology. Despite the many similarities between mouse and human embryo development there are also key-differences in molecular, cellular, and dynamic events between the two species (Cockburn and Rossant, 2010).

Generally, miRNA sequences are highly conserved throughout evolution and that is one of the characteristics that makes them ideal molecules for research. Nevertheless the miR-290-295 cluster studied in the previous chapters is mouse-specific and is one of the earliest clusters that is transcribed by the embryo, with rising expression levels as early as the zygotic genome activation at the 4-cell stage (Tang et al., 2007). This cluster has homologue miRNAs in humans and in specific the five miRNAs transcribed share active seed sequences with the miR-371-373 cluster (Wu et al., 2014). Therefore theoretically miRNAs miR-371, -372, and -373 are candidate matches for further experiments that are based on the mouse cluster miR-290-295.

Interestingly, the human miR-371-373 cluster is highly conserved in the Bos taurus species with a conservation score of 91% (Kiezun et al., 2012). The best characterised and annotated miRNA of this cluster is the bta-miR-371 which is transcribed by a sequence of 23 nucleotides. The only difference between human and bovine is found in the 13th base which in humans in a cytosine and in bovine a guanine (www.mirbase.org). Considering this high conservation in the sequence, the bovine embryo could be a candidate animal model to further investigate and ideally reproduce the findings between apoptosis and miRNA release in the mouse embryo.

Although the mouse model offers many advantages as a study organism in embryology, the potential of the bovine as a model organism must not be overlooked. Cattle embryos are used widely in studies due to the many similarities in biochemical and regulatory processes with the human. For example, polyadenylation of mRNA in oocyte maturation, glucose utilisation and amino acid metabolism in early embryos are more similar between human and bovine compared to murine embryos (Ménézo and Hérubel, 2002). Additionally, histone modification changes and the enrichment motif profiles at accessible chromatin sites established at the ZGA stage are remarkably similar between cattle and humans, even though the actual timing of ZGA occurs earlier in human embryos (Halstead et al., 2020). The reproductive physiology of cows is also close to the human especially in the follicular development stages and the fact that they are both mono-ovulatory species. Ultimately the choice of the animal model to study human reproduction depends on the process under study.

In regards to miRNA roles there is no firm evidence supporting the use of one species over the other for applications in the human. Interestingly, miRNA secretion by embryos has only been published for bovine embryos as described in detail in Chapter 1. This could be due to the higher genomic conservation in miRNA sequences

between bovine and human species. The study by Lin and colleagues (2019) is the only large-scale genomic study in secreted miRNAs by bovine embryos that found a minimum number of 294 miRNAs present in culture media after embryo culture. Two previous small-scale studies also found that bovine blastocysts secrete miRNAs in the surrounding media (Kropp et al., 2014, Gross et al., 2017b). Specifically for miR-371 there is no available information about its exact roles in bovine embryo development although it is known that blastocysts express miR-371 in their blastomeres suggesting thus functions at these stages (Goossens et al., 2013). Nevertheless, there is no evidence yet that bovine blastocysts actually release miR-371. This needs to be verified in the present study before any associations with embryo quality can be made.

5.2 Rationale and hypothesis

Initially it was hypothesised that bovine embryos release miR-371 in spent culture media. This was based on previous evidence on miR-371 expression inside blastocyst cells and reports that bovine embryos secrete miRNAs. Secondly, the hypothesis was that bovine embryos which have a high degree of DNA fragmentation in their blastomeres release significantly higher amounts of miR-371 in the surrounding micro-environment. This hypothesis was proposed on the initial findings in murine embryos and the existing knowledge that miRNAs are highly conserved molecules across evolution, not only in the genomic sequences but also in the actions that they serve. Although small, this experiment was designed to bridge the gap between the findings in mouse embryos and biological roles of miRNA secretion in human embryos.

5.3 Aims and objectives

The aim of the experiment presented here was to reproduce the findings of Chapter 3 and more specifically to investigate whether the association between extended apoptosis and miRNA release is maintained in other mammalian species. To study this, bovine embryos were produced *in vitro*, cultured to the blastocyst stage, stained for apoptosis detection, and the culture media samples were analysed for the presence of miR-371. The bovine model was used here to study miR-371 which belongs to the homologue cluster of the mouse-specific miR-290-295. This miRNA is the most annotated of the cluster in the bovine genome and carries a homology score of 91% with only one base replacement compared to the human genome. Definitely one of the advantages of the bovine model over the mouse in the presented thesis is the higher genomic homology in miRNAs with the human. Ultimately the goal of this experiment was to examine whether there is an association between apoptosis with this miRNA which is highly conserved to the human genome. If this is found then it would set a stronger basis for studying the biological roles behind embryo-released miRNAs, not only in model organisms but also in humans.

5.4 Methodology

5.4.1 Bovine *in vitro* oocyte maturation, fertilisation, and embryo culture

Bovine embryos were produced *in vitro* in two consecutive repetitions. All media were purchased by IVF Bioscience, UK unless otherwise stated. Ovaries were collected from the local abattoir and transported to the laboratory within 1 h in a thermos flask containing Dulbecco's PBS (Sigma-Aldrich, USA) at 37ºC. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2-10mm diameter) and oocytes with ≥3 layers of cumulus cells were collected for *in vitro* maturation (IVM). The COCs were washed three times in *Search* media (S199) in groups of 45 and cultured in 4-well dishes (Nunclon, Denmark) previously prepared with BO-IVM and equilibrated at 38.8ºC, 6% CO_2 for 24 h. Frozen semen from Holstein and Friesian bulls (Sexing Technologies, USA) was thawed at 38ºC for 10 seconds. Motility and concentration were assessed and the sperm was washed twice by centrifugation in 2ml of BO-SemenPrep, 200g for 5 minutes. The pellets were re-suspended in 350µl of BO-IVF and the motility and concentration were determined. The groups of matured oocytes were washed three times in oocyte wash medium and once in BO-IVF medium. The oocytes were transferred to 4-well dishes previously prepared with 400µl of BO-IVF, 38.8ºC, 6% CO_2. An aliquot of sperm suspension was added to each fertilisation well, calculated according to the semen concentration, giving a total concentration of $10x10^6$ spermatozoa/ml. The gametes were incubated overnight at 38.8ºC, 6% CO_2 in a humidified incubator. The presumptive zygotes were washed in BO-Wash, vortexed for 2 min in 1ml of BO-Wash, washed three times in BO-Wash and once in BO-IVC. The zygotes were then placed individually in wells of Embryoslides™ previously prepared with BO-IVC overlaid with BO-Oil and equilibrated at 38.8ºC and 6% CO_2, 6% O_2. Embryo culture was carried out in the

time-lapse incubator until the embryos reached the full blastocyst stage (~170hrs psi). Blastomere lysis was annotated in the time-lapse videos using EmbryoViewer (Vitrolife, Sweden).

5.4.2 Blastocyst staining

The embryos were cultured individually up to the full blastocyst stage, the blastocysts were stained with the triple staining protocol (2.2) and visualised with the appropriate filters with fluorescent imaging (2.3). Sex determination with H2AK119Ub staining was attempted due to the previously reported sexual dimorphism in miRNA secretion in the bovine (Gross et al., 2017b). The degree of apoptosis was calculated by dividing the number of apoptotic cells with the total cell count and expressed as a percentage.

5.4.3 Culture media analysis

From each culture well 20 μL culture media were collected and stored at -80^{o}C until further analysis. miRNAs were extracted using the same protocol as with the mouse experiment in Chapter 3 with the addition of cel-miR-39 spike-in control for normalisation of the Ct values (2.5.1). miRNAs miR-371 and miR-39 were quantified with single-tube real-time qPCR (2.7 and 2.9) with the respective miRNA probes detailed in Table 5.1.

Table 5.1 miRNA assays used for amplifying each miRNA. The target miRNA and the mature sequence are displayed.

TaqMan miRNA probes			
Target	**Assay name**	**MiRBase accession number**	**Mature miRNA sequence (5'-3')**
miR-371	bta-miR-371	MIMAT0009301	AAGUGCCGCCAUGUUUUGAGUGU
miR-39	cel-miR-39-3p	MIMAT0000010	UCACCGGGUGUAAAUCAGCUUG

5.4.4 Statistical analysis

All data were analysed using the SPSS Statistics 26 software (IBM, USA) and graphs were created with GraphPad PRISM 8. Data distributions were checked using the Shapiro-Wilk test for normality and visual examination of the histograms. Values greater than twice the standard deviation were considered outliers and excluded from further analysis. The normalised miRNA values were converted with the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008) and were tested against time-lapse parameters and apoptotic index with Spearman's or Pearson's correlation tests. A *p* value of <0.05 was considered significant for all tests.

5.5 Results

5.5.1 miR-371 expression in spent blastocyst media

After IVM of 72 bovine oocytes, 60 cleaved (83%), 46 formed morulae and developed into blastocysts (64%) by 170 h post-insemination. From the culture media collected 36 were successfully processed for miRNA extraction and amplification. miR-371 was present in detectable levels in all samples (100% amplification rate) and only one sample was assessed as an outlier and excluded. After normalisation with miR-39, the average ΔCt value for miR-371 was 6.2 (±1.8). Blank samples, namely water and culture media not exposed to embryo culture, were also run in parallel and showed no amplification for the miR-371. The average and standard error of mean of the raw Ct values are shown in Table 5.2.

Table 5.2 Average Ct value and standard error of mean for each miRNA in the spent media.

miRNA	Ct values
miR-371	26.5 (±1.4)
cel-miR-39	20.5 (±0.9)

5.5.2 Blastocyst staining

The methods that were successfully applied for embryo staining in mouse embryos were also used in this experiment. Unlike mouse embryos, bovine blastocysts did not show the characteristic Xi patches of FITC dye in their blastomeres and therefore it was impossible to assess embryonic sex through this method. Nevertheless, the TUNEL staining worked as expected and it was possible to calculate the apoptotic index in each embryo by fluorescent imaging (Figure 5.1). A total of 34 blastocysts were stained successfully and the average total cell number was 132 cells with an average apoptotic index of 5%, ranging from <1 to 16%.

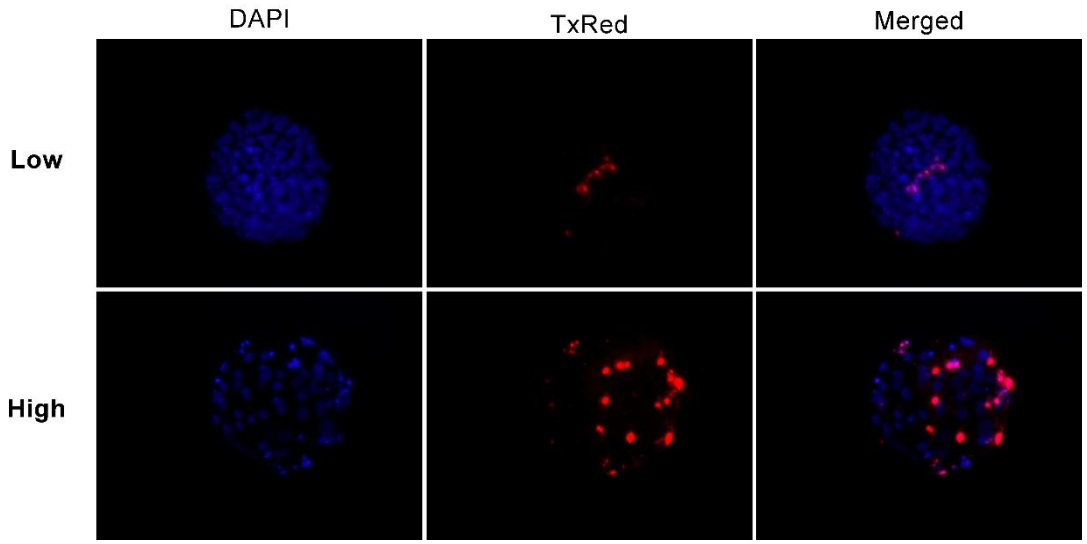

Figure 5.1 Bovine blastocysts with different apoptotic indexes (x40 magnification). DAPI nuclear dye was used to count the TCN and the TxRed dye specifically binds to broken DNA, indicating this way cell death. The apoptotic extent was calculated by dividing the apoptotic cells with the TCN (%). The embryo on the top row consists of 234 cells out of which 2 have fragmented DNA whereas the embryo below comprises 58 cells out of which 9 are apoptotic.

5.5.3 miR-371 and apoptosis

To exclude the possibility that miR-371 is leaked from damaged cells, the embryos were cultured in continuous monitoring equipment (EmbryoScope) and cell lysis at any stage of development was annotated. Blastomere lysis was observed in 6 blastocysts and the SCM samples deriving from these embryos were compared against the population average for miR-371 expression (t-test). The average ΔCt for the blastocysts with lysed cells was 6.8 ± 2.3 and for the rest of the population (*N=30*) 6 ± 1.7, which was not significantly different (*p>0.05*). Therefore, lysis was not considered a confounding factor further tests were not adjusted for lysis. Regarding the association of miR-371 in SCM and apoptosis, Pearson's correlation coefficient revealed a significant positive relationship between these variables (*r=0.555, p< 0.001, N=33*) (Figure 5.2). The power of the study was found 0.93 in the post-hoc analysis with GPower (Faul et al., 2009).

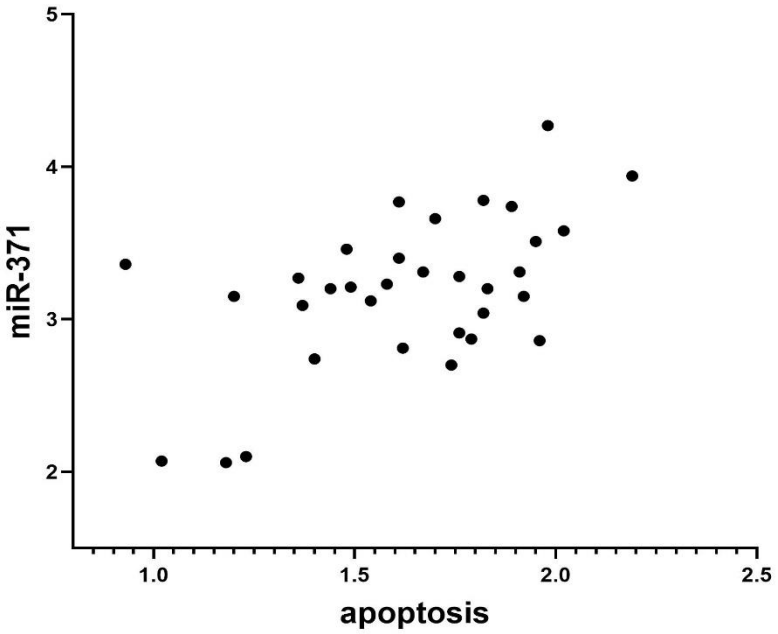

Figure 5.2 Scatter plot of miR-371 in relation to apoptosis. The variables are transformed using log10 (x*10) transformations to achieve a Gaussian distribution. The transformed values are used as inputs for the x and y axis. MiR-371 and apoptosis are significantly correlated (*N=33, p<0.001)* in a positive relationship (*r=0.555*).

5.6 Discussion

Although small this experiment aimed to show that, similarly to mouse embryos, bovine blastocysts that are highly apoptotic release specific miRNAs in the surrounding environment which can be detected and quantified. In line with the hypothesis it was found that bovine embryos secrete miR-371 in increasing levels relating to the extent of apoptosis inside their blastomeres.

5.6.1 Bovine blastocysts release miR-371 in culture media

Even though there are no reports about the specific miRNA, it was not surprising that miRNAs would be present in spent culture media samples. In fact the very first report in this topic by Kropp and colleagues (2014) used the bovine species to study embryo-secreted miRNAs. This study is described in detail in Chapter 1 and Appendix A; however the main implication was that miRNAs are secreted by embryos and they can be quantified with potential use as non-invasive biomarkers of embryo quality (Kropp et al., 2014). A more recent study in bovine embryos analysed culture media samples with the use of large-scale sequencing. The presence of at least 294 miRNAs, 180 of which were novel, was identified in spent media which implies that these molecules possibly have more important roles than previously thought at the peri-implantation period (Lin et al., 2019).

Another interesting finding in this experiment was the high amplification rate of miR-371 in culture media samples collected from each embryo. All of the samples had detectable levels of this miRNA which translates to 100% amplification rate. Not only this, but the Ct values were approximately 1 cycle earlier in bovine samples compared to the mouse which means that it is released in higher levels by bovine embryos. This could be due to the cellular number that comprises a blastocyst in

bovine, meaning that more cells secrete higher amounts of miRNAs thus resulting in elevated levels in the collected sample. Regardless, the finding that all samples had detectable levels of miR-371 and only one was considered an outlier suggests that the sensitivity of SCM miRNA analysis is higher than it was thought based on the mouse findings.

5.6.2 X chromosome inactivation is not identified through histone staining at the blastocyst stage in bovine

The triple staining protocol used in Chapter 3 was also carried out in bovine blastocysts to simultaneously identify embryonic sex and assess the apoptotic percentage. Although expression of the homologue cluster in mouse did not differ between male and female embryos, sexual dimorphic expression and release of molecules is known for bovine embryos. Male embryos release more extracellular vesicles containing transcription factors NFE2L2, superoxide dismutase 1, and NOTCH1 when exposed to oxidative stress compared to female embryos (Taqi et al., 2019). Evidence for miRNAs also suggests sexual dimorphism in miRNA secretion, with at least 8 miRNAs differentially released by male and female blastocysts (Gross et al., 2017b). Unfortunately, H2AK911Ub staining did not work in bovine blastocysts with the FITC dye being diffused inside the blastomeres in all embryos stained. Instead it was expected that in some of the embryos a nuclear patch of FITC would mark the inactivated condensed chromatin, characteristic of the X chromosome inactivation (XCI) in female embryos.

The reason behind this could be the diverse patterns of XCI initiation among mammalian embryos. XCI takes place at a later stage in bovine embryos, thus antibodies against histone modifications (like H2AK911Ub or H3K27me3) of the

inactivated chromosome do not bind to their targets. Indeed it is estimated that in bovine embryos XCI is partially achieved by the blastocyst stage (Bermejo-Alvarez et al., 2010). Although the *XIST* transcript whose actions lead to X chromosome-wide gene silencing is upregulated from the morula stage, its colocalisation with histone modifications at the X-inactivated chromosome is first detected in day 7 blastocysts (Yu et al., 2020). This is also seen in pig blastocysts (Zou et al., 2019), which further highlights the need for tailored protocols according to the species studied. It is also possible that molecular mechanisms of XCI differ between murine and bovine and thus the protocol used in this chapter was not efficient for the purpose of embryo sexing.

Despite the inability to assess embryonic sex it is not believed that miR-371 expression and secretion differs between male and female embryos. This assumption is based first and foremost on the mouse findings in which all embryos released similar levels of the homologue miR-290-295 cluster, as presented in Chapter 3. In addition, the miR-371 -373 is located on chromosome 18 in the *Bos taurus* species and not on a sex chromosome, which means it is more likely that this cluster is expressed similarly in both male and female embryos. Lastly, array-based studies in human embryos comparing a panel of 754 miRNAs found no differences in the expression or secretion of miR-371, -372, and -373 between male and female blastocysts (Rosenbluth et al., 2014, Rosenbluth et al., 2013).

5.6.3 Highly apoptotic bovine blastocysts release high levels of miR-371 into the surrounding media

This experiment was carried out for a very specific reason: to show that the correlation between apoptosis and the miR-290-295 cluster is conserved in another species with higher miRNA homology with the human genome. In agreement with the hypothesis, a significant positive correlation was found in bovine blastocysts. Embryos with more apoptotic cells released significantly higher levels of the homologue miR-371 in spent media. This miRNA was detectable in all samples meaning that it could serve as a marker of poor embryonic quality in a non-invasive manner.

There is very limited evidence on embryo-released miRNAs and their value as quality markers – the relevant studies are described in detail in Chapters 1 and 3. Briefly, Kropp and Khatib (2015) first showed that degenerate embryos release at least 11 miRNAs in higher levels compared to blastocysts. Moreover, the study by Lin and colleagues (2019) which also used bovine embryos found 2 miRNAs, miR-10b and miR-45, highly released by degenerate embryos. Notably, the two studies were not in agreement in regards to the miRNAs that could be used as biomarkers of quality – this could be due to different experimental protocols. However, the elevated levels of miRNAs in spent media from degenerate embryos could be a result of passive release from damaged membranes rather than an active, regulated process. Moreover it is important to point out that samples were pooled in both studies and analysed in groups which increases the risk of false positive/negative findings (Kropp and Khatib, 2015, Lin et al., 2019). In contrast with the above, in the present experiment cell lysis was annotated for each embryo and no correlation was found with the levels of miR-371

in spent media. Additionally, the experiments presented so far were all carried out with individual sample analysis in order to generate detailed data and avoid false findings.

The association behind increased apoptosis and intensive miR-371 release by embryos is difficult to interpret. Specifically for this miRNA in pre-implantation development, it is known that early bovine blastocysts express detectable levels of this miRNA in their blastomeres (Goossens et al., 2013) and human embryos release this miRNA, along with miR-372 and miR-373, in the surrounding media (Rosenbluth et al., 2014). No studies have assessed embryonic apoptosis in correlation to miRNA levels in spent media. The only study using TUNEL for apoptosis assessment in fact transfected embryos with a miRNA mimic (miR-30c) and then stained the embryos, concluding that increased levels inside the embryonic cells cause apoptosis (Lin et al., 2019). This approach is the opposite from the one presented here thus making the findings incomparable. Nevertheless, considering the positive correlation between apoptosis and miR-371 media levels, the lack of association between blastomere lysis and miRNA levels, and the findings of Chapters 3 and 4 it seems logical to suggest that release of miR-371 by bovine blastocysts is an active process and that apoptosis-triggered mechanisms are responsible for the extra-cellular release of this miRNA.

5.7 Limitations

The findings presented in this chapter are limited in bovine embryos produced with in vitro maturation protocols and further validation is needed for other species including human embryos. For reasons discussed in the previous section, embryo sexing was unsuccessful and thus, although unlikely, sexual dimorphism in miRNA secretion cannot be ruled out. Lastly, these results are specific for miR-371 although it is likely that other miRNAs are also packaged and released by cells in response to intra-cellular stress. Experiments using large-scale platforms with high sensitivity could provide more information on miRNA profiles in relation to embryo quality.

5.8 Conclusions

Bovine blastocysts release elevating levels of miR-371 in response to extended blastomere apoptosis. This association was consistent between chapters 3 and 5 despite key-differences. Not only were the species different, the mouse embryos came from inbred strains with genetic uniformity whereas the cattle were outbred from heterogenous populations with genetic variance and the embryo production protocols were different (hyperstimulation/IVF in mouse IVM/IVP in bovine). Thereby it is encouraging to observe the same mechanisms conserved in homologue miRNAs across different species. Because the results were reproducible it is also more likely that similar associations will be found in other species, with specific focus on human embryos and the homologue miR-371-373 cluster. Further experiments will possibly decipher the biological roles behind miRNA release at the peri-implantation period.

6. CHAPTER SIX: TRANSCRIPTOMIC RESPONSE OF HUMAN ENDOMETRIAL CELLS TO MIR-371

6.1 Introduction

Implantation of the developing embryo into the maternal endometrium is a highly coordinated process that is necessary for the establishment of a successful pregnancy. Implantation is achieved through the actions of a plethora of factors acting in a spatiotemporal fashion to control this process. Initially, the maternal endometrium undergoes major molecular and morphological changes during the so-called window of implantation (WOI). These changes are collectively known as decidualisation and are initiated by the increase of maternal steroid hormones, namely progesterone and oestrogen (Dunn et al., 2003). Decidualisation is initiated regardless of the presence of an embryo; however, molecules secreted by the growing blastocyst also guide this transformation (e.g. chorionic gonadotropin and interleukin-1β) throughout the invasion of the trophoblast to establish implantation (Massimiani et al., 2019).

As the blastocyst orientates towards the uterine epithelium, it interacts with cellular protrusions found on the apical surface of endometrial epithelial cells, known as pinopodes. This interaction results in adhesion of the blastocyst and is quickly followed by penetration and invasion of the cyncytiotrophoblast through the epithelial layer into the stromal cells (Davidson and Coward, 2016). The process of implantation is therefore a sequence of distinct events that occur shortly after decidualisation: apposition of the blastocyst to the implantation site, adhesion of trophoblast cells to the endometrial epithelium, and invasion of the cyncytiotrophoblast to the endometrial stroma.

Collectively these events form the embryo-maternal interaction which is necessary for the establishment of a successful pregnancy. Each stage is under the regulation of both maternally- and embryo-secreted factors, which are known as the embryo-maternal interactome. The molecular interactions at the uterine interface create a dynamic equilibrium of factors that promotes or inhibits implantation. For this, embryonic and uterine cells produce molecules and release them to the extra-cellular environment to exert their paracrine (or sometimes autocrine) actions. Therefore, the endometrial cells and the embryo also possess receptors of specific molecules which upon ligand binding activate a cascade of molecular pathways.

There is a variety of molecules involved in the embryo-maternal interactome, including adhesion molecules, signalling molecules, inflammatory response proteins, and growth factors. Initially, a molecular dialogue via chemokines acts for the orientation of the blastocyst at the site of adhesion. The embryo and the uterine cells produce chemokines and they also express chemokine receptors to enable this communication. Moreover, blastocyst adhesion is promoted by cadherins which are present in both trophoblast and epithelial cells and are essential for the attachment and robust adhesion of the embryo to the uterus. Insuline-like growth factors are involved in the communication between the embryo and the endometrium and regulate endometrial proliferation and decidualisation as well as trophoblast invasion. Additionally, members of the interleukin family are released by the endometrium and the embryo to intermediate the inflammatory response which is necessary for a healthy implantation (Mourik et al., 2009, Massimiani et al., 2019).

Although the actions of hormones, proteins, and growth factors at the embryo-maternal interactome have been studied in the past, there is very limited information to date regarding the role of miRNAs in implantation. Limited data (mainly from two

studies; one in humans the other in cattle) indicate that miRNAs have a role in embryo-maternal communication.

In the study of Cuman and colleagues (2015), high levels of miR-661 SCM were linked to implantation failure in humans. *In silico* predicted targets, namely *PVRL1*, *MTA*, and *EPHB2*, were identified as potential targets of miR-661 in endometrial epithelial cells (EECs) using qPCR. Indeed, the expression of these genes was altered by miR-661 and these authors reported that the adhesion process of implantation is affected by reduced levels of PVRL1 (Cuman et al., 2015).

In a later study, 68 miRNAs were differentially expressed according to embryonic sex in cattle. Gene regulation of the progesterone receptor (*PGR*) by three of these miRNAs was investigated by qPCR in endometrial cells. Both miR-122 and miR-320a altered the expression of *PGR*, and these authors suggested a sex-specific transcriptomic response of the uterine cells in the presence of a blastocyst. By dysregulating the progesterone receptor it was suggested that these miRNAs influence implantation, as progesterone is a key-hormone regulating the receptivity of the endometrium at the WOI (Gross et al., 2017b).

Although the findings of the above studies are encouraging, to date there is no information about miRNA gene targets in endometrial cells using large-scale platforms. In addition, there are currently no publications identifying specific embryo-secreted miRNAs in relation to DNA quality which in turn regulate uterine factors and the implantation of the embryo itself. This experiment was based on previous findings and aimed to provide complete information about miRNA roles in embryo-maternal communication.

6.2 Rationale and hypothesis

This experiment was carried out to investigate if embryo-released miRNAs have active roles in the implantation of the embryo. Indeed, it is logical to assume that miRNAs are part of the embryo-maternal interactome. Firstly because, as described in Chapter 1, miRNAs are released by mammalian embryos and studies reported associations of the presence/absence and/or levels of specific miRNAs with embryo quality and reproductive outcomes. Secondly, because miRNAs share similar characteristics with other cell signalling molecules, for example they regulate the expression of numerous genes, they are packaged into extra-cellular vehicles or are bound to proteins or lipids, and they are actively released by cells and travel within biological fluids to other parts of the body. Thirdly, because the actual process of miRNA packaging and release is energy-demanding, there must be therefore a biological reason behind their release by embryos. Indeed, for a biological system like the mammalian embryo, energy resources are limited and every process is under tight regulation until the embryo implants and the maternal nutritional support begins.

This experiment investigated whether there is a biological reason that miR-371 is released by apoptotic embryos. Based on previous findings with bovine embryos (Chapter 5) it was proposed that miR-371 impairs the implantation of such embryos, perhaps in order to save energy investment required for establishing a pregnancy with a low-survival embryo. Overall, it was hypothesised that miR-371a changes the transcriptome of endometrial cells which results in molecular and phenotypic changes that are consistent with a non-receptive endometrium and impaired implantation.

6.3 Aims and objectives

The aim of this experiment was to investigate whether there is a biological reason that poor quality embryos release specific miRNAs extra-cellularly. Based on the previous findings that extended apoptosis triggers the release of miR-294/371 in culture medium of mammalian embryos (Makri et al., 2020), the experiment presented here investigated the transcriptomic changes in endometrial cells caused by this specific miRNA. Specifically, a human endometrial cell line was chosen to study the actions of the specific miRNA in human cells, which could provide preliminary information for human implantation. For this, the miR-371a mimic was transfected in human endometrial stromal cells. The miR-371a sequence in the human genome corresponds to the miR-371 in bovine with only one base difference between the two species and these miRNAs are homologue to the mouse-specific miR-290-295 cluster studied in Chapter 3.

Endometrial stromal cells were chosen for transfection instead of epithelial cells or tissue for two reasons. Firstly, because stromal cells are involved in all stages of implantation except adhesion, which means that more information about the whole implantation process will come from studying these cells. Secondly tissue culture was avoided in order to study the direct actions of miR-371a in the absence of factors normally present *in vivo* that might hinder our ability to interpret such actions. Moreover, gene profiles were analysed with microarray technology followed by bioinformatic analysis. Large-scale analysis was chosen to analyse gene changes at the whole genome level. miRNAs are regulatory molecules and a single miRNA can have thousands of gene targets, which in turn interact with each other in complex networks. This is the reason why some *in silico* predicted gene targets are in fact not

altered in mimic experiments. Therefore, large-scale gene analysis followed by bioinformatics was carried out to study miR-371a actions in endometrial cells.

6.4 Methodology

6.4.1 Endometrial Stromal Cells Culture

Human endometrial stromal cells (CRL-4003) were purchased and cultivated in T-75 flasks according to manufacturer's recommendations (ATCC, USA). All medium components were purchased from Sigma-Aldrich (USA), unless otherwise stated. The cells were cultured in complete growth medium comprising a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 3.1 g/L glucose and 1 mM sodium pyruvate without phenol red supplemented with 1.5 g/L sodium bicarbonate, 1% ITS + Premix, 500 ng/ml puromycin, 90%; charcoal/dextran treated foetal bovine serum, 10%. The medium was renewed every 2-3 days and sub-culturing was carried out every 5-6 days at a ratio of 1:6. After 8 consecutive passages, the transfection procedure was carried out.

6.4.2 miRNA transfection

The Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher, UK) was used for delivery of the *mir*Vana hsa-miR-371a-3p mimic (Invitrogen, UK) in the endometrial cells. The *mir*Vana miR-1 Positive Control (Invitrogen, UK) was used to monitor the transfection efficiency, as recommended by the manufacturer. Optimisation was required to establish the following: cell confluency at the time of transfection, lipofectamine concentration, miRNA mimic concentration, and optimal transfection period. Additionally any deleterious effects of Lipofectamine, Opti-MEM, or the mimic (combined or separately) were investigated in preparation of the experiment and no apparent phenotypic changes were found in the cells. Based

on the proliferation speed and population growth, the optimal cell confluency for transfection in this study was strictly 25-30%. Firstly, the cells were seeded in 24-well plates in a concentration of approximately 20% and allowed to settle and attach overnight. The next day the plates were checked to assess the attachment and confluency and the growth medium was renewed with 450 µl of fresh medium. The miRNA-lipofectamine complexes were then formed in preparation for transfection. For this, the miRNA mimic and lipofectamine were separately diluted in Opti-MEM medium with reduced FBS (ThermoFisher, UK) and then mixed at a 1:1 ratio and incubated at 37^{o}C for 15-20 min. The optimal concentrations and volumes for 24-well plate transfections are found in Table 6.1.

Table 6.1 Steps and component concentrations for miRNA mimic transfection in human endometrial cells.

Steps	Component	24-well plate
Seed cells one day before transfection	Adherent cells	25-30% confluency
Dilute Lipofectamine RNAiMAX in Opti-MEM Medium	Opti-MEM Medium	25 µl
	Lipofectamine	1.5 µl
Dilute miRNA mimic in Opti-MEM Medium	Opti-MeM Medium	25 µl
	miRNA mimic (10 µM)	1.5 µl (15 pmol)
Mix diluted miRNA with diluted Lipofectamine (1:1 ratio)	Diluted Lipofectamine	25 µl
	Diluted miRNA mimic	25 µl
Incubate	Incubate 15-20mins, 37^{o}C	
Add miRNA-lipid complexes to cells	miRNA-lipid complex per well	50 µl
	Final miRNA/well	15 pmol (30 nM)
	Final Lipofectamine/well	1.5 µl
Culture cells	Incubate for 72hrs at 37^{o}C	

The miRNA-lipid complexes were then added in individual wells of the plate (50 µl/well – total volume = 500 µl, mimic concentration = 30 nM) and the cells were incubated at 37^{o}C for 72 h without changing the growth medium. Each plate contained miR-371a-3p-transfected wells, positive control wells (miR-1), and two types of negative controls (type 1: cells in 450 µl growth medium + 50 µl Opti-MEM, type 2: cells and growth medium, 500 µl). Transfections were carried out in 4 consecutive repetitions, in triplicate plates. For each repetition, a plate with ECs was run in parallel in which the cells were transfected with fluorescently-tagged transfection efficiency controls. Cells were transfected with 30nM of Cy3™ Dye-Labeled Pre-miR Negative Control, and 30nm of BLOCK-iT™ Fluorescent Oligo Alexa Fluor Red and FITC transfection efficiency controls (ThermoFisher, USA). Every 24 h the wells were examined under the microscope to assess cell survival and proliferation.

6.4.3 Cell collection, RNA extraction, RNA clean-up, cDNA libraries

Culture medium was removed from the wells and a mix of 1% β-mercaptoethanol in RLT buffer was added in each well (200 µl) and left for 1 min. The cells were mechanically disrupted using the pipet and the RLT mix containing the cells were moved in sterile tubes. To achieve enough RNA concentration for the microarray, 3 wells with the same culture conditions were combined in a single tube. The tubes were stored at -80^{o}C until extraction. RNA extraction was carried out using the RNeasy Mini Kit according to the protocol provided (Qiagen, USA) with a minor modification at the final step where 20 µl of H_2O were used for elution (2.6). An extra procedure for RNA clean-up was carried out due to the high guanidine salts: RNA concentration ratio which interferes with the amplification steps of the microarray protocol (Section 2.6.1). After RNA extraction and clean-up, the positive and negative

control samples were processed to produce cDNA libraries using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, USA) following the protocol provided in Section 2.8.

6.4.4 Transfection efficiency

The transfection efficiency was assessed by transfecting a positive miRNA mimic control under the same experimental conditions. Downregulation of the expected targets for the specific miRNA was then examined to assess the mimic delivery efficiency. As recommended by the manufacturer (Invitrogen, UK), miR-1 was delivered to stromal cells as described above and the expression of predicted target TWF1 was examined. The PCR plates were prepared for analysis using the following volumes per well: 5 µl TaqMan Universal PCR Master Mix (no AmpErase UNG), 0.5 µl TaqMan Gene Expression Assay (ThermoFisher, USA), 2.5 µl H_2O, and 2 µl cDNA product in triplicates for each sample. The assay for TWF1 was used for checking the transfection efficiency and 18S ribosomal RNA was used as a normaliser. The 96-well plates were run following the protocol described in Section 2.10. The amplification plots were visually examined and the TWF1 Ct values were normalised against 18S using the ΔCt method (2.11). The percentage of downregulation in the positive control samples compared to the negative controls was then calculated.

Transfection efficiency was also assessed qualitatively with fluorescent microscopy, by verifying the uptake of the Cy3-tagged pre-miR mimic negative control, which does not cause any identifiable molecular effects upon miRNA transfection, and the BLOCK-iT Alexa Fluor Red and FITC fluorescent controls that are specifically designed to monitor lipid-mediated transfection efficiency. After 72 hours of transfection, the supernatant was removed and 500 µl of 70% ethanol were

added in each well. The plate was kept in the fridge for 3-4 hours, the ethanol was removed and mounting medium containing DAPI (Vectashield, USA) was added until it covered the surface of each well. After 30 min, the cells were mechanically detached from the surface using rigorous pipetting and a small amount of PBS solution. The cells were placed in microscope slides for fluorescent microscopy. The settings described previously were adjusted for detecting the fluorescent signals with the appropriate filters for each dye (542/620 nm TRITC, 475/530 nm FITC, 559/630 TxRed, 350/470 DAPI).

6.4.5 Microarray analysis

Total RNA was isolated from endometrial cells using the RNeasy Mini Kit, as described above. The quality and integrity of the total RNA were evaluated on the Agilent-2100 Bioanalyzer system. Only samples surpassing the minimal quality threshold (RIN > 8.0) were used in the subsequent transcriptomic assessment. cDNA was prepared from 50 ng/µl of total RNA as per the GeneChip™ WT-PLUS Reagents (ThermoFisher Scientific/Affymetrix, UK) and followed by *in vitro* transcription to produce cRNA, end-labelled and hybridised for 16 h at 45^{o}C to Clariom™ S Human Arrays (ThermoFisher Scientific/Affymetrix, UK). Washing and staining steps were performed by a GeneChip Fluidics station 450 and scanning was done using ™ Scanner 3000 7G System (ThermoFisher Scientific/Affymetrix, UK) according to manufacturer's instructions at the Nottingham Arabidopsis Stock Centre (NASC), School of Biosciences, University of Nottingham. The raw CEL files were normalised using RMA background correction with quantile normalisation, log base 2 transformation with adjustment for GC content. Differentially expressed genes were considered significant when p-value was <0.05, false discovery rate, FDR ≤ 0.05 with Benjamini-Hochberg correction, and fold-change of >1.0 or < -1.0.

6.4.6 *In vitro* validation

For each sample that was analysed by microarray, an appropriate volume of RNA product was stored at -80^{o}C to be used for validation of the microarray results. cDNA templates were created and PCR was carried out as described above. The Ct values were normalised with 18S, the ΔCt values were used for group comparisons and the ΔΔCt values were used for calculating the fold changes between control and transfected samples (Schmittgen and Livak, 2008). T-tests were used for comparing the expression of each gene across the two groups. Fold change values from the microarray and PCR were input in a correlation test to determine the consistency between the two amplification methods. For all tests a p value of <0.05 was considered significant.

6.4.7 Gene Set Enrichment Analysis (GSEA)

The top gene list contained only 45 genes, thus it was not possible to run a robust enrichment analysis with this low number. After consultation with the bioinformatician, GSEA analysis of the genes that differed significantly ($p<0.05$) was carried out using WebGestalt 2019 version (Liao et al., 2019). Gene ontology (GO, Biological Process noRedundant) and pathway (KEGG) analyses were carried out using the following parameters: minimum number of genes for a category = 3, Significance level = TOP 10, Number of Permutations = 1000, Collapse Method = Mean. GSEA was also carried out using a second bioinformatics tool to ensure that no significant categories are missed due to access in online databases. Therefore, the gene list was analysed using Enrichr to generate GO and pathway lists (Chen et al., 2013), and the results are represented in graphs using combined information from both WebGestalt and Enrichr. Lastly, Mammalian Phenotype (MP) analysis was carried out to identify phenotypic changes relevant to implantation caused by miR-371a addition.

6.5 Results

6.5.1 Transfection efficiency

After optimisation, the protocol for miRNA transfection was established and the experiment was carried out. Endometrial cells were examined on a daily basis to assess cell survival and proliferation and also observe if any adverse effects caused by the treatment occurred (e.g. precipitation in the medium, abnormal cell morphology and clumping). As shown in the picture below, no such effects were apparent. However, macroscopically it was observed that cell confluency in the transfection wells differed from the control wells. More specifically, at 72 h the transfection wells were confluent by about 60-70% compared to >90% in the control wells.

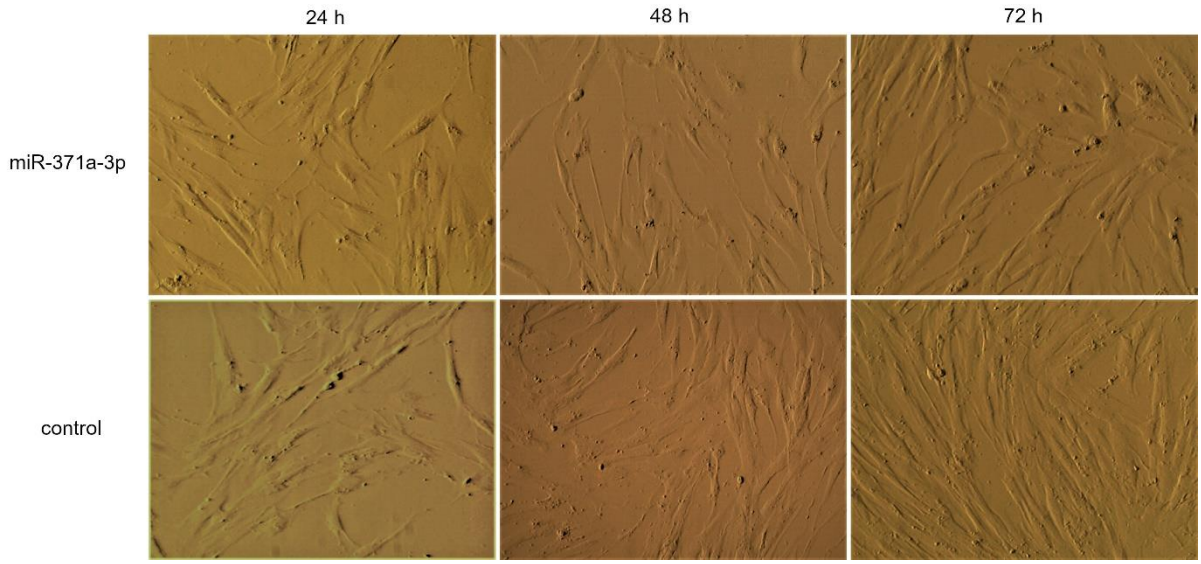

Figure 6.1 Human endometrial cells at 24, 48, and 72 hours (h) post-transfection. The images on the upper row are from wells that were transfected with 15 pmol of mimic and the images on the lower row are negative control wells with no added lipofectamine or mimic. The pictures were taken at a 10X magnification.

Furthermore, transfection efficiency was assessed by determining the downregulation of miR-1 target, TWF1, in the positive and negative control samples. According to the manufacturer, downregulation of the target for >75% is required for successful transfection experiments. From the 4 repetitions of the transfection experiment, repetition number 3 was the one where transfection efficiency was acceptable and therefore samples from this repetition were used for microarray analysis. All samples were positive and amplified at the same PCR cycle for the reference gene 18S, and more specifically the positive controls amplified at 14.1 cycles and the negative controls at 14.5 cycles. Moreover, the positive controls amplified for TWF1 at 30.1 cycles and the negative samples at 27.8 cycles. The ΔCt and $2^{-\Delta Ct}$ values were calculated and the percentage of downregulation of the target was determined using the following formula:

$$\%\ downregulation = \left(\frac{(positive\ control\ 2^{-\Delta Ct} - \text{negative control}\ 2^{-\Delta Ct})}{positive\ control\ 2^{-\Delta Ct}}\right) \text{x } 100$$

Target downregulation was verified in each cell culture plate. On average, TWF1 was downregulated by 82% in the miR-1 transfected wells compared to the negative controls (Figure 6.2).

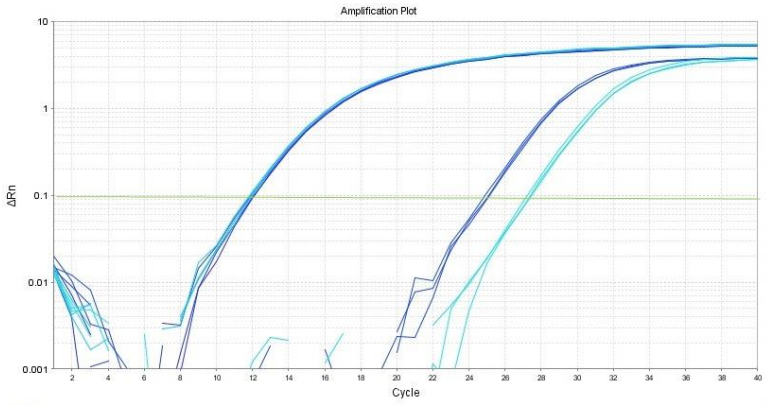

Figure 6.2 Amplification plot of the positive and negative controls. The x axis contains the number of PCR cycles and the y axis the log ΔRn values (ΔRn: reporter fluorescent signal/baseline). The threshold line is shown in green. The dark blue lines represent the amplification of the fluorescent signals from the negative control and the light blue lines are from the positive control (triplicates). All samples are amplifying for 18S (reference gene) at the same cycle (~14 cycles). For TWF1, the negative controls amplify earlier (~28 cycles) than the positive controls (~30cycles).

In agreement with the PCR results, fluorescent microscopy also verified the transfection efficiency. The Cy3, Alexa Fluor Red, and FITC signals were clearly visible inside the endometrial cells (Figure 6.3) and therefore samples from repetition 3 were sent for downstream analysis.

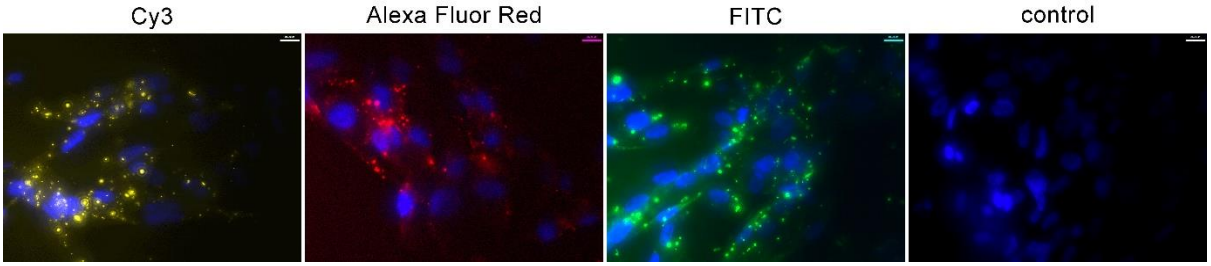

Figure 6.3 Endometrial cells observed with fluorescent microscopy (x40 magnification) with the appropriate filters for each dye. Transfection efficiency was verified by detecting fluorescence inside the cells. Scale bars (10.00 nm) are added on the top right corner of each picture.

6.5.2 Quality control

The RNA products achieved a concentration of >50 ng RNA/μL, with acceptable 260/280 and 260/230 purity values (all close to 2) when measured with NanoDrop™ 1000 Spectrophotometer (ThermoFisher, USA). All samples had RIN scores of 10 which allowed the use of the 3 mimic and 3 control samples for further analysis.

6.5.3 Partek analysis

The first step for interpreting the microarray results was to visually examine the principal components analysis (PCA) plot. A PCA plot containing 6 samples in total (3 transfected, 3 control) was generated (Figure 6.4). 3D concentration ellipsoids were fitted to the graph enabling the visualisation of the homo- and hetero-geneity of expression profiles within and between the groups. The two ellipsoids are clearly separated (not intermingled) and distanced in the graph, which means that there is a prominent difference in gene expression between the two groups. The dots within each

ellipsoid (samples within each group) are tightly concentrated which means that these samples have similar expression profiles and should therefore be included in the downstream analysis. Regarding the transfected samples, this is an additional verification that the transfection was consistent and equally efficient across the culture plates.

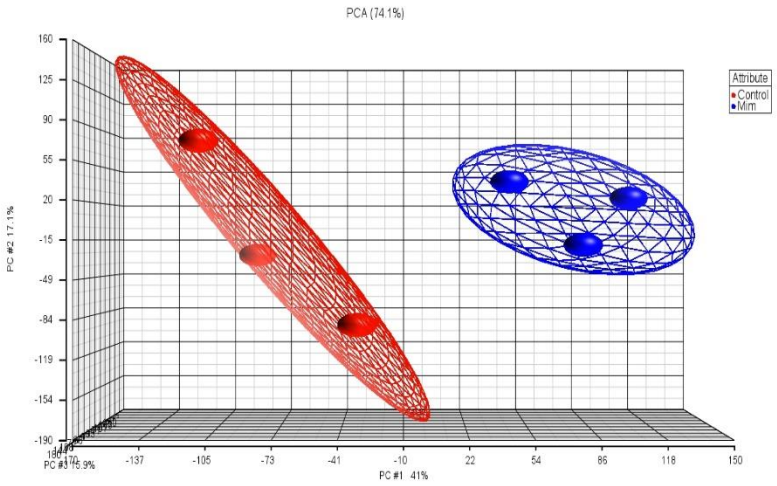

Figure 6.4 PCA plot of the samples analysed with microarray. Each sample is represented by a dot and each colour represents a group. The red dots and ellipsoid represent 3 control samples while the blue dots and ellipsoid represent miR-371a-3p mimic-transfected samples.

Moreover, a volcano plot was generated to identify the data points (genes) which display large magnitude changes that are also statistically significant (Figure 6.5). The volcano plot represents each gene with a dot and combines a measure of statistical significance from a statistical test, in this case a 3-way ANOVA between transfected and control samples, with the magnitude of the fold change. In the following figure, numerous genes are either significantly downregulated or significantly upregulated in the transfected samples compared to the controls. In particular 4.760 genes differed significantly ($p<0.05$) between the control and the transfected cells. The p values varied from <0.001 to ≤0.05 and the FC from -3.7 to 4 across the 4.760 genes. Furthermore, the top gene list was generated using Partek. In total 45 genes were differentially expressed between the groups with stricted criteria applied (Figure 6.6).

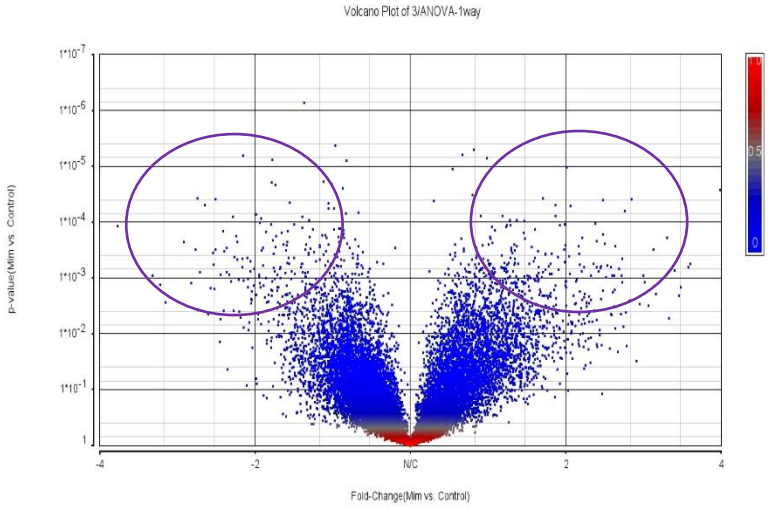

Figure 6.5 Volcano plot of 21.448 genes analysed with the microarray chip. The fold change (FC) is displayed on the x axis and the p-value calculated with 3-way ANOVA is given on the y axis. The calculations are given for the transfected samples compared to the controls (baseline). Each gene is represented with a dot. The genes that are further apart on the x axis and on the top of the y axis are the ones that are significantly differentially expressed between transfected and control samples. Manually drawn purple ellipsoids enclose candidate top differentially expressed genes.

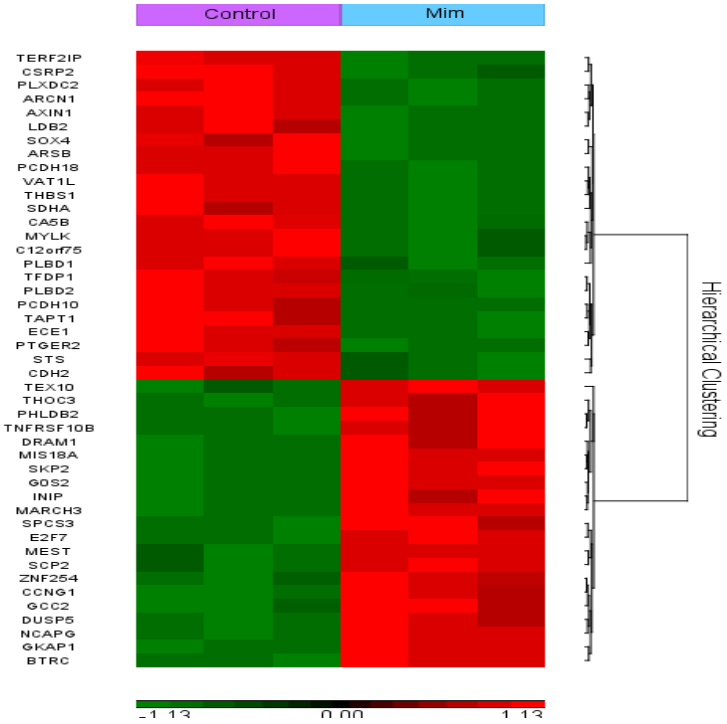

Figure 6.6 Heatmap of the top differentially expressed genes. Mimic and control samples are clustered in the 6 rows of the map. The gene names are given for every column. Over-expressed genes are displayed in read and under-expressed genes in green. The genes are clustered based on the FC values.

6.5.4 *In vitro* validation

Six genes of interest were chosen for *in vitro* validation based on the mechanisms involved and the fold change values. Specifically, downregulated genes *CDH2*, *PCDH10*, and *SOX4* and upregulated genes *E2F7*, *G0S2*, and *TNFRSF10* were examined in the same samples with PCR. The expression of all genes was significantly different between control and transfected groups ($p<0.001$ for all). Moreover, Pearson's correlation test revealed a statistically significant positive association of the fold change values generated by microarray and by PCR ($r=0.985$, $p<0.001$). These results show consistency across microarray and PCR for the specific genes which implies that the findings are accurate, reliable, and reproducible. Table 6.2 summarises the expression profiles and fold change values for the 6 genes as generated by Affymetrix and the validation *in vitro* by PCR.

Table 6.2 List of the selected genes for validation of the microarray results with PCR. The nature of regulation and fold change difference is given for the microarray in comparison to the PCR values. The p values from t-test comparisons for each gene between the two methods are also given.

Gene symbol	Discovery set by microarray	Validation *in vitro*	Fold change by Affymetrix	Fold change by RT-qPCR
CDH2	Down	Down	-1.78	-1.17
SOX4	Down	Down	-1.99	-1.34
PCDH10	Down	Down	-2.31	-1.65
TNFRSF10	Up	Up	2.28	3.75
G0S2	Up	Up	2.01	5.02
E2F7	Up	Up	2.36	5.30

6.5.5 Gene Set Enrichment Analysis (GSEA)

GO analysis was carried out for the 4.760 differentially expressed genes (noRedundant). Firstly, the genes were categorised based on their biological, cellular, and molecular functions. Figure 6.7, Figure 6.8, and Figure 6.9 are slim summaries of these analyses and contain combined results from WebGestalt and Enrichr.

Reproduction was one of the biological processes affected by miR-371a transfection with 356 affected genes in this category. Other affected biological processes which are likely involved in normal endometrial functions and implantation are: response to stimulus (2.124 genes), cell communication (1.501 genes), cell proliferation (552 genes), growth (268 genes), and DNA damage response (32 genes).

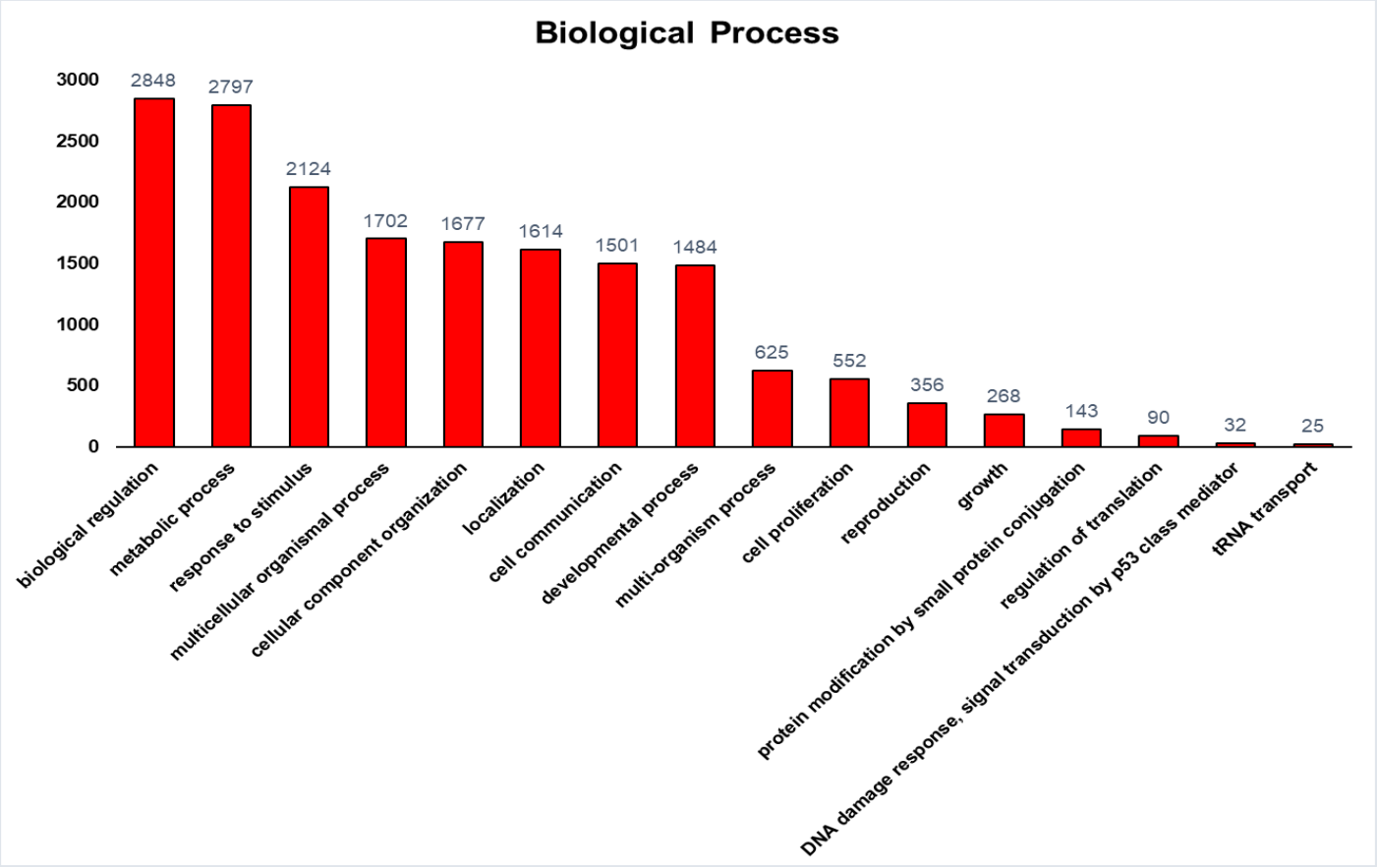

Figure 6.7 Gene ontology biological process summary for the differentially expressed genes. The height of the bar represents the number of genes in each process and the exact number of genes involved is given above each bar.

Moreover, the membrane was the anatomical cellular part which was mostly altered by miR-371a, with 2.112 gene alterations post-transfection. Other cellular components relevant to implantation were affected, including nucleus (1.915 genes), cytoskeleton (597 genes), and extracellular matrix (124 genes) changes.

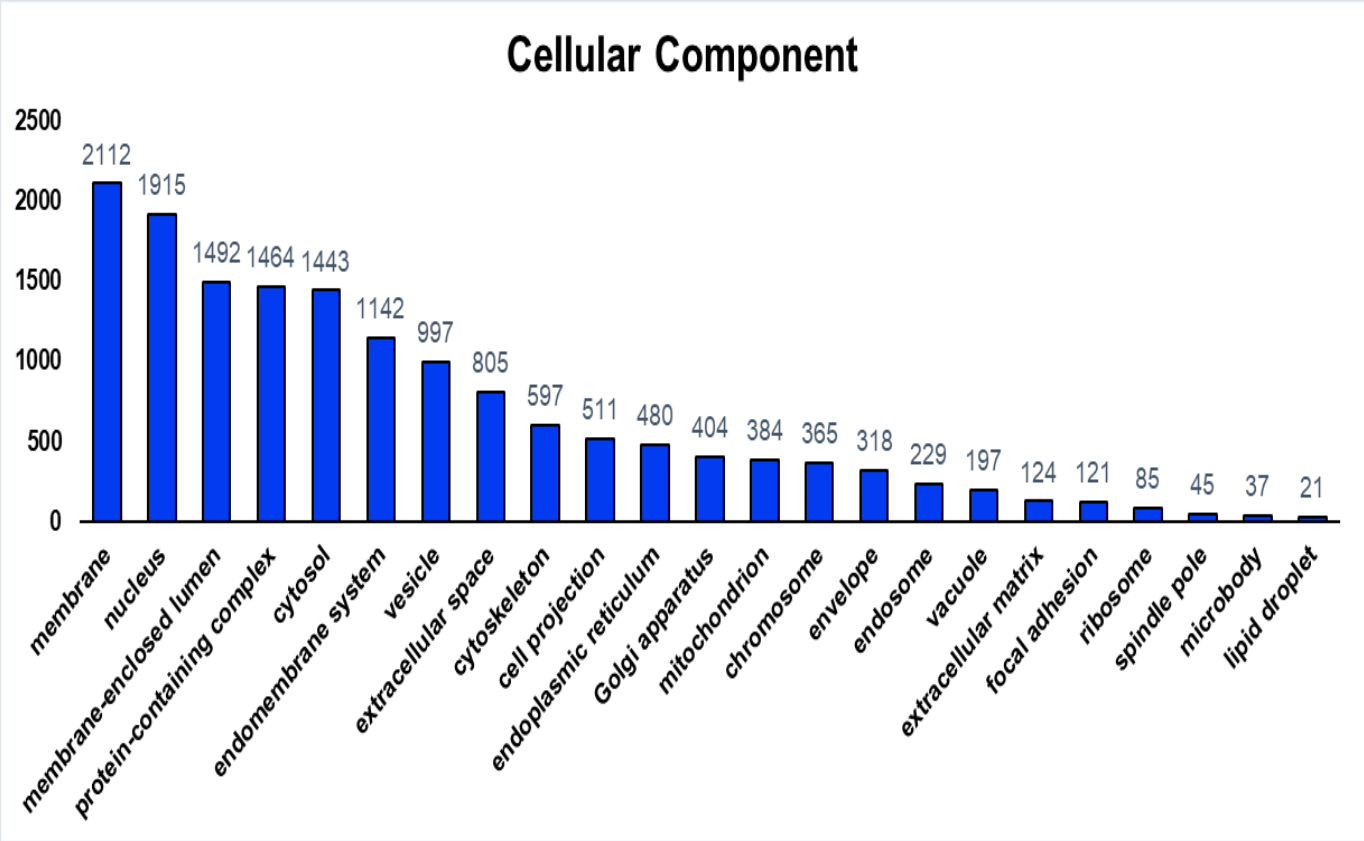

Figure 6.8 Gene ontology cellular component summary for the differentially expressed genes. The height of the bar represents the number of genes in each process and the exact number of genes involved is given above each bar.

The majority of molecular function changes were related to binding activities, with the mostly affected category being protein binding (2.967 genes) followed by other relevant categories like ion binding (1.513 genes) and nucleic acid binding (1.067 genes), lipid binding (174 genes), kinase binding (135 genes), and cadherin binding (111 genes). Enzyme activity changes were also prominent, with hydrolase (688 genes), transferase (620 genes), and ATPase (71 genes) activities significantly affected by miR-371a.

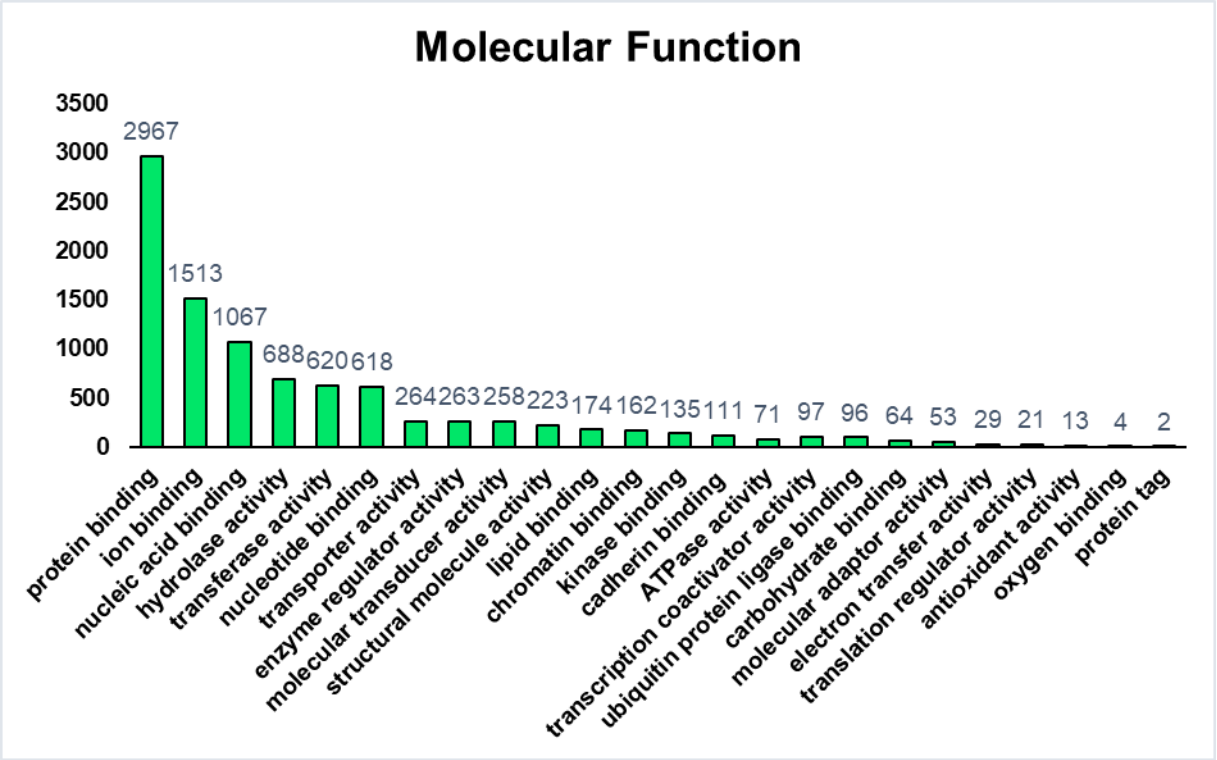

Figure 6.9 Gene ontology molecular function summary for the differentially expressed genes. The height of the bar represents the number of genes in each process and the exact number of genes involved is given above each bar.

From the original input list containing genes that differed significantly ($p<0.05$), 3.646 genes were annotated to selected functional categories by WebGestalt and were used for the gene set enrichment analysis. Based on the parameters set for analysis, 114 positive related and 45 negative related categories were identified as enriched categories. A bar chart was generated, in which the enriched biological processes were ranked using the normalised enrichment scores (NES) calculated by WebGestalt (Figure 6.10). In relation to normal endometrial cell functions, processes such as RNA catabolism (3.1 NES), negative cell cycle regulation (3 NES), apoptotic signalling (2.5 NES), proteolysis (2 NES), and cycle arrest (1.9 NES) were upregulated in endometrial cells whereas cell proliferation (-2.1 NES), cellular component maintenance (-2.1 NES), cell adhesion (-2 and -2.1 NES), immune and inflammatory responses (-2.6 and -2.5 NES) were downregulated after miR-371a addition.

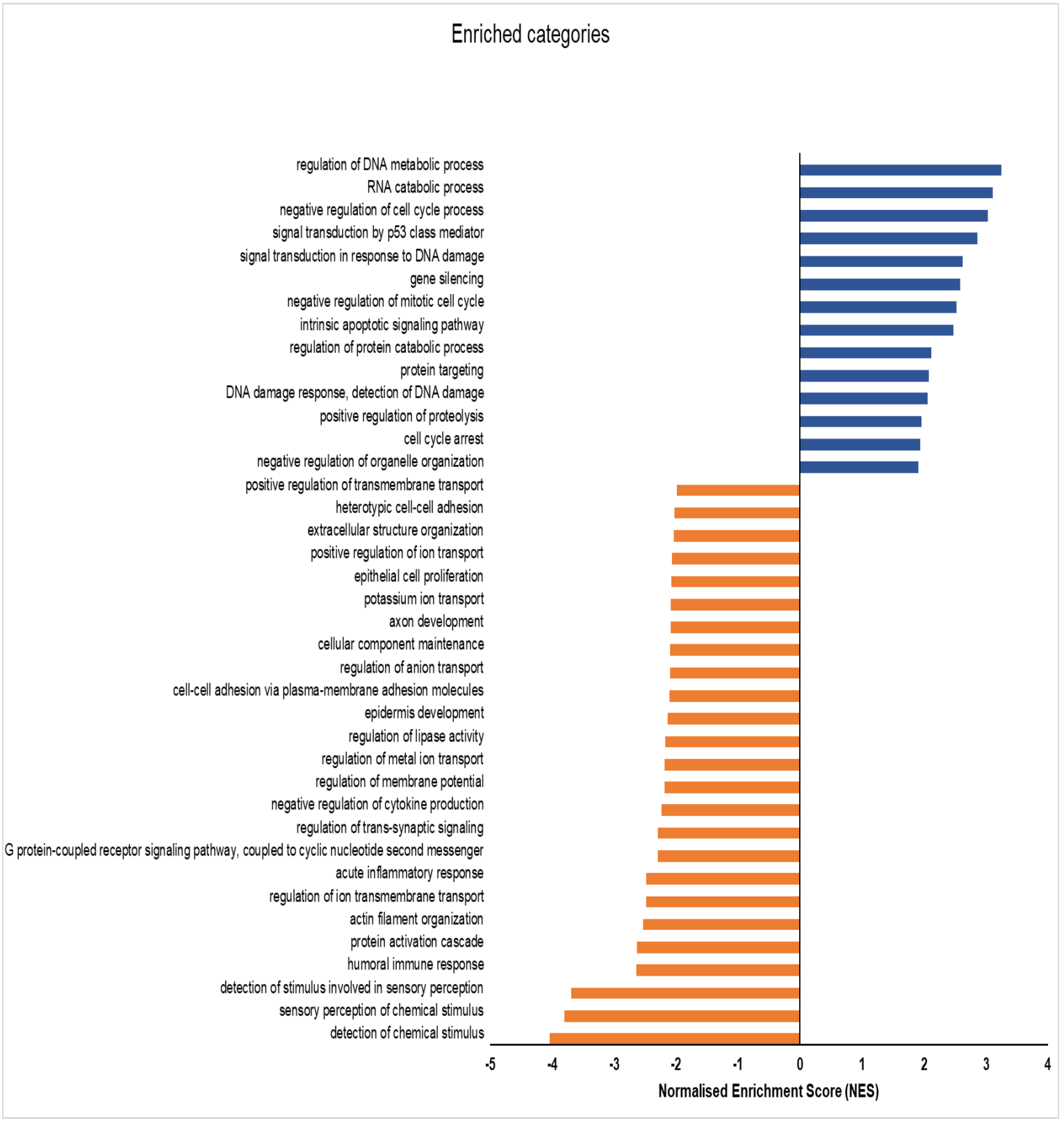

Figure 6.10 Bar chart of the GSEA. The x axis shows the normalised enrichment score given by WebGestalt. Blue bars represent biological process enhanced in the miRNA-transfected samples and yellow bars represent processes downregulated compared to the control samples. FDR was ≤0.05 for all categories. The figure above has been edited to contain processes relevant to the subject of the present experiment.

6.5.6 Pathway analysis

The Kyoto Encyclopaedia of Genes and Genomes (KEGG) 2019 was used as a database for pathway analysis. The differentially expressed genes were involved in 305 pathways in total including pathways relevant to endometrial functions such as signalling pathways (MAPK, chemokine, p53, Wnt, TNF, and oestrogen), cell cycle (54 genes), apoptosis (52 genes), cytokine interactions (58 genes), and cell adhesion molecules (36 genes) . A summary of the most relevant pathways is given in the figure below.

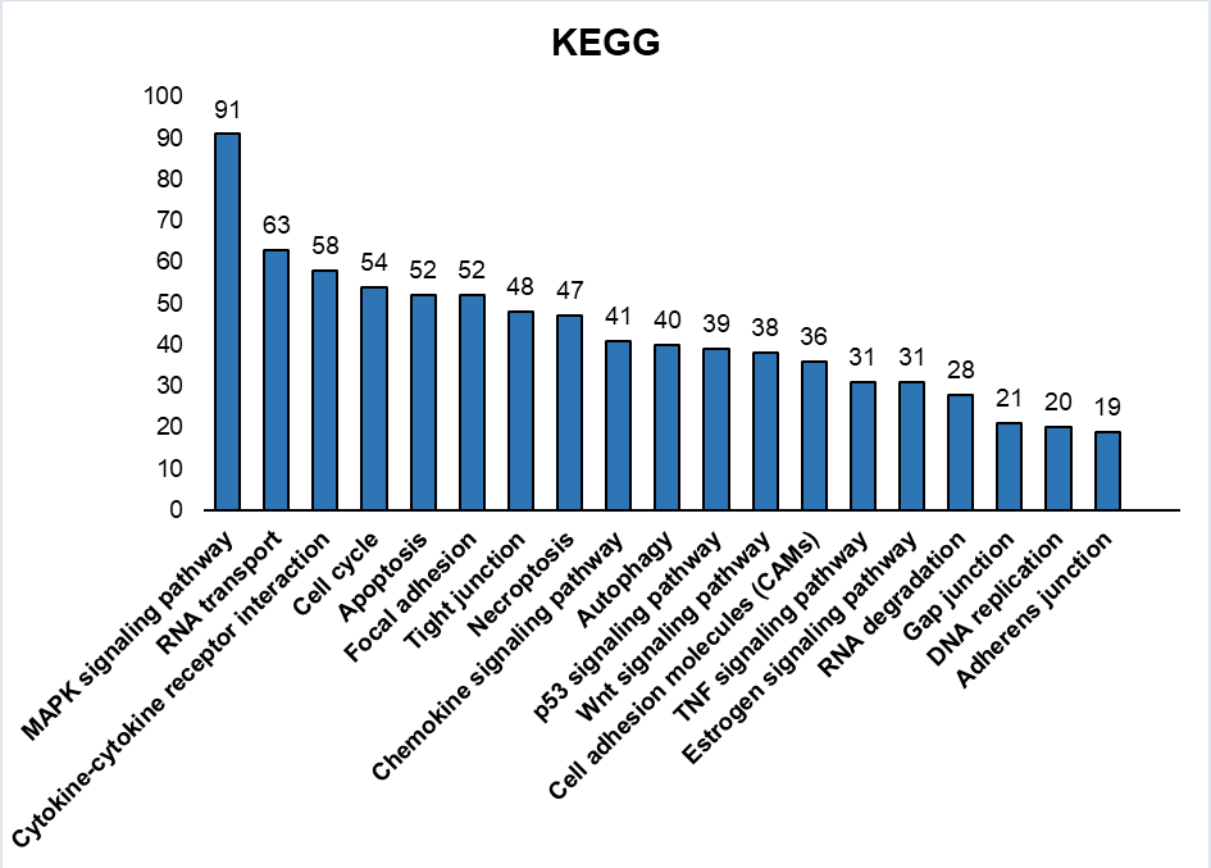

Figure 6.11 KEGG pathways in the miRNA-transfected cells. The height of the bar represents the number of genes in each process and the exact number of genes involved is also given above each bar.

6.5.7 Mammalian Phenotype

Input of the differentially expressed genes in MP ontology analysis showed that 4.818 phenotypes were affected by miR-371a transfection. From these, 13 phenotypes have direct effects in human implantation are described in Figure 6.12. The phenotype affected the most from miR-371a presence was the decreased endometrial cell proliferation (58 gene changes), followed by increased apoptosis (48 genes), failure of embryo implantation (7 genes), abnormal decidualisation (5 genes), and abnormal endometrium morphology (3 genes).

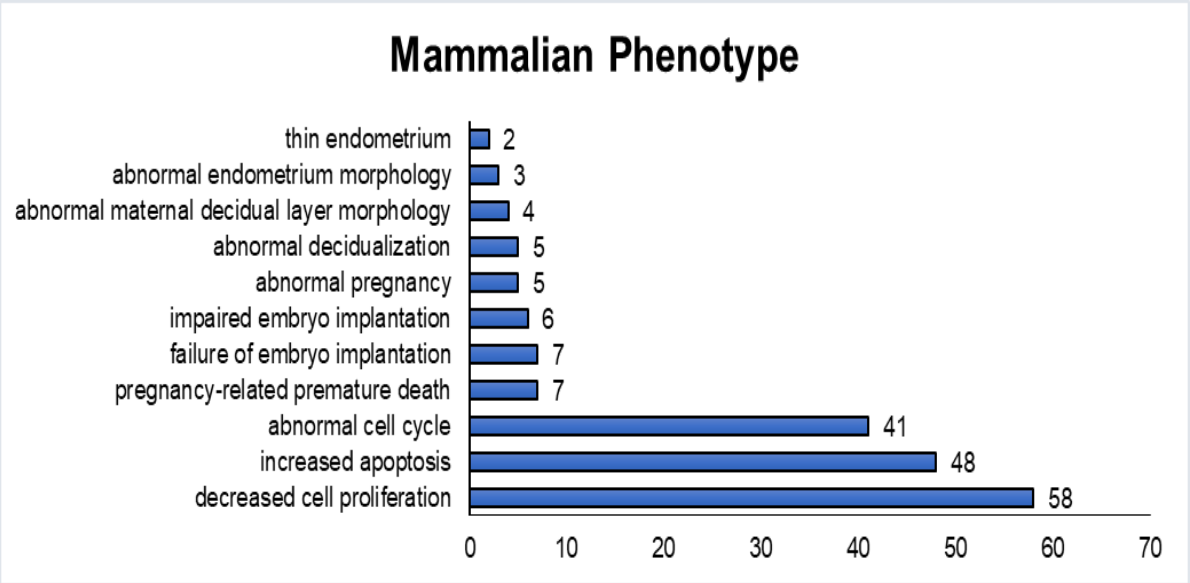

Figure 6.12 Mammalian phenotypes affected by miR-371a. The length of the bar represents the number of genes in each process and the exact number of genes involved is also given next to each bar.

6.5.8 Implantation-related genes

Furthermore, a thorough literature review discovered that numerous genes altered by miR-371a are involved in all processes of embryo implantation. More specifically, genes regulating decidualisation of the stromal cells, uterine cell communication, embryo orientation, adhesion, invasion, and establishment of the implantation site were dysregulated by miR-371a. The following tables 6.3-6.6 list the affected genes according to the process involved. The literature sources are given in discussion where gene regulation is described in detail.

Table 6.3 Decidualisation-related genes that were altered by miR-371a. The gene family and the gene symbols are given for each gene along with the nature of dysregulation caused by miR-371a.

Family	Genes	
	Upregulated	**Downregulated**
Prolactin	PRLR	PRLH
Progesterone		PGR
Vascular Endothelial Growth Factors (VEGF)		VEGFA;VEGFB
Cluster of Differentiation antigens (CD)		CD44
Insulin Growth Factors (IGF)	IGF2;IGFBP4	IGF1;IRS2
Vimentin		VIM
Snail		SNAI2
Catenins	CTNNB1	
Intermediate filaments	DES	

Table 6.4 Genes regulating the stroma-epithelium communication at the window of implantation. The gene family and the gene symbols are given for each gene along with the nature of dysregulation caused by miR-371a.

Family	Genes	
	Upregulated	**Downregulated**
Hand And Neural crest Derivatives (HAND)		HAND2
Progesterone		PGR
Fibroblast Growth Factors (FGF)	FGF2	FGF-1;-5;-7;-9;-17;-20
Insulin Growth Factors (IGF)		IGF1

Table 6.5 Genes controlling the adhesion phase of embryo implantation. The gene family and the gene symbols are given for each gene along with the nature of dysregulation caused by miR-371a.

Family	Genes	
	Upregulated	**Downregulated**
Cadherins (CDH, PCDH)	PCDH20	PCDH-7;-B8;-9;-B9;-10;-11X;-B11;-B14;-18;-19 CDH-2;-3;-4;-6;-16
Integrins (ITG)	ITGA3;ITGB3BP	ITGB5;ITGB1BP2;ITGBL1
Cell Adhesion Molecules (CAMs)		ICAM3;NCAM2
Connexins		GJB6
Catenins	CTNNB1	
Tight Junction (TJ)	TJP2;TJAP1	
Desmocollins		DSC2
Cluster of Differentiation antigens (CD)	CD166 (ALCAM)	CD44
Trophinin	TRO	
Galectins		LGALS7B;LGALS9C;LGALS9B
Proteases	ADAM-9;-17;-32;-TS1;-TS12	ADAM-7;-19;-22;-30;-TS7;-TS13;-TS19;-TSL4;-TSL5
Wingless/Int-1 (WNT)		WNT9B
SRY-related HMG-box (SOX)		SOX-2 ;-4 ;-5 ;-12;-18
Epidermal Growth Factors (EGF)	HBEGF;ERBB2IP	EGFL6;ERBB2
Fibroblast growth factors (FGF)	FGF2	FGF-1;-5;-7;-9;-17;-20
Progesterone receptor		PGR
Homeobox (HOX)	HOXC8	HOX-A2;-A11;-A-AS3;-C13;-D3;-D11
B-Cell Lymphoma (BCL)	BCL-2L1;-2L12;-11A;-AF1	BCL-7A;-2L14
Cyclooxygenase (COX)	COX-7C ;-7A2L ;-X17 SYNJ2BP-COX16	COX1;COX7A1
Neurogenic locus Notch Homologue (NOTCH)		NOTCH3
Thyroid Hormone	THRA;THRAP3	
Interferons (IFN)		IFNL1
Leukemia inhibitory factors (LIF)	LIFR	

Table 6.6 Genes involved in the invasion of the trophoblast to the uterine lining. The gene family and the gene symbols are given for each gene along with the nature of dysregulation caused by miR-371a.

Family	Genes	
	Upregulated	**Downregulated**
Integrins (ITG)	ITGA3;ITGB3BP	ITGB5;ITGB1BP2;ITGBL1
Interleukins (IL)	IL-1R1;-F2;-4R;-13RA1;-15	IL-20RA;-34;-16;-3RA;-31;-9R;-2RB;-10;-5;-33
Epidermal growth factors (EGF)		EGFL6;ERBB2
Cytokines/Chemokines		CXCL-6;-8;-12;-16,CLCF1,CCL-2;-13,LEP
Vascular Endothelial Growth Factors (VEGFs)		VEGFB
Insulin Growth Factors (IGFs)	IGF2BP2	IGF-1;-2;-BP3;-BP4;-BP5;-BP6;-BP7
Tumour Necrosis Factors (TNF)	TNF-RSF9;-RSF10A;-RSF10B; -SF15;-RSF10D;-RSF1B;C1QTNF6	TNF-RSF1A;-RSF13B;-RSF17;-SF12;-SF13;-SF12;C1QTNF7
Mucins (MUC)		MUC4;MUC16
Cell Adhesion Molecules (CAMs)		ICAM3
Prolactin	PRLR	PRLH
Dickkopf (Wnt signalling)		DKK1
Leukaemia inhibitory factors (LIF)	LIFR	

6.6 Discussion

The aim of this experiment was to investigate whether miR-371a causes molecular changes in human endometrial cells that could have an impact on implantation. Since this miRNA is released by both mouse and bovine embryos that have extended apoptosis in their blastomeres (Makri et al., 2020), it was hypothesised that miR-371a might act as a messenger molecule to interact with endometrial cells and inhibit implantation of the incoming embryo. The hypothesis was based on existing knowledge regarding the embryo-maternal communication via a plethora of molecules that regulate implantation in the mouse and human species (Massimiani et al., 2019). As described in detail in the introduction, limited information on miRNA actions on human and bovine endometrial cells were also used as preliminary evidence for the completion of the present study (Cuman et al., 2015, Gross et al., 2017b). In agreement with the hypothesis, the main findings of the present experiment are that miR-371a dysregulated the expression of genes in human endometrial cells and this dysregulation is overall consistent with impaired implantation of the embryo.

6.6.1 miR-371a is predicted to cause biological, cellular, and molecular changes in human endometrial cells

Biological processes that are generally involved in cell survival were predicted to be altered by miR-371a. These include metabolic processes, cell communication, and protein modification and translation. Reproduction-relevant processes in endometrial cells such as cell proliferation, response to stimulus, growth, and development were also predicted to be altered in transfected cells. Moreover, miR-371a was predicted to cause changes in a number of cellular components with the most enriched group being the membrane followed by changes in the nucleus, cytoskeleton, extracellular matrix, focal adhesion, ribosome, and spindle amongst others.

Additionally, many molecular processes were predicted to be affected by miR-371a with leading category the protein binding functions followed by cadherin, ion, nucleic acid, lipid, and chromatin binding and enzyme activities such as hydrolase, transferase, and ATPase and enzyme regulation.

Further analysis found numerous enriched categories in endometrial cells after miR-371a addition. Many of these have essential roles in implantation, such as the cell-cell adhesion, cell proliferation, cytokine production, immune and inflammatory responses, and detection of chemical stimulus (Mourik et al., 2009) and all of these processes were negatively enriched by miR-371a. Positively enriched processes included the negative regulation of cell cycle, cell cycle arrest, p53-mediated signalling pathway, apoptotic signalling pathway, response to DNA damage, and enhanced proteolysis. These are processes which are generally associated with impaired cellular functions and low survival.

KEGG analysis identified additional pathways predicted to be affected including cytokine signalling, RNA degradation, WNT signalling, and oestrogen signalling pathways. These pathways are important in all stages of implantation. For example, oestrogen signalling is necessary to prepare the endometrium to gain a receptive state (Young, 2013). Embryo-maternal communication via cytokine signalling is essential for the orientation of the blastocyst towards the site of implantation at the uterine epithelium (Dominguez et al., 2005). Moreover, WNT signalling activates factors in the trophoblast cells which promote invasion of the blastocyst cells into the endometrium (Chen et al., 2009). Taken together these findings reveal that while processes that are essential for implantation are predicted to be downregulated, other pathways that impair signalling and promote degradation and cell death are predicted to be enhanced by miR-371a.

6.6.2 miR-371a is predicted to induce phenotypic alterations in endometrial stromal cells consistent with impaired implantation

The MP analysis revealed 13 predicted phenotypic changes related to implantation that were caused by aberrant gene expression due to miR-371a addition. The most affected category was proliferation in which 58 genes were identified that cause decreased cellular proliferation. Stromal cell proliferation is essential during the initial stages of decidualisation when intense cell proliferation is followed by morphophysical changes of the stroma cells (Pan-Castillo et al., 2018). Indeed, decreased cell numbers and increased number of dead cells were observed under the microscope while this experiment was carried out (Figure 6.1). This is also in agreement with the second most affected phenotype predicted, increased apoptosis, which was a result of 48 genes being dysregulated. Another predicted altered phenotype was abnormal decidualisation of the stromal cells which has deleterious effects on embryo implantation as the uterine cells cannot adequately support the establishment of an embryo-maternal interface at the invasion part of implantation (Pan-Castillo et al., 2018). Overall, impaired implantation and failure of implantation were predicted for miR-371a regulation which is in agreement with the hypothesis of the present study.

6.6.3 Effects on stroma cell decidualisation

In vivo, endometrial stromal cells undergo a series of morphological, cellular, and molecular changes at the WOI, collectively known as decidualisation. Endometrial remodelling is a prerequisite for successful invasion of trophoblast cells, establishment of implantation, and normal growth of the embryo throughout pregnancy. Any disturbance in this process could impair endometrial receptivity, implantation, and pregnancy (Jeeyeon et al., 2012, Pan-Castillo et al., 2018).

Decidualisation is initially triggered by progesterone which exerts its actions via binding to the progesterone receptor (PGR). Upon binding the receptors undergo conformational changes and gain affinity for various transcription factors causing overall changes in gene transcription. In the present study the *PGR* gene was downregulated in stromal cells (by 1.9 FC). It is therefore possible that while the blastocyst invades through the epithelial layer, secreted miR-371a causes downregulation of *PGR* expression in the underlying stromal cells which could impair their cellular transition.

Another molecular pathway that could affect decidua competence to establish implantation is the Hyaluronan (HA) – CD44 interaction. Decidualised stromal cells (DSCs) continuously synthesise and secrete HA and express its receptor CD44 on their cell surface membranes. Upon HA binding to CD44, activation of PI3K and MAPK signalling pathways promote DSC proliferation while inhibiting apoptosis, both of which are thought to be beneficial for maintaining normal pregnancy. Indeed, human decidua coming from patients with miscarriages have significantly decreased *CD44* levels compared to normal pregnancy controls (Zhu et al., 2013). Interestingly, miR-371a significantly downregulated *CD44* expression by 1.1 FC in human stromal cells in the current experiment. Possibly increased miR-371a levels at the embryo attachment site could diminish the ability of stromal cells to respond to HA via reduction of CD44 binding sites on the cell surface membranes.

Maintenance and proliferation of DSCs are partly regulated by the insulin-like growth factor (IGF) network. The expression of these factors and their binding proteins alters upon endometrial differentiation. For example, *IGF-2*, *IGFBP-4*, and the insulin receptor *IRS-2* are highly expressed after decidualisation (Ganeff et al., 2009). Notably, supressed expression of *IGF-2*, *IGFBP-4*, and *IRS-2* was caused by

miR-371a (-1.1, -1.1, and -1.7 FC respectively). Dysregulation of this particular pathway at the site of implantation could have negative effects on the competence of DSCs to establish a healthy foetal-maternal interface.

6.6.4 Effects on stromal-epithelial communication

Equally important is the stromal-epithelial crosstalk which prepares the uterine cells to accept an invading embryo. Oestrogen and progesterone together with growth factors trigger and coordinate the stroma-epithelium communication. Dysregulation of mediators of the stroma-epithelial crosstalk often leads to pregnancy loss and infertility, highlighting thus the importance of inter-cellular communication for implantation (Hantak et al., 2014).

As in decidualisation, PGR plays a pivotal role in the luminal-stromal communication. Progesterone receptors are found in both epithelial and stromal cells; however, *PGR* expression is higher in the latter cell type at the WOI (Kurita et al., 1998). These receptors are transcription factors that are found in the cytoplasm in absence of progesterone. Upon binding, these receptors translocate to the nucleus where they bind to specific genomic sites to activate or repress the expression of genes. Additionally, PGR regulate gene expression via targeting other transcription factors (Hantak et al., 2014). With regards to epithelial-stromal communication, the stromal PGR mediates the inhibitory effect of progesterone on epithelial DNA synthesis and proliferation (Kurita et al., 1998). Moreover, lactoferrin and *Pgr* expression in mouse epithelial cells is also mediated via PGR in the stromal cells in response to progesterone (Kurita et al., 2000). Interestingly, in mice stromal PGR are necessary and sufficient to mediate the increase of Indian Hedgehog (IHH), a cell signalling protein, in epithelial cells whereas PGR in the epithelium play no role in *Ihh* induction

upon progesterone activation (Simon et al., 2009). Considering the above, it is probable that miR-371a affects PGR-mediated molecular pathways involved in stromal-epithelial communication. This could have deleterious effects in the healthy cooperation of the uterine cell populations to establish a pregnancy.

Stroma-derived growth factors also regulate epithelial functions. For instance, members of the fibroblastic growth factor (FGF) family are important for the activation of kinases in signalling cascades. *FGF* expression in human stroma cells is partly induced by elevating levels of oestrogen at the WOI and FGFs are then released and bind to FGF receptors at the surface of epithelial cells (Hantak et al., 2014). This binding activates extracellular signal-regulated kinases 1 and 2 (ERK1/2) signalling cascade in the cytoplasm of epithelial cells (Eswarakumar et al., 2005) that overall promotes epithelial proliferation (Li et al., 2011). Specifically, FGF-1, -2, -9, and -18 are produced in stromal cells and trigger signalling cascades in EECs which enhances epithelial proliferation (Li et al., 2011, Tsai et al., 2002). In the present experiment, the expression of seven *FGF* genes was altered by miR-371a addition, with *FGF-1*, *-5*, *-7*, *-9*, *-17*, *-20* being significantly downregulated and *FGF-2* upregulated.

IGF-1 is another factor which mediates the stromal-epithelial communication. In humans, elevating oestrogen enhances the transcription of *IGF-1* in stromal cells which is then released and binds to the respective receptor (IGF-1R) at the surface of epithelial cells (Hantak et al., 2014). Upon binding, it triggers the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signalling pathway which stimulates cell cycle progression in EECs acting thus as a paracrine factor to enhance epithelial proliferation (Zhu and Pollard, 2007, Walker et al., 2010). MiR-371a in the present study lowered *IGF-1* expression in stromal cells by 1.2 FC which could disturb the paracrine regulation of the luminal epithelium with effects on implantation.

A tight temporal interplay of oestrogen and progesterone regulates the uterine environment during pregnancy. While oestrogen enhances epithelial proliferation during the first two days of pregnancy, elevating levels of progesterone counteract and sustain proliferation on day 4 of pregnancy. A key-player of this exit from the cell cycle is the heart and neural crest derivatives-expressed protein 2 (HAND2). HAND2 is a progesterone-driven factor that is induced in stroma by day 3 of pregnancy while it is absent in the epithelium (Huyen and Bany, 2011). HAND2 directly inhibits the actions of FGFs which trigger signalling cascades that promote proliferation (Li et al, 2011). More specifically, the ERK1/2 pathway is directly affected by HAND2 which leads to cessation of proliferation (Li et al., 2011). Whereas *FGF* expression is necessary for the first days of implantation, their levels need to be sustained after day 3 to slow down epithelial proliferation and promote cellular differentiation. Indeed *Hand2* knockout mice show persistent epithelial proliferation with uteri failing to achieve a receptive state compared to controls (Li et al., 2011). Notably, miR-371a significantly decreased *HAND2* expression by 1.3 FC in stromal cells which could directly affect the FGF crosstalk with the uterine epithelium and impair implantation *in vivo*.

6.6.5 Effects on embryo attachment and adhesion

Although stromal cells were transfected in the present study, it is likely that similar gene expression changes are observed in endometrial epithelial cells; therefore, effects on the process of embryo adhesion are briefly discussed. The initial analysis showed that membrane structure was the cellular component mostly affected by miR-371a and moreover cell adhesion processes were downregulated. Additionally, key-genes involved in embryo attachment and adhesion were generally dysregulated by miR-371a.

As the embryo approaches the uterine wall, a level of polarity orientates the blastocyst in relation to the uterine epithelium. In humans, the ICM is directed at the site of attachment (Lee and Demayo, 2004) through a chemokine gradient (Dominguez et al., 2005). Chemokines and their receptors are found in both uterus and the blastocyst, suggesting a bi-directional communication via chemokines at this stage (Hannan et al., 2006). Interestingly, KEGG analysis found that the chemokine signalling pathway was significantly altered by miR-371a and 5 chemokines (*CCL-2*, *-13* and *CXCL-6*, *-12*, *-16*) were significantly downregulated. While the roles of these molecules in embryo attachment is not yet established, they all serve chemoattractant properties in human cells (www.uniprot.org) and are thus mentioned within this context.

Furthermore, cell adhesion molecules (CAMs) and junctional complexes at the epithelium are responsible for binding with other cells or with the extracellular matrix (ECM) during implantation. Cadherins (CDH) and protocadherins (PCDH) are transmembrane glycoproteins that form adherens junctions with the embryo (Singh and Aplin, 2009, Dey et al., 2004), whereas connexins form gap junctions between cells and create channels which allow the cytoplasmic exchange of molecules (Grummer and Winterhager, 2011). Furthermore, immunoglobulin CAMs (NCAMs, ICAMs, CCAMs) form non-classical junctions between cells (Singh and Aplin, 2009). ICAMs additionally act as ligands for integrins; transmembrane receptors that mediate cell-ECM adhesion and trigger signalling cascades for cell cycle regulation and cytoskeleton organisation (Kimber and Spanswick, 2000). Another important cell surface receptor is CD44, the receptor for hyaluronan (HA). The interaction between CD44 and HA is important for the initial docking of the embryo to the uterus (Berneau et al., 2019). Lastly, galectins (LGALS) are secreted by cells and act via cross-linking

carbohydrate chains on cells surfaces and the ECM, thus having functional roles in embryo adhesion (Aplin and Singh, 2008, Smalley and Ley, 2005, Shimizu et al., 2008). In the present experiment, miR-371a caused downregulation of 5 *CDH* genes (*CDH-2*, *-3*, *-4*, *-6*, *-16*) and 10 *PCDHs* (*PCDH-7*, *-B8*, *-9*, *-B9*, *-10*, *-B11*, *-11X*, *-B14*, *-18*, *-19*) and upregulation of *PCDH-20*. Moreover, KEGG analysis showed significant alteration of CAMs actions in endometrial cells and expression of *connexin-30* (*Cx-30*) and *ICAM-3* and *NCAM-2* were downregulated. Integrin family members *ITG-B5*, *-B1BP2*, *-L1* were downregulated and *ITG-A3* and *ITG-B3BP* upregulated. Furthermore, *CD44* and *LGALS-7B*, *-9C*, *9B* were downregulated.

Signalling molecules that mediate embryo adhesion were also dysregulated. The epidermal growth factor family (EGF) members mediate signalling with matrix metalloproteinases MMPs and ADAMs (Qiu et al., 2004, Gee and Knowlden, 2003), which are responsible for molecule assembly and degradation at the embryo-uterine interface to facilitate adhesion (Singh and Aplin, 2009). Additionally, EGFs interfere with the expression of the neurogenic locus notch proteins (Notch) (Massimiani et al., 2019) which mediate signalling networks involved in cell proliferation, invasion, adhesion, apoptosis, and differentiation (Bray, 2006). Lastly, EGF and WNT members form signalling cascades (Civenni et al., 2003) which are important for embryo orientation at the site of apposition (Goad et al., 2017). Regarding miR-371a regulation, signalling pathways were generally affected including MAPK and Wnt signalling. Moreover, *EGF-L6*, the EGF receptor *ERBB-2*, *ADAM-7*, *-19*, *-22*, *-30*, *-TSL4*, *-TSL5*, *-TS7*, *-TS13*, *-TS19*, *MMP-3*, *-12*, *-14*, *Notch-3*, and *Wnt-9B* were all significantly downregulated whereas *ADAM-9*, *-17*, *-32*, *-TS1*, *-TS12* were upregulated.

Lastly, increasing levels of progesterone at the WOI cause structural and molecular changes to the uterine epithelium in order to achieve a receptive state. In adhesion, progesterone upregulates the expression of MUC-1 at the apical surface of uterine epithelia (Meseguer et al., 2001) which acts as a ligand for selectins expressed by human blastocysts at the time of adhesion (Carson et al., 2006). Progesterone also controls the expression of cadherins in the luminal epithelium which are essential CAMs for embryo adhesion as described above (Jha et al., 2006). In the present experiment miR-371a significantly decreased the expression of the progesterone receptor *PGR* which could cause reduced response of the epithelial cells to progesterone stimulus.

6.6.6 Effects on trophoblast invasion, inflammatory response, and establishment of the implantation site

In humans, embryo invasion triggers an inflammatory response which needs to be controlled in order for the semi-allogeneic embryo to be accepted by the maternal endometrium. The balance between pro- and anti-inflammation is key to establish a healthy pregnancy. Immune cells are necessary to promote tolerance of the invading blastocyst and these cells in synergy with the endometrial cells produce cytokines and growth factors which are essential for the maternal acceptance of the embryo (Sharma et al., 2016, Mourik et al., 2009). In regards to this experiment, miR-371a generally impaired immune response and acute inflammatory response processes in endometrial cells.

Interleukins (ILs) are key-players of the inflammatory response in implantation. In human decidua IL-1, IL-6, and IL-11 form complex signalling networks with other cytokines and factors to regulate implantation (Dimitriadis et al., 2005). Knockout

experiments for these ILs show reduced implantation or complete implantation failure in mice (Sakurai et al., 2012). Interestingly, miR-371a changed the expression of eight ILs with upregulation of *IL-15* and downregulation of *IL-34*, *-33*, *-31*, *-16*, *-10*, *-8*, and *-5*. Although the roles of these interleukins are not yet clear in relation to implantation, this does not mean that these molecules have no functions. For example, IL-5, IL-8, and IL-10 are all secreted in high levels by decidualised cells (Sharma et al., 2016, Robb et al., 1998) and IL-5, IL-10 have anti-inflammatory roles promoting immunotolerance in other cell types (Hara et al., 2001, Tran et al., 2012). Additionally, IL-8 acts as a pro-inflammatory chemokine and also enhances angiogenesis (David et al., 2016). Downregulation of these interleukins might impair the ability of the maternal tissue to tolerate the invading embryo.

Chemokines control the migration and positioning of immune cells in tissues having as such pivotal roles in immune response. Several chemokines and chemokine receptors are expressed at the embryo-maternal interface to regulate the inflammatory response in implantation (Dimitriadis et al., 2005). In this study, miR-371a downregulated the expression of five chemokines, *CCL2*, *CCL13*, *CXCL6*, *CXCL12*, and *CXCL16*. Of these, it is known that *CXCL6* and *CXCL16* are expressed highly by human decidua (Red-Horse et al., 2001, Jones et al., 2004). Disturbance of the chemokine signalling network could affect the ability of the decidua to trigger an inflammatory response.

Another pro-inflammatory protein involved in implantation is leptin (LEP). Leptin is expressed in human endometrial stroma cells and exerts its actions mainly as a modulator of the expression of immune factors (Gonzalez et al., 2003). *In vitro*, LEP upregulates components of the IL-1 family (IL-1β, IL-1Ra, IL-1R1) (Gonzalez and Leavis, 2001, Gonzalez et al., 2004). In addition, LEP enhances the expression of the

leukaemia inhibitory factor (LIF) in stromal cells which is critical for the invasion of the trophoblast in the endometrium (Song et al., 2000, Robb et al., 2002). Low *LIF* expression in endometrium is associated with implantation failure in humans (Wu et al., 2013) and *Lif*-deficient mice are unable to establish implantation (Song et al., 2000). Leptin also enhances the production of integrin β3 (ITGB3) by endometrial cells, a transmembrane adhesion receptor that mediates cell-cell and cell-extracellular matrix adhesion and induces bidirectional signalling (Gonzalez and Leavis, 2001). ITGB3 is important for maintaining the physical interaction and cellular communication during the invasion of the trophoblast. Endometrial tissues from women with unexplained infertility have low *ITGB3* levels (Dorostghoal et al., 2017) and *Itgb3*-knockout mice show reduced implantation sites (Illera et al., 2000). Considering all, the decreased expression of *LEP* in this experiment could suggest an inhibitory role of miR-371a on implantation through disturbance of the trophoblast invasion.

Moreover, the IGF signalling networks are essential for establishing the immune response balance by stimulating and limiting inflammation. IGFBP-1 is expressed by stromal cells and has a major role in implantation, specifically through regulation of invasion via IGF-2 communication of the trophoblast cells (Fazleabas et al., 2004). The roles of other IGF family members in implantation is not clear yet; however it is likely that IGFs form complex molecular pathways mediating the embryo-maternal crosstalk (Mourik et al., 2009). With regards to miR-371a effects, the expression of IGF2BP-2 was upregulated whereas IGFBP-3, -4, -5, -6, -7 and IGF-1, -2 were significantly downregulated. Disruption of the IGF network could have effects on the ability of the cells to balance the immune response during invasion.

Lastly, a plethora of growth factors at the WOI are essential for the establishment of a nutrient supply system. Members of the vascular endothelial growth factor family (VEGF) possibly act at the last stages of trophoblast invasion to stimulate the formation of blood vessels (angiogenesis) which will establish placentation and support an ongoing pregnancy. Ligands and receptors of VEGF members are found in trophoblast and endometrial cells, suggesting a bidirectional embryo-maternal communication via VEGF signalling (Sharma et al., 2016, Matsui et al., 2004). Additionally, VEGFs are increasingly produced and released by endometrial cells during decidualisation (Sharma et al., 2016) and the expression is also influenced by the site of implantation which implies a specific role of VEGF regulation in implantation (Hayashi et al., 2019). The mode of *VEGF* regulation in implantation is not clear yet; although VEGFs promote vascular permeability and endothelial cell proliferation and migration through the extracellular matrix, they inhibit trophoblast cell invasiveness in *in vitro* experiments (Fitzpatrick et al., 2003). The existing literature suggests that VEGFs are anti-invasive factors that are however expressed in high levels to balance the pro-invasive actions of other molecules and establish an equilibrium for trophoblast invasion. Regarding the present experiment, *VEGFB* in stromal cells was significantly downregulated by miR-371a (-1.3 FC), which could disturb the balance of pro- and anti-invasive factors at the WOI with adverse effects on implantation.

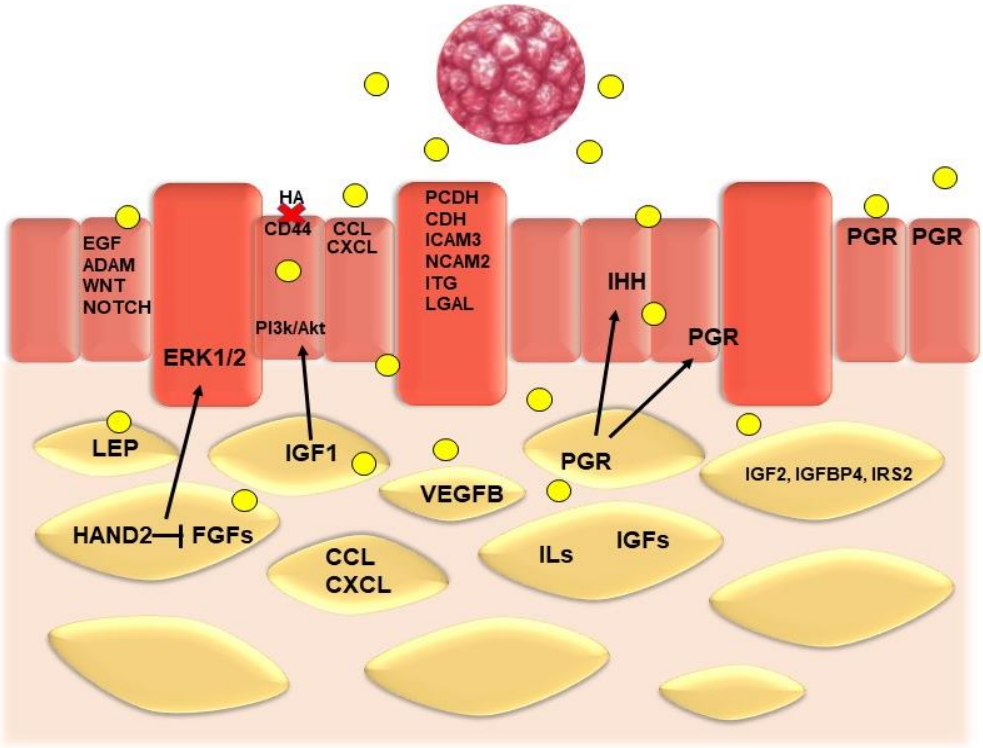

Figure 6.13 Summary of predicted genes altered by miR-371. A highly apoptotic embryo releases miR-371 which is uptaken by epithelial cells, possibly changing the expression of genes responsible for embryo attachment and adhesion. Levels of this miRNA could then reach the stromal layer and alter the expression of genes involved in stroma-epithelium communication, invasion of the trophoblast, inflammation response and establishment of the implantation site.

6.7 Limitations

These findings are limited to the actions of miR-371a in human endometrial stromal cells. Possibly other miRNAs released by embryos interact with proteins, transcription factors, and RNAs and form molecular networks which regulate implantation. Additional experiments using epithelial cells could confirm the predicted actions of miR-371a in embryo attachment and adhesion. In this sense, embryo adhesion assays could be performed in cells transfected with miR-371a to confirm the phenotypic changes in embryo adhesion. Moreover, co-cultures with endometrial cells and trophoblast cells could be carried out to observe any changes in trophoblast invasion. Experiments where endometrial tissue is transfected with this miRNA could provide evidence on the transcriptomic changes at the tissue level.

Finally, although literature suggests that miRNAs are taken up by the endometrium, this was not proven for the specific miRNA as transfection was carried out with chemical reagents. It is worth noting that experiments addressing these points were already under way before the labs shut down due to Covid-19 lockdown restrictions.

6.8 Conclusions

The findings strongly support the hypothesis that miR-371a changes the expression of genes in uterine cells with possible effects on implantation. The major transcriptomic changes suggest that miRNAs may have important roles as mediators of the embryo-maternal communication. This study has generated the first large-scale evidence of miRNA involvement in embryo-maternal communication. Although it is focused on miR-371a, other miRNAs likely exert similar actions and form complex networks with other molecules to control embryo implantation.

7. CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSIONS

The present thesis focused on the roles of microRNAs in the pre-implantation embryo. miRNAs are relatively newly-discovered RNAs with distinct characteristics that make them ideal molecules for study. Nevertheless, the actions of miRNAs in the early life stages are not yet clear which is why this topic was chosen. Initially a literature review was conducted to set the specific objectives of the thesis and establish the technical approach for the studies. Thereafter a series of experiments were carried out to test the hypotheses. Each consecutive experiment was designed based on the findings of the previous experiment in order to direct the research towards providing a in-depth analyses of the subject.

Although this thesis initially explored the potential of miRNA analysis for embryo assessment, the focus shifted towards developing our basic understanding of the role of miRNAs released by embryos during the peri-implantation period. Firstly, we investigated whether miRNAs released from individual embryos into the surrounding media can be detected and quantified. After some trials, protocols were optimised, and it was shown that this is possible. We then investigated whether the levels of miRNAs are indicative of the developmental competence and DNA quality of the embryos. Strong evidence for the latter was found for a specific miRNA. The mechanism of release of this miRNA was then investigated to understand the reason behind this phenomenon. Lastly, the effects of the specific miRNA on uterine cells were examined in order to elucidate whether microRNAs are part of the embryo-maternal interactome during the peri-implantation window.

The findings reported in this thesis are novel and form a strong basis for future research on mammalian embryo development and pregnancy establishment. The main findings and conclusions are listed below:

1. Mouse embryos release microRNAs into the surrounding micro-environment. Thus the mouse could be a useful as a model organism for relevant studies.
2. MicroRNAs in culture media of single-cultured embryos are detectable and quantifiable using current PCR technology.
3. The levels of specific miRNAs in spent media are informative of the embryonic competence. Evidence presented in Chapter 3 linked miR-124 levels with blastulation duration, although these results were overall considered weak.
4. Specific miRNAs could be used as biomarkers of DNA quality. As presented in Chapter 3, the levels of miR-294 are directly correlated with the extent of apoptosis in the embryonic blastomeres of mouse embryos.
5. The release of miRNAs by embryos is likely an active process which is triggered as a downstream cellular response to a stimulus. It was shown in Chapter 4 that extended apoptosis triggers the release of miR-294 by mouse embryos and passive release was rejected as a mechanism for this specific miRNA.
6. Bovine embryos also release miRNAs from the homologue cluster in response to a high apoptotic index. The same association is conserved across different mammalian species and it is possible that it is maintained in human embryos due to the high homology of miRNA sequences in bovine and human; although this remains to be determined.
7. MicroRNAs in the uterine environment change the endometrial transcriptome and can promote or inhibit embryo implantation. Gene expression changes can be significant due to the numerous targets of miRNAs.
8. In keeping with other molecules, miRNAs are part of the embryo-maternal interactome and form complex regulatory networks to control implantation-related processes. miRNAs exert secretory and inter-cellular communication actions at the peri-implantation period.

The original findings that mouse and bovine embryos release higher levels of specific miRNAs in direct correlation with the extent of DNA fragmentation in their blastomeres can form a strong basis for future research on non-invasive embryo assessment methods. The use of available technology for analysing miRNAs in media from single-cultured embryos further strengthens the suggestion that these molecules could be valuable biomarkers for embryo (de-)selection purposes. Indeed, these results were peer-reviewed and published recently in a reproduction-focused journal (Makri et al., 2020). It is likely that, in future, miRNA analysis could be integrated in multi-omics platforms to score embryos based on the molecular profile of culture media.

One of the noteworthy findings of this thesis was that embryo-released miRNAs can have adverse effects on implantation. To date there is limited evidence on the involvement of miRNAs in implantation-related processes. However it is recognised that as regulatory molecules miRNAs are pivotal for the normal development of the early embryo, its viability and, by extension, its potential to robustly adhere to and invade into the uterine wall (Liang et al., 2017). Interestingly, an *in vitro* study in mouse peri-implantation embryos found that those with potential to implant (outgrowth embryos) expressed at least 10 miRNAs differentially compared to those with no implantation potential (non-outgrowth embryos) and blastocysts (control). According to the authors, these miRNAs are likely involved in embryo attachment and the interaction between the embryo and the endometrium which are necessary for the implantation of the incoming embryo (Kim et al., 2019).

Moreover, there is limited knowledge regarding the association of extra-cellular miRNA profiles with implantation outcomes. Initially, the study by Cuman and colleagues (2015) found distinct profiles for at least 19 miRNAs released by implanted

embryos and 22 miRNAs exclusively released by non-implanted embryos. Thus it was suggested that specific miRNAs could be used as predictors of implantation in human assisted reproduction cycles (Cuman et al., 2015). Moreover, a small study by Borges *et al.* (2016) found that embryos that failed to implant had significantly higher levels of miR-142-3p in the spent culture media. It was therefore proposed that this miRNA could be a biomarker of implantation failure (Borges et al., 2016). Therefore, taken together, these two studies showed that quantification of miRNAs in spent media could be of clinical value for predicting implantation outcomes in humans. Even more interesting was the preliminary evidence they provided on the roles of extra-cellular miRNAs on implantation.

During the last few years the biological functions of microRNAs at the embryo-maternal interface have been studied. It is known that these molecules circulate in biological fluids where they are protected by their carriers and can exert secretory functions in other cell types (Boon and Vickers, 2013). Exosomes are the main carriers of miRNAs and these vesicles are released by both uterine and embryonic cells, which indicated the existence of an inter-cellular communication network via miRNAs (Saadeldin et al., 2015). This suggestion is further supported by evidence that extra-cellular vesicles (EVs) with miRNA cargoes are internalised by embryos and endometrial cells.

In early pregnancy in sheep EVs mediate conceptus-maternal interactions. When vesicles purified from uterine luminal fluid are fluorescently tagged and infused back to the lumen of pregnant sheep, after some days these EVs are located in the conceptus trophectoderm. Inversely, EVs released by conceptuses are rich in proteins and RNAs and are uptaken by uterine cells (Burns et al., 2016). Moreover, human endometrial epithelium specifically releases at least 6 miRNAs during the window of implantation,

with miR-30d being the most differentially-released. This miRNA is in exosomes and is internalised by embryos (tested with *in* vitro mouse assays) either exosome-bound or in a free form via the trophectoderm. There it causes overexpression of genes encoding for adhesion molecules like integrins and cadherins. Embryo adhesion is notably increased in an embryo adhesion assay with miR-30d supplementation (Vilella et al., 2015).

On the other hand, when bound-free miRNAs are added to culture media at the morula stage they are uptaken by embryos. Kropp and Khatib (2015) tested this with the miR-24 mimic in bovine embryos and not only found significantly elevated levels in the blastocyst cells (by 44 FC) but this uptake was associated with repression of the *CDKN1b* gene which could have effects on embryonic development and implantation competence. Moreover, in the study of Cuman and colleagues (2015), human trophoblast cells were transfected with a tagged miRNA mimic, the refreshed conditioned media was collected after 24 hours and transferred to cultures of endometrial epithelial cells. Interestingly, the endometrial cells contained significantly higher levels of the specific miRNA (Cuman et al., 2015). This protocol mimicked as closely as possible the *in vivo* conditions where miRNAs are bound by trophectoderm cells in complexes or enclosed in EVs which are then internalised by other cell types. Thus it enabled trophoblast cells to package and release miRNAs which were later recognised and uptaken "naturally" (i.e., without transfection reagents), by endometrial cells. Inside the endometrial cells the miRNA reduced the levels of MTA2 and PVRL1 proteins which blocked embryo adhesion, an effect which was also verified with an adhesion assay (Cuman et al., 2015).

In agreement with the above studies, miRNA-mediated transcriptomic changes in human endometrium were also found in the experiment presented in Chapter 6. In

this experiment miR-371a transfection in endometrial cells caused major dysregulation in gene expression. Changes in biological and cellular processes, as well as predicted phenotypic changes associated with disruption of the implantation process, were caused by the embryo-secreted miR-371a. Additionally, a plethora of key-genes regulating decidualisation, stroma-epithelium communication, adhesion, trophoblast invasion, and inflammatory response were affected by this miRNA.

Considering all the above it is evident that small RNAs released by embryos exert inter-cellular communication actions by regulating gene expression inside the endometrial cells. These changes can directly inhibit or promote implantation of the blastocyst. These findings are interesting because cytokines and growth factors were until recently considered the predominant regulators of implantation. However, as more and more information becomes available, miRNAs have now been acknowledged as key-mediators of embryo implantation, with roles in uterine receptivity, embryonic competence, adhesion, trophoblast invasion, and beyond (Gross et al., 2017a, Liang et al., 2017).

Undoubtedly the most interesting suggestion of this thesis is that embryos of poor quality could impair their implantation via secreted miRNAs. As discussed previously miRNA expression, packaging, and exocytosis is an active and energy-demanding process. The embryo has limited resources at the very early stages of development and therefore there must be a biological reason why apoptotic embryos release intensely miR-371. Moreover, the transfection experiment in endometrial cells demonstrated that implantation is impaired after addition of this miRNA. Thus, it is logical to suggest that miR-371a interacts with the endometrium to reject an embryo with low probability of survival. Signalling the endometrium to reject a defective embryo saves the maternal energy investment required for implantation.

Mechanisms contributing to active de-selection of human embryos at implantation have been described. For example, arresting human embryos trigger a strong response in decidual secretory profiles characterised by selective inhibition of IL-1β, -6, -10, -17, -18, eotaxin, and HB-EGF secretion. The decidualising endometrial cells selectively recognise the presence of a developmentally impaired embryo and respond by inhibiting the secretion of key implantation mediators and immunomodulators (Teklenburg et al., 2010). The authors here suggest that this could be a mechanism evolved to avoid the energy investment from the maternal side to establish a pregnancy with an embryo that has reduced probabilities of survival and instead promote the beginning of a new menstrual cycle in which a good quality embryo will implant resulting in a healthy live birth (Teklenburg et al., 2010).

Moreover, low-quality embryos cause a heightened and prolonged Ca^{2+} response in endometrial epithelial cells. A stress response is also observed when mouse uteri are exposed *in vivo* to culture medium conditioned by poor-quality embryos. Conversely, developmentally competent human embryos trigger a short-lived Ca^{2+} response fluxes and conditioned media from these embryos activate a focused gene network enriched in metabolic enzyme and implantation factors (Brosens et al., 2014).

Thus, enhancing and inhibiting mechanisms activated by embryo-derived signals contribute to active selection at implantation. It is clear that the embryo-maternal dialogue is essential for the initiation of implantation and the state of the blastocyst influences the success of implantation (Zhang et al., 2013b). Nevertheless, the roles of miRNAs as factors of this interactome are not yet clear. Although there is a lack of published literature regarding this topic, the actions of extra-cellular vesicles with RNA cargoes in cell signalling receive more and more attention in reproductive

science and preliminary findings are already presented in international conferences. So far it seems that EVs are key-mediators not only during the peri-implantation period but furthermore to the conceptus-maternal communication.

Interestingly, supporting evidence for embryo selection mechanisms triggered by miRNAs came to light only recently. In the study by Berkhout and colleagues (2020), human ICSI-fertilised embryos were cultured to Day 4, media samples were pooled according to the morphological quality (high-quality: ≤20% fragmentation, low-quality: >20% fragmentation), and analysed for miRNA profiles using a PCR-array. It was found that high-quality embryos exclusively secreted miR-320a whereas low-quality embryos exclusively secreted miR-19b-3p and miR-19a-3p. Mimics and inhibitors of these miRNAs were transfected into decidualised human endometrial stromal cells to identify phenotypical changes caused by them. It was found that miR-320a significantly enhances endometrial cell migration and further experiments on the gene targets of this miRNA showed changes in 223 genes. Gene ontology analysis identified effects on cell adhesion, regulation of cytoskeleton organisation, and cell migration caused by miR-320a presence. As a conclusion the authors suggested that by secreting miR-320a, high-quality embryos directly influence endometrial cells to prime the endometrium at the implantation site for successful implantation (Berkhout et al., 2020).

The results described above are in good agreement with the findings of the present thesis, that embryos with different quality cause gene changes in neighbouring endometrial cells through secreted miRNAs, and these changes can affect the ability of the endometrium to establish implantation. Although the effect of miR-371a was not verified in a clinical study here, Cuman and colleagues (2015) detected the presence of this miRNA exclusively in samples deriving from embryos that failed to

implant. Considering the findings of the present thesis and the available literature it seems that the embryo-secreted miR-371a has a signalling role in the implantation period.

During the peri-implantation period the decidualised endometrium acts as a sensor of embryonic quality. To be reproductively successful, the endometrium must be receptive as well as selective. This is proposed as a maternal strategy that has evolved to prevent inappropriate investment in poorly viable embryos (Macklon and Brosens, 2014). Regarding reproduction-related processes, as increasingly more evidence becomes available it is expected that the roles of miRNAs as triggering molecules for embryo selection by the endometrium will be deciphered.

7.1 General conclusions

In conclusion, mammalian pre-implantation embryos express, package, and release miRNAs to the surrounding micro-environment. There are specific miRNAs released by embryos of differing developmental competence and DNA quality which can be quantified in spent culture media derived from single-cultured embryos. Analysis of spent media for miRNAs could be of clinical value for embryo (de-)selection purposes, alongside other -omics platforms that are currently developed.

High DNA fragmentation triggers the release of specific miRNAs belonging to the miR-290-295 cluster by murine embryos and the release of the homologue miR-371-373 cluster by bovine blastocysts. MiR-371a triggers major transcriptomic changes in human endometrial cells which are overall consistent with impaired implantation. Human studies show a link between miR-371a release and implantation failure. Overall, it is suggested that miR-371a acts as a mediator to prevent implantation of an abnormal embryo with low chance of survival.

The novel findings presented here show secretory functions of miRNAs at the embryo-maternal interface. As further research is carried out it is expected that miRNAs will be acknowledged as important mediators of implantation. Implantation success depends on the complex interplay between a receptive endometrium and a competent embryo. Implantation-related processes remain largely unknown making implantation a true “black box” in developmental biology. Future research on molecules mediating the first dialogue between the embryo and the mother is necessary to shed light on the mechanisms controlling implantation. This will enrich the scientific knowledge and can have clinical applications in human assisted reproduction.

REFERENCES

ABD EL NABY, W. S., HAGOS, T. H., HOSSAIN, M. M., SALILEW-WONDIM, D., GAD, A. Y., RINGS, F., CINAR, M. U., THOLEN, E., LOOFT, C., SCHELLANDER, K., HOELKER, M. & TESFAYE, D. 2013. Expression analysis of regulatory microRNAs in bovine cumulus oocyte complex and preimplantation embryos. *Zygote,* 21**,** 31-51.

ABDELHAFEZ, F., XU, J., GOLDBERG, J. & DESAI, N. 2011. Vitrification in open and closed carriers at different cell stages: assessment of embryo survival, development, DNA integrity and stability during vapor phase storage for transport. *BMC Biotechnology,* 11**,** 29.

AHMED, K., LAPIERRE, M. P., GASSER, E., DENZLER, R., YANG, Y., RUELICKE, T., KERO, J., LATREILLE, M. & STOFFEL, M. 2017. Loss of microRNA-7a2 induces hypogonadotropic hypogonadism and infertility. *Journal of Clinical Investigation,* 127**,** 1061-1074.

ALLES, J., FEHLMANN, T., FISCHER, U., BACKES, C., GALATA, V., MINET, M., HART, M., ABU-HALIMA, M., GRÄSSER, F. A., LENHOF, H.-P., KELLER, A. & MEESE, E. 2019. An estimate of the total number of true human miRNAs. *Nucleic Acids Research,* 47**,** 3353-3364.

ALVES, M. B. R., DE ARRUDA, R. P., DE BEM, T. H. C., FLOREZ-RODRIGUEZ, S. A., SÁ FILHO, M. F., BELLEANNÉE, C., MEIRELLES, F. V., DA SILVEIRA, J. C., PERECIN, F. & CELEGHINI, E. C. C. 2019. Sperm-borne miR-216b modulates cell proliferation during early embryo development via K-RAS. *Scientific Reports,* 9.

AMANAI, M., BRAHMAJOSYULA, M. & PERRY, C. F. A. 2006. A restricted role for sperm-borne microRNAs in mammalian fertilization. *Biology of Reproduction,* 75**,** 877-884.

APLIN, J. D. & SINGH, H. 2008. Bioinformatics and transcriptomics studies of early implantation. *Assessment Of Human Reproductive Function,* 1127**,** 116-120.

ARROYO, J. D., CHEVILLET, J. R., KROH, E. M., RUF, I. K., PRITCHARD, C. C., GIBSON, D. F., MITCHELL, P. S., BENNETT, C. F., POGOSOVA-AGADJANYAN, E. L., STIREWALT, D. L., TAIT, J. F. & TEWARI, M. 2011. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proceedings of the National Academy of Sciences - PNAS,* 108**,** 5003-5008.

BALABAN, B., BRISON, D., CALDERÓN, G., CATT, J., CONAGHAN, J., COWAN, L., EBNER, T., GARDNER, D., HARDARSON, T., LUNDIN, K., CRISTINA MAGLI, M., MORTIMER, D., MORTIMER, S., MUNNÉ, S., ROYERE, D., SCOTT, L., SMITZ, J., THORNHILL, A., VAN BLERKOM, J. & VAN DEN ABBEEL, E. 2011. † The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting †. *Human Reproduction,* 26**,** 1270-1283.

BAO, J., LI, D., WANG, L., WU, J., HU, Y., WANG, Z., CHEN, Y., CAO, X., JIANG, C., YAN, W. & XU, C. 2012. MicroRNA-449 and microRNA-34b/c function redundantly in murine testes by targeting E2F transcription factor-retinoblastoma protein (E2F-pRb) pathway. *The Journal of Biological Chemistry,* 287**,** 21686.

BARRIE, A., HOMBURG, R., MCDOWELL, G., BROWN, J., KINGSLAND, C. & TROUP, S. 2017. Examining the efficacy of six published time-lapse imaging embryo selection algorithms to predict implantation to demonstrate the need for the development of specific, in-house morphokinetic selection algorithms. *Fertility and Sterility,* 107**,** 613-621.

BASHIRI, A., HALPER, K. I. & ORVIETO, R. 2018. Recurrent Implantation Failure-update overview on etiology, diagnosis, treatment and future directions. *Reproductive Biology and Endocrinology,* 16**,** 121-18.

BEHM-ANSMANT, I., REHWINKEL, J., DOERKS, T., STARK, A., BORK, P. & IZAURRALDE, E. 2006. mRNA degradation by miRNAs and GW182 required both

CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes & Development,* 20, 1885-1898.

BELLEANNÉE, C., CALVO, E., THIMON, V., CYR, D. G., LÉGARÉ, C., GARNEAU, L. & SULLIVAN, R. 2012. Role of MicroRNAs in Controlling Gene Expression in Different Segments of the Human Epididymis (Region-Specific miRNA Signatures in the Epididymis). *PLoS ONE,* 7**,** e34996.

BERKHOUT, R. P., KEIJSER, R., REPPING, S., LAMBALK, C. B., AFINK, G. B., MASTENBROEK, S. & HAMER, G. 2020. High-quality human preimplantation embryos stimulate endometrial stromal cell migration via secretion of microRNA hsa-miR-320a. *Human Reproduction (Oxford),* 35**,** 1797-1807.

BERMEJO-ALVAREZ, P., RIZOS, D., RATH, D., LONERGAN, P., GUTIERREZ-ADAN, A. & SEIDEL, G. 2010. Sex Determines the Expression Level of One Third of the Actively Expressed Genes in Bovine Blastocysts. *Proceedings of the National Academy of Sciences - PNAS,* 107**,** 3394-3399.

BERNEAU, S. C., RUANE, P. T., BRISON, D. R., KIMBER, S. J., WESTWOOD, M. & APLIN, J. D. 2019. Investigating the role of CD44 and hyaluronate in embryo-epithelial interaction using an in vitro model. *Molecular Human Reproduction,* 25**,** 265-273.

BERNSTEIN, E., KIM, S. Y., CARMELL, M. A., MURCHISON, E. P., ALCORN, H., LI, M. Z., MILLS, A. A., ELLEDGE, S. J., ANDERSON, K. V. & HANNON, G. J. 2003. Dicer is essential for mouse development. *Nature Genetics,* 35**,** 215-217.

BOON, R. A. & VICKERS, K. C. 2013. Intercellular Transport of MicroRNAs. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 33**,** 186-192.

BORGES, E., SETTI, A. S., BRAGA, D., GERALDO, M. V., FIGUEIRA, R. D. S. & IACONELLI, A. 2016. miR-142-3p as a biomarker of blastocyst implantation failure A pilot study. *Jornal Brasileiro De Reproducao Assistida,* 20**,** 200-205.

BOSCH, E., LABARTA, E., KOLIBIANAKIS, E., ROSEN, M. & MELDRUM, D. 2016. Regimen of ovarian stimulation affects oocyte and therefore embryo quality. *Fertility and Sterility,* 105**,** 560-570.

BRATTON, S. B. & SALVESEN, G. S. 2010. Regulation of the Apaf-1-caspace-9 apoptosome. *Journal of Cell Sciene,* 123, 3209-3214.

BRAUN, J. E., TRUFFAULT, V., BOLAND, A., HUNTZINGER, E., CHANG, C.-T., HAAS, G., WEICHENRIEDER, O., COLES, M. & IZAURRALDE, E. 2012. A direct interaction between DCP1 and XRN1 couples mNRA decapping to 5′ exonucleolytic degradation. *Nature Structural & Molecular Biology,* 19, 1324-1331.

BRAY, S. J. 2006. Notch signalling: a simple pathway becomes complex. *Nature Reviews Molecular Cell Biology,* 7**,** 678-689.

BROSENS, J. J., SALKER, M. S., TEKLENBURG, G., NAUTIYAL, J., SALTER, S., LUCAS, E. S., STEEL, J. H., CHRISTIAN, M., CHAN, Y.-W., BOOMSMA, C. M., MOORE, J. D., HARTSHORNE, G. M., ŠUĆUROVIĆ, S., MULAC-JERICEVIC, B., HEIJNEN, C. J., QUENBY, S., GROOT KOERKAMP, M. J., HOLSTEGE, F. C. P., SHMYGOL, A. & MACKLON, N. S. 2014. Uterine Selection of Human Embryos at Implantation. *Scientific Reports,* 4**,** 3894.

BURNS, G. W., BROOKS, K. E. & SPENCER, T. E. 2016. Extracellular Vesicles Originate from the Conceptus and Uterus During Early Pregnancy in Sheep. *Biology of Reproduction,* 94**,** 56.

BUTLER, A. E., RAMACHANDRAN, V., HAYAT, S., DARGHAM, S. R., CUNNINGHAM, T. K., BENURWAR, M., SATHYAPALAN, T., NAJAFI-SHOUSHTARI, S. H. & ATKIN, S. L. 2019. Expression of microRNA in follicular fluid in women with and without PCOS. *Scientific Reports,* 9**,** 1-9.

CAMPBELL, A., FISHEL, S., BOWMAN, N., DUFFY, S., SEDLER, M. & HICKMAN, C. F. L. 2013. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. *Reproductive BioMedicine Online,* 26**,** 477-485.

CAO, R., WU, W. J., ZHOU, X. L., XIAO, P., WANG, Y. & LIU, H. L. 2015. Expression and preliminary functional profiling of the let-7 family during porcine ovary follicle atresia. *Molecules and Cells,* 38**,** 304.

CAPALBO, A., RIENZI, L., CIMADOMO, D., MAGGIULLI, R., ELLIOTT, T., WRIGHT, G., NAGY, Z. P. & UBALDI, F. M. 2014. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. *Human Reproduction,* 29**,** 1173-1181.

CAPALBO, A., UBALDI, F. M., CIMADOMO, D., NOLI, L., KHALAF, Y., FARCOMENI, A., ILIC, D. & RIENZI, L. 2016. MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment. *Fertility and Sterility,* 105**,** 225-235.e3.

CARLETTI, M. Z., FIEDLER, S. D. & CHRISTENSON, L. K. 2010. MicroRNA 21 blocks apoptosis in mouse periovulatory granulosa cells. *Biology of Reproduction,* 83**,** 286.

CERNILOGAR, F. M., ONORATI, M.C., KOTHE, G. O., BURROUGHS, A. M., PARSI, K. M., BREILING, A., LO SARDO, F., SAXENA, A., MIYOSHI, K., SIOMI, M. C., CARNINCI, P., GILMOUR, D. S., CORONA, D. F. V. & ORLANDO, V. 2011. Chromatin-associated RNA interference components contribute to transcriptional regulation in Drosophila. *Nature,* 480, 391-395.

CARSON, D. D., JULIAN, J., LESSEY, B. A., PRAKOBPHOL, A. & FISHER, S. J. 2006. MUC1 is a scaffold for selectin ligands in the human uterus. *Frontiers in Bioscience,* 11**,** 2903-2908.

CASTANOTTO, D., ZHANG, X., ALLUIN, J., ZHANG, X., RÜGER, J., ARMSTRONG, B., ROSSI, J., RIGGS, A. & STEIN, C. A. 2018. A stress-induced response complex (SIRC) shuttles miRNAs, siRNAs, and oligonucleotides to the nucleus. *Proceedings of the National Academy of Sciences - PNAS,* 115**,** E5756-E5765.

CASTILLO, C. M., HARPER, J., ROBERTS, S. A., O'NEILL, H. C., JOHNSTONE, E. D. & BRISON, D. R. 2020. The impact of selected embryo culture conditions on ART treatment cycle outcomes: a UK national study. *Human Reproduction Open,* 2020**,** hoz031.

CETINKAYA, M., PIRKEVI, C., YELKE, H., COLAKOGLU, Y., ATAYURT, Z. & KAHRAMAN, S. 2015. Relative kinetic expressions defining cleavage synchronicity are better predictors of blastocyst formation and quality than absolute time points. *Official Publication of ALPHA, Scientists in Reproductive Medicine,* 32**,** 27-35.

CHAPUIS, A., GALA, A., FERRIÈRES-HOA, A., MULLET, T., BRINGER-DEUTSCH, S., VINTEJOUX, E., TORRE, A. & HAMAMAH, S. 2017. Sperm quality and paternal age: effect on blastocyst formation and pregnancy rates. *Basic and Clinical Andrology,* 27**,** 2.

CHEN, E. Y., TAN, C. M., KOU, Y., DUAN, Q., WANG, Z., MEIRELLES, G. V., CLARK, N. R. & MA'AYAN, A. 2013. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics,* 14**,** 128.

CHEN, Q., ZHANG, Y., LU, J., WANG, Q., WANG, S., CAO, Y., WANG, H. & DUAN, E. 2009. Embryo–uterine cross-talk during implantation: the role of Wnt signaling †. *Molecular Human Reproduction,* 15**,** 215-221.

CHEN, X., LI, X., GUO, J., ZHANG, P. & ZENG, W. 2017. The roles of microRNAs in regulation of mammalian spermatogenesis. *Journal of Animal Science and Biotechnology,* 8**,** 779-786.

CHEN, X., XIE, M., LIU, D. & SHI, K. 2015. Downregulation of microRNA-146a inhibits ovarian granulosa cell apoptosis by simultaneously targeting interleukin-1 receptor-associated kinase and tumor necrosis factor receptor-associated factor 6. *Molecular Medicine Reports,* 12**,** 5155-5162.

CHEN, Y.-N., DAI, J.-J., WU, C.-F., ZHANG, S.-S., SUN, L.-W. & ZHANG, D.-F. 2018. Apoptosis and developmental capacity of vitrified parthenogenetic pig blastocysts. *Animal Reproduction Science,* 198**,** 137-144.

CHRISTIE, M., BOLAND, A., HUNTZINGER, E., WEICHENRIEDER, O. & IZAURRALDE, E. 2013. Structure of the PAN3 pseudokinase reveals the basis for

interactions with the PAN2 deadenylase and the GW182 proteins. *Molecular Cell,* 51, 360-373.

CIMADOMO, D., CAPALBO, A., UBALDI, F. M., SCARICA, C., PALAGIANO, A., CANIPARI, R. & RIENZI, L. 2016. The Impact of Biopsy on Human Embryo Developmental Potential during Preimplantation Genetic Diagnosis. *BioMed Research International,* 2016**,** 7193075-10.

CIMADOMO, D., FABOZZI, G., VAIARELLI, A., UBALDI, N., UBALDI, F. M. & RIENZI, L. 2018. Impact of Maternal Age on Oocyte and Embryo Competence. *Frontiers in Endocrinology,* 9**,** 327.

CIRAY, H. N., CAMPBELL, A., AGERHOLM, I. E., AGUILAR, J., CHAMAYOU, S., ESBERT, M. & SAYED, S. 2014. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. *Human Reproduction,* 29**,** 2650-2660.

CIVENNI, G., HOLBRO, T. & HYNES, N. E. 2003. Wnt1 and Wnt5a induce cyclin D1 expression through ErbB1 transactivation in HC11 mammary epithelial cells. *EMBO Reports,* 4**,** 166-171.

COCKBURN, K. & ROSSANT, J. 2010. Making the blastocyst: lessons from the mouse. *The Journal of Clinical Investigation,* 120**,** 995-1003.

COLLINO, F., DEREGIBUS, M. C., BRUNO, S., STERPONE, L., AGHEMO, G., VILTONO, L., TETTA, C. & CAMUSSI, G. 2010. Microvesicles Derived from Adult Human Bone Marrow and Tissue Specific Mesenchymal Stem Cells Shuttle Selected Pattern of miRNAs. *PloS One,* 5**,** e11803-e11803.

CONDORELLI, G., LATRONICO, M. V. G. & CAVARRETTA, E. 2014. microRNAs in Cardiovascular Diseases: Current Knowledge and the Road Ahead: Current Knowledge and the Road Ahead. *Journal of the American College of Cardiology,* 63**,** 2177-2187.

CORRAL-VAZQUEZ, C., SALAS-HUETOS, A., BLANCO, J., VIDAL, F., SARRATE, Z. & ANTON, E. 2019. Sperm microRNA pairs: new perspectives in the search for male fertility biomarkers. *Fertility and Sterility,* 112**,** 831-841.

CORTEZ, M. A., BUESO-RAMOS, C., FERDIN, J., LOPEZ-BERESTEIN, G., SOOD, A. K. & CALIN, G. A. 2011. MicroRNAs in body fluids—the mix of hormones and biomarkers. *Nature Reviews Clinical Oncology,* 8**,** 467-477.

CRUZ, M., GARRIDO, N., HERRERO, J., PÉREZ-CANO, I., MUÑOZ, M. & MESEGUER, M. 2012. Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reproductive BioMedicine Online,* 25**,** 371-381.

CUI, L., FANG, L., SHI, B., QIU, S. & YE, Y. 2015. Spermatozoa micro ribonucleic acid–34c level is correlated with intracytoplasmic sperm injection outcomes. *Fertility and Sterility,* 104**,** 312-317.e1.

CUMAN, C., VAN SINDEREN, M., GANTIER, M. P., RAINCZUK, K., SORBY, K., ROMBAUTS, L., OSIANLIS, T. & DIMITRIADIS, E. 2015. Human Blastocyst Secreted microRNA Regulate Endometrial Epithelial Cell Adhesion. *EBioMedicine,* 2**,** 1528-1535.

DA SILVEIRA, J. C., DE ANDRADE, G. M., NOGUEIRA, M. F. G., MEIRELLES, F. V. & PERECIN, F. 2015. Involvement of miRNAs and Cell-Secreted Vesicles in Mammalian Ovarian Antral Follicle Development. *Reproductive Sciences,* 22**,** 1474-1483.

DAL CANTO, M., COTICCHIO, G., MIGNINI RENZINI, M., DE PONTI, E., NOVARA, P. V., BRAMBILLASCA, F., COMI, R. & FADINI, R. 2012. Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. *Reproductive BioMedicine Online,* 25**,** 474-480.

DAVID, J. M., DOMINGUEZ, C., HAMILTON, D. H., PALENA, C. & WHITESIDE, T. L. 2016. The IL-8/IL-8R Axis: A Double Agent in Tumor Immune Resistance. *Vaccines,* 4**,** 22.

DAVIDSON, L. M. & COWARD, K. 2016. Molecular mechanisms of membrane interaction at implantation. *Birth Defects Research Part C: Embryo Today,* 108**,** 19-32.

DE MATEO, S. & SASSONE-CORSI, P. 2014. Regulation of spermatogenesis by small non-coding RNAs: Role of the germ granule. *Seminars in Cell and Developmental Biology,* 29**,** 84-92.

DEY, S. K., LIM, H., DAS, S. K., REESE, J., PARIA, B. C., DAIKOKU, T. & WANG, H. 2004. Molecular cues to implantation. *Endocrine Reviews,* 25**,** 341-373.

DI PALO, A., SINISCALCHI, C., SALERNO, M., RUSSO, A., GRAVHOLT, C. H. & POTENZA, N. 2020. What microRNAs could tell us about the human X chromosome. *Cellular and Molecular Life Sciences : CMLS,* 77**,** 4069-4080.

DIMITRIADIS, E., WHITE, C. A., JONES, R. L. & SALAMONSEN, L. A. 2005. Cytokines, chemokines and growth factors in endometrium related to implantation. *Human Reproduction Update,* 11**,** 613-630.

DOMINGUEZ, F., YÁÑEZ-MÓ, M., SANCHEZ-MADRID, F. & SIMÓN, C. 2005. Embryonic implantation and leukocyte transendothelial migration: different processes with similar players? *FASEB Journal,* 19**,** 1056-1060.

DONADEU, F. X., MOHAMMED, B. T. & IOANNIDIS, J. 2017. A miRNA target network putatively involved in follicular atresia. *Domestic Animal Endocrinology,* 58**,** 76-83.

DOROSTGHOAL, M., GHAFFARI, H. O. A., SHAHBAZIAN, N. & MIRANI, M. 2017. Endometrial expression of β3 integrin, calcitonin and plexin-B1 in the window of implantation in women with unexplained infertility. *International Journal of Reproductive BioMedicine,* 15**,** 33-40.

DU, Y., WANG, X., WANG, B., CHEN, W., HE, R., ZHANG, L., XING, X., SU, J., WANG, Y. & ZHANG, Y. 2014. Deep sequencing analysis of microRNAs in bovine sperm. *Molecular Reproduction and Development,* 81**,** 1042-1052.

DUNN, C. L., KELLY, R. W. & CRITCHLEY, H. O. 2003. Decidualization of the human endometrial stromal cell: an enigmatic transformation. *Reproductive BioMedicine Online,* 7**,** 151-161.

EBNER, T., YAMAN, C., MOSER, M., SOMMERGRUBER, M., PÖLZ, W. & TEWS, G. 2001. Embryo fragmentation in vitro and its impact on treatment and pregnancy outcome. *Fertility and Sterility,* 76**,** 281-285.

EGEA, R., ESCRIVÁ, M., PUCHALT, N. & VARGHESE, A. 2014. OMICS: Current and future perspectives in reproductive medicine and technology. *Journal of Human Reproductive Sciences,* 7**,** 73-92.

ESHRE. 2020. ART fact sheet.

ESWARAKUMAR, V. P., LAX, I. & SCHLESSINGER, J. 2005. Cellular signaling by fibroblast growth factor receptors. *Cytokine and Growth Factor Reviews,* 16**,** 139-149.

FAUL, F., ERDFELDER, E., BUCHNER, A. & LANG, A.-G. 2009. Statistical power analyses using GPower 3.1: Tests for correlation and regression analyses. *Behavior Research Methods,* 41**,** 1149-1160.

FAZLEABAS, A. T., KIM, J. J. & STRAKOVA, Z. 2004. Implantation: embryonic signals and the modulation of the uterine environment--a review. *Placenta,* 25 Suppl A**,** S26-31.

FIEDLER, S. D., CARLETTI, M. Z., HONG, X. & CHRISTENSON, L. K. 2008. Hormonal regulation of MicroRNA expression in periovulatory mouse mural granulosa cells. *Biology of Reproduction,* 79**,** 1030-1037.

FIGUEIRA, R. C. S., SETTI, A. S., BRAGA, D. P. A. F., FERREIRA, R. C., IACONELLI, A. & BORGES, E. 2012. Blastocyst morphology holds clues concerning the chromosomal status of the embryo. *International Journal of Fertility & Sterility,* 98**,** 215-220.

FISHEL, S., CAMPBELL, A., MONTGOMERY, S., SMITH, R., NICE, L., DUFFY, S., JENNER, L., BERRISFORD, K., KELLAM, L., SMITH, R., AMP, APOS, CRUZ, I. & BECCLES, A. 2017. Live births after embryo selection using morphokinetics versus conventional morphology: a retrospective analysis. *Reproductive BioMedicine Online***,** 407-416.

FITZPATRICK, T. E., LASH, G. E., YANAIHARA, A., CHARNOCK-JONES, D. S., MACDONALD-GOODFELLOW, S. K. & GRAHAM, C. H. 2003. Inhibition of breast carcinoma and trophoblast cell invasiveness by vascular endothelial growth factor. *Experimental Cell Research,* 283**,** 247-255.

FORMAN, J. J., LEGESSE-MILLER, A. & COLLER, H. A. 2008. A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 14879-14884.

FU, J., QU, R.-G., ZHANG, Y.-J., GU, R.-H., LI, X., SUN, Y.-J., WANG, L., SANG, Q. & SUN, X.-X. 2018. Screening of miRNAs in human follicular fluid reveals an inverse relationship between microRNA-663b expression and blastocyst formation. *Reproductive BioMedicine Online,* 37**,** 25-32.

FURUTA, M., KOZAKI, K.-I., TANAKA, S., ARII, S., IMOTO, I. & INAZAWA, J. 2010. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis,* 31**,** 766-776.

GANEFF, C., CHATEL, G., MUNAUT, C., FRANKENNE, F., FOIDART, J. M. & WINKLER, R. 2009. The IGF system in in-vitro human decidualization. *Molecular Human Reproduction,* 15**,** 27-38.

GARCÍA-LÓPEZ, J., ALONSO, L., CÁRDENAS, D. B., ARTAZA-ALVAREZ, H., HOURCADE, J. D. D., MARTÍNEZ, S., BRIEÑO-ENRÍQUEZ, M. A. & DEL MAZO, J. 2015. Diversity and functional convergence of small noncoding RNAs in male germ cell differentiation and fertilization. *RNA,* 21**,** 946-962.

GARCÍA-LÓPEZ, J. & DEL MAZO, J. 2012. Expression dynamics of microRNA biogenesis during preimplantation mouse development. *Gene Regulatory Mechanisms,* 1819**,** 847-854.

GARDNER, D. K. & BALABAN, B. 2016. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important? *Molecular Human Reproduction,* 22**,** 704-718.

GARDNER, D. K., LANE, M., STEVENS, J., SCHLENKER, T. & SCHOOLCRAFT, W. B. 2000. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertility and Sterility,* 73**,** 1155-1158.

GARDNER, D. K. & SCHOOLCRAFT, W. B. 1999. In vitro culture of human blastocysts. *In:* GARDNER, D. K., SCHOOLCRAFT, W. B., JANSEN, R. & MORTIMER, D. (eds.) *Toward Reproductive Certainty: Fertility and Genetics Beyond.* London: London Parthenon Publishing, 378-388.

GEE, J. M. W. & KNOWLDEN, J. M. 2003. ADAM metalloproteases and EGFR signalling. *Breast Cancer Research,* 5**,** 223-224.

GHORAI, A. & GHOSH, U. 2014. miRNA gene counts in chromosomes vary widely in a species and biogenesis of miRNA largely depends on transcription or post-transcriptional processing of coding genes. *Frontiers in Genetics,* 5**,** 100-100.

GILAD, S., MEIRI, E., YOGEV, Y., BENJAMIN, S., LEBANONY, D., YERUSHALMI, N., BENJAMIN, H., KUSHNIR, M., CHOLAKH, H., MELAMED, N., BENTWICH, Z., HOD, M., GOREN, Y. & CHAJUT, A. 2008. Serum MicroRNAs Are Promising Novel Biomarkers. *PloS One,* 3**,** e3148.

GOAD, J., KO, Y.-A., KUMAR, M., SYED, S. M. & TANWAR, P. S. 2017. Differential Wnt signaling activity limits epithelial gland development to the anti-mesometrial side of the mouse uterus. *Developmental Biology,* 423**,** 138-151.

GONZALEZ, R. R., LEARY, K., PETROZZA, J. C. & LEAVIS, P. C. 2003. Leptin regulation of the interleukin1 system in human endometrial cells. *Molecular Human Reproduction,* 9**,** 151-158.

GONZALEZ, R. R. & LEAVIS, P. 2001. Leptin upregulates beta3-integrin expression and interleukin-1beta, upregulates leptin and leptin receptor expression in human endometrial epithelial cell cultures. *Endocrine,* 16**,** 21-28.

GONZALEZ, R. R., RUEDA, B. R., RAMOS, M. P., LITTELL, R. D., GLASSER, S. & LEAVIS, P. C. 2004. Leptin-induced increase in leukemia inhibitory factor and its

receptor by human endometrium is partially mediated by interleukin 1 receptor signaling. *Endocrinology,* 145**,** 3850-3857.

GOODMAN, L. R., GOLDBERG, J., FALCONE, T., AUSTIN, C. & DESAI, N. 2016. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. *Fertility and Sterility,* 105**,** 275-285.e10.

GOOSSENS, K., MESTDAGH, P., LEFEVER, S., VAN POUCKE, M., VAN ZEVEREN, A., VAN SOOM, A., VANDESOMPELE, J. & PEELMAN, L. 2013. Regulatory microRNA Network Identification in Bovine Blastocyst Development. *Stem Cells and Development,* 22**,** 1907-1920.

GRADY, R., ALAVI, N., VALE, R., KHANDWALA, M. & MCDONALD, S. D. 2012. Elective single embryo transfer and perinatal outcomes: a systematic review and meta-analysis. *Fertility and Sterility,* 97**,** 324-331.

GRAHAM, J., HAN, T., PORTER, R., LEVY, M., STILLMAN, R. & TUCKER, M. J. 2000. Day 3 morphology is a poor predictor of blastocyst quality in extended culture. *Fertility and Sterility,* 74**,** 495-497.

GRANDJEAN, V., GOUNON, P., WAGNER, N., MARTIN, L., WAGNER, K. D., BERNEX, F., CUZIN, F. & RASSOULZADEGAN, M. 2009. The miR-124-Sox9 paramutation: RNA-mediated epigenetic control of embryonic and adult growth. *Development,* 136**,** 3647-3655.

GROSS, N., KROPP, J. & KHATIB, H. 2017a. MicroRNA Signaling in Embryo Development. *Biology,* 6**,** 34.

GROSS, N., KROPP, J. & KHATIB, H. 2017b. Sexual Dimorphism of miRNAs Secreted by Bovine I*n vitro*-produced Embryos. *Frontiers in Genetics,* 8**,** 39.

GRUMMER, R. & WINTERHAGER, E. 2011. Blastocyst-mediated induction of endometrial connexins: an inflammatory response? *Journal Of Reproductive Immunology,* 90**,** 9-13.

GU, K. L., ZHANG, Q., YAN, Y., LI, T. T., DUAN, F. F., HAO, J., WANG, X. W., SHI, M., WU, D. R., GUO, W. T. & WANG, Y. M. 2016. Pluripotency-associated miR-290/302 family of microRNAs promote the dismantling of naive pluripotency. *Cell Research,* 26**,** 350-366.

GUAN, Y., LIANG, G., HAWKEN, P. A. R., MALECKI, I. A., COZENS, G., VERCOE, P., E, MARTIN, G. B. & GUAN, L. L. 2015. Roles of small RNAs in the effects of nutrition on apoptosis and spermatogenesis in the adult testis. *Scientific Reports,* 5**,** 10372.

GULYAEVA, L. F. & KUSHLINSKIY, N. E. 2016. Regulatory mechanisms of microRNA expression. *Journal of Translational Medicine,* 14**,** 143.

GÜMÜRDÜ, A., YILDIZ, R., EREN, E., KARAKÜLAH, G., ÜNVER, T., GENÇ, Ş. & PARK, Y. 2017. MicroRNA exocytosis by large dense-core vesicle fusion. *Scientific Reports,* 7**,** 45661-45661.

HA, M. & KIM, V. N. 2014a. Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology,* 15**,** 509.

HA, M. & KIM, V. N. 2014b. Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology,* 15**,** 509-524.

HALSTEAD, M. M., MA, X., ZHOU, C., SCHULTZ, R. M. & ROSS, P. J. 2020. Chromatin remodeling in bovine embryos indicates species-specific regulation of genome activation. *Nature Communications,* 11**,** 4654-4654.

HANNAN, N. J., JONES, R. L., WHITE, C. A. & SALAMONSEN, L. A. 2006. The chemokines, CX3CL1, CCL14, and CCL4, promote human trophoblast migration at the feto-maternal interface. *Biology of Reproduction,* 74**,** 896-904.

HANTAK, A. M., BAGCHI, I. C. & BAGCHI, M. K. 2014. Role of Uterine Stromal-Epithelial Crosstalk in Embryo Implantation. *International Journal of Developmental Biology,* 58**,** 139-46.

HARA, M., KINGSLEY, C. I., NIIMI, M., READ, S., TURVEY, S. E., BUSHELL, A. R., MORRIS, P. J., POWRIE, F. & WOOD, K. J. 2001. IL-10 is required for regulatory

T cells to mediate tolerance to alloantigens in vivo. *Journal of Immunology,* 166**,** 3789-3796.

HARDARSON, T., VAN LANDUYT, L. & JONES, G. 2012. The blastocyst. *Human Reproduction,* 27**,** i72-i91.

HARPER, J., JACKSON, E., SERMON, K., AITKEN, R. J., HARBOTTLE, S., MOCANU, E., HARDARSON, T., MATHUR, R., VIVILLE, S., VAIL, A. & LUNDIN, K. 2017. Adjuncts in the IVF laboratory: where is the evidence for 'add-on' interventions? *Human Reproduction,* 32**,** 485-491.

HASUWA, H., UEDA, J., IKAWA, M. & OKABE, M. 2013. miR-200b and miR-429 function in mouse ovulation and are essential for female fertility. *Science,* 341**,** 71-73.

HAYASHI, K.-G., HOSOE, M., FUJII, S., KANAHARA, H. & SAKUMOTO, R. 2019. Temporal expression and localization of vascular endothelial growth factor family members in the bovine uterus during peri-implantation period. *Theriogenology,* 133**,** 56-64.

HE, L., HE, X. Y., LIM, L. P., DE STANCHINA, E., XUAN, Z. Y., LIANG, Y., XUE, W., ZENDER, L., MAGNUS, J., RIDZON, D., JACKSON, A. L., LINSLEY, P. S., CHEN, C. F., LOWE, S. W., CLEARY, M. A. & HANNON, G. J. 2007. A microRNA component of the p53 tumour suppressor network. *Nature,* 447**,** 1130-1134.

HE, Z., JIANG, J., KOKKINAKI, M., TANG, L., ZENG, W., GALLICANO, I., DOBRINSKI, I. & DYM, M. 2013. MiRNA-20 and mirna-106a regulate spermatogonial stem cell renewal at the post-transcriptional level via targeting STAT3 and Ccnd1. *Stem Cells,* 31**,** 2205-2217.

HE, Z., KOKKINAKI, M., PANT, D., GALLICANO, G. I. & DYM, M. 2009. Small RNA molecules in the regulation of spermatogenesis. *Reproduction,* 137**,** 901-911.

HEITMANN, R., HILL, M., RICHTER, K., DECHERNEY, A. & WIDRA, E. 2013. The simplified SART embryo scoring system is highly correlated to implantation and live birth in single blastocyst transfers. *Journal of Assisted Reproduction and Genetics,* 30**,** 563-567.

HFEA. 2021. Code of Practice. 8th edition.

HODES-WERTZ, B., LEE, H. L., ADLER, A., AMPELOQUIO, E., CLARKE-WILLIAMS, M. & GRIFO, J. A. 2013. Single euploid embryo transfer improves IVF pregnancy, miscarriage, and multiple gestation outcomes compared to national society for assisted reproductive technology database (SART) outcomes. *Fertility and Sterility,* 100**,** S209.

HOSSAIN, M. M., SALILEW-WONDIM, D., SCHELLANDER, K. & TESFAYE, D. 2012. The role of microRNAs in mammalian oocytes and embryos. *Animal Reproduction Science,* 134**,** 36-44.

HUSZAR, J. M. & PAYNE, C. J. 2013. MicroRNA 146 (Mir146) modulates spermatogonial differentiation by retinoic acid in mice. *Biology of Reproduction,* 88**,** 15.

HUYEN, D. V., BANY, B. M. 2011. Evidence for a conserved function of heart and neural crest derivatives expressed transcript 2 in mouse and human decidualization. *Reproduction,* 142, 353-368.

ILLERA, M. J., CULLINAN, E., GUI, Y., YUAN, L., BEYLER, S. A. & LESSEY, B. A. 2000. Blockade of the alpha(v)beta(3) integrin adversely affects implantation in the mouse. *Biology of Reproduction,* 62**,** 1285-1290.

IPSARRO, J. J., & JOSHUA-TOR, L. 2015. From guide to target: molecular insights into eukaryotic RNA-interference machinery. *Nature Structural & Molecular Biology*, 22, 20-28.

JAROUDI, S. & SENGUPTA, S. 2007. DNA repair in mammalian embryos. *Mutation Research,* 635**,** 53-77.

JEEYEON, C., XIAOFEI, S. & SUDHANSU, K. D. 2012. Mechanisms of implantation: strategies for successful pregnancy. *Nature Medicine,* 18**,** 1754-1767.

JHA, R. K., TITUS, S., SAXENA, D., KUMAR, P. G. & LALORAYA, M. 2006. Profiling of E-cadherin, β-catenin and Ca 2+ in embryo–uterine interactions at implantation. *FEBS Letters,* 580**,** 5653-5660.

JONES, L. R., HANNAN, J. N., KAITU'U, J. T. U., ZHANG, A. J. & SALAMONSEN, A. L. 2004. Identification of Chemokines Important for Leukocyte Recruitment to the Human Endometrium at the Times of Embryo Implantation and Menstruation. *The Journal of Clinical Endocrinology & Metabolism,* 89**,** 6155-6167.

JONAS, S. & IZAURRALDE, E. 2015. Towards a moleular understanding of microRNA-mediated gene silencing. *Nature Review Genetics,* 16, 421-433.

KAHRAMAN, S., SAHIN, Y., YELKE, H., KUMTEPE, Y., TUFEKCI, M. A., YAPAN, C. C., YESIL, M. & CETINKAYA, M. 2020. High rates of aneuploidy, mosaicism and abnormal morphokinetic development in cases with low sperm concentration. *Journal of Assisted Reproduction and Genetics,* 37**,** 629-640.

KIEZUN, A., ARTZI, S., MODAI, S., VOLK, N., ISAKOV, O. & SHOMRON, N. 2012. miRviewer: a multispecies microRNA homologous viewer. *BMC Research Notes,* 5**,** 92.

KIM, J., LEE, J. & JUN, J. H. 2019. Identification of differentially expressed microRNAs in outgrowth embryos compared with blastocysts and non-outgrowth embryos in mice. *Reproduction, Fertility and Development,* 31**,** 645-657.

KIMBER, S. J. & SPANSWICK, C. 2000. Blastocyst implantation: the adhesion cascade. *Seminars in Cell and Developmental Biology,* 11**,** 77-92.

KIRKEGAARD, K., AGERHOLM, I. E. & INGERSLEV, H. J. 2012. Time-lapse monitoring as a tool for clinical embryo assessment. *Human Reproduction,* 27**,** 1277-1285.

KROPP, J. & KHATIB, H. 2015. Characterization of microRNA in bovine in vitro culture media associated with embryo quality and development. *Journal of Dairy Science,* 98**,** 6552-6563.

KROPP, J., KHATIB, H. & SALIH, S. M. 2014. Expression of microRNAs in bovine and human pre-implantation embryo culture media. *Frontiers in Genetics,* 5**,** 91.

KUMAR, P., DEZSO, Z., MACKENZIE, C., OESTREICHER, J., AGOULNIK, S., BYRNE, M., BERNIER, F., YANAGIMACHI, M., AOSHIMA, K. & ODA, Y. Circulating miRNA biomarkers for Alzheimer's disease. *PLoS One,* 8**,** e69807.

KURITA, T., LEE, K., COOKE, P. S., LYDON, J. P. & CUNHA, G. R. 2000. Paracrine regulation of epithelial progesterone receptor and lactoferrin by progesterone in the mouse uterus. *Biology of Reproduction,* 62**,** 831-838.

KURITA, T., YOUNG, P., BRODY, J. R., LYDON, J. P., O'MALLEY, B. W. & CUNHA, G. R. 1998. Stromal progesterone receptors mediate the inhibitory effects of progesterone on estrogen-induced uterine epithelial cell deoxyribonucleic acid synthesis. *Endocrinology,* 139**,** 4708-4713.

LAL, A., NAVARRO, F., MAHER, C. A., MALISZEWSKI, L. E., YAN, N., AMP, AMP, APOS, DAY, E., CHOWDHURY, D., DYKXHOORN, D. M., TSAI, P., HOFMANN, O., BECKER, K. G., GOROSPE, M., HIDE, W. & LIEBERMAN, J. 2009. miR-24 Inhibits Cell Proliferation by Targeting E2F2, MYC, and Other Cell-Cycle Genes via Binding to "Seedless" 3′UTR MicroRNA Recognition Elements. *Molecular Cell,* 35**,** 610-625.

LANDGRAF, P., RUSU, M., SHERIDAN, R., SEWER, A., IOVINO, N., ARAVIN, A., PFEFFER, S., RICE, A., KAMPHORST, A. O., LANDTHALER, M., LIN, C., SOCCI, N. D., HERMIDA, L., FULCI, V., CHIARETTI, S., FOÀ, R., SCHLIWKA, J., FUCHS, U., NOVOSEL, A., MÜLLER, R.-U., SCHERMER, B., BISSELS, U., INMAN, J., PHAN, Q., CHIEN, M., WEIR, D. B., CHOKSI, R., DE VITA, G., FREZZETTI, D., TROMPETER, H.-I., HORNUNG, V., TENG, G., HARTMANN, G., PALKOVITS, M., DI LAURO, R., WERNET, P., MACINO, G., ROGLER, C. E., NAGLE, J. W., JU, J., PAPAVASILIOU, F. N., BENZING, T., LICHTER, P., TAM, W., BROWNSTEIN, M. J., BOSIO, A., BORKHARDT, A., RUSSO, J. J., SANDER, C., ZAVOLAN, M. & TUSCHL, T. 2007. A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing. *Cell,* 129**,** 1401-1414.

LEE, K. Y. & DEMAYO, F. J. 2004. Animal models of implantation. *Reproduction,* 128**,** 679-695.

LEE, S.-H., CHEN, T.-Y., DHAR, S. S., GU, B., CHEN, K., KIM, Y. Z., LI, W. & LEE, M. G. 2016. A feedback loop comprising PRMT7 and miR-24-2 interplays with Oct4, Nanog, Klf4 and c-Myc to regulate stemness. *Nucleic Acids Research,* 44**,** 10603-10618.

LEI, L., JIN, S., GONZALEZ, G., BEHRINGER, R. R. & WOODRUFF, T. K. 2010. The regulatory role of Dicer in folliculogenesis in mice. *Molecular and Cellular Endocrinology,* 315**,** 63-73.

LESSER, M. P., KRUSE, V. A. & BARRY, T. M. 2003. Exposure to ultraviolet radiation causes apoptosis in developing sea urchin embryos. *Journal of Experimental Biology,* 206**,** 4097-4103.

LI, C., CHEN, C., CHEN, L., CHEN, S., LI, H., ZHAO, Y., RAO, J. & ZHOU, X. 2016a. BDNF-induced expansion of cumulus-oocyte complexes in pigs was mediated by microRNA-205. *Theriogenology,* 85**,** 1476-1482.

LI, C., CHEN, S., LI, H., CHEN, L., ZHAO, Y., JIANG, Y., LIU, Z., LIU, Y., GAO, S., WANG, F., YU, J., WANG, H., RAO, J. & ZHOU, X. 2016b. MicroRNA-16 Modulates Melatonin-Induced Cell Growth in the Mouse-Derived Spermatogonia Cell Line GC-1 spg Cells by Targeting Ccnd1. *Biology of Reproduction,* 95**,** 57.

LI, L., ZHANG, X., ZHAO, L., XIA, X. & WANG, W. 2012. Comparison of DNA apoptosis in mouse and human blastocysts after vitrification and slow freezing. *Molecular Reproduction and Development,* 79**,** 229-236.

LI, Q., KANNAN, A., DEMAYO, F. J., LYDON, J. P., COOKE, P. S., YAMAGISHI, H., SRIVASTAVA, D., BAGCHI, M. K. & BAGCHI, I. C. 2011. The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. *Science,* 331**,** 912-916.

LI, Y., SHAN, Z., LIU, C., YANG, D., WU, J., MEN, C. & XU, Y. 2017. MicroRNA-294 Promotes Cellular Proliferation and Motility through the PI3K/AKT and JAK/STAT Pathways by Upregulation of NRAS in Bladder Cancer. *Biochemistry (Mosc),* 82**,** 474-482.

LIAN, J., TIAN, H., LIU, L., ZHANG, X. S., LI, W. Q., DENG, Y. M., YAO, G. D., YIN, M. M. & SUN, F. 2010. Downregulation of microRNA-383 is associated with male infertility and promotes testicular embryonal carcinoma cell proliferation by targeting IRF1. *Cell Death & Disease,* 1**,** e94.

LIANG, J., WANG, S. & WANG, Z. 2017. Role of microRNAs in embryo implantation. *Reproductive Biology and Endocrinology,* 15**,** 90.

LIAO, Y., WANG, J., JAEHNIG, E. J., SHI, Z. & ZHANG, B. 2019. WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Research,* 47**,** W199-W205.

LIN, F., LI, R., PAN, Z. X., ZHOU, B., YU, D. B., WANG, X. G., MA, X. S., HAN, J., SHEN, M. & LIU, H. L. 2012. miR-26b promotes granulosa cell apoptosis by targeting ATM during follicular atresia in porcine ovary. *PloS One,* 7**,** e38640.

LIN, X., BECKERS, E., MC CAFFERTY, S., GANSEMANS, Y., JOANNA SZYMAŃSKA, K., CHAITANYA PAVANI, K., CATANI, J. P., VAN NIEUWERBURGH, F., DEFORCE, D., DE SUTTER, P., VAN SOOM, A. & PEELMAN, L. 2019. Bovine Embryo-Secreted microRNA-30c Is a Potential Non-invasive Biomarker for Hampered Preimplantation Developmental Competence. *Frontiers in Genetics,* 10**,** 315.

LIU, W.-M., PANG, T. K. R., CHIU, C. N. P., WONG, P. C. B., LAO, K., LEE, K.-F. & YEUNG, S. B. W. 2012. Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proceedings of the National Academy of Sciences,* 109**,** 490-494.

LUJAMBIO, A., ROPERO, S., BALLESTAR, E., FRAGA, M. F., CERRATO, C., SETIÉN, F., CASADO, S., SUAREZ-GAUTHIER, A., SANCHEZ-CESPEDES, M., GIT, A., GITT, A., SPITERI, I., DAS, P. P., CALDAS, C., MISKA, E. & ESTELLER, M. 2007. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Research,* 67**,** 1424-1429.

LUO, Z., LIU, Y., CHEN, L., ELLIS, M., LI, M., WANG, J., ZHANG, Y., FU, P., WANG, K., LI, X. & WANG, L. 2015. microRNA profiling in three main stages during porcine spermatogenesis. *Journal of Assisted Reproduction and Genetics,* 32**,** 451-460.

MA, T., JIANG, H., GAO, Y., ZHAO, Y., DAI, L., XIONG, Q., XU, Y., ZHAO, Z. & ZHANG, J. 2011. Microarray analysis of differentially expressed microRNAs in non-regressed and regressed bovine corpus luteum tissue; microRNA-378 may suppress luteal cell apoptosis by targeting the interferon gamma receptor 1 gene. *Journal of Applied Genetics,* 52**,** 481-486.

MACKLON, N. S. & BROSENS, J. J. 2014. The human endometrium as a sensor of embryo quality. *Biology of Reproduction,* 91**,** 98.

MAKRI, D., EFSTATHIOU, P., MICHAILIDOU, E. & MAALOUF, W. E. 2020. Apoptosis triggers the release of microRNA miR-294 in spent culture media of blastocysts. *Journal of Assisted Reproduction and Genetics,* 37**,** 1685-1694.

MARSON, A., LEVINE, S. S., COLE, M. F., FRAMPTON, G. M., BRAMBRINK, T., JOHNSTONE, S., GUENTHER, M. G., JOHNSTON, W. K., WERNIG, M., NEWMAN, J., CALABRESE, J. M., DENNIS, L. M., VOLKERT, T. L., GUPTA, S., LOVE, J., HANNETT, N., SHARP, P. A., BARTEL, D. P., JAENISCH, R. & YOUNG, R. A. 2008. Connecting microRNA Genes to the Core Transcriptional Regulatory Circuitry of Embryonic Stem Cells. *Cell,* 134**,** 521-533.

MARTINEZ, R. M., LIANG, L., RACOWSKY, C., DIONI, L., MANSUR, A., ADIR, M., BOLLATI, V., BACCARELLI, A. A., HAUSER, R. & MACHTINGER, R. 2018. Extracellular microRNAs profile in human follicular fluid and IVF outcomes. *Scientific Reports,* 8**,** 1-10.

MASSIMIANI, M., LACCONI, V., LA CIVITA, F., TICCONI, C., RAGO, R. & CAMPAGNOLO, L. 2019. Molecular Signaling Regulating Endometrium-Blastocyst Crosstalk. *International Journal of Molecular Sciences,* 21**,** 23.

MATSUI, N., KAWANO, Y., NAKAMURA, S. & MIYAKAWA, I. 2004. Changes in vascular endothelial growth factor production associated with decidualization by human endometrial stromal cells in vitro. *Acta Obstetricia et Gynecologica Scandinavica,* 83**,** 138-143.

MCBRIDE, D., CARRÉ, W., SONTAKKE, S. D., HOGG, C. O., LAW, A., DONADEU, F. X. & CLINTON, M. 2012. Identification of miRNAs associated with the follicular-luteal transition in the ruminant ovary. *Reproduction,* 144**,** 221-233.

MCCALLIE, B., SCHOOLCRAFT, W. B. & KATZ-JAFFE, M. G. 2010. Aberration of blastocyst microRNA expression is associated with human infertility. *Fertility and Sterility,* 93**,** 2374-2382.

MCCUBBIN, N. I., MCCALLIE, B. R., PARKS, J. C., SCHOOLCRAFT, W. B. & KATZ-JAFFE, M. 2017. Disrupted sperm mirna expression profiles revealed a fingerprint of impaired spermatogenesis in oligozoospermia males. *Fertility and Sterility,* 108**,** e139-e139.

MCIVER, S. C., STANGER, S. J., SANTARELLI, D. M., ROMAN, S. D., NIXON, B. & MCLAUGHLIN, E. A. 2012. A Unique Combination of Male Germ Cell miRNAs Coordinates Gonocyte Differentiation. *PLoS One,* 7**,** e35553.

MENDELL, JOSHUA T. & OLSON, ERIC N. 2012. MicroRNAs in Stress Signaling and Human Disease. *Cell,* 148**,** 1172-1187.

MENEZO, Y. J. R. & HERUBEL, F. 2002. Mouse and bovine models for human IVF. *Reproductive BioMedicine Online,* 4, 170-175.

MENG, F.-L., WANG, W. & JIA, W.-D. 2014. Diagnostic and prognostic significance of serum miR-24-3p in HBV-related hepatocellular carcinoma. *Medical Oncology,* 31**,** 1-6.

MESEGUER, M., APLIN, J. D., CABALLERO-CAMPO, P., O'CONNOR J. E., MARTIN, J. C., REMOHI, J., PELLICER, A. & SIMON, C. 2001. Human endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the human blastocyst. *Biology of Reproduction,* 64, 590-601.

MESEGUER, M., HERRERO, J., TEJERA, A., HILLIGSØE, K. M., RAMSING, N. B. & REMOHÍ, J. 2011. The use of morphokinetics as a predictor of embryo implantation. *Human Reproduction,* 26**,** 2658-2671.

MILEWSKI, R., KUĆ, P., KUCZYŃSKA, A., STANKIEWICZ, B., ŁUKASZUK, K. & KUCZYŃSKI, W. 2015. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. *Journal of Assisted Reproduction and Genetics,* 32**,** 571-579.

MITCHELL, P. S., PARKIN, R. K., KROH, E. M., FRITZ, B. R., WYMAN, S. K., POGOSOVA-AGADJANYAN, E. L., PETERSON, A., NOTEBOOM, J., O'BRIANT, K. C., ALLEN, A., LIN, D. W., URBAN, N., DRESCHER, C. W., KNUDSEN, B. S., STIREWALT, D. L., GENTLEMAN, R., VESSELLA, R. L., NELSON, P. S., MARTIN, D. B. & TEWARI, M. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 10513-10518.

MOKÁNSZKI, A., MOLNÁR, Z., TÓTHNÉ, E. V., BODNÁR, B., JAKAB, A., BÁLINT, B. L. & BALOGH, I. 2019. Altered microRNAs expression levels of sperm and seminal plasma in patients with infertile ejaculates compared with normozoospermic males. *Human Fertility***,** 246-255.

MOMENI, A., NAJAFIPOUR, R., HAMTA, A., JAHANI, S. & MOGHBELINEJAD, S. 2020. Expression and Methylation Pattern of hsa-miR-34 Family in Sperm Samples of Infertile Men. *Reproductive Sciences,* 27**,** 301-308.

MOTATO, Y., DE LOS SANTOS, M. J., ESCRIBA, M. J., RUIZ, B. A., REMOHÍ, J. & MESEGUER, M. 2016. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertility and Sterility,* 105**,** 376-384.e9.

MOURIK, M. S. M., MACKLON, N. S. & HEIJNEN, C. J. 2009. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *Journal of Leukocyte Biology,* 85**,** 4-19.

MUMUSOGLU, S., OZBEK, I. Y., SOKMENSUER, L. K., POLAT, M., BOZDAG, G., PAPANIKOLAOU, E. & YARALI, H. 2017. Duration of blastulation may be associated with ongoing pregnancy rate in single euploid blastocyst transfer cycles. *Reproductive BioMedicine Online,* 35**,** 633-639.

NISHI, K., NISHI, A., NAGASAWA, T., UI-TEI, K. 2013. Human TNRC6A is an Argonaute-navigator protein for micro*RNA*-mediated gene silencing in the nucleus. *RNA,* 19,17-35.

NIU, Z., GOODYEAR, M. S., RAO, S., WU, X., TOBIAS, W. J., AVARBOCK, R. M. & BRINSTER, L. R. 2011. MicroRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences,* 108**,** 12740-12745.

NIXON, B., STANGER, S. J., MIHALAS, B. P., REILLY, J. N., ANDERSON, A. L., TYAGI, S., HOLT, J. E. & MCLAUGHLIN, E. A. 2015. The microRNA signature of mouse spermatozoa is substantially modified during epididymal maturation. *Biology of Reproduction,* 93**,** 91.

O'BRIEN, J., HAYDER, H., ZAYED, Y. & PENG, C. 2018. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Frontiers in Endocrinology,* 9**,** 402.

OHNISHI, Y., TOTOKI, Y., TOYODA, A., WATANABE, T., YAMAMOTO, Y., TOKUNAGA, K., SAKAKI, Y., SASAKI, H. & HOHJOH, H. 2010. Small RNA class transition from siRNA/piRNA to miRNA during pre-implantation mouse development. *Nucleic Acids Research,* 38**,** 5141-5151.

OTSUKA, M., ZHENG, M., HAYASHI, M., LEE, J.-D., YOSHINO, O., LIN, S. & HAN, J. 2008. Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. *Journal of Clinical Investigation,* 118**,** 1944-1954.

OU, Z., CHEN, Z., YIN, M., DENG, Y., LIANG, Y., WANG, W., YAO, Y. & SUN, L. 2020. Re-analysis of whole blastocysts after trophectoderm biopsy indicated chromosome aneuploidy. *Human Genomics,* 14**,** 3.

PAN, B., TOMS, D. & LI, J. 2018. MicroRNA-574 suppresses oocyte maturation via targeting hyaluronan synthase 2 in porcine cumulus cells. *American Journal of Physiology. Cell Physiology,* 314**,** C268-C277.

PAN-CASTILLO, B., GAZZE, S. A., THOMAS, S., LUCAS, C., MARGARIT, L., GONZALEZ, D., FRANCIS, L. W. & CONLAN, R. S. 2018. Morphophysical dynamics of human endometrial cells during decidualization. *Nanomedicine: Nanotechnology, Biology, and Medicine,* 14**,** 2235-2245.

POTHOF, J., VERKAIK, N., IJCKEN, W., WIEMER, E., TA, V., HORST, G., JASPERS, N., GENT, D., HOEIJMAKERS, J. & PERSENGIEV, S. 2009. MicroRNA-mediated gene silencing modulates the UV-induced DNA-damage response. *The EMBO Journal,* 28**,** 2090-2099.

QI, L., HONGJUAN, H., NING, G., ZHENGBIN, H., YANJIANG, X., TIEBO, Z., ZHIJUN, H. & QIONG, W. 2013. miR-370 is stage-specifically expressed during mouse embryonic development and regulates Dnmt3a. *FEBS Letters,* 587**,** 775-781.

QIU, Q., YANG, M., TSANG, B. K. & GRUSLIN, A. 2004. EGF-induced trophoblast secretion of MMP-9 and TIMP-1 involves activation of both PI3K and MAPK signalling pathways. *Reproduction,* 128**,** 355-363.

RADTKE, A., DIECKMANN, K. P., GROBELNY, F., SALZBRUNN, A., OING, C., SCHULZE, W. & BELGE, G. 2019. Expression of miRNA-371a-3p in seminal plasma and ejaculate is associated with sperm concentration. *Andrology,* 7**,** 469-474.

RED-HORSE, K., DRAKE, P. M., GUNN, M. D. & FISHER, S. J. 2001. Chemokine Ligand and Receptor Expression in the Pregnant Uterus. *The American Journal of Pathology,* 159**,** 2199-2213.

REZA, A. M. M. T., CHOI, Y.-J., HAN, S. G., SONG, H., PARK, C., HONG, K. & KIM, J.-H. 2019. Roles of microRNAs in mammalian reproduction: from the commitment of germ cells to peri-implantation embryos. *Biological Reviews of the Cambridge Philosophical Society,* 94**,** 415-438.

RIENZI, L., CIMADOMO, D., DELGADO, A., MINASI, M. G., FABOZZI, G., GALLEGO, R. D., STOPPA, M., BELLVER, J., GIANCANI, A., ESBERT, M., CAPALBO, A., REMOHÌ, J., GRECO, E., UBALDI, F. M. & MESEGUER, M. 2019. Time of morulation and trophectoderm quality are predictors of a live birth after euploid blastocyst transfer: a multicenter study. *Fertility and Sterility,* 112**,** 1080-1093.e1.

ROBB, L., DIMITRIADIS, E., LI, R. & SALAMONSEN, L. A. 2002. Leukemia inhibitory factor and interleukin-11: cytokines with key roles in implantation. *Journal of Reproductive Immunology,* 57**,** 129-141.

ROBB, L., LI, R., HARTLEY, L., NANDURKAR, H. H., KOENTGEN, F. & BEGLEY, C. G. 1998. Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. *Nature Medicine,* 4**,** 303-308.

ROMERO, Y., MEIKAR, O., PAPAIOANNOU, M. D., CONNE, B., GREY, C., WEIER, M., PRALONG, F., DE MASSY, B., KAESSMANN, H., VASSALLI, J.-D., KOTAJA, N. & NEF, S. 2011. Dicer1 Depletion in Male Germ Cells Leads to Infertility Due to Cumulative Meiotic and Spermiogenic Defects. *PLoS One,* 6**,** e25241.

ROSENBLUTH, E. M., SHELTON, D. N., SPARKS, A. E. T., DEVOR, E., CHRISTENSON, L. & VAN VOORHIS, B. J. 2013. MicroRNA expression in the human blastocyst. *Fertility and Sterility,* 99**,** 855-861.

ROSENBLUTH, E. M., SHELTON, D. N., WELLS, L. M., SPARKS, A. E. T. & VAN VOORHIS, B. J. 2014. Human embryos secrete microRNAs into culture media—a potential biomarker for implantation. *Fertility and Sterility,* 101**,** 1493-1500.

ROTKRUA, P., SHIMADA, S., MOGUSHI, K., AKIYAMA, Y., TANAKA, H. & YUASA, Y. 2013. Circulating microRNAs as biomarkers for early detection of diffuse-type gastric cancer using a mouse model. *British Journal of Cancer,* 108**,** 932-940.

RUBIO, C., RIENZI, L., NAVARRO-SÁNCHEZ, L., CIMADOMO, D., GARCÍA-PASCUAL, C. M., ALBRICCI, L., SOSCIA, D., VALBUENA, D., CAPALBO, A., UBALDI, F. & SIMÓN, C. 2019. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications. *Fertility and Sterility,* 112**,** 510-519.

SAADELDIN, I. M., OH, H. J. & LEE, B. C. 2015. Embryonic–maternal cross-talk via exosomes: potential implications. *Stem Cells and Cloning: Advances and Applications,* 8**,** 103-107.

SAKURAI, T., TAKAI, R., BÜRGIN, H., ISHIHARA, K., SAKAMOTO, Y., AMANO, J., HIGUCHI, Y., CHIBA, S., SINGER, T., KAWAMURA, A., SUZUKI, M. & MÜLLER, L. 2012. The effects of interleukin-6 signal blockade on fertility, embryo-fetal development, and immunization in vivo. *Birth Defects Research Part B, Developmental and Reproductive Toxicology,* 95**,** 304-317.

SALILEW-WONDIM, D., AHMAD, I., GEBREMEDHN, S., SAHADEVAN, S., HOSSAIN, M. D. M., RINGS, F., HOELKER, M., THOLEN, E., NEUHOFF, C., LOOFT, C., SCHELLANDER, K. & TESFAYE, D. 2014. The expression pattern of microRNAs in granulosa cells of subordinate and dominant follicles during the early luteal phase of the bovine estrous cycle. *PloS One,* 9**,** e106795.

SANG, Q., YAO, Z., WANG, H., FENG, R., WANG, H., ZHAO, X., XING, Q., JIN, L., HE, L., WU, L. & WANG, L. 2013. Identification of microRNAs in human follicular fluid: characterization of microRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. *The Journal of Clinical Endocrinology and Metabolism,* 98**,** 3068-3079.

SANTANGELO, A., IMBRUCÈ, P., GARDENGHI, B., BELLI, L., AGUSHI, R., TAMANINI, A., MUNARI, S., BOSSI, A. M., SCAMBI, I., BENATI, D., MARIOTTI, R., DI GENNARO, G., SBARBATI, A., ECCHER, A., RICCIARDI, G. K., CICERI, E. M., SALA, F., PINNA, G., LIPPI, G., CABRINI, G. & DECHECCHI, M. C. 2017. A microRNA signature from serum exosomes of patients with glioma as complementary diagnostic biomarker. *Journal of Neuro-Oncology,* 136**,** 51-62.

SANTONOCITO, M., VENTO, M., GUGLIELMINO, M. R., BATTAGLIA, R., WAHLGREN, J., RAGUSA, M., BARBAGALLO, D., BORZÌ, P., RIZZARI, S., MAUGERI, M., SCOLLO, P., TATONE, C., VALADI, H., PURRELLO, M. & DI PIETRO, C. 2014. Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. *Fertility and Sterility,* 102**,** 1751-1761.e1.

SAZONOVA, A., KÄLLEN, K., THURIN-KJELLBERG, A., WENNERHOLM, U.-B. & BERGH, C. 2013. Neonatal and maternal outcomes comparing women undergoing two in vitro fertilization (IVF) singleton pregnancies and women undergoing one IVF twin pregnancy. *Fertility and Sterility,* 99**,** 731-737.

SCALICI, E., TRAVER, S., MULLET, T., MOLINARI, N., FERRIÈRES, A., BRUNET, C., BELLOC, S. & HAMAMAH, S. 2016. Circulating microRNAs in follicular fluid, powerful tools to explore in vitro fertilization process. *Scientific Reports,* 6**,** 24976.

SCHMITTGEN, T. D. & LIVAK, K. J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols,* 3**,** 1101-1108.

SCHOOLCRAFT, W. B. M. D. 2016. Importance of embryo transfer technique in maximizing assisted reproductive outcomes. *Fertility and Sterility,* 105**,** 855-860.

SEN, A., PRIZANT, H., LIGHT, A., BISWAS, A., HAYES, E., LEE, H.-J., BARAD, D., GLEICHER, N. & HAMMES, R. S. 2014. Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. *Proceedings of the National Academy of Sciences,* 111**,** 3008-3013.

SHARMA, S., GODBOLE, G. & MODI, D. 2016. Decidual Control of Trophoblast Invasion. *American Journal of Reproductive Immunology,* 75**,** 341-350.

SHEN, G., WU, R., LIU, B., DONG, W., TU, Z., YANG, J., XU, Z. & PAN, T. 2014. Upstream and downstream mechanisms for the promoting effects of IGF-1 on differentiation of spermatogonia to primary spermatocytes. *Life Sciences,* 101**,** 49-55.

SHIMIZU, Y., KABIR-SALMANI, M., AZADBAKHT, M., SUGIHARA, K., SAKAI, K. & IWASHITA, M. 2008. Expression and localization of galectin-9 in the human uterodome. *Endocrine Journal,* 55**,** 879-887.

SIMON, L., SPIEWAK, K. A., EKMAN, G. C., KIM, J., LYDON, J. P., BAGCHI, M. K., BAGCHI, I. C., DEMAYO, F. J. & COOKE, P. S. 2009. Stromal progesterone receptors mediate induction of Indian Hedgehog (IHH) in uterine epithelium and its downstream targets in uterine stroma. *Endocrinology,* 150**,** 3871-3876.

SINGH, H. & APLIN, J. D. 2009. Adhesion molecules in endometrial epithelium: tissue integrity and embryo implantation. *Journal of Anatomy,* 215**,** 3-13.

SMALLEY, D. M. & LEY, K. 2005. L-selectin: mechanisms and physiological significance of ectodomain cleavage. *Journal of Cellular and Molecular Medicine,* 9**,** 255-266.

SMORAG, L., ZHENG, Y., NOLTE, J., ZECHNER, U., ENGEL, W. & PANTAKANI, D. V. K. 2012. MicroRNA signature in various cell types of mouse spermatogenesis: evidence for stage-specifically expressed miRNA-221, -203 and -34b-5p mediated spermatogenesis regulation. *Biology of the Cell,* 104**,** 677-692.

SOHEL, M. M. H., HOELKER, M., NOFERESTI, S. S., SALILEW-WONDIM, D., THOLEN, E., LOOFT, C., RINGS, F., UDDIN, M. J., SPENCER, T. E., SCHELLANDER, K., TESFAYE, D. & BUSSON, P. 2013. Exosomal and Non-Exosomal Transport of Extra-Cellular microRNAs in Follicular Fluid: Implications for Bovine Oocyte Developmental Competence. *PLoS One,* 8**,** e78505.

SONG, C., YAO, J., CAO, C., LIANG, X., HUANG, J., HAN, Z., ZHANG, Y., QIN, G., TAO, C., LI, C., YANG, H., ZHAO, J., LI, K. & WANG, Y. 2016. PPARγ is regulated by miR-27b-3p negatively and plays an important role in porcine oocyte maturation. *Biochemical and Biophysical Research Communications,* 479**,** 224-230.

SONG, H., LIM, H., DAS, S. K., PARIA, B. C. & DEY, S. K. 2000. Dysregulation of EGF family of growth factors and COX-2 in the uterus during the preattachment and attachment reactions of the blastocyst with the luminal epithelium correlates with implantation failure in LIF-deficient mice. *Molecular Endocrinology,* 14**,** 1147-1161.

SONTAKKE, S. D., MOHAMMED, B. T., MCNEILLY, A. S. & DONADEU, F. X. 2014. Characterization of microRNAs differentially expressed during bovine follicle development. *Reproduction,* 148**,** 271-283.

SUH, N., BAEHNER, L., MOLTZAHN, F., MELTON, C., SHENOY, A., CHEN, J. & BLELLOCH, R. 2010. MicroRNA Function Is Globally Suppressed in Mouse Oocytes and Early Embryos. *Current Biology,* 20**,** 271-277.

SVOBODA, P., FRANKE, V. & SCHULTZ, R. M. 2015. Sculpting the Transcriptome During the Oocyte-to-Embryo Transition in Mouse. *Current Topics in Developmental Biology,* 113**,** 305-349.

TANG, F., KANEDA, M., O'CARROLL, D., HAJKOVA, P., BARTON, S. C., SUN, Y. A., LEE, C., TARAKHOVSKY, A., LAO, K. & SURANI, M. A. 2007. Maternal microRNAs are essential for mouse zygotic development. *Genes & Development,* 21**,** 644-648.

TAQI, M. O., SAEED-ZIDANE, M., GEBREMEDHN, S., SALILEW-WONDIM, D., KHDRAWY, O., RINGS, F., NEUHOFF, C., HOELKER, M., SCHELLANDER, K. & TESFAYE, D. 2019. Sexual dimorphic expression and release of transcription factors in bovine embryos exposed to oxidative stress. *Molecular Reproduction and Development,* 86**,** 2005-2019.

TEKLENBURG, G., SALKER, M., MOLOKHIA, M., LAVERY, S., TREW, G., AOJANEPONG, T., MARDON, H. J., LOKUGAMAGE, A. U., RAI, R., LANDLES, C., ROELEN, B. A. J., QUENBY, S., KUIJK, E. W., KAVELAARS, A., HEIJNEN, C. J., REGAN, L., BROSENS, J. J. & MACKLON, N. S. 2010. Natural Selection of Human Embryos: Decidualizing Endometrial Stromal Cells Serve as Sensors of Embryo Quality upon Implantation. *PloS One,* 5**,** e10258.

TESFAYE, D., WORKU, D., RINGS, F., PHATSARA, C., THOLEN, E., SCHELLANDER, K. & HOELKER, M. 2009. Identification and expression profiling of microRNAs during bovine oocyte maturation using heterologous approach. *Molecular Reproduction and Development,* 76**,** 665-677.

TONG, M.-H., MITCHELL, D. A., MCGOWAN, S. D., EVANOFF, R. & GRISWOLD, M. D. 2012. Two miRNA clusters, Mir-17-92 (Mirc1) and Mir-106b-25 (Mirc3), are involved in the regulation of spermatogonial differentiation in mice. *Biology of Reproduction,* 86**,** 72.

TRAN, G. T., HODGKINSON, S. J., CARTER, N. M., VERMA, N. D., PLAIN, K. M., BOYD, R., ROBINSON, C. M., NOMURA, M., KILLINGSWORTH, M. & HALL, B. M. 2012. IL-5 promotes induction of antigen-specific CD4+CD25+ T regulatory cells that suppress autoimmunity. *Blood,* 119**,** 4441-4450.

TRIPURANI, S. K., XIAO, C., SALEM, M. & YAO, J. 2010. Cloning and analysis of fetal ovary microRNAs in cattle. *Animal Reproduction Science,* 120**,** 16-22.

TSAI, S.-J., WU, M.-H., CHEN, H.-M., CHUANG, P.-C. & WING, L.-Y. C. 2002. Fibroblast growth factor-9 is an endometrial stromal growth factor. *Endocrinology,* 143**,** 2715-2721.

TULAY, P. & SENGUPTA, S. 2016. MicroRNA expression and its association with DNA repair in preimplantation embryos. *Journal of Reproduction and Development,* 62**,** 225-234.

UHDE, K., VAN TOL, H. T. A., STOUT, T. A. E. & ROELEN, B. A. J. 2017. MicroRNA Expression in Bovine Cumulus Cells in Relation to Oocyte Quality. *Non-coding RNA,* 3**,** 12.

VAN DEN ABBEEL, E., BALABAN, B., ZIEBE, S., LUNDIN, K., CUESTA, M. J. G., KLEIN, B. M., HELMGAARD, L. & ARCE, J.-C. 2013. Association between blastocyst morphology and outcome of single-blastocyst transfer. *Reproductive BioMedicine Online,* 27**,** 353-361.

VANNUCCINI, S., CLIFTON, V. L., FRASER, I. S., TAYLOR, H. S., CRITCHLEY, H., GIUDICE, L. C. & PETRAGLIA, F. 2016. Infertility and reproductive disorders: impact of hormonal and inflammatory mechanisms on pregnancy outcome. *Human Reproduction Update,* 22**,** 104-115.

VASILEVA, A., TIEDAU, D., FIROOZNIA, A., MÜLLER-REICHERT, T. & JESSBERGER, R. 2009. Tdrd6 Is Required for Spermiogenesis, Chromatoid Body Architecture, and Regulation of miRNA Expression. *Current Biology,* 19**,** 630-639.

VASUDEVAN, S. & STEITZ, J. A. 2007. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. *Cell,* 128, 1105-1118.

VERMEESCH, J. R., VOET, T. & DEVRIENDT, K. 2016. Prenatal and pre-implantation genetic diagnosis. *Nature Reviews Genetics,* 17**,** 643-656.

VICHERA, G., MORO, L. N., BUEMO, C. & SALAMONE, D. 2014. DNA fragmentation, transgene expression and embryo development after intracytoplasmic injection of DNA–liposome complexes in IVF bovine zygotes. *Zygote,* 22**,** 195-203.

VILELLA, F., MORENO-MOYA, J. M., BALAGUER, N., GRASSO, A., HERRERO, M., MARTÍNEZ, S., MARCILLA, A. & SIMÓN, C. 2015. Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. *Development,* 142**,** 3210-3221.

VISWANATHAN, S. R., MERMEL, C. H., LU, J., LU, C. W., GOLUB, T. R. & DALEY, G. Q. 2009. microRNA Expression during Trophectoderm Specification. *Plos One,* 4**,** e6143.

WALKER, M. P., DIAUGUSTINE, R. P., ZERINGUE, E., BUNGER, M. K., SCHMITT, M., ARCHER, T. K. & RICHARDS, R. G. 2010. An IGF1/insulin receptor substrate-1 pathway stimulates a mitotic kinase (cdk1) in the uterine epithelium during the proliferative response to estradiol. *Journal of Endocrinology,* 207**,** 225-235.

WAN, G., MATHUR, R., HU, X., ZHANG, X. & LU, X. 2011. MicroRNA response to DNA damage. *Trends in Biochemical Sciences,* 36**,** 478-484.

WANG, L. & QIAN, L. 2014. miR-24 regulates intrinsic apoptosis pathway in mouse cardiomyocytes. *PLoS One,* 9**,** e85389.

WANG, S., ZHANG, R., CLARET, F. X. & YANG, H. 2014. Involvement of microRNA-24 and DNA methylation in resistance of nasopharyngeal carcinoma to ionizing radiation. *Molecular Cancer Therapeutics,* 13**,** 3163-3174.

WANG, Y., MEDVID, R., MELTON, C., JAENISCH, R. & BLELLOCH, R. 2007. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nature Genetics,* 39**,** 380-385.

WANG, Y., ZHOU, T., WAN, J. Y., YANG, Y., CHEN, X. J., WANG, J. Y., ZHOU, C., LIU, M. X., LING, X. F. & ZHANG, J. Q. 2016. Comparative transcriptome analysis reveals a regulatory network of microRNA-29b during mouse early embryonic development. *Oncotarget,* 7**,** 53772-53782.

WIJNHOVEN, B. P. L., MICHAEL, M. Z. & WATSON, D. I. 2007. MicroRNAs and cancer. *British Journal of Surgery,* 94**,** 23-30.

WINTER, J., JUNG, S., KELLER, S., GREGORY, R. I. & DIEDERICHS, S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nature Cell Biology,* 11**,** 228-234.

WU, M., YIN, Y., ZHAO, M., HU, L. & CHEN, Q. 2013. The low expression of leukemia inhibitory factor in endometrium: Possible relevant to unexplained infertility with multiple implantation failures. *Cytokine,* 62**,** 334-339.

WU, S., AKSOY, M., SHI, J. & HOUBAVIY, H. B. 2014. Evolution of the miR-290-295/miR-371-373 cluster family seed repertoire. *PloS One,* 9**,** e108519-e108519.

XI, C., YI, B., LIJIA, M., XING, C., YUAN, Y., KEHUI, W., JIGANG, G., YUJING, Z., JIANGNING, C., XING, G., QIBIN, L., XIAOYING, L., WENJING, W., YAN, Z., JIN, W., XUEYUAN, J., YANG, X., CHEN, X., PINGPING, Z., JUANBIN, Z., RUIQIANG, L., HONGJIE, Z., XIAOBIN, S., TING, G., GUANG, N., JUN, W., KE, Z., JUNFENG, Z. & CHEN-YU, Z. 2008. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Research,* 18**,** 997-1006.

XIE, R., LIN, X., DU, T., XU, K., SHEN, H., WEI, F., HAO, W., LIN, T., LIN, X., QIN, Y., WANG, H., CHEN, L., YANG, S., YANG, J., RONG, X., YAO, K., XIAO, D., JIA, J. & SUN, Y. 2016. Targeted Disruption of miR-17-92 Impairs Mouse Spermatogenesis by Activating mTOR Signaling Pathway. *Medicine,* 95**,** e2713.

XIONG, L., WENBI, Z., JING, F., YAN, X., RUIHUAN, G., RONGGUI, Q., LU, L., YIJUAN, S. & XIAOXI, S. 2019. MicroRNA-451 is downregulated in the follicular fluid of women with endometriosis and influences mouse and human embryonic potential. *Reproductive Biology and Endocrinology,* 17**,** 1-11.

XU, H., WANG, X., WANG, Z., LI, J., XU, Z., MIAO, M., CHEN, G., LEI, X., WU, J., SHI, H., WANG, K., ZHANG, T. & SUN, X. 2020. MicroRNA expression profile analysis in sperm reveals hsa-mir-191 as an auspicious omen of in vitro fertilization. *BMC Genomics,* 21**,** 165.

XU, L., YANG, B. F. & AI, J. 2013. MicroRNA transport: A new way in cell communication. *Journal of Cellular Physiology,* 228**,** 1713-1719.

XU, Y.-W., WANG, B., DING, C.-H., LI, T., GU, F. & ZHOU, C. 2011. Differentially expressed micoRNAs in human oocytes. *Journal of Assisted Reproduction and Genetics,* 28**,** 559-566.

YANG, Q., LIN, J., LIU, M., LI, R., TIAN, B., ZHANG, X., XU, B., LIU, M., ZHANG, X., LI, Y., SHI, H. & WU, L. 2016. Highly sensitive sequencing reveals dynamic modifications and activities of small RNAs in mouse oocytes and early embryos. *Science Advances,* 2**,** e1501482.

YANG, Q.-E., RACICOT, K. E., KAUCHER, A. V., OATLEY, M. J. & OATLEY, J. M. 2013a. MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. *Development,* 140**,** 280-290.

YANG, S., WANG, S., LUO, A., DING, T., LAI, Z., SHEN, W., MA, X., CAO, C., SHI, L., JIANG, J., RONG, F., MA, L., TIAN, Y., DU, X., LU, Y., LI, Y. & WANG, S. 2013b.

Expression patterns and regulatory functions of microRNAs during the initiation of primordial follicle development in the neonatal mouse ovary. *Biology of Reproduction,* 89**,** 126.

YAO, G., YIN, M., LIAN, J., TIAN, H., LIU, L., LI, X. & SUN, F. 2010. MicroRNA-224 is involved in transforming growth factor-beta-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4. *Molecular Endocrinology,* 24**,** 540-551.

YOUNG, S. L. 2013. Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. *Reproductive BioMedicine Online,* 27**,** 497-505.

YU, B., VAN TOL, H. T. A., STOUT, T. A. E. & ROELEN, B. A. J. 2020. Initiation of X Chromosome Inactivation during Bovine Embryo Development. *Cells,* 9**,** 1016.

YU, M., MU, H., NIU, Z., CHU, Z., ZHU, H. & HUA, J. 2014. miR-34c Enhances Mouse Spermatogonial Stem Cells Differentiation by Targeting Nanos2. *Journal of Cellular Biochemistry,* 115**,** 232-242.

YUAN, L., LI, S., ZHOU, Q., WANG, D., ZOU, D., SHU, J. & HUANG, Y. 2017. MiR-124 inhibits invasion and induces apoptosis of ovarian cancer cells by targeting programmed cell death 6. *Oncology Letters,* 14**,** 7311-7317.

YUAN, S., SCHUSTER, A., TANG, C., YU, T., ORTOGERO, N., BAO, J., ZHENG, H. & YAN, W. 2016. Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development. *Development,* 143**,** 635-647.

ZENG, B., LI, Z., CHEN, R., GUO, N., ZHOU, J., ZHOU, Q., LIN, Q., CHENG, D., LIAO, Q., ZHENG, L. & GONG, Y. 2012. Epigenetic regulation of miR-124 by Hepatitis C Virus core protein promotes migration and invasion of intrahepatic cholangiocarcinoma cells by targeting SMYD3. *FEBS Letters,* 586**,** 3271-3278.

ZERNICKA-GOETZ, M., MORRIS, S. A. & BRUCE, A. W. 2009. Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. *Nature Reviews, Genetics,* 10**,** 467-477.

ZHANG, H., JIANG, X., ZHANG, Y., XU, B., HUA, J., MA, T., ZHENG, W., SUN, R., SHEN, W., COOKE, H. J., HAO, Q., QIAO, J. & SHI, Q. 2014. microRNA 376a regulates follicle assembly by targeting Pcna in fetal and neonatal mouse ovaries. *Reproduction,* 148**,** 43-54.

ZHANG, J., WANG, Y., LIU, X., JIANG, S., ZHAO, C., SHEN, R., GUO, X., LING, X. & LIU, C. 2015. Expression and Potential Role of microRNA-29b in Mouse Early Embryo Development. *Cellular Physiology and Biochemistry,* 35**,** 1178-1187.

ZHANG, Q., SUN, H., JIANG, Y., DING, L., WU, S., FANG, T., YAN, G., HU, Y. & FRANKS, S. 2013a. MicroRNA-181a Suppresses Mouse Granulosa Cell Proliferation by Targeting Activin Receptor IIA. *PLoS One,* 8**,** e59667.

ZHANG, S., LIN, H., KONG, S., WANG, S., WANG, H., WANG, H. & ARMANT, D. R. 2013b. Physiological and molecular determinants of embryo implantation. *Molecular Aspects of Medicine,* 34**,** 939-980.

ZHAO, G., LIU, L., ZHAO, T., JIN, S., JIANG, S., CAO, S., HAN, J., XIN, Y., DONG, Q., LIU, X. & CUI, J. 2015. Upregulation of miR-24 promotes cell proliferation by targeting NAIF1 in non-small cell lung cancer. *Tumor Markers, Tumor Targeting and Translational Cancer Research,* 36**,** 3693-3701.

ZHENG, G. X. Y., RAVI, A., CALABRESE, J. M., MEDEIROS, L. A., KIRAK, O., DENNIS, L. M., JAENISCH, R., BURGE, C. B. & SHARP, P. A. 2011. A Latent Pro-Survival Function for the Mir-290-295 Cluster in Mouse Embryonic Stem Cells (A Novel Anti-Apoptotic Role for Mir-290-295). *PLoS Genetics,* 7**,** e1002054.

ZHU, L. & POLLARD, W. J. 2007. Estradiol-17β regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling. *Proceedings of the National Academy of Sciences,* 104**,** 15847-15851.

ZHU, R., WANG, S.-C., SUN, C., TAO, Y., PIAO, H.-L., WANG, X.-Q., DU, M.-R., DA-JIN, L. & SANCHEZ-MARGALET, V. 2013. Hyaluronan-CD44 Interaction

Promotes Growth of Decidual Stromal Cells in Human First-Trimester Pregnancy. *PLoS One,* 8**,** e74812.

ZOU, H., YU, D., DU, X., WANG, J., CHEN, L., WANG, Y., XU, H., ZHAO, Y., ZHAO, S., PANG, Y., LIU, Y., HAO, H., ZHAO, X., DU, W., DAI, Y., LI, N., WU, S. & ZHU, H. 2019. No imprinted XIST expression in pigs: biallelic XIST expression in early embryos and random X inactivation in placentas. *Cellular and molecular life sciences : CMLS,* 76**,** 4525-4538.

Online sources

HFEA (2020) *Fertility treatment 2018: trends and figures.* Available at: www.hfea.gov.uk/about-us/publications/research-and-data/fertility-treatment-2018-trends-and-figures/ (accessed 1 March 2021)

miRBase (2021). Available at: www.mirbase.org (accessed 1 March 2021)

UniProt (2021). Available at: www.uniprot.org (accessed 1 June 2021)

targetscan (2021) Available at: www.targetscan.org (2021) (accessed 1 March 2021)

WHO (2021) *Infertility*. Available at: www.who.int/news-room/fact-sheets/detail/infertility (accessed: 1 March 2021)

APPENDICES

A. Studies on miRNAs as non-invasive biomarkers of embryo quality and predictors of clinical outcomes.

Reference	Type of study	Aim	Species	Methodology	Sample size	Findings	Implications	Limitations
Kropp *et al.* (2014)	Experimental	a) To study whether miRNAs are released by bovine and human blastocysts b) To study whether released miRNAs play a role in bovine embryo development	bovine, human	i) IVP bovine embryos cultured in groups to the morula stage, compacted and degenerate embryos were further cultured in individual drops in SOF with BSA (Exp.A) or without (Exp.B). Blastocysts (B) and degenerate (D) embryos and their SCM were collected and analysed with qPCR for miR-25, -302c, -196A1, -181A1, -370 ii) human blastocyst SCM from individual drops were collected	Exp.A: pools of n=83 B and n=88 SCM, n=54 D embryos and n=69 SCM Exp.B: pools of n=39 B and n=39 SCM, n=40 D embryos and n=40 SCM Exp.C: pool of n=5 SCM from human blastocysts	i) D bovine embryos express higher levels of all miRNAs ($p<0.05$) except miR-370 ii) miR-25 is released by bovine and human embryos	Quantification of miRNAs secreted in SCM could be used for non-invasive embryo quality assessment	i) contamination of SCM from serum additives and proteins ii) analysis of pooled samples might not be reflective of the status of each individual embryo iii) 5 miRNAs tested iv) possible false discovery rate due to Ct threshold = 34

Rosenbluth *et al.* (2014)	Experimental	a) To study whether human blastocysts secrete miRNAs into SCM b) To study whether miRNAs in SCM reflect on embryo ploidy and can predict IVF outcomes	human	i) PN embryos cultured to blastocyst stage, SCM collected, TE biopsies analysed with aCGH, SCM analysed with TaqMan Low Density Arrays ii) PN embryos cultured to blastocyst stage, SCM collected, TE biopsies analysed with aCGH, SCM analysed with single assay qPCR for confirmation of identified miRNA (from Exp.1) and association with euploidy iii) SCM collected from day 4 and 5 eSET cycles, PCR quantification of identified miRNAs (Exp.1 and 2) and analysed based on pregnancy	i) n=18 TE biopsies, n=15 pooled SCM ii) n=17 TE biopsies, n=13 SCM pooled together with n=15 SCM from Exp.1 for confirmation iii) n=55 SCM	i) miR-372 and miR-191 released by human embryos, miR-645 present in control media but not in SCM ii) miR-191 highly released by aneuploid embryos ($p<0.05$, FC 4.7) iii) miR-191 and miR-372 in higher levels in SCM of day 5 ICSI vs IVF embryos ($p<0.05$, FC 4.4 and 7.1 respectively) but similar in day 4 SCM iv) miR-372 highly concentrated in day 5	MiRNAs in SCM are candidate markers of aneuploidy and clinical outcomes	i) miRNAs not associated with specific type of chromosomal aneuploidy ii) SCM contamination from added serum protein substitute iii) possible miRNA leakage from ICSI opening iv) possible false discovery rate due to Ct threshold = 38

				outcome, day of culture, and insemination method		SCM compared to day 4 (*p<0.05*, FC 5.4), miR-191 and miR-372 higher in day 5 SCM of ICSI embryos compared to day 4 (*p<0.05*, FC 1.9 and 12 respectively) v) miR-191, miR-645, miR-372 higher (*p<0.05*, FC 5.1, 6, 7.1 respectively) in day 5 SCM of IVF-inseminated embryos that failed to lead to a live birth (n=9 failed, n=18 live birth)		

Kropp and Khatib (2015)	Experimental	To characterise and identify miRNA in SCM of embryos of differing developmental competence	bovine	i) IVP bovine embryos cultured in groups to the morula stage, compacted and degenerate embryos were further cultured in individual drops in SOF without BSA, SCM from blastocysts (B) and degenerate (D) embryos were collected, pooled, and analysed with small RNA-Sequencing ii) miR-24 mimic was supplemented at the morula stage, embryos were collected and analysed for miR-24 and *CDKN1b* expression with qPCR	N=126 SCM from B and n=240 SCM from D	i) D embryos release higher levels ($p<0.05$) of 11 miRNAs (miR-2887, -24-3p, -22, -423-5p, -146a, -191, -2904, -286, -148a, -192, and uncharacterised PC-3p-21760); miR-24, -191, -148a were validated with qPCR ii) addition of the miR-24 mimic causes 27.3% decline in blastocyst formation ($p<0.0001$), the grown blastocysts have higher miR-24 levels (44.29 FC), and *CDKN1b* levels are repressed by 33%	i) sequencing is beneficial for the annotation of multiple miRNAs in relation to embryo development ii) miRNAs in SCM are potential biomarkers of embryo quality iii) miRNA mimics are useful to study roles in embryo development iv) miRNAs in the extra-cellular environment are up-taken by embryos	i) miRNA contamination even in media without serum supplementation ii) possible miRNA leakage from degenerate/lysed embryos iii) mimic addition cannot explain the reason miRNAs are secreted

						(*p<0.01*) in these embryos	and can cause mRNA changes- possible implications for media manufacturers	
Cuman *et al.* (2015)	Experimental	To identify miRNAs secreted by human embryos in relation to their implantation potential	human	i) human ICSI embryos were cultured to the blastocyst stage, SCM were collected and pooled in groups from implanted and non-implanted embryos and analysed with qPCR miRNA array ii) fluorescein-tagged miR-661 mimic was transfected to trophoblast cells, the conditioned media (CM) was collected, exosomes in CM	pooled n=8 SCM samples from implanted and n=8 samples from non-implanted; n=10 individual SCM samples were also analysed to verify results	i) 47 miRNAs are exclusively detected in SCM, 22 solely in control media, 19 miRNAs secreted by the implanted group, 22 miRNAs from the non-implanted group ii) miR-661 is the highest differentially expressed miRNA in the non-implanted SCM	i) miRNAs in SCM can be used for prediction of implantation, specifically for miR-661 as predictor of implantation failure ii) embryo-secreted miRNAs are internalised by endometrial cells	i) possible miRNA leakage from ICSI opening ii) contamination in control media iii) information only about one miRNA while the other miRNAs were not investigated further, *in vivo* conditions might differ as miRNAs form complex networks

				were isolated with ultracentrifugation and transferred in cultures of human endometrial epithelial cells (hEECs) iii) *in silico* analysis was carried out to identify mRNA targets iv) hEECs were analysed for mRNA expression changes v) spheroid adhesion assay was performed using hEECs and miR-661 mimic and inhibitor		(verified by single assay qPCR) and verified by analysis of the 10 individual SCM samples (5 implanted, 5-non-implanted) iii) increased up-take of miR-661 from hEECs iv) *MTA2* and *PVRL1* are downregulated in hEECs by miR-661 v) miR-661 blocks adhesion in hEECs via PVRL1 regulation	suggesting roles in embryo-maternal communication iii) internalised miRNAs cause transcriptomic changes in endometrial cells which can change their competence to establish implantation iv) miRNAs in the embryo-maternal interface can affect the initial adhesion of the blastocyst to the uterus	iv) implantation outcome might be affected by patient characteristics v) 784 miRNAs studied

Capalbo *et al.* (2016)	Prospective cohort	To assess whether miRNAs in SCM can be used as embryonic biomarkers	human	i) spare embryos were cultured to the blastocyst stage, TE was biopsied, SCM were collected, the TE and SCM samples analysed with TaqMan Low Density miRNA Array, miRNA profiles were compared between TE and SCM and results cross-validated with single assays ii) SCM from cleavage, morula, and expanded blastocysts were collected from SET IVF cycles, TE biopsies and 24-chromosome screening was performed iii) SCM were collected from euploid implanted blastocysts	i) n=5 TE and n=5 SCM samples ii) n=3 SCM from the same embryos in each stage iii) SCM from n=25 implanted and n=28 non-implanted	i) 96.6% concordance rate in miRNA profiles between SCM and TE, mRNA targets in embryo implantation, apoptosis, cell proliferation, communication, and differentiation ii) cleavage and morula stages similar to blank controls, SCM from blastocysts consistently expressing 34 miRNAs iii) miR-20a and miR-30c higher in SCM from implanted blastocysts ($p<0.05$),	i) origin of miRNAs in SCM is mainly the TE ii) miRNAs can be consistently detected in SCM only after blastulation iii) specific miRNAs in SCM can be predictive of positive implantation outcome, specifically miR-20a and miR-30c	i) small sample size ii) blank samples showed consistent amplification for few miRNAs iii) pooled samples, although authors tested for biological variation between distinct SCM samples iv) patient characteristics are possible confounders of implantation v) potentially false positive signals (Ct threshold = 35) iv) 381 miRNAs studied

				and non-implanted embryos and miRNA profiles were compared iv) *in silico* target prediction was carried out		gene targets involved in cell adhesion, communication, signalling, and growth		
Borges *et al.* (2016)	Prospective cohort	To find whether miRNAs in SCM can be used as biomarkers of implantation	human	ICSI-fertilised embryos cultured to day 5 and transferred, SCM collected on day 3 and analysed only for blastocysts that were transferred, samples were split in pools according to implantation outcome and 7 miRNAs analysed with single TaqMan assays (qPCR)	n=18 SCM from positive implantation group and n=18 from negative implantation group	4/7 miRNAs detected in SCM (miR-21, miR-19b, miR-92a, miR-142-3p) miR-142-3p highly expressed in the negative implantation group ($p<0.001$)	miR-142-3p is potential biomarker of implantation failure	i) small case basis ii) pooled samples iii) 7 miRNAs studied iv) possible miRNA leakage from ICSI opening v) SCM samples collected on day 3 when miRNA release is minimum
Lin *et al.* (2019)	Experimental	To investigate if miRNAs in SCM have potential as	bovine	i) IVP bovine embryos cultured individually to blastocyst stage, embryos divided in 3 groups	i) n=167 embryos/replicate, 3	i) FB and FD group excluded due to low concentration	i) miRNAs in SCM can be used as biomarkers of	i) low sample size (3 replicates/group)

		non-invasive biomarkers for pre-implantation developmental competence		according to the first cleavage pattern in “fast” (F), “intermediate” (I), and “slow” (S) groups then further divided in 2 sub-groups in degenerate embryos (D) and blastocysts (B) making 6 groups in total: FB, IB, SB, FD, ID, SD, SCM collected and pooled according to group, miRNAs analysed with small RNA-Sequencing ii) miR-30c mimic supplemented to embryo culture, embryos cultured to blastocyst stage, collected for qPCR, WB, or stained for apoptosis with TUNEL	replicates from each group were analysed	ii) I embryos produce significantly more blastocysts than S embryos i) 294 miRNAs present in SCM, miR-30c and miR-10b higher in S compared to I ($p<0.001$), miR-10b, miR-45 higher in D than in B SCM ($p<0.001$), miR-113 and miR-139 higher in B than in D SCM ($p<0.001$) – results confirmed with intra-cellular analysis	developmental potential ii) miR-10b is a candidate biomarker of poor developmental potential and miR-30c is a potential marker of impaired development and extended apoptosis	ii) low miRNA concentration in SCM for sequencing iii) pooled samples iv) mimic supplementation does not explain the mechanism behind miR-30c release

						ii) embryo development, signalling, and apoptosis pathways affected by the differentially expressed miRNAs between IB and SB iii) miR-30c is up-taken by embryos and increases apoptosis at the blastocyst stage by 8%, blastocyst formation rate is similar iv) miR-30c downregulates *CDK12* which alters DDR		

						pathways as confirmed by qPCR and WB		

SCM: spent culture media, PN: pronuclear, aCGH: array comparative genome hybridisation, TE: trophectoderm, (e)SET: (elective) single embryo transfer, FC: fold change, SOF: synthetic oviductal fluid, BSA: bovine serum albumin, WB: western blotting

B. TaqMan probes used for miRNA amplification. The assay name, miRNA accession number and the mature miRNA sequence are given for each target miRNA.

TaqMan miRNA probes			
Target	**Assay name**	**MiRBase accession number**	**Mature miRNA sequence (5'-3')**
miR-291a	mmu-mir-291a-3p	MIMAT0000368	AAAGUGCUUCCACUUUGUGUGC
miR-294	mmu-mir-294-3p	MIMAT0000372	AAAGUGCUUCCCUUUUGUGUGU
miR-24	hsa-miR-24-3p	MIMAT0000080	UGGCUCAGUUCAGCAGGAACAG
miR-124	mmu-mir-124-3p	MIMAT0000134	UAAGGCACGCGGUGAAUGCC
let-7b	hsa-let-7b-5p	MIMAT0000063	UGAGGUAGUAGGUUGUGUGGUU
let-7b	hsa-let-7b-3p	MIMAT0004482	CUAUACAACCUACUGCCUUCCC
miR-320a	hsa-miR-320a-3p	MIMA0000510	AAAAGCUGGGUUGAGAGGGCGA
miR-193	mmu-miR-193b-5p	MIMAT0017271	CGGGGUUUUGAGGGCGAGAUGA
miR-34c	mmu-miR-34c-5p	MIMAT0000381	AGGCAGUGUAGUUAGCUGAUUGC
miR-122	mmu-miR-122-5p	MIMAT0000246	UGGAGUGUGACAAUGGUGUUUG
miR-26a	mmu-miR-26a-5p	MIMAT0000533	UUCAAGUAAUCCAGGAUAGGCU
miR-10a	mmu-miR-10a-5p	MIMAT0000648	UACCCUGUAGAUCCGAAUUUGUG
miR-103a	hsa-miR-103a-3p	MIMAT0000101	AGCAGCAUUGUACAGGGCUAUGA
miR-17	mmu-miR-17-5p	MIMAT0000649	CAAAGUGCUUACAGUGCAGGUAG
U6 snRNA*	hsa-u6snra	N/A	~150 nucleotides

*Although not a miRNA, U6 small nuclear RNA has often been used as a reference RNA in published literature and was examined as a potential normaliser of the miRNA expression in this study.

C. Presence of miRNAs in KSOM culture medium without embryo culture and spent culture medium where embryos were cultured for 5 days.

miRNA	**KSOM**	**SCM**
miR-291a-3p	-	+
miR-294-3p	-	+
miR-24-3p	-	+
miR-124-3p	-	+
let-7b-5p	+	N/A
let-7b-3p	+	N/A
miR-320a-3p	+	N/A
miR-193b-5p	-	-
miR-34c-5p	-	-
miR-122-5p	-	-
miR-26a-5p	-	-
miR-10a-5p	-	-
miR-103a-3p	+	N/A
miR-17-5p	-	-
U6 snRNA	-	-