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Characterisation of stem cell-specific miRNA expression in chicken PGCs and rabbit preimplantation embryos

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TITTLE: CHARACTERISATION OF STEM CELL-SPECIFIC MIRNA EXPRESSION IN CHICKEN PGCS AND RABBIT PREIMPLANTATION EMBRYOS

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List of abbreviations

AGO – Argonaute protein
ANOVA – Analysis of Variance
AKT1 – AKT Serine/Threonine Kinase 1
ART – Assisted Reproductive Technology
ASH1L – ASH1 Like Histone Lysine Methyltransferase
ATM – Ataxia Telangiectasia Mutated
ATR – Ataxia Telangiectasia and Rad3-related

BMP – Bone Morphogenetic Protein
BNIP3L (NIX) – BCL2 Interacting Protein 3 Like
BSA – Bovine Serum Albumin

$CaCl_2$ – Calcium Chloride
CCNG2 – Cyclin G2
cDNA – Complementary DNA
CDKN1A (p21) – Cyclin-Dependent Kinase Inhibitor 1A
CHD1-Z – Chromodomain Helicase DNA-binding protein 1 (Z chromosome)
CHD1-W – Chromodomain Helicase DNA-binding protein 1 (W chromosome)
circRNA – Circular RNA
CO_2 – Carbon Dioxide
Ct – Threshold Cycle

DAZL – Deleted in Azoospermia-Like
DDX4 – DEAD-box Helicase 4 (VASA)
DKK1 – Dickkopf WNT Signaling Pathway Inhibitor 1
DGCR8 – DiGeorge Syndrome Critical Region 8
DMEM – Dulbecco's Modified Eagle Medium
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic Acid
dpc– days post coitum

ENA – European Nucleotide Archive
eRNA – Enhancer RNA
EMT – Epithelial–Mesenchymal Transition
ERK – Extracellular Signal-Regulated Kinase
ESC – Embryonic Stem Cell

FACs medium – Avian-KO-DMEM-based medium for FACS-sorted PGC culture
FACS – Fluorescence-Activated Cell Sorting
FAO – Fatty Acid Oxidation
FGF – Fibroblast Growth Factor
FGF2 – Fibroblast Growth Factor 2
FGFR – Fibroblast Growth Factor Receptor

GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase
GPI3 – Glucose-6-Phosphate Isomerase 3
GSK3β – Glycogen Synthase Kinase 3 Beta

H3K4me3 – Trimethylation of Histone H3 at Lysine 4
H3K27me3 – Trimethylation of Histone H3 at Lysine 27
HSC – Hematopoietic Stem Cell

ICM – Inner Cell Mass
IL-6 – Interleukin-6
iPSC / iPS – Induced Pluripotent Stem Cell
ITS – Insulin–Transferrin–Selenium
IVF – *In Vitro* Fertilization

JAK1 – Janus Kinase 1
JAK2 – Janus Kinase 2

KO-DMEM – Knock-Out Dulbecco's Modified Eagle Medium

LATS2 – Large Tumor Suppressor Kinase 2
LEF – Lymphoid Enhancer Factor
LEFTY – Left-Right Determination Factor (LEFTY1 / LEFTY2)
LIF – Leukemia Inhibitory Factor
LIFR – Leukemia Inhibitory Factor Receptor
lncRNA – Long Non-Coding RNA

MAPK – Mitogen-Activated Protein Kinase
MBD2 – Methyl-CpG Binding Domain Protein 2
MEF – Mouse Embryonic Fibroblast
MEK – MAPK/ERK Kinase, Mitogen-Activated Protein Kinase
miRNA – MicroRNA
miRISC – MicroRNA-Induced Silencing Complex
mRNA – Messenger RNA
MMP14 – Matrix Metallopeptidase 14 (Membrane-Type 1 Matrix Metalloproteinase, MT1-MMP)

NANOG – Nanog Homeobox Transcription Factor
ncRNA – Non-Coding RNA
NEAA – Non-Essential Amino Acids
NFκBIB – Nuclear Factor Kappa B Inhibitor Beta

OCT4 (POU5F1) – Octamer-Binding Transcription Factor 4
OxPhos – Oxidative Phosphorylation

P2– Second culture passage
PCR – Polymerase Chain Reaction
PGC – Primordial Germ Cell
PGTA – Preimplantation Genetic Testing for Aneuploidy
PI3K – Phosphoinositide 3-Kinase
PMAIP1 (NOXA) – Phorbol-12-Myristate-13-Acetate-Induced Protein 1
pSTAT3 – Phosphorylated Signal Transducer and Activator of Transcription 3
piRNA– Piwi-interacting RNA
PRC2 – Polycomb Repressive Complex 2
pri-miRNA – Primary MicroRNA Transcript

qPCR – Quantitative Polymerase Chain Reaction
qRT-PCR – Quantitative Reverse Transcription Polymerase Chain Reaction

Raf – Rapidly Accelerated Fibrosarcoma kinase
RBL2 – Retinoblastoma-Like Protein 2
REF – Rabbit Embryonic Fibroblast
RISC – RNA-Induced Silencing Complex
RLC – RISC-Loading Complex
RNA – Ribonucleic Acid
RNase – Ribonuclease
RT – Reverse Transcription

SCF – Stem Cell Factor
SD – Standard Deviation
siRNA – Small Interfering RNA
SMAD – Small Mothers Against Decapentaplegic proteins
SNO RNA (snoRNA) – Small Nucleolar RNA
SOM – Self-Organising Map
SOX2 – SRY-Box Transcription Factor 2
SOX11 – SRY-Box Transcription Factor 11
SRA – Sequence Read Archive
STAT3 – Signal Transducer and Activator of Transcription 3

TAE – Tris-Acetate-EDTA Buffer
TβR-I – Transforming Growth Factor Beta Receptor Type I
TβR-II – Transforming Growth Factor Beta Receptor Type II
TCA cycle – Tricarboxylic Acid Cycle
TCF – T-Cell Factor
TET1/2/3 – Ten-Eleven Translocation Enzymes
TGF-β – Transforming Growth Factor Beta
tRNA – Transfer RNA
TP53INP1 – Tumor Protein p53 Inducible Nuclear Protein 1

UTR – Untranslated Region

WNT – Wingless/Integrated Signalling Protein

ZMAT3 (WIG-1) – Zinc Finger Matrin-Type 3

1. INTRODUCTION AND OBJECTIVES

1.1. Importance of the field

microRNAs (miRNAs) are powerful post-transcriptional regulators capable of influencing cell fate decisions, cell proliferation, differentiation, and apoptosis. Their involvement in controlling embryonic development and maintaining stem cell properties highlights their potential as key molecular determinants of pluripotency and self-renewal.

This thesis is based on two main approaches. First, miRNA expression will be investigated in chicken primordial germ cells (PGCs) before and after cryopreservation in order to evaluate cellular resilience under stressful conditions. Second, rabbit embryos will be studied across different developmental stages, with particular attention to the presence of miRNAs in the culture medium and their potential relevance for embryo assessment.

The investigation of miRNAs in chicken primordial germ cells (PGCs), with particular emphasis on their potential role in modulating cellular stress responses under unfavorable conditions, may provide important insights into the molecular mechanisms underlying early developmental processes. Moreover, it may facilitate the identification and characterization of specific miRNA functions involved in germ cell specification in chickens.

Furthermore, the study of miRNAs has broader biomedical significance. In the rabbit model, this research may contribute to the development of alternative strategies for embryo quality assessment. Infertility is an increasingly important global health problem. According to the World Health Organization, around 17.5% of adults worldwide experience infertility at some point in their reproductive life, indicating that infertility is becoming a common condition and public health concern. Assisted reproductive technologies (ART) have become increasingly important in reproductive medicine. ART includes a range of laboratory and clinical procedures designed to help achieve pregnancy by handling oocytes, sperm, or embryos outside the body. Among these techniques, *in vitro* fertilization (IVF) is the best known and most widely used. In IVF, oocytes are retrieved from the ovaries, fertilized with sperm in the laboratory, and the resulting embryo is transferred into the uterus (Njagi et al., 2023). IVF is now a major contributor to births in several countries, showing how important it has become in modern fertility care. In the United States, the American Society for Reproductive Medicine reported that 432,641 IVF cycles were performed in 2023, leading to 95,860 babies, which accounted for 2.6% of all U.S. births. In the United Kingdom, the HFEA reported that IVF contributed to about 1 in 32 births in 2023. These figures show that IVF is no longer a marginal intervention, but a substantial part of contemporary reproductive healthcare (Fertility treatment 2023, 2025; Tipton & Hovey, 2025). The effectiveness of IVF has improved over time, but the success remains limited by biological and clinical factors. One of the major unresolved problems in IVF is embryo assessment and selection. In routine practice, embryos are usually selected for transfer based on their developmental stage and morphological grading. Although morphology assessment is widely used and standardized, its ability to predict implantation and live birth is still imperfect and generates emotional expectations, waste of biological resources and time. More advanced approaches such as preimplantation genetic testing for aneuploidy (PGT-A) may provide additional information, but they are more expensive and involve embryo biopsy, making them partly invasive and still controversial for routine use in all patients. These limitations create a major challenge in IVF: clinicians want to identify the embryo with the highest developmental potential while avoiding the transfer of multiple embryos or losing biological resources (Coticchio et al., 2025). For this reason, there is growing interest in non-invasive biomarkers that could improve embryo selection (Caponnetto et al., 2025).

Within this context, the present study aims to contribute to the understanding of the dynamic relationship between miRNA expression patterns, stem cell behaviour, and embryo development. Such insights may advance the fields of developmental biology and reproductive biotechnology and may also support future applications in embryo assessment, disease modelling, and regenerative medicine.

1.2.Objectives

To conduct a bioinformatic analysis of embryonic stem cell (ESC)-associated miRNA clusters in the rabbit and chicken genomes
The aim was to examine how these miRNA clusters are organised within the genome, how well they are conserved between species, and how effectively their expression patterns can be used as molecular signatures. In particular, this analysis sought to determine whether specific miRNA profiles could reliably distinguish between different developmental stages, stress conditions, and embryo types based on miRNA expression.

To analyse the miRNA expression profile in chicken primordial germ cells (PGCs)
The first objective of this study was to characterise the miRNA expression profile of chicken primordial germ cells under standard culture conditions and following cryopreservation-induced stress. This analysis aimed to identify miRNAs involved in stress responses, pluripotency maintenance, and post-thaw cellular recovery, thereby elucidating miRNA-mediated regulatory mechanisms influencing PGC viability and function.

To characterise miRNA expression patterns in rabbit preimplantation embryos
This objective aimed to investigate the miRNA expression profiles of rabbit preimplantation embryos across selected developmental stages, including both intracellular and extracellular miRNA populations. Particular emphasis was placed on identifying developmentally regulated miRNAs and evaluating their secretion into the culture medium as potential non-invasive biomarkers of embryo quality and developmental competence.

2. LITERATURE REVIEW

2.1.Stem cells

In living organisms, stem cells are unspecialised cells capable of differentiating into any cell of the organism (potency) and renewing itself (proliferate in an undifferentiated state) (Zakrzewski et al., 2019).

Stem cells can be categorised as embryonic stem cells, induced pluripotent stem cells, and nonembryonic or somatic stem cells which are known as adult stem cells (Bartold & Ivanovski, 2022; Poliwoda et al., 2022).

2.1.1. Stem cell potency and plasticity

Potency is a term which refers to the stem cell's potential to give rise to several differentiated cells. The stem cell potency can vary according to the tissue type and the origin and necessity of specific stem cell types. Stem cells in animal systems can be either totipotent, pluripotent or multipotent (Martinez-Agosto et al., 2007; A. Singh et al., 2015).

Plasticity is related mostly to adult stem cells, which is the capacity to acquire different identities and functions beyond their typical lineage, especially in response to specific signals during development, tissue homeostasis and regeneration by cell dedifferentiation or cell trans differentiation (Cammareri & Myant, 2023). For example, hematopoietic stem cells in normal conditions, generate blood cells, nevertheless, under different conditions, hematopoietic stem cells have shown plasticity by differentiating into neurons, liver cells, and muscle cells (Camargo et al., 2004; Merrell & Stanger, 2016; Strauss et al., 2012).

In unfavourable conditions, cell plasticity allows healthy cells to adopt new phenotypic and functional features by transiting across distinct cell states that contribute to tumour initiation, progression, metastasis and resistance to therapy. Understanding cell plasticity and its molecular mechanisms in cancer can be beneficial for discovering new, efficient cancer treatments (Pérez-González et al., 2023).

2.1.2. Embryonic stem cell source

Fecundation is the beginning of numerous cell divisions where embryonic stem cells are generated. Stem cells are undifferentiated cells, which are highly important for the embryo development and long-term maintenance of functional tissue types in any living organism by self-renewal, commitment to differentiate, and senescence or cell death (Martinez-Agosto et al., 2007).

A fertilized egg is considered the ultimate stem cell. In an early stage, each cell keeps totipotency and each single cell can give rise to progeny (Sell, 2013).

Totipotency as a concept was proposed by the Austrian botanist Gottlieb Haberlandt to describe the ability of a cell to develop into any type of cell, including embryonic and extra-embryonic tissues. Totipotency describes the potency to generate the whole animal (Niwa, 2009; M. B. Singh & Bhalla, 2006)

During early blastulation, the mitotically dividing cells are called progenitors. Progenitors are pluripotent cells which can start all lineages of the somatic cell type of a mature organism.

Pluripotency describes the ability to differentiate into a variety of cells that belong to all three germ layers, with at least one cell type for each. In this stage, two layers of cells can be differentiated trophectoderm, which will generate the trophoblast and the inner cell mass, or ICM, which retains pluripotency until the next stage of development (Niwa, 2009; Sell, 2013). There are two distinct states of pluripotent stem cells characterized by differences in their developmental potential and epigenetic profiles: Naïve and primed pluripotent stem cells. Embryonic Stem cells or ESC are derived from the inner cell mass of the preimplantation blastocyst and are considered Naïve pluripotent stem cells (Hassani et al., 2019).

During the early gastrula stage, progenitors continuously divide, generating primary germinal layers. The cells in the blastocyst's outer cell layer or trophoblast become specific for extraembryonic structures such as the placenta and extra-embryonic tissues. The ICM differentiates into two major layers: the epiblast and the hypoblast. The hypoblast forms extraembryonic tissues, as well (Sheng, 2015).

Epiblast-derived stem cells or EpiSCs rise from the post-implantation epiblast and are considered primed pluripotent stem cells (S. Takahashi et al., 2018). Other layers from the epiblast are formed called ectoderm, endoderm, and mesoderm, which are mostly multipotent cells (Martinez-Agosto et al., 2007).

Multipotency is the ability to yield multiple cell types that belong to specific germ layers, not all three (Niwa, 2009). Ectoderm further differentiates to skin and brain; endoderm to the gastrointestinal tract and internal organs; and mesoderm to connective tissue, bone, blood vessels, and blood-forming tissue (Sell, 2013; Williams & Bhatia, 2014).

Naïve pluripotent stem cells maintain a high developmental potential and can contribute to the formation of chimeric embryos. Naïve cells typically exhibit a round, dome-shaped morphology. In addition, naïve cell cultures do not require external signalling like Activin or FGF (Fibroblast growth factors), which distinguishes them from primed states (S. Takahashi et al., 2018).

Primed pluripotent stem cells exhibit a more limited developmental potential compared to naïve stem cells. The gene expression profiles of primed cells show distinct differences compared to naïve cells, showing epigenetic limitations in reprogramming to transition back to a naïve state. Moreover, chimeric embryos cannot be formed from primed pluripotent stem cells, and the maintenance of primed cell culture requires Activin and FGF to activate specific pathways (S. Takahashi et al., 2018).

2.1.3. Germinal Stem cells source

During early embryogenesis, just a few cells are destined to become germinal cells and migrate into the gonad to form egg or sperm. Consequently, these cells are going to have female chromosomes or male chromosomes (Sell, 2013). A germ cell alone are considered as unipotent but after fusion with another germ cell (fecundation) a totipotent cell is formed (Mummery et al., 2021).

2.1.4. Adult stem cells

Adult stem cells refer to tissue-resident undifferentiated cells. Adult stem cells are responsible for tissue homeostasis maintenance, tissue growth and replacement of differentiated cells that die in tissue damage (Ahsan et al., 2008; Loya, 2014; Mills et al., 2019).

The first adult stem cells were found in bone marrow in 1961. These cells were capable of producing different types of blood cells, which led to the identification of hematopoietic stem cells (HSCs) responsible for blood cell formation. Stem cell niches are specialized microenvironments found within tissues and organs. These vascular niches (regions with blood vessels) provide signals that regulate: Cell renewal (self-replication of stem cells) and Cell differentiation (conversion into specialized cell types). The origin of the niche (tissue type) influences which cell type the stem cell will become. This is why adult stem cells have limited differentiation potential; they are considered multipotent, meaning they can only turn into a specific set of related cell types.

However, some studies suggest an unexpected plasticity, some adult stem cells can differentiate into unrelated cell types under certain conditions (Hansen & Inselman, 2011; Utikal et al., 2015).

2.1.5. Induced pluripotent stem cell

Induced pluripotent stem (iPS) cells are a type of pluripotent stem similar to embryonic pluripotent stem cells, derived from adult somatic. Years ago, adult cells were thought to keep their differentiated characteristics under stable conditions. Differentiated cells have in their genetic material dormant genes that are associated with pluripotency, several pluripotency-associated transcription factors, including OCT4 (encoded by *POU5F1*), NANOG, LIN28, and members of the SOX family (notably SOX2), have been shown to reverse cellular differentiation, a reversible process called reprogramming, resulting in induced pluripotent cells. The absence of the previous factors makes induction impossible (A. Singh et al., 2015; Smith, 2009; Yamanaka, 2020; Ye et al., 2013).

iPSC has given a novel understanding of cell biology, disease modelling, drug discovery and regenerative medicine, avoiding bioethical controversy. Nevertheless, iPSC still faces challenges: during reprogramming, some mutations are accumulated in the somatic cell, and those mutations are present as well in iPSC after reprogramming. Other challenges regarding reprogramming efficiency, iPSC behaviour in scalation, immune compatibility, and application are still being investigated (Moy et al., 2023; Samruan et al., 2020).

2.2.Experimental stem cell species

Despite their significant contributions to scientific inquiry, the use of animals in research remains a controversial topic due to ethical considerations. To address these concerns, various organizations advocate for the implementation of the 3Rs (Replacement, Reduction, and Refinement) at upholding high standards of animal care while conducting high-quality research. Concurrently, alternative methodologies are being explored, such as the utilization of stem cells, to further minimize the ethical implications related to animal use in research (Workman et al., 2010).

2.2.1. Rabbits stem cells

Rabbits offer several advantages as animal models for research, particularly in areas such as immune system studies, pharmaceutical testing, and biomedical research. These benefits stem from their biological similarities to humans, short reproductive periods, genetic characteristics, manageable size, and ease of handling (Kamaruzaman et al., 2013; Y. Wang et al., 2014).

Nowadays, rabbits are useful animal models to extrapolate animal studies to humans because rabbits are phylogenetically closer to primates than rodents. The rabbit model offers a more diverse genetic background than genetically modified rodent strains, which makes the model a better overall approximation of humans (Bosze Zs. & Houdebine L.M., 2010; Shiomi, 2009)

Generally, Pluripotent stem cells from rabbits are derived from inner cell mass cells of early blastocysts. They are cultured on feeder cells and form flat colonies. Another alternative to obtain pluripotent stem cells from rabbits is by reprogramming rabbit somatic cells. Rabbit pluripotent cells have potential applications in cellular therapies and disease modelling. Additionally, rabbit pluripotent stem cells serve as valuable tools for transgenesis, as they can be genetically modified *in vitro* and screened for mutations before generating transgenic rabbit strains. To enhance this process, alternative methods, such as differentiating these cells into male or female germ cells or using them as nuclear donors for cloning, need to be developed. While these techniques require further testing in rabbits, they could significantly improve cloning efficiency, which remains low in this species. Finally, studying rabbit pluripotent cells provides insights into pluripotency maintenance in a model species beyond rodents and primates. Rabbits are particularly suitable for this research due to the accessibility of their early embryonic development and many advances in iPSC (Samruan et al., 2020).

2.2.2.Mouse stem cells

Mouse stem cells remain a fundamental resource for advancing the understanding of stem cell biology and its applications in medicine and biotechnology. Mice stem cell was the first to be isolated. Moreover, mouse studies have been useful to explore biological fundaments, development biology, transgenesis, cloning and others. Mice have been used as a primary model organism to investigate the principles of stem cell biology due to their genetic similarity to humans. Moreover, mice show advantages like a relatively short life cycle, easy maintenance, and reproductive features making them ideal for studying developmental processes (Guasch et al., 2008).

Mouse embryonic stem cells are isolated from the inner cell mass of early embryos and have the ability to proliferate indefinitely while retaining the ability to differentiate into all types of somatic cells, in fact, mouse embryonic stem cells can generate the entire organism except the trophoblast cells (Guasch et al., 2008).

Significant advancements in the culture media technology for embryonic stem cells have led to the development of commercial media that utilize a combination of inhibitors, such as MEK and GSK3β inhibitors. These inhibitors effectively maintain the pluripotent state of stem cells, allowing for their continued self-renewal and undifferentiated growth. Traditionally, mouse embryonic stem cells relied on leukaemia inhibitory factor (LIF) as a critical supplement in their culture. However, the new formulations have made it possible to culture these cells without serum, representing a notable evolution in stem cell maintenance practices, and their use in several applications (Dupont, 2024; Lin & Talbot, 2011).

2.2.3.Chicken stem cells

Avian stem cells are classified regarding to their origin and differentiation capacity:

Embryonic stem cells (ESCs) are derived from the blastoderm or early-stage embryo and exhibit pluripotent characteristics, with the capacity to differentiate into derivatives of all three germ layers. However, their practical application is limited by reduced long-term stability in culture and poor germline contribution, which restricts their suitability for studies involving heritable gene transmission (Pain et al., 1996).

Somatic stem cells can be isolated from embryonic or adult avian tissues, including muscle, bone marrow, skin, and neural tissue. These cells are typically multipotent rather than pluripotent (Mozdziak et al., 2005).

Chicken primordial germ cells (PGCs). These cells are significant in developmental biology and have numerous biotechnological applications. For instance, the preservation of PGCs has been extensively studied, leading to the development of several effective long-term conservation methods. Preservation of PGCs is particularly important for gene conservation. While sperm samples exhibit satisfactory stability during freezing and can effectively maintain their genetic information, this approach has a limitation: it only preserves the genetic material associated with the Z-chromosome, omitting the W-chromosome information from female chickens. Conversely, preserving eggs or embryos for extended periods is challenging due to their relatively large size, as well as their high water and protein content, which complicate long-term storage. Continued research into PGC preservation holds promise for comprehensive genetic conservation strategies in poultry and other avian species (Ecker et al., 2024).

The chicken is a well-established vertebrate model organism with several experimental advantages, including external embryonic development, accessibility of embryos, rapid development, and well-characterised staging systems. Importantly, avian PGCs can be isolated, cultured long-term, cryopreserved, and reintroduced into host embryos while maintaining germline competency (Farzaneh et al., 2017).

2.3. Cell stemness and self-renewal mechanism

Ernst Haeckel (1868), August Weismann (1892), Alexander Maximow (1909), Leroy Stevens (1953), John Gurdon (1954) studied cells and their ability to retain full developmental potential, initiating the study of cell stemness and self-renewal mechanisms. In 1981, the first mouse embryonic stem cell line was generated. Since then, significant efforts have been made to understand the differences and similarities in signalling pathways involved in maintaining pluripotency and initiating differentiation (Tancos et al., 2012).

2.3.1.Key molecular and genetic mechanisms in cell stemness and self-renewal

The maintenance of an undifferentiated, highly plastic, and self-renewing stem cell state is reinforced by multiple interconnected mechanisms, including core transcription factors, epigenetic regulators, key signalling pathways, specialised metabolic programs, and non-coding RNAs.

2.3.1.1. Transcription factors

In the 2000s, the fundamental mechanisms controlling the self-renewal of pluripotent cells began to be investigated, some of which are conserved across different species. The first transcription factors discovered that maintain the pluripotency, self-renewal, genome surveillance and cell fate determination of embryonic stem cells, were OCT3/4, SOX2 and NANOG. Other transcription factors have been added like KLF4, c-MYC, ESRRB, and SALL4 (Avilion et al., 2003; Chambers et al., 2003; Loh et al., 2006; Nichols et al., 1998; K. Takahashi & Yamanaka, 2006).

OCT4 (octamer-binding transcription factor 4), SOX2 (Sex determining region Y-box 2) and NANOG (homeobox transcription factor) are important regulators of pluripotency. These factors activate and repress a network of genes that promote self-renewal and avoid differentiation, in this way pluripotency is maintained in embryonic stem cells. In Addition, KLF4, c-MYC, Esrrb, and SALL4 support pluripotency and reprogramming, and other factors that participate in stemness, are described in the Table 2-1 (Lee et al., 2013; Pan & Thomson, 2007; Rosner & Hengstschläger, 2024; Zhang, 2014).

Shinya Yamanaka described OCT4, SOX2, KLF4, c-MYC as factors which can be used to induce pluripotency in somatic cells in a process called reprogramming. Nowadays, another set of factors called Thomson factors were discovered that can be used for reprogramming which includes OCT4, SOX2, NANOG and, LIN28 (Han & Yoon, 2011; K. Takahashi & Yamanaka, 2006).

Table 2-1 Interaction between auxiliary and primary transcription factors associated with pluripotency.

Gene	**Transcriptional interaction with core factors**
Esrrb	Interacts with OCT4, NANOG, SOX2 and NCOA3.
	Activate NANOG, OCT4 and other genes.
	Target of NANOG and TCF3.
Nr5a2	Interacts with DAX1 and RARΓ.
	Activates OCT4 and NANOG.
	Direct target of OCT4, β-Catenin and Tcf3.
Dax1	Interacts with NANOG, OCT4 and Nr5a2.
	Activate OCT4 expression.
	Target of STAT3, OCT4, Esrrb, SOX2, Nr5a2, NANOG and β-catenin.
GCNF	Repress OCT4 and NANOG upon differentiation.
Klf4	Interacts with OCT4 and SOX2.
	Activate NANOG, Esrrb and other genes.
	Downstream target of LIF/Stat3 signaling.
Sall4	Interact with NANOG, OCT4, SOX2, Esrrb, DAX1, MTA2 and NAC1;
	Activate OCT4 expression;
	Linked to TGF-β and WNT signaling through Usp9X and Cxxc5;
	Involved in transcriptional repression, cell cycle regulation (via binding to cyclin D1).
FoxD3	Activate NANOG through cooperating with OCT4.
	Target of OCT4.
	High level of FoxD3 can inhibit itself.
FoxO1	Essential for human ESC maintenance;
	Activate OCT4 and SOX2 in human ESCs.
Zfp206	Interact with OCT4, SOX2, Zscan4 and Zfp110.
	Activate NANOG and OCT4.
	Target of OCT4 and NANOG.
Zfp296	Activate OCT4.
	Enhance reprogramming.
Zfp281	Interact with OCT4, SOX2 and NANOG.
	Activate NANOG.
Rex1	Target of OCT4, SOX2, NANOG, KLF4, DAX1 and NACI.
	Maintains X-activation, imprinting, cell cycle, and mitochondrial fission in ESCs.
Zscan4	Overexpression or knockdown Zscan4 did not alter the transcriptome dramatically.
	Transient expression of Zscan4 lead to telomere elongation and can restore the developmental potency of ESC.
Dppa2 & Dppa4	Essential for ESC maintenance;
	Putative target of OCT4.
Tbx3	Activate Nanog, OCT4, SOX2, SALL4, LEFTY1, LEFTY2, Zfp42, Klf2, Klf4, Klf5 , n-MYC and c-MYC;
	Target of NANOG and TCF3;
	Partially activated by PI3-kinase but inhibited by MAP-kinase;
	Repress the expression of cell cycle regulators NFκBIB and p14.
PRDM14	Activate OCT4 In human ESCs.
	Repress differentiation-related genes.
	Interact with PRC2 complex to repress gene expression.
L1td1	Interacts with Lin28 to modulate levels of OCT4;
	Target of OCT4, SOX2, and NANOG in human ESCs.
Utf1	Direct target of OCT4 and SOX2.
	Involved in chromatin-associated transcriptional repression.

(Lee et al., 2013)

2.3.1.2. Epigenetics

Epigenetic modification has also been related to the regulation of pluripotency genes, especially DNA methylation and Histone marks. In pluripotent stem cells, the promoters and enhancers of *OCT4* (POU5F1), *SOX2, NANOG* and other pluripotency-related genes are typically unmethylated. In stemness, the robust expression of pluripotency-related genes is essential to maintain pluripotency and self-renewal. Nevertheless, hypermethylation of those genes has been seen during differentiation (Bar & Benvenisty, 2019)

Another important epigenetic mechanism involved in maintaining stemness is the presence of both H3K4me3 (trimethylation of histone H3 at lysine) and H3K27me3 (trimethylation at lysine 27) at certain genomic regions, which can indicate a bivalent state. H3K4me3 and H3K27me3 support the balance between self-renewal and differentiation, allowing cells to maintain pluripotency while also being ready to differentiate quickly when differentiation is required (Shanak & Helms, 2020).

H3K4me3 is a critical marker that identifies the promoters of actively transcribed genes, signalling regions of the genome that are engaged in transcriptional activity and contributing to the regulation of gene expression. For instance, promoters of key pluripotency factors are enriched with H3K4me3, allowing their robust expression, which is essential for stem cell pluripotency, the absence of H3K4me3 in promoters of pluripotency genes is associated with differentiation (Shanak & Helms, 2020).

H3K27me3 marks genes which are inactive or silenced, maintaining cellular identity and regulating developmental processes. While the pluripotent cell is being differentiated, H3K27me3 is commonly found at the promoters of pluripotency genes, blocking their expression and activity (Shanak & Helms, 2020).

Ten-Eleven Translocation (TET) enzymes consist of a protein family with three members: TET1, TET2 and TET3. The main function of these enzymes is to remove methylation marks, allowing cells to transition between different states and genes that were previously silenced to be reactivated. In embryonic stem cells, the enzymes TET1 and TET2 are highly expressed. Usually, TET1 and TET2 prevent the unusual silencing of pluripotent genes, removing methyl groups from their promoters and maintaining pluripotent gene expressions of NANOG, OCT4, and ESRRB (Rasmussen & Helin, 2016).

2.3.1.3.Signalling pathways

External signals from the environment support the cell stemness by different key signalling pathways such as: LIF/STAT3 Pathway, FGF/ERK Pathway Wnt/β-Catenin Pathway, and TGF-β/Smad Pathway.

2.3.1.3.1.LIF/STAT3 pathway

Leukaemia Inhibitory Factor or LIF is a member of the interleukin-6 (IL-6) family of cytokines. Embryonic stem cells express the LIF receptor (LIFR) on the surface and it has affinity to bind to LIF, after LIF binds to LIFR, LIFR dimerizes activating the Janus Kinases (JAK1 and JAK2). JAK1 and JAK2 phosphorylate and activate Signal Transducer and Activator of Transcription 3 (STAT3). When STAT3 got a phosphorylated state (pSTAT3), it can be translocated to the nucleolus, once in the nucleolus, pSTAT3 binds to specific DNA elements in the promoters and enhancers of target genes and activating the transcription of OCT4, NANOG, and SOX2. When LIF is removed the embryonic stem cell starts differentiation process (Wong et al., 2018).

2.3.1.3.2.FGF/ERK pathway

FGF/ERK pathway is involved in the regulation of pluripotency, balancing self-renewal and differentiation processes according to intrinsic signals and external signals from the cellular environment. Fibroblast Growth Factors (FGFs) are a family of 4 ligands, and it has a kinase domain. In embryonic stem cells FGF2 binds specifically to fibroblast growth factor receptors (FGFRs), leading to the dimerization of two receptor molecules, activating the receptor's kinase domain of the receptor, and several autophosphorylation events. The activation of FGFR stimulates the Raf kinase, which activate the kinase called MEK(Mitogen-Activated Protein Kinase) and activates, which ERK (extracellular signal-regulated kinase). ERK in activated state, is translocated to the nucleus, where it influences the expression of key pluripotency factors such as OCT4, NANOG, and SOX2. In addition, ERK supports the self-renewal of ESCs, enabling them to proliferate without differentiating prematurely. FGF/ERK pathway also guides cells toward differentiation when necessary. For instance, the emission of FGF signalling can drive a group of embryonic stem cells toward differentiation into specific lineages, such as the mesoderm or endoderm. The balance between proliferation and differentiation is dynamically regulated by FGF signaling. When FGF levels are appropriate, pluripotency is enhanced; however, as these levels change, differentiation can be triggered. This responsiveness is vital for embryo development and tissue homeostasis (Soszyńska et al., 2019).

2.3.1.3.3.Wnt/β-Catenin pathway

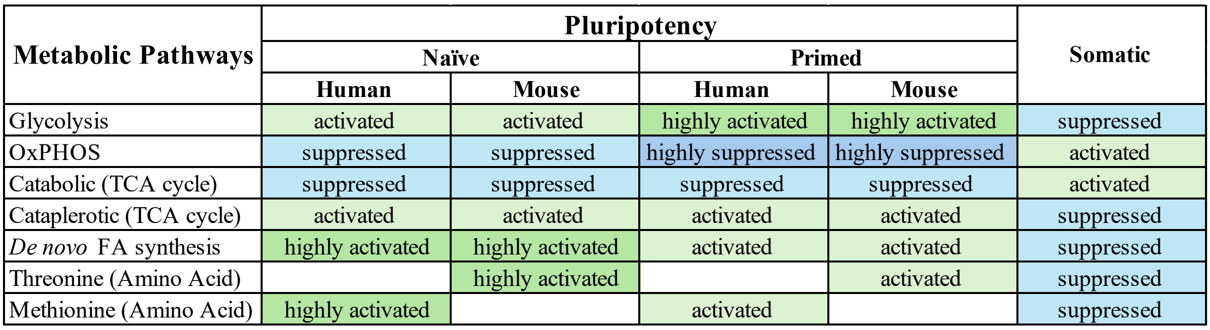
WNT proteins are extracellular ligands that bind to Frizzled receptors on the cell membrane. Once, WNT/Frizzed receptor are together, destruction complex is inhibited. The function of destruction complex is the regulation of β-catenin levels within the cell and avoid the accumulation of β-catenin in the cytoplasm. When destruction complex is inhibited, β-catenin is accumulated and stabilized, and it is translocated to the nucleus where β-catenin interacts with T-cell Factor (TCF) and Lymphoid Enhancer Factor (LEF) which are considered as transcription factors. This interaction activates the transcription of target genes related to cell proliferation, differentiation, and survival (Liu et al., 2022).

2.3.1.3.4.TGFβ/Smad pathway

Transforming Growth Factor-beta (TGF-β) is a pleiotropic cytokines superfamily which includes Activin and Nodal protein. TGF-βs bind to binds to a heteromeric receptor complex consisting of TGF-β type I (TβR-I) and type II (TβR-II) receptors. The binding triggers the activation of TβR-II, which then phosphorylates TβR-I. TβR-I phosphorylates receptor-regulated SMADS (SMAD2 and SMAD3). Phosphorylated SMAD proteins suffer a conformational change, and dissociate from the receptor complex, forming Smad4 or Smad complex. The Smad complex translocate into the nucleus, where with other transcription factors and co-factors regulate the expression of genes involved in processes such as cell differentiation, growth, apoptosis, and numerous other cellular functions (Papanayotou & Collignon, 2014; M.-K. Wang, 2012).

2.3.1.4.Metabolic pathway in pluripotent stem cells

Pluripotent stem cells, such as embryonic stem cells and induced pluripotent stem cells, develop sophisticated metabolic characteristics to maintain their pluripotency and differentiation capabilities. Key metabolic pathways which are crucial for pluripotent stem cell function are glycolysis, the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OxPhos), *De novo* FA synthesis, Threonine, Methionine and fatty acid oxidation (FAO). Figure 2-1 illustrates the major metabolic pathways involved in naïve and primed pluripotent stem cells from human and mice, and their difference compared to somatic stem cells (Tsogtbaatar et al., 2020).



Metabolic Pathways	**Pluripotency**				**Somatic**
	Naïve		**Primed**		
	Human	**Mouse**	**Human**	**Mouse**	
Glycolysis	activated	activated	highly activated	highly activated	suppressed
OxPHOS	suppressed	suppressed	highly suppressed	highly suppressed	activated
Catabolic (TCA cycle)	suppressed	suppressed	suppressed	suppressed	activated
Cataplerotic (TCA cycle)	activated	activated	activated	activated	suppressed
De novo FA synthesis	highly activated	highly activated	activated	activated	suppressed
Threonine (Amino Acid)		highly activated		activated	suppressed
Methionine (Amino Acid)	highly activated		activated		suppressed

Figure 2-1: Major metabolic pathway differences found in pluripotent stem cells and somatic cells. (Tsogtbaatar et al., 2020).

2.3.1.5.Non-coding RNAs involved in cell stemness and self-renewal

The genome contains sequences that are transcribed and ultimately translated into proteins, as outlined by the central dogma of molecular biology. However, it is important to note that non-coding regions of the genome are also transcribed, giving rise to various non-coding RNAs that do not translate into proteins, such as:

- MicroRNAs (miRNAs)
- Long Non-Coding RNAs (lncRNAs)
- Circular RNAs (circRNAs)
- Small Interfering RNAs (siRNAs)
- Piwi-Interacting RNAs (piRNAs)
- Small Nuclear RNAs (snRNAs)
- Small Nucleolar RNAs (snoRNAs)
- Transfer RNAs (tRNAs)
- Enhancer RNAs (eRNAs)

These non-coding RNAs play crucial roles in various biological processes, highlighting the complexity of gene regulation beyond protein-coding sequences, chromatin modification, and other cellular processes. Transcriptome analyses have shown that non-coding RNAs are considerably present in different cells and tissues (Nemeth et al., 2024; Wu & Kuo, 2020).

Non-coding RNAs expression controls efficiently different signalling pathways which function as regulatory elements in determining the fate of stem cells. Non-coding RNAs involved in stemness and self-renewal are miRNAs, lncRNAs, circRNAs, siRNAs and piRNAs (Mishra et al., 2023; Wu & Kuo, 2020).

2.4.miRNAs involved in cell stemness and self-renewal

2.4.1.Definition

MicroRNAs (miRNA) are short non-coding RNAs which are known as part of the gene expression regulation at the post-transcriptional level (Wei et al., 2017). miRNAs have the ability to interact with 3' untranslated region (3'UTR) of target messengers (mRNAs) RNA by complementary sequence to induce mRNA degradation and translational repression. In addition, miRNAs also interact with 5'UTR, coding sequences and gene promotors. MiRNAs are evolutionarily conserved across numerous species and play fundamental roles in various biological processes, including cell fate decisions, the pathogenesis of numerous diseases, cell cycle regulation, apoptosis, aging, and diverse signalling pathways (Maraghechi et al., 2013, 2023).

2.4.2.miRNA biogenesis and RISC formation

The biogenesis of microRNA involves three key steps: transcription, maturation, and the formation of the RNA-induced silencing complex (RISC), Figure 2-2. During this process, the miRNA undergoes a series of enzymatic cleavages, ultimately resulting in the distinctive structure characteristic of a mature miRNA Figure 2-3.

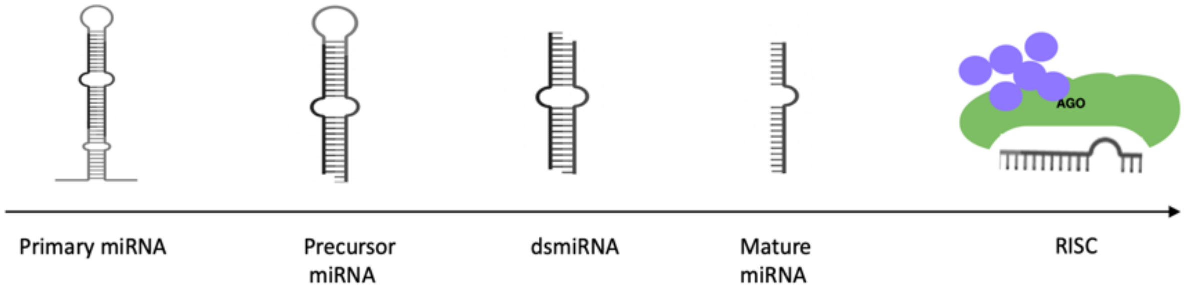


Figure 2-2: Changes in the molecular structure of miRNA during its biogenesis.

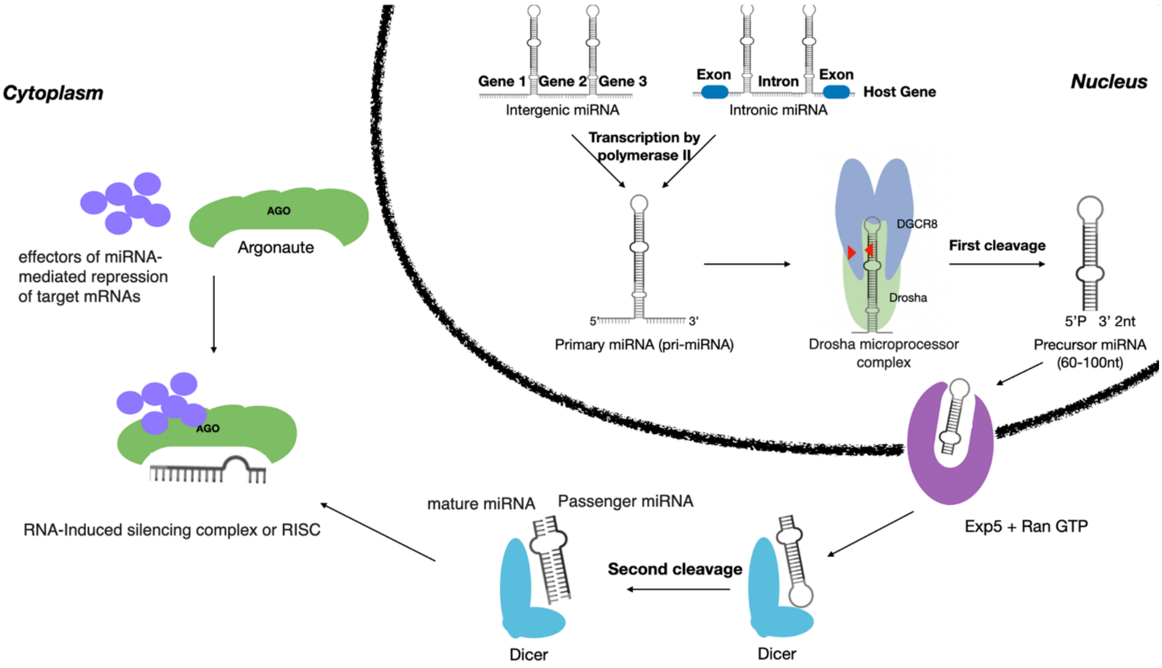


Figure 2-3: miRNA biogenesis: transcription (nucleolus), maturation (cytoplasm), and RISC formation (cytoplasm).

The transcription (Figure 2-3) occurs in the nucleus carried by polymerase II, the transcript usually contains several thousand nucleotides, and it is called primary miRNA or pri-RNA (Maraghechi et al., 2023).

The maturation involves 2 sequential cleavages carried by Drosha and Dicer.
Cleavage 1: Following transcription, the primary miRNA, or pri-miRNAs, is going to form a hairpin or stem-loop structure. This structure has 2 domains that are recognized by Drosha microprocessor complex. The complex consists of an RNase III enzyme called Drosha and a cofactor called DGCR8. Drosha recognizes and cleaves the stem of the hairpin and produces a shorter hairpin with around 60-100 nucleotides. The shorter hairpin is called now pre-miRNA and it has 2 nucleotides overhang at the 3-end (Maraghechi et al., 2023; O'Brien et al., 2018).

Two proteins called Exportin 5 and Ran-GTP translocate the pre-miRNA from the nucleus into the cytoplasm, where a second cleavage takes place (Maraghechi et al., 2023).
Cleavage 2: once the pre-miRNA is in the cytoplasm, an RNase type III protein called Dicer, recognizes the pre-miRNA loop and eliminates it. The cleavage produces a double-strand mature miRNA, one strand is going to be the mature miRNA and the other is denominated as a passenger

miRNA strand. The mature miRNA can now promote the formation of RISC (RNA-Induced silencing complex)(O'Brien et al., 2018).

Dicer and other proteins form a complex called the RISC-loading complex (RLC), allowing the incorporation of miRNA into the RISC. The Argonaute or AGO proteins are the main components of the RISC complex followed by the effectors of miRNA-mediated repression of target mRNAs. Once the RISC is formed, miRNA can start to block or silence mRNAs (Davis-Dusenbery & Hata, 2010; O'Brien et al., 2018).

There is another miRNA biogenesis process where the biogenesis occurs through a Drosha/DGCR8 and/or Dicer-independent manner when miRNAs originate from introns (mirtrons) or other small RNAs, such as snoRNAs, shRNAs, tRNAs, tRNase Z, and endo-siRNA. In addition, short hairpin RNAs, produced in a Dicer-independent pathway, can be loaded into AGO2 directly (Maraghechi et al., 2023).

2.4.3.Mechanism of action

MiRNA can stop the expression of the protein in 2 ways: Transcript Degradation and Translation Inhibition.

Once miRISC (mature miRNA RISC complex) is built, miRNA can bind to 3′-UTR of an mRNA by complementary sequences and consequently block the translation. A perfect base pairing results in target degradation, and an imperfect base pairing results in sequestration of a target Figure 2-4. The miRNA structure contains a seed that is a part of the sequence well conserved among species. Analysis *in silico* has shown that the seed has an important role in the matching to mRNA and the further mRNA translation silencing. In addition, there is evidence that miRISC can be transported to the nucleus and binds to ncRNAs including pri-miRNA to repress their processing or interfere with their functions (Bizuayehu & Babiak, 2014).

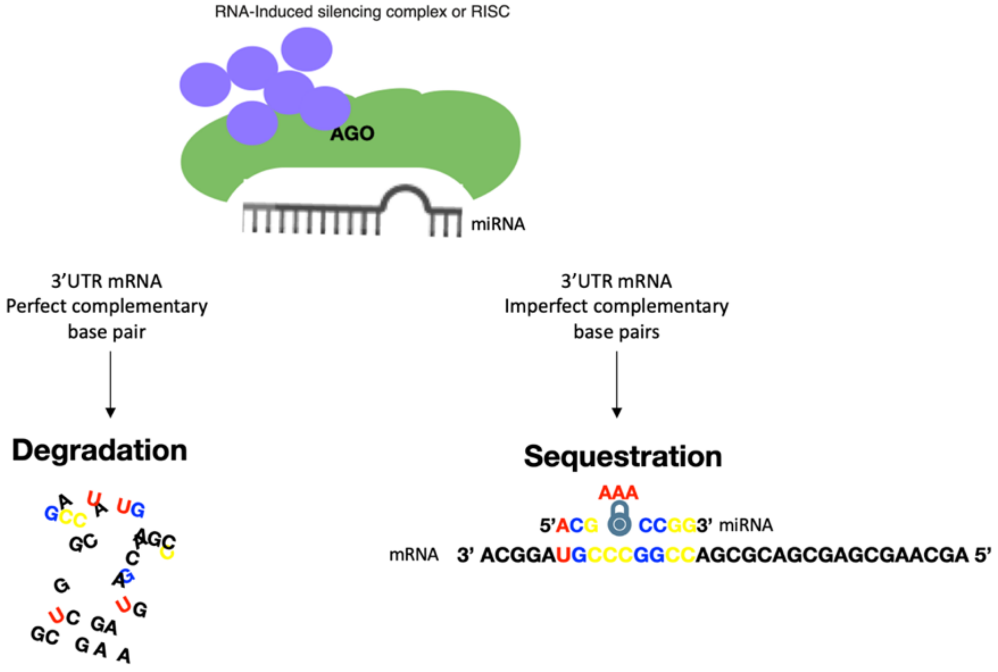


Figure 2-4 : RISC Mechanism of action.

2.4.4. miRNA families and clusters are involved in stemness and self-renewal

MiRNAs play essential regulatory roles in establishing and maintaining stem cell identity across species. By modulating transcriptional networks, signalling pathways, epigenetic states, and cell-cycle dynamics, specific miRNA clusters help sustain pluripotency, promote self-renewal, or initiate differentiation depending on their expression patterns.

A subset of miRNAs is particularly critical for maintaining pluripotency and self-renewal in stem cells, exhibiting distinct expression profiles that vary according to the specific type of stem cell. This diversity in miRNA expression underscores their importance in regulating stem cell identity and functionality (Chen et al., 2015; Gangaraju & Lin, 2009; Maraghechi et al., 2013).

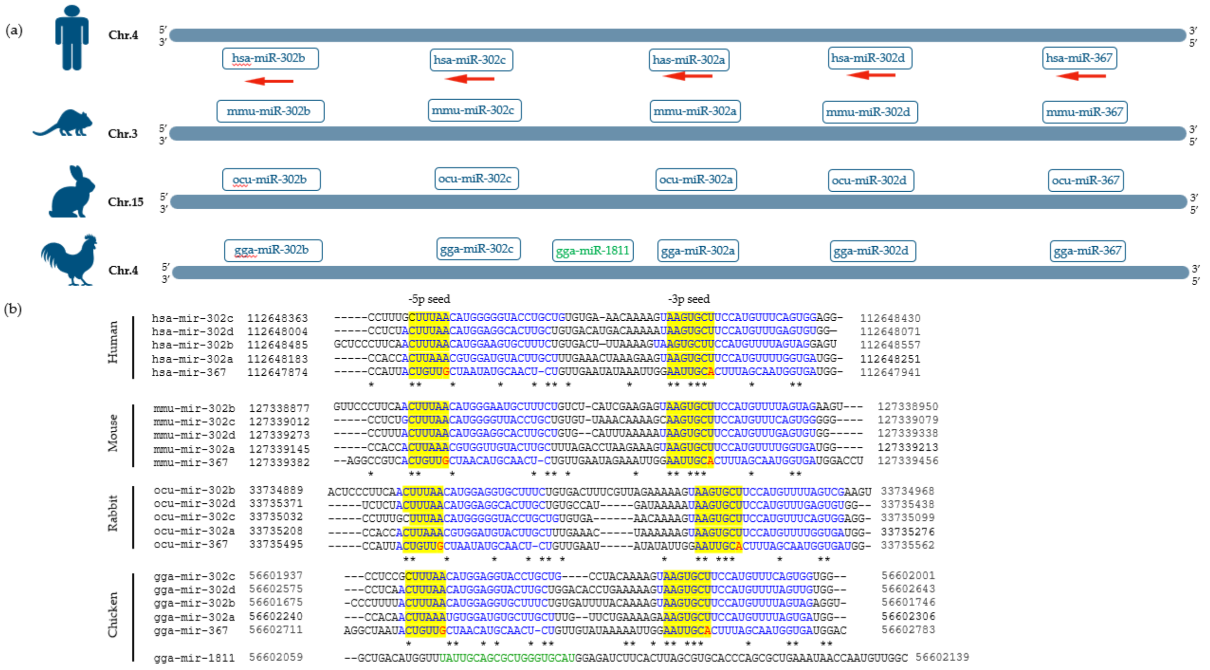
Several miRNAs have been identified to work during early embryonic development. Moreover, sequencing studies show that miRNA related to embryogenesis are common sequences found in vertebrates and mostly are conforming families and/or clusters in the genome.

A miRNA family consists of miRNAs that share a common seed sequence. For instance, a seed sequence is crucial for recognizing and binding to target mRNAs. The presence of the seed suggests that the members of the family may regulate similar biological processes or pathways. On the other hand, miRNA clusters refer to groups of miRNAs that are found close to each other on the same chromosome and are often co-expressed. This closeness leads a coordinated regulation of gene expression, offering a mechanism for fine-tuning cellular responses to various signals (Maraghechi et al., 2023).

2.4.4.1. MiR-302/ 367

The miR-302/367 cluster is conserved among vertebrates, exhibiting a similar structural arrangement across multiple species, as illustrated in the accompanying Figure 2-5 (a). Notably, in chickens, an additional miRNA, gga-miR-1811, is present, bearing slight similarities to the other members of the cluster. The expression level in early embryos, the function, the signalling pathway and the cluster targets are detailed in the Table 2-2. This suggests an intriguing evolution of miRNA families and their functional implications across different vertebrate lineages (Maraghechi et al., 2023).

The cluster contains ten miRNAs: miR-302a-3p, miR-302a-5p, miR-302b-3p, miR-302b-5p, miR-302c-3p, miR-302c-5p, miR-302d-3p, miR-302d-5p, miR-367-3p, miR-367-5p, and miR-1811.



```
hsa-mir-302c  112648363  -----CCUUUGCUUUAACAUGGGGUACCUGCUGUGUGA-AACAAAAGUAAGUGCUUCCAUGUUUCAGUGGAGG-  112648430
hsa-mir-302d  112648004  -----CCUCUACUUUAACAUGGAGGCACUUGCUGUGACAUGACAAAAAUAAGUGCUUCCAUGUUUGAGUGUGG—  112648071
hsa-mir-302b  112648485  GCUCCCUUCAACUUUAACAUGGAAGUGCUUUCUGUGACU—UUAAAAGUAAGUGCUUCCAUGUUUUAGUAGGAGU  112648557
hsa-mir-302a  112648183  -----CCACCACUUAAACGUGGAUGUACUUGCUUUGAAACUAAAGAAGUAAGUGCUUCCAUGUUUUGGUGAUGG-  112648251
hsa-mir-367   112647874  -----CCAUUACUGUUGCUAAUAUGCAACU-CUGUUGAAUAUAAAUUGGAAUUGCACUUUAGCAAUGGUGAUGG-  112647941
                              *     **    *      *    * ** *        *    **  ***     *     **

mmu-mir-302b  127338877  GUUCCCUUCAACUUUAACAUGGGAAUGCUUUCUGUCU-CAUCGAAGAGUAAGUGCUUCCAUGUUUUAGUAGAAGU---  127338950
mmu-mir-302c  127339012  -----CCUCUGCUUUAACAUGGGGUUACCUGCUGUGU-UAAACAAAAGCAAGUGCUUCCAUGUUUCAGUGGGGG----  127339079
mmu-mir-302d  127339273  -----CCUUUACUUUAACAUGGAGGCACUUGCUGUG--CAUUUAAAAAUAAGUGCUUCCAUGUUUGAGUGUGG-----  127339338
mmu-mir-302a  127339145  -----CCACCACUUAAACGUGGUUGUACUUGCUUUAGACCUAAGAAAGUAAGUGCUUCCAUGUUUUGGUGAUGG----  127339213
mmu-mir-367   127339382  --AGGCCGUCACUGUUGCUAACAUGCAACU-CUGUUGAAUAGAAAUUGGAAUUGCACUUUAGCAAUGGUGAUGGACCU  127339456
                              *     **    *           * ** *         *   **  ***     *     **

ocu-mir-302b  33734889   ACUCCCUUCAACUUUAACAUGGAGGUGCUUUCUGUGACUUUCGUUAGAAAAAGUAAGUGCUUCCAUGUUUUAGUCGAAGU  33734968
ocu-mir-302d  33735371   -----UCUCUACUUUAACAUGGAGGCACUUGCUGUGCCAU-----GAUAAAAAUAAGUGCUUCCAUGUUUGAGUGUGG--  33735438
ocu-mir-302c  33735032   -----CCUUUGCUUUAACAUGGGGGUACCUGCUGUGUGA------AACAAAAGUAAGUGCUUCCAUGUUUCAGUGGAGG-  33735099
ocu-mir-302a  33735208   -----CCACCACUUAAACGUGGAUGUACUUGCUUUGAAAC-----UAAAAAAGUAAGUGCUUCCAUGUUUUGGUGAUGG-  33735276
ocu-mir-367   33735495   -----CCAUUACUGUUGCUAAUAUGCAACU-CUGUUGAAU-----AUAUAUUGGAAUUGCACUUUAGCAAUGGUGAUGG-  33735562
                                    **    *      *    * ** *              *    ** ***     *     **

gga-mir-302c  56601937   ---CCUCCGCUUUAACAUGGAGGUACCUGCUG----CCUACAAAAGUAAGUGCUUCCAUGUUUCAGUGGUGG--      56602001
gga-mir-302d  56602575   ---CCUCAACUUUAACAUGGAGGUACUUGCUGGACACCUGAAAAAGUAAGUGCUUCCAUGUUUUAGUUGUGG--      56602643
gga-mir-302b  56601675   -CCCUUUUACUUUAACAUGGAGGUGCUUUCUGUGAUUUUACAAAAGUAAGUGCUUCCAUGUUUUAGUAGAGGU-      56601746
gga-mir-302a  56602240   ---CCACAACUUAAAUGUGGAUGUGCUUGCUUUG--UUCUGAAAAGAAAGUGCUUCCAUGUUUUAGUGAUGG--      56602306
gga-mir-367   56602711   AGGCUAAUACUGUUGCUAACAUGCAACU-CUGUUGUAUAAAAAUUGGAAUUGCACUUUAGCAAUGGUGAUGGAC      56602783
                                  *     **   * *    * **          **  * ** ***      *    **   **

gga-mir-1811  56602059   --GCUGACAUGGUUUAUUGCAGCGCUGGGUGCAUGGAGAUCUUCACUUAGCGUGCACCCAGCGCUGAAAUAACCAAUGUUGGC 56602139
```

Figure 2-5 Genome organisation of MiR-302/ 367 cluster.

Figure 2-5 provides a comprehensive overview of the miRNA members’ positions within the genomes of humans, mice, rabbits, and chickens. Additionally, panel (b) of Figure 2-5 highlights the structure of the pre-miRNA, along with its notation and the defining seed sequence (highlight in yellow). This detailed representation emphasises the evolutionary conservation and structural characteristics of these miRNAs across various species (Maraghechi et al., 2023).

2.4.4.2. C19MiRNA cluster

The C19miRNA or C19MC cluster is a group of 56 mature miRNAs that are uniquely found in primate species located on chromosome 19. However, cluster homologous regions have been found recently in the rabbit genome. Figure 2-6 (a) shows the genomic distribution of the cluster in Humans and rabbits. In panel (b), the miRNA sequences are compared among the cluster members (Maraghechi et al., 2023).

This cluster is predominantly expressed in embryonic stem cells and placental tissues, and it is associated with various cancers. Its selective expression pattern suggests a significant role in both early development and tumorigenesis, highlighting its potential as a key regulator in these biological processes. The expression level in early embryos, the function, the signalling pathway and the cluster targets are detailed in the Table 2-2 (Maraghechi et al., 2023; Mong et al., 2020).

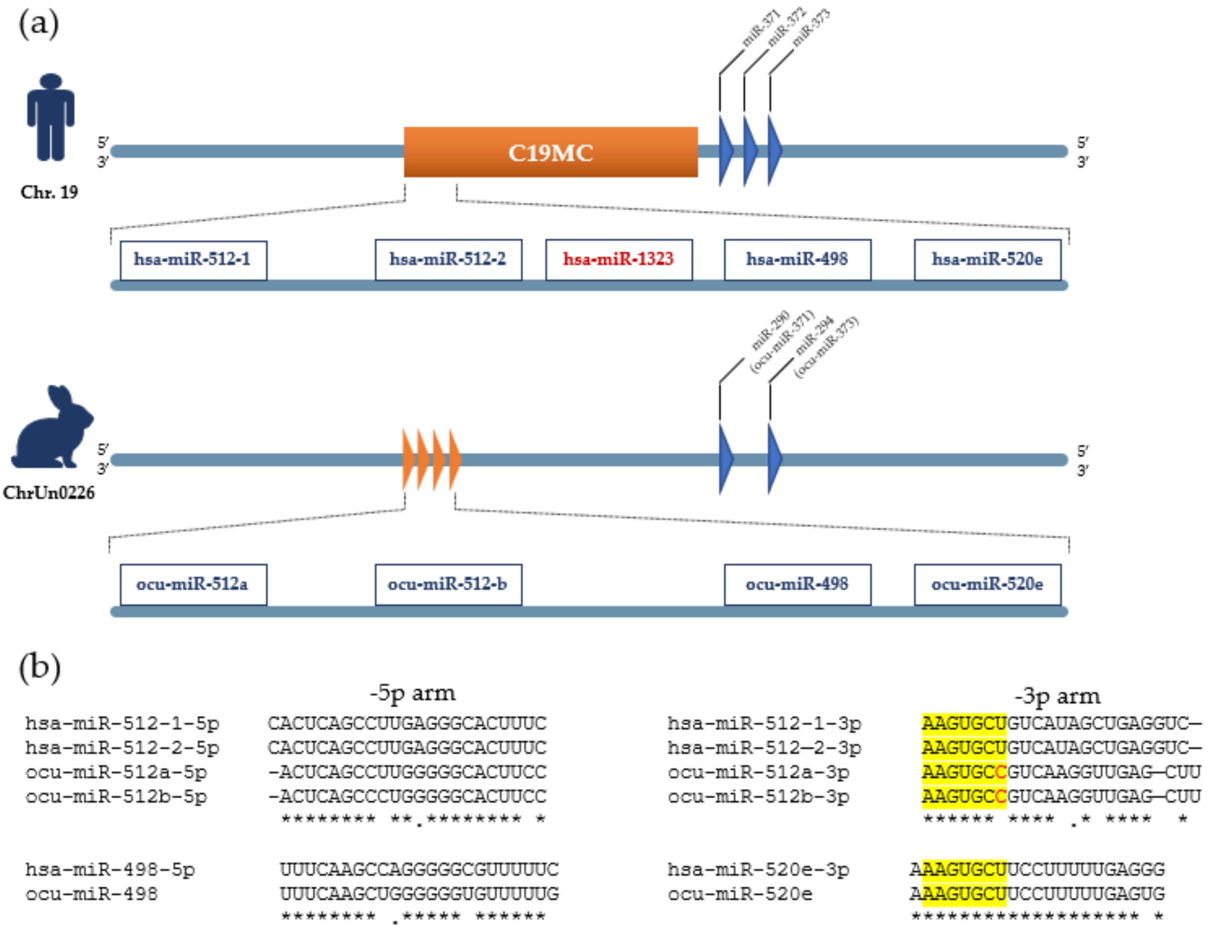


Figure 2-6: Genome organization of C19MC cluster.

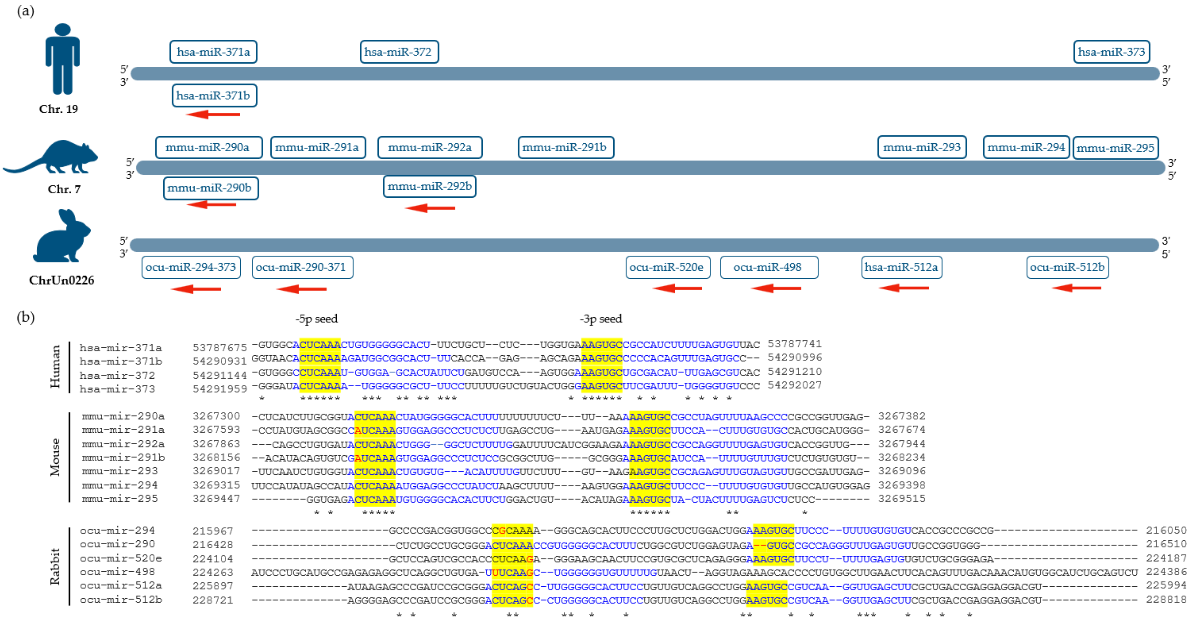
2.4.4.3.Mmu-miR-290/295, hsa-miR-371/373, and ocu-miR-290/295 clusters

The mmu-miR-290/295, has-miR-371/373, and you-miR-290/295 clusters are conserved in humans, mice, rabbits, chimpanzees, rats, dogs, and cows, with some important differences such as structure, number of members, and genomic location (Maraghechi et al., 2023).

In mouse, mmu-miR-290/295 cluster is located in chromosome 7; it is the most abundant miRNA cluster in embryonic stem cell in mice. The cluster contains 14 mature miRNAs: miR-290-5p , miR-290-3p, miR-291a-5p, miR-291a-3p, miR-291b-5p, miR-291b-3p, miR-292-5p, miR-292-3p, miR-293-5p, miR-293-3p, miR-294-5p, miR-294-3p, miR-29-5p, and miR-295-3p.

MiR-371/373, a human ortholog of mmu-miR-290/295 cluster and contains 6 members: miR-371a-3p, miR-371a-5p, miR-371b-3p, miR-371b-5p, miR-372, and miR-373.. Rabbit homologs of the mmu-miR-290/295 cluster is known as ocu-miR-290/295 and contains 3 miRNAs: ocu-miR290-5p, ocu-miR-292-3p, and ocu-miR-294-3p.

Figure 2-7 (a) shows the genomic distribution of the cluster in humans, mice and rabbits and the seed of each one, and in panel (b), the sequences of the miRNA members are compared between humans, mice and rabbits (Maraghechi et al., 2023).



```
hsa-mir-371a  53787675 -GUGGCACUCAAACUGUGGGGGCACU-UUCUGCU--CUC---UGGUGAAAGUGCCGCCAUCUUUUGAGUGUUAC 53787741
hsa-mir-371b  54290931 GGUAACACUCAAAAGAUGGCGGCACU-UUCACCA--GAG---AGCAGAAAGUGCCCCCACAGUUUGAGUGCC-- 54290996
hsa-mir-372   54291144 -GUGGGCCUCAAAU-GUGGA-GCACUAUUCUGAUGUCCA---AGUGGAAAGUGCUGCGACAU-UUGAGCGUCAC 54291210
hsa-mir-373   54291959 -GGGAUACUCAAAA--UGGGGGCGCU-UUCCUUUUUGUCUGUACUGGGAAGUGCUUCGAUUU-UGGGGUGUCCC 54292027
                        *      ******   ***  ** ** ***              * ******  * *    * * * *

mmu-mir-290a  3267300  -CUCAUCUUGCGGUACUCAAACUAUGGGGGCACUUUUUUUUUUCU---UU--AAAAAGUGCCGCCUAGUUUUAAGCCCCGCCGGUUGAG- 3267382
mmu-mir-291a  3267593  -CCUAUGUAGCGGCCAUCAAAGUGGAGGCCCUCUCUUGAGCCUG----AAUGAGAAAGUGCUUCCA--CUUUGUGUGCCACUGCAUGGG- 3267674
mmu-mir-292a  3267863  ---CAGCCUGUGAUACUCAAACUGGG--GGCUCUUUUGGAUUUUCAUCGGAAGAAAAGUGCCGCCAGGUUUUGAGUGUCACCGGUUG--- 3267944
mmu-mir-291b  3268156  --ACAUACAGUGUCGAUCAAAGUGGAGGCCCUCUCCGCGGCUUG-----GCGGGAAAGUGCAUCCA--UUUUGUUUGUCUCUGUGUGU-- 3268234
mmu-mir-293   3269017  -UUCAAUCUGUGGUACUCAAACUGUGUG---ACAUUUUGUUCUUU---GU--AAGAAGUGCCGCAGAGUUUGUAGUGUUGCCGAUUGAG- 3269096
mmu-mir-294   3269315  UUCCAUAUAGCCAUACUCAAAAUGGAGGCCCUAUCUAAGCUUUU----AAGUGGAAAGUGCUUCCC--UUUUGUGUGUUGCCAUGUGGAG 3269398
mmu-mir-295   3269447  --------GGUGAGACUCAAAUGUGGGGCACACUUCUGGACUGU----ACAUAGAAAGUGCUA-CUACUUUUGAGUCUCUCC-------- 3269515
                               * *   *****                             ******        **        *

ocu-mir-294   215967   --------------------GCCCCGACGGUGGCCCGCAAAA--GGGCAGCACUUCCCUUGCUCUGGACUGGAAAGUGCUUCCC--UUUUGUGUGUCACCGCCCGCCG--------------------- 216050
ocu-mir-290   216428   ----------------------CUCUGCCUGCGGGACUCAAACCGUGGGGGCACUUUCUGGCGUCUGGAGUAGA--GUGCCGCCAGGGUUUGAGUGUUGCCGGUGGG------------------------ 216510
ocu-mir-520e  224104   --------------------GCUCCAGUCGCCACCCUCAAGA--GGGAAGCAACUUCCGUGCGCUCAGAGGGAAAGUGCUUCCU--UUUUGAGUGUGUCUGCGGGAGA--------------------- 224187
ocu-mir-498   224263   AUCCCUGCAUGCCGAGAGAGGCUCAGGCUGUGA-UUUCAAGC--UGGGGGGUGUUUUGUAACU--AGGUAGAAAGCACCCCUGUGGCUUGAACUUCACAGUUUGACAAACAUGUGGCAUCUGCAGUCU 224386
ocu-mir-512a  225897   --------------AUAAGAGCCCGAUCCGCGGGACUCAGCC-UUGGGGGCACUUCCUGUUGUCAGGCCUGGAAGUGCCGUCAA--GGUUGAGCUUCGCUGACCGAGGAGGACGU------------- 225994
ocu-mir-512b  228721   --------------AGGGGAGCCCGAUCCGCGGGACUCAGCC-CUGGGGGCACUUCCUGUUGUCAGGCCUGGAAGUGCCGUCAA--GGUUGAGCUUCGCUGACCGAGGAGGACGU------------- 228818
                                      * *     *      **     ** *    *              **   *  *      ***     *   * *    *
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Figure 2-7: Genome organisation and multiple sequence alignment of mmu-miR-290/295, ocu-miR-290/295, and hsa-miR-371/373.

2.4.4.4.Summary of embryonic stem cells-specific miRNAs and their function

The following Table 2-2 summarises key miRNAs expressed in embryonic stem cells (ESCs) and germ cell lines from multiple species, highlighting their expression levels, functional roles, associated signalling pathways, and validated molecular targets involved in stemness regulation (Maraghechi et al., 2023).

Recent discoveries regarding pluripotency mechanisms have revealed that pluripotency is governed by a sophisticated core regulatory network comprised of key factors such as OCT4, SOX2, and NANOG shown (M. Li & Izpisua Belmonte, 2018). These factors work in concert to modulate a vast and interconnected array of pluripotency-associated genes, both in embryonic stem cells and other pluripotent cells.

Table 2-2: Summary of ESC-specific miRNAs and their relative function.

Cell type	miRNAs	Expression level	Function	Signaling Pathways	Targets
Mouse ESCs	miR-302/367 cluster	Low expression	Regulation of pluripotency self-renewal and reprogramming	CDKN1A TGF-β signaling pathway BMP signaling pathway	NA LATS2
	miR-290/295 cluster	High expression	Regulation of cell cycle progression and naïve pluripotency	ATM/ATR chek2-p53 pathway Wnt signalling pathway MEK Pathway activation	Cyclin D1, p21, LATS2 DKK-1 AKT1
			Early phases of differentiation	DNA methylation Hox gene inactivation	RBL2 ASH1L
Human ESCs	miR-302/367 cluster	High expression	Regulation of pluripotency, self-renewal and reprogramming	Cyclin D1/D2 and Cdk2 Nodal-activin pathway	LEFTY BNIP3L/Nix
	miR-371/373 cluster	High expression	Cell cycle regulation Reprogramming	G1/S transition P53 pathway	P21 MBD2
	C19MC	High expression in naïve hESCs and iPCs	Pluripotency maintenance	Chromatin structure modifications pathway	NA
			Prevent differentiation	ECM- related pathway	NA
			Derivation and maintenance of human trophoblast stem cells	P53 pathway	CCNG2 DKN1A PMAIP1 ZMAT3 TP53INP1
Rabbit ES-like cells	*miR-302/367 cluster*	High expression	Regulation of pluripotency and self-renewal	Nodal-activin pathway	LEFTY
	miR-290/295 cluster	Low expression	Early embryonic development	NA	NA
	C19MC	Low expression	Early embryonic development	NA	NA
Chicken PGCs line	miR-302b miR-302a	High expression	Cell proliferation Prevent differentiation	Glycolysis metabolism Somatic gene silencing	GPI3 SOX11

(Maraghechi et al., 2023)

In conclusion, the maintenance of pluripotent states involves multiple regulatory mechanisms, including transcriptional and post-transcriptional regulation, cellular signalling pathways, bioenergetic processes, epigenetic modifications, and transcriptional diversity. This complex network is showed in Figure 2-8 (M. Li & Izpisua Belmonte, 2018).

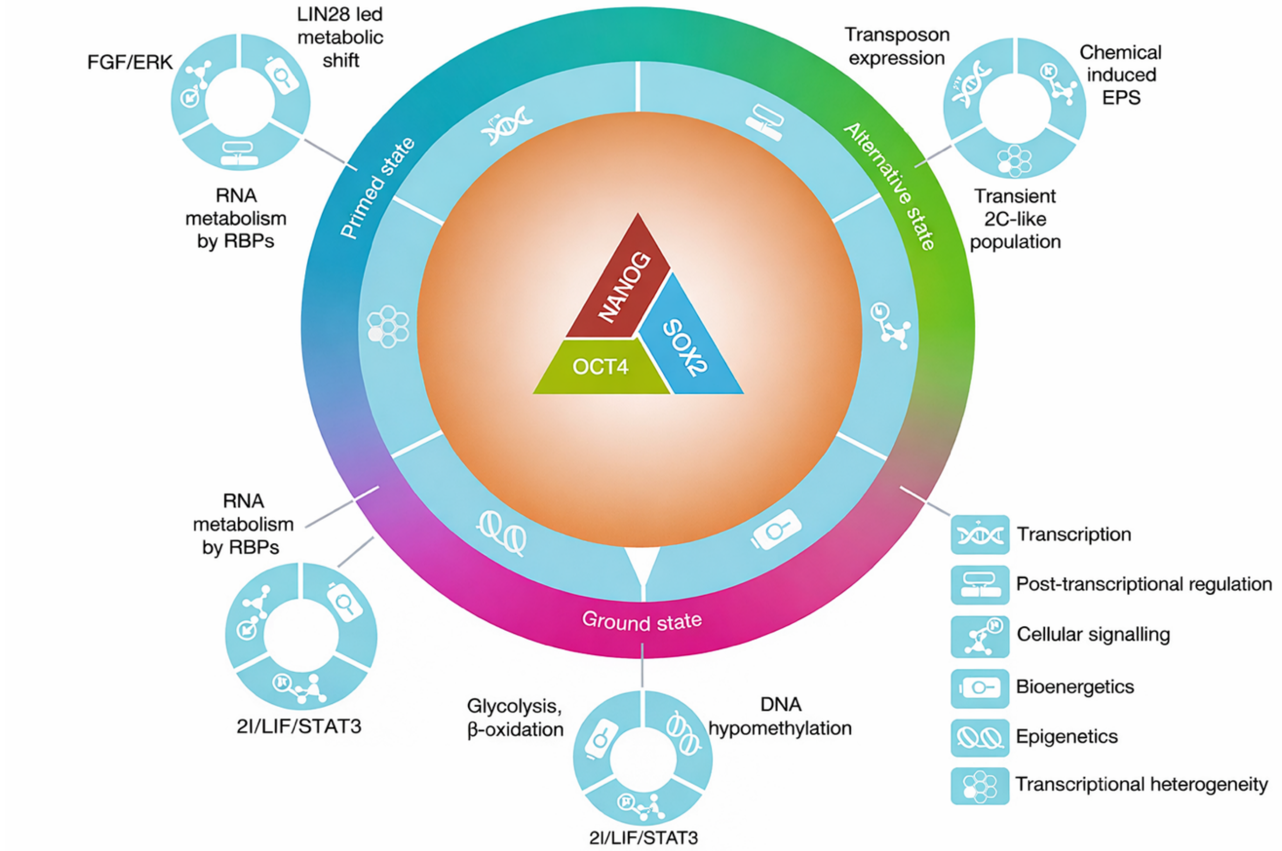

Figure 2-8 : Core transcription factors and regulatory crosstalks of the pluripotency gene regulatory network.

2.5.The importance of stem cells and miRNAs in modern biology

miR-184 Suppresses Apoptosis in Ovarian Granulosa Cells (Shan et al., 2024)

Shan *et al.,* (2024) published an article where miR-184 has demonstrated to be a strong regulator of ovarian granulosa cells apoptosis. More women have postponed the motherhood age due to the new roles where the women are involved nowadays. It is acknowledgeable that women reproductive capacity decreases after the age of 37 due to ovarian aging and functional recession. Regarding the ovary, its principal function is to support follicular development, ovulation, and hormone secretion. Studies have found that 40% of infertile cases are caused by follicular development disorder and anovulation. Although, primordial follicle pool is storage, studies demonstrated that most of the follicles undergo atresia and degeneration at each stage during development, with less than 1% being able to mature and ovulate. Follicular atresia is the degeneration and loss of ovarian follicles before they reach full maturity. A key mechanism behind follicular atresia is apoptosis. When too many granulosa cells undergo apoptosis, the follicle collapses and the oocyte is lost. Granulosa cells are very important somatic cells that surround the developing oocyte inside the ovarian follicle. They provide nutrients, produce hormones such as estrogen, and create a supportive microenvironment that allows the oocyte to grow and mature properly. Without healthy granulosa cells, the oocyte cannot survive or develop correctly. Therefore, controlling apoptosis in granulosa cells is essential for maintaining ovarian reserve and fertility.

The study showed that miR-184 plays a protective role against apoptosis in granulosa cells, when miR-184 levels were reduced, granulosa cells showed increased markers of apoptosis. On the other hand, when miR-184 was overexpressed (increased artificially in the laboratory), granulosa cell survival improved and apoptosis decreased. This clearly demonstrates that miR-184 acts as an anti-apoptotic regulator in ovarian cells.

SMAD3 (Mothers Against Decapentaplegic Homolog 3) signalling pathway was identified as a key target influenced by miR-184. SMAD3 is an important molecule in the TGF-β (transforming growth factor-beta) signalling pathway, In ovarian follicles, TGF-β/SMAD3 signalling is essential for proper follicular development and hormone production. miR-184 enhances SMAD3 activity, which promotes survival signals inside granulosa cells and reduces activation of apoptotic pathways such as caspase signalling or death signals.

This study provides evidence that a specific microRNA can regulate ovarian cell survival, controlling reproductive biology. In fact, miR-184 can be considered as a therapeutic tool contributing to conditions such as premature ovarian insufficiency, diminished ovarian reserve, age-related fertility decline and endometriosis-associated infertility. miR-184 could also be considered as a biomarker helping the prediction of follicle health and oocyte quality in assisted reproductive technologies.

MiRNA alterations in sperm of infertile men (Shunmuga Sundram et al., 2025)

Shunmuga Sundram et al., (2025) demonstrated that miRNA can be used as a biomarker of male infertility.

Male infertility contributes to nearly half of infertility cases in couples and can result from hormonal imbalances, structural abnormalities, or genetic factors. Recent research has shown that miRNAs play an important role in sperm development and function.

In this study, the researchers investigated four specific miRNAs miR-139, miR-34b, miR-296, and miR-942, to determine whether their expression levels are associated with common sperm abnormalities. Sperm samples were collected from men with low sperm count (oligozoospermia), poor sperm movement (asthenozoospermia), and abnormal sperm shape (teratozoospermia), and compared with samples from men with normal sperm parameters.

The levels of the selected miRNAs were measured using real-time PCR. The results showed that miR-139 was increased in men with reduced sperm motility, miR-34b was elevated in men with abnormal sperm morphology, and miR-942 was increased in all infertile groups.

In contrast, miR-296 was decreased in infertile men compared to controls. Statistical analysis suggested that miR-139 and miR-942, in particular, may serve as potential biomarkers for diagnosing certain types of male infertility. Overall, the study indicates that changes in specific miRNAs are linked to sperm abnormalities and may improve understanding and diagnosis of male infertility at the molecular level.

Targeted editing of miRNAs can control stem cell differentiation (Truong et al., 2024)

In this study, the researchers developed a new method to specifically edit primary miRNAs (pri-miRNAs), which are the early forms of miRNAs before they become mature and active. While RNA editing tools have been widely used to modify messenger RNA (mRNA), they have not been effectively applied to control miRNAs. To solve this problem, the authors created a new system called REPRESS (RNA Editing of Pri-miRNA for Efficient Suppression of miRNA). This tool allows scientists to precisely modify pri-miRNAs and reduce the levels of mature miRNAs without affecting other genes.

The researchers tested this system in different cell types, including adipose-derived stem cells (ASCs). They showed that editing pri-miRNAs successfully lowered mature miRNA levels while keeping the overall gene expression profile stable. They then developed an improved version called iREPRESS, which enhanced and prolonged the editing effect for at least 10 days, with minimal changes to other cellular RNAs.

Importantly, when they edited pri-miR-21 in stem cells, they were able to change the way the stem cells differentiated. The modified stem cells showed improved cartilage formation in laboratory conditions and enhanced bone regeneration in animal models. These findings demonstrate that targeted editing of miRNAs can control stem cell differentiation and improve tissue repair. Overall, this study highlights a new strategy to regulate miRNAs in stem cells, offering promising applications in regenerative medicine and tissue engineering.

3. METHODOLOGY

3.1.Investigation of chicken PGCs: miRNA expression before and after freezing

Oravka chicken eggs were obtained from the National Agricultural and Food Centre, Research Institute for Animal Production (Nitra, Slovakia). The establishment of primordial germ cells (PGCs), sex determination, and proliferation analyses were conducted by the project team; related findings are being prepared separately for a manuscript and publication.

The methodology comprised two main steps: PGC isolation and miRNA analysis, as illustrated in Figure 3-1.

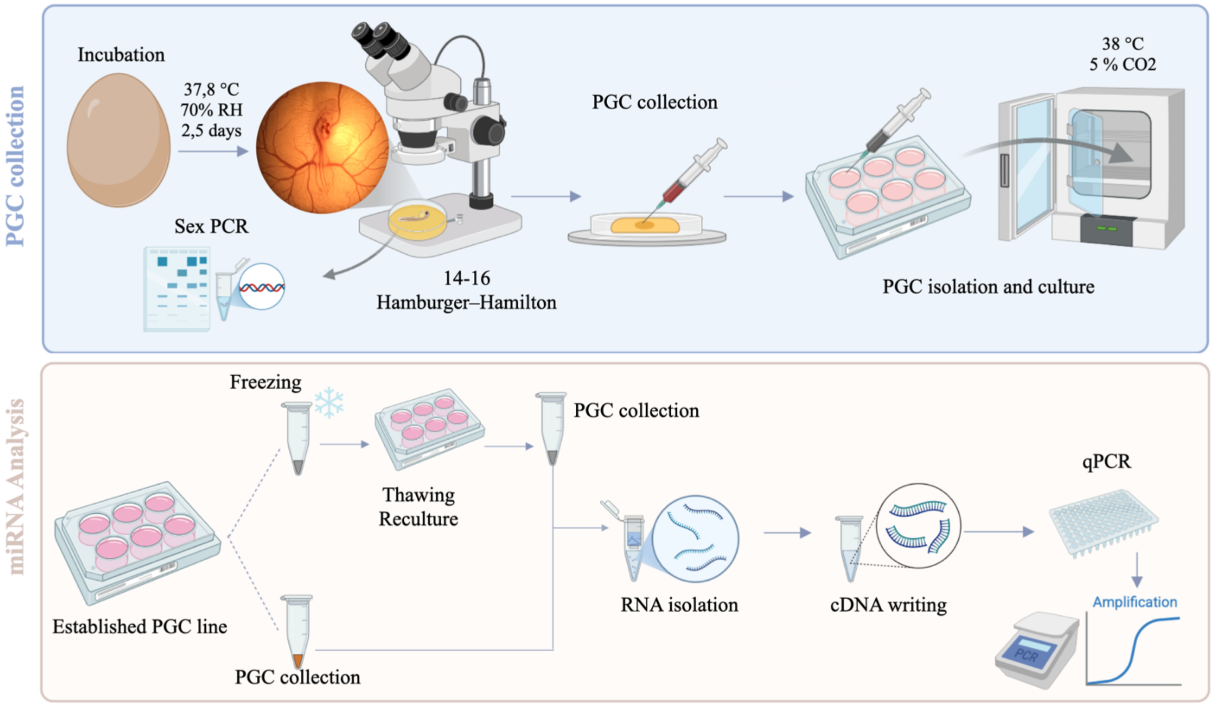

Figure 3-1 Schematic flowchart summarising the steps of the experimental process. Created with BioRender.com.

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3.1.1. PGC isolation

Oravka chicken eggs were incubated at 37.8 °C and 70% relative humidity in MIDI F500S hatchery machine (PL Machine Ltd., Tárnok, Hungary) for 2.5 days, this timepoint is at Hamilton-Hamburger HH 14-16 stage(Hamburger & Hamilton, 1951), where the dorsal aorta, pumping heart, and peripheral vessels are clearly visible under the stereomicroscope.

The eggs were sanitized by wiping with 70% ethanol. The eggs were open carefully, and the embryo and other components were placed in a petri dish.
1-3 µL of blood was taken with a sterile glass microcapillary and a mouth-controlled pipette from the dorsal aorta under the stereomicroscope Leica M205FCA supplied with DFC7000-T Leica camera. The collected blood sample was transferred to a 48-well cell culture plate containing 300 µL of chicken PGC medium supplemented with chicken serum, as developed by McGrew and colleagues (see Section 3.1.2, PGC medium, PGC establishment and maintenance). The

cultures were incubated at 38 °C under 5% CO_2 in a Sanyo MCO-19AIC (UV) CO_2 incubator (Sanyo, Osaka, Japan; model 10040162).

After the blood samples was taken, a portion of embryo tissue was taken for sex determination.

3.1.2. Sex determination

After the blood samples was taken, a portion of embryo tissue was collected to determine gender. Sex determination of primordial germ cell (PGC) samples was performed by genomic DNA isolation followed by sex-specific conventional PCR and agarose gel electrophoresis.

PGC samples stored at -70 °C were lysed in lysis buffer (100µL) supplemented with proteinase K (1 µL) and incubated at 56 °C for 3 hours and 700 rpm to achieve complete proteolytic digestion. Proteinase K activity was terminated by heat inactivation at 100 °C for 15 min, after which samples were centrifuged at 13,000 × g to remove insoluble debris. Genomic DNA was purified by sequential phenol (50µL) and chloroform (50µL) extraction under sterile conditions; the samples were centrifuged 13,000 × g for 10 minutes at 4 °C. Nucleic acids were precipitated by the addition of an equal volume of ice-cold isopropanol supplemented with GlycoBlue (0,75µL) (Thermo Fisher Scientific) as a co-precipitant. Following incubation at -20 °C for 30 min, DNA was pelleted by centrifugation at 13,000 × g for 5 min at 4 °C, washed with 75% (v/v) ethanol (500µL), air-dried, and resuspended in 20-40 µL of nuclease-free water. DNA concentration and purity were assessed using a NanoDrop spectrophotometer, and all samples were diluted to a final concentration of 25 ng/µL prior to PCR amplification.

Sex-specific PCR amplification was performed using primers targeting the CHD1-Z and CHD1-W loci described in Table 3-1, generating amplicons of 461 bp and 322 bp, respectively. Reactions were carried out in a MyTaq-based master mix containing MyTaq DNA polymerase and other components described in Table 3-2. The thermocycle conditions are described in Table 3-3.

Table 3-1: Forward and reverse primer sequences for amplification of CHD1-Z and CHD1-W genes.

Primer	Refer ID	Sequence		(bp)
CHD1-Z	NC_006127.4	FW	TATCGTCAGTTTCCTTTTCAGGT	461
		RV	CCTTTT ATTGATCCATCAAGCCT	
CHD1-W	NC_006126.4	FW	TATCGTCAGTTTCCTTTTCAGGT	322
		RV	CCTTTT ATTGATCCATCAAGCCT	

Table 3-2: PCR master mix composition for amplification of CHD1-Z and CHD1-W loci.

Sex-specific PCR Master mix Components	1x
Reaction buffer MyTaq	6,75 µL
Forward primer	0,5 µL
Reverse primer	0,5 µL
Nuclease-free water	4,25 µL
DNA 25 ng/ µL	1 µL

Table 3-3: Thermal cycling conditions for sex-specific PCR amplification

Temperature	Time	Cycle
95 °C	1 min	1x
95 °C	15 sec	28x
48 °C	30 sec	
72 °C	10 sec	
72 °C	5 min	1x

PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel prepared in 1× TAE (Tris Acetate EDTA) buffer and stained with ethidium bromide. DNA fragments were visualized under ultraviolet illumination, and the presence of sex-specific bands enabled unambiguous determination of the genetic sex of PGC samples.

3.1.3. PGC medium, PGC establishment and maintenance

The culture medium used for primordial germ cells (PGCs) was developed by Whyte and colleagues and is referred to as Avian-KO-DMEM basal media, commonly known as FACs medium (because Whyte et al. used it to culture FACS-sorted PGCs) (Whyte et al., 2015). This formulation is a modification of the Knock-out DMEM produced by Life Technologies, designed specifically to support avian cell cultures. The medium is enriched with a range of key components designed to optimise the growth and viability of PGCs, the function of each component is summarised in the Table 3-4.

FACs medium includes a 50x B-27 supplement, which provides essential vitamins and hormones important for cell growth. It also contains 100x GlutaMax and 100x non-essential amino acids (NEAA) to enhance nutrient availability and cellular metabolism. To promote cell survival and prevent oxidative stress, 50 mM beta-mercaptoethanol is incorporated.

In addition, the medium is supplemented with 100 mM DNA nucleotides to support DNA synthesis and repair, alongside 100x pyruvate, which serves as an energy source. To maintain a sterile environment, 100x Penicillin/Streptomycin is included to inhibit bacterial growth. This comprehensive formulation also features 20% ovalbumin, which acts as a protein source, and 50 mg/mL heparin sulphate, known for its role in cellular signalling and growth.

In addition to these foundational components, the medium is further enhanced with growth factors, including Human Activin A at a concentration of 25 ng/µL (PeproTech) and Human FGF2 at 10 ng/µL (R&D Biosystems, Sigma). These factors play a critical role in promoting cellular proliferation and differentiation. The inclusion of chicken serum provides additional nutrients and growth factors that are crucial for the successful culture of avian primordial germ cells. The prepared medium was filtered through a 0.2 μm pore size syringe (Merck-Millipore) and the medium was aliquoted into 50 mL tubes. These tubes were wrapped with aluminium foil to keep them protected from the light. The medium was stored at 4 °C.

Table 3-4: Components of FACs Medium and Their Functions in Avian PGC Culture.

Component	**Function**
KO-DMEM (avian-modified)	Provides nutrients, balanced osmolarity, supports undifferentiated growth
FGF2	Drives PGC proliferation and self-renewal
Activin A	Prevents differentiation; enhances stemness
Low % chicken serum	Growth support without inducing differentiation
BSA	Buffers proteins; improves survival after FACS sorting
NEAA, pyruvate	Enhance energy metabolism and protein synthesis
L-glutamine / GlutaMAX	Supports rapid growth and reduces metabolic stress
β-mercaptoethanol	Protects against oxidative stress
ITS (optional)	Supports metabolism and antioxidant defense
Pen/Strep	Prevents contamination
SCF (optional)	Boosts PGC proliferation in some species

The PGC cultures were examined daily to assess cell morphology and to identify contaminating non-PGC populations. Cells displaying morphology inconsistent with PGCs were selectively removed to enrich the cultures. The medium was replaced every two days to support PGC growth and survival. All PGC lines were maintained in the same incubator under the environmental conditions described in Section 3.1.1. This enrichment process was continued until the cell lines achieved high purity, as evidenced by a homogeneous PGC population with appropriate cell numbers and characteristic morphology, according to the method described in Section 3.1.4. Once the culture was established, aproximately 1-2 × $10^{\wedge 5}$ cells were taken for RNA isolation, while the rest of the cells were frozen.

3.1.4. Cell counting and proliferation assay

The cell counting and proliferation assay were done in our laboratory. Cell viability and cell counting of the primordial germ cells were performed before freezing using the Arthur Novel Fluorescence Cell Counter (NanoEnTek, Pleasanton, USA). Each side of the specialized Arthur slide allows a maximum of 25 µL of cell suspension to be measured. For each cell line, two measurements were taken, and the average was calculated. A cell proliferation assay was performed using a PICO machine over a period of two days. Approximately 10–15 µL of cell suspension was loaded into a 96-well cell culture plate [Greiner Bio-One, Cat. No. 655180] for the assay.

3.1.5. PGC freezing

Primordial germ cells (PGCs) were cryopreserved using a protocol consisting of suspension, freezing, and long-term storage. In this study, the cells were not stored but thawed to check the gene expression differences before and after freezing. Cultivated cells were first gently resuspended and transferred from culture wells into 1.5 mL sterile Eppendorf tubes. The suspensions were centrifuged at 1300 rpm for 4 minutes at room temperature. Following centrifugation, the supernatant was carefully aspirated to avoid disturbing the pellet. The cell pellets were resuspended in 250 µL of pre-warmed Avian-KO-DMEM, which was prepared with the components detailed in Table 3-5. An equal volume (250 µL) of freshly prepared freezing medium, components detailed in Table 3-6, was then added dropwise with gentle mixing to minimise osmotic shock.

Table 3-5: Avian-KO-DMEM. The following components were mixed and filtered.

Component	Proportion	Catalogue number
DMEM	2	Life technologies cat: 21068-028
Sterile water	1	Invitrogen Tissue Culture grade
Sodium Pyruvate	4 µL/ 1 mL	cat: 11360-039

Table 3-6: The following Freezing medium components were mixed and filtered.

Component	Volume
Avian-KO-DMEM	5 mL
DMSO	400µL (final concentration 8%)
Chicken serum	500 µL
CaCl2 100 mM	7.5 µL

The cell suspensions were immediately transferred into labelled cryovial tubes. Samples were placed at -70 °C overnight to allow controlled cooling. For long-term preservation, vials were transferred the following day to -150 °C or liquid nitrogen storage. The freezing rate properly for preserving cells is the 1 °C per minute, with this the cells avoid rapid ice crystal formation, reduce osmotic stress, and reduce DMSO toxicity (BAUST et al., 2000) .

3.1.6. PGC thawing

After long-term freezing, the cryovials were thawed at room temperature, submerging in the water bath for approximately 15 seconds. DMSO is toxic to PGCs, so the thawing was taking as fast as possible. The cells were transferred to a new 15mL tube containing 2 mL of PGC culture medium. The tube was centrifuged at 1400 rpm for 4 minutes (Jouan GR412, radius 12.5-18 cm). The supernatant was discarded, keeping the bottom. The pellet that contains the cells was suspended in PGC medium (300 µL/well) and transferred to a 48-well culture plate. The cells were maintained for approximately one week until optimal viability was achieved.

3.1.7. RNA isolation from PGC

RNA was isolated from PGC using RNAqueous micro kit RNA isolation from microscale and LCM samples (Invitrogen by Thermo Fisher Scientific, catalogue number: AM1931).

PGCs amples were initially treated with 100 µL of lysis buffer and stored for at least one night. Subsequently, 50 µL of ethanol was added, and the mixture was vortexed briefly. A total of 150 µL of the resulting solution was transferred into a spin filter column placed inside a collection tube.

The column was centrifuged at maximum speed for 10 seconds to allow RNA to bind to the filter membrane. Next, 180 µL of Wash Solution 1 was added to the column, followed by centrifugation at maximum speed (13,000 x g) for 10 seconds at 4 °C.

The column was then washed twice with 180 µL of Wash Solution 2. Each wash was followed by centrifugation at maximum speed (13,000 x g) for 10 seconds at 4 °C, ensuring that RNA remained bound to the filter.

To remove any residual wash buffer, the empty column was centrifuged at maximum speed (13,000 x g) for 60 seconds at 4 °C with a clean collection tube.

The spin filter was then transferred to a new 1.5 mL microcentrifuge tube. For RNA elution, 10 µL of preheated (75 °C) Elution Solution was applied directly to the centre of the membrane. After a 1-minute incubation at room temperature, the tube was centrifuged for 30 seconds (13,000 x g) at 4 °C to collect the eluted RNA.

The isolated RNA was quality checked using a NanoDrop (ND-1000, Thermo Fisher Scientific, Waltham, MA, UV-Vis). The purified RNA samples were then stored at -70 °C to ensure their integrity for future experiments.

3.1.8. cDNA writing for markers

RNA was diluted until it reached a concentration of 25 ng/mL. High-capacity RNA-to-cDNA kit (Applied biosystems by Thermo Fisher Scientific, catalogue number: 4387406) was used to generate cDNA from the isolated RNA.

The following components described in Table 3-7 were prepared:

Table 3-7: Master mix reagents of High-Capacity RNA-to-cDNA Kit

High-capacity RNA-to-cDNA component	1x 1 sample
2x RT Buffer mix	10 µL
20x RT Enzyme mix	1 µL
RNA Sample 25 ng/mL	9 µL

Table 3-8: Retro-transcriptase thermal cycling conditions.

Setting	Temperature (°C)	Time
Step 1	37	60 min
Step 2	95	5 min
Step 3	4	hold

9 µL of RNA Sample 25 ng/mL was added to 11 mL of the master mix in an individual PCR tube.

The ProFlex PCR System (Applied Biosystems, Life Technologies) was used to perform the reverse transcription reaction under the conditions detailed in Table 3-8. The cDNA samples were stored at 2-8°C for the short term.

3.1.9. cDNA writing for miRNA

RNA samples were diluted to 5 ng/mL. TaqMan® Advanced miRNA cDNA Synthesis kit (Applied Biosystems by Thermo Fisher Scientific, catalogue number: A28007) was used to generate cDNA from the isolated Additionally, for all the steps the ProFlex PCR System (Applied Biosystems, Life Technologies) was used. The mentioned kit consists of the following four steps: Poly(A) tailing reaction, adaptor ligation reaction, reverse transcription (RT) reaction, and miR-Amp reaction.

Poly(A) tailing reaction

The 3′ end of all mature miRNAs was enzymatically polyadenylated, where a string of adenosine nucleotides was added. The polyadenylation reaction mixture was prepared according to Table 3-9. A 2 µL sample was transferred to a PCR tube, and 3 µL of the reaction mix was added to each sample. After brief vortexing, the tubes were incubated for 45 minutes at 37 °C, and the reaction was stopped at 65 °C for 10 minutes. The thermocycle conditions are described in Table 3-10.

Table 3-9: Poly(A) tailing reaction components

Component	Volume (µL)
10× Poly(A) buffer	0.5
ATP	0.5
Poly(A) enzyme	0.3
RNase-free water	1.7
Total	3.0

Table 3-10: Thermal conditions for Poly(A) tailing reaction.

Step	(°C)	Time
Polyadenylation	37	45 min
Stop reaction	65	10 min
Hold	4	Hold

Adaptor ligation reaction

Following polyadenylation, a universal RNA adaptor was ligated to the 5′ end of the miRNAs. The adaptor ligation reaction mixture was prepared according to Table 3-11. 10 µL of the adaptor ligation reaction mix was added to the previous preparation. After thorough vortexing, the ligation reaction was incubated at 16 °C for 60 minutes. The thermocycle conditions are described in Table 3-12.

Table 3-11: Adaptor ligation reaction components

Component	Volume (µL)
5× DNA ligase buffer	3.0
50% PEG 8000	4.5
25× Ligation adaptor	0.6
RNA ligase	1.5
RNase-free water	0.4
Total	10.0

Table 3-12: Thermal cycling conditions for adaptor ligation

Step	Temperature (°C)	Time
Ligation	16	60 min
Hold	4	Hold

Reverse transcription (RT) reaction

Due to the addition of the RNA adaptor, a universal RT primer could bind to the poly(A) tail and adaptor-ligated RNA. Reverse transcriptase synthesised cDNA from the mature miRNA templates. The reverse transcription mixture was prepared according to Table 3-13. 15 µL of the RT reaction mixture was added to the previous tube and incubated at 42 °C for 15 minutes. The thermocycle conditions are described in Table 3-14.

Table 3-13: Reverse transcription reaction components.

Component	Volume (µL)
5× RT buffer	6.0
dNTP mix (25 mM)	1.2
20× universal RT primer	1.5
10× RT enzyme mix	3.0
RNase-free water	3.3
Total	15.0

Table 3-14: Thermal cycling conditions for reverse transcription.

Step	Temp (°C)	Time
Reverse transcription	42	15 min
Stop reaction	85	5 min
Hold	4	Hold

miR-Amp reaction

miRNAs were amplified to increase the cDNA levels of low-abundance targets. The miR-Amp reaction mixture was prepared according to Table 3-15. 45 µL of the miR-Amp reaction mix was transferred into clean Eppendorf tubes, and 5 µL of the reverse transcription product was added. The mixture was incubated under the conditions detailed in Table 3-16.

Table 3-15: miR-Amp reaction components

Component	Volume (µL)
2× miR-Amp master mix	25.0
20× miR-Amp primer mix	2.5
RNase-free water	17.5
Total	45.0

Table 3-16: miR-Amp thermal conditions.

Cycle	(°C)	Time	Step
1×	95	5 min	Enzyme activation
14×	95	3 sec	Denaturation
1×	60	30 sec	Annealing/ extension
1×	99	10 min	Stop Reaction
1×	4	hold	Hold

Storage

The cDNA samples were ready for real-time amplification and were stored at -20 °C. The remaining reverse transcription product was also stored at -20 °C.

3.1.10. qPCR for markers

TaqMan™ universal PCR master mix (Applied Biosystems by Thermo Fisher Scientific) was utilised to study the PGCs marker expression. The master mix was prepared according to the manufacturer's instructions and described in Table 3-17. The qPCR was performed by Eppendorf MasterCycler Realplex thermal cycler with the conditions described in Table 3-18. The Taq primers used are described in the following Table 3-19.

Table 3-17: TaqMan™ PCR mix for PGC markers

TaqMan™PCRmix(14 µL)	1x/sample
RNase free water (NFW)	5.75 µL
Master Mix universal TaqMan™	7.5 µL
TaqMan ™ Primer	0.75 µL

Table 3-18:Thermal conditions for PGC markers qPCR

Cycle	(°C)	Time	Step
1x	95	10 min	Enzyme activation
40x	95	20 sec	Denaturation
	60	1 min 05 sec	Annealing

Table 3-19: Primer for PGC markers.

Primer	Assay ID	Catalogue number
cGAPDH	Gg03346984_g1	4351372
cPOUV	Gg03813787_s1	4351372
cDDX4	Gg03338555_m1	4351372
cDAZL	Gg03363982_m1	4351372

In a PCR plate 14 µL of TaqMan PCR mix was loaded and 1 µL of cDNA was added. The qPCR plate was covered and centrifuged for 1 minute (300 x g), mixed for 10-20 seconds with a thermomixer (100 x g) and centrifuged for 1 minute (300 x g).

3.1.11. qPCR for miRNA

The cDNA was diluted in 1:10 rate. miRNA qPCR was performed with TaqMan® Fast advanced master mix (applied biosystems by Thermo Fisher Scientific, catalogue number: 4444557).

The master mix was prepared according to the manufacturer's instructions and described in Table 3-20. The qPCR was performed by Eppendorf MasterCycler Realplex thermal cycler with the conditions detailed in Table 3-21. The primers used are described in Table 3-22.

Table 3-20: Preparation of TaqMan Fast Advanced Master mix.

TaqMan® Fast Advanced Master mixture (15 µL)	**1x/sample**
TaqMan® Fast Advanced Master mix	10 µL
TaqMan® Advanced miRNA Assay (primer)	1 µL
Free RNasa water	4 µL

Table 3-21: qPCR Cycling Conditions

Cycle	**(°C)**	**Time**	**Step**
1x	95	20 sec	Enzyme activation
45x	95	3 sec	Denaturation
	60	30 sec	Annealing

Table 3-22: List of TaqMan Advanced miRNA Assays and Catalogue Numbers.

TaqMan® Advanced miRNA Assay (primer)	**Access**	**Catalogue number**
hsa-miR-92a-3p	477827_mir ADV	A25576
hsa-miR-138-5P	477905_mir ADV	A25576
hsa-miR-302c-3P	478509_mir ADV	A25576

15 µL TaqMan® Fast Advanced Master mix containing the corresponding miRNA primer was added to each position of the qPCR plate, and then 5 µL of the samples (1:10) were added; additionally, 5 µL of RNase-free water was added as well as negative control.

For each sample, the reaction was performed in 3 parallels for the tested miRNAs.
The qPCR plate was covered and centrifuged for 1 minute (300 x g), mixed for 10-20 seconds with a thermomixer (100 x g) and centrifuged for 1 minute (300 x g).

3.1.12. qPCR analyses

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and miR-92a-3p were selected as the internal control, or housekeeping gene, for the expression calculation of PCG markers.

GAPDH and miR-92a-3p are well-known for its stable expression across different tissues and conditions. For instance, GAPDH and miR-92a-3p were used for accurate normalisation of the data and to determine the relative expression levels of the target PGC-specific markers. In the same way miR-92a-3p was used as an internal control for accurate normalization of the data to calculate relative expression levels of miRNAs.

In each qRT-PCR reaction, three technical replicates (parallels) were analyzed to assess the consistency and reliability of the amplification results.
The resulting data were processed and analysed using the GenEx 7 software program (MultiD Analyses AB, Göteborg, Sweden), which facilitated relative quantification of the expression levels for each gene examined.

The threshold cycle (Ct) values obtained from the qRT-PCR reactions were analysed, and the expression levels of the target miRNAs were calculated using the comparative Ct method. This method allows for the quantification of relative expression levels by comparing the Ct of the target miRNAs to that of the internal control.

The formula used was $2^{-\Delta\Delta Ct}$ where:
$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal control}}$

To evaluate the expression differences between various experimental conditions, ΔΔCt was calculate where:

$\Delta\Delta Ct = \Delta Ct_{\text{test sample}} - \Delta Ct_{\text{control sample}}$

T-test (type=2, tail=2) in GenEx 7.0 software was used to evaluate statistical differences between the groups. T-test compares the means of two independent groups to determine whether there is a statistically significant difference between them in either direction.
A p-value of less than 0.05 was considered statistically significant, indicating a meaningful difference in gene expression levels.

3.2.Investigation in rabbit embryos: From studying developmental embryonic stages to verifying the presence of miRNAs in culture media.

The methodology comprised three main steps: miRNA selection, embryo production, and miRNA analysis, as illustrated in Figure 3-2.

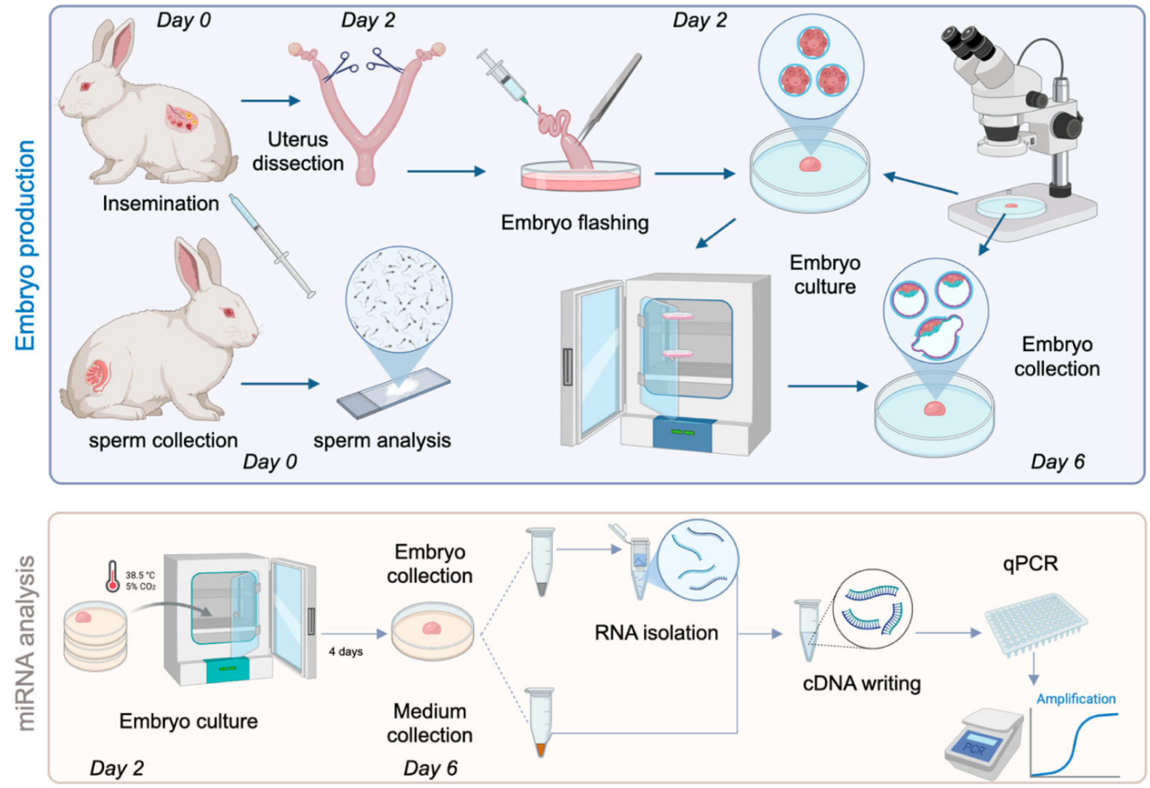


Figure 3-2: Schematic flowchart summarising the steps of the experimental process. Created with BioRender.com

3.2.1.miRNA selection

Libraries were prepared from total RNA previously isolated from rabbit embryo (6,7 and 14 days post coitum), ES cells, and germinal cells, and sequenced at the Institute of Plant Genomics, Human Biotechnology and Bioenergetics of the Bay Zoltán Applied Research Foundation (Szeged, Hungary) using the SOLiD 3 System (Applied Biosystems). SOLiD System is a small RNA analysis pipeline Tool (RNA2MAP, version 0.5), which includes read filtering, mapping, and quantification steps. The resulting sequencing data were deposited in the European Nucleotide Archive (ENA) under accession number ERP002216 via the SRA Webin submission system (Maraghechi et al., 2013).

The previous library were used for miRNA identification, the RNA sequences were annotated using a comparative approach based on known human (hsa), bovine (bta), and mouse (mmu) miRNA sequences obtained from miRBase (https://mirbase.org). This cross-species annotation strategy was applied to identify conserved miRNAs in rabbit. From the annotated dataset, miRNA sequences were extracted, and their relative abundance was assessed in mouse embryonic fibroblast, rabbit embryonic fibroblasts, rabbit embryonic stem cells, rabbit embryo of 6 and 7 dpc (days post coitum), and rabbit PGCs 14 dpc. For clarity and easier interpretation of the results, the abundance data are presented in the following chapter, in Table 4-15.

The miRNAs were ordered with the catalogue numberA25576 details in the following Table 3-23:

Table 3-23 miRNAs with Corresponding Assay IDs and Sequences.

Name of miRNAs	ID number	miRNA sequency
mmu-miR-302b-3p	481677_mir	UAAGUGCUUCCAUGUUUUAGUAG
hsa-miR-92a-3p#	477827_mir	UAUUGCACUUGUCCCGGCCUGU
hsa-miR-378a-3p	478349_mir	ACUGGACUUGGAGUCAGAAGGC
hsa-miR-372-3P	478071_mir	AAAGUGCUGCGACAUUUGAGCGU
hsa-miR-371a-5p	478851_mir	ACUCAAACUGUGGGGGCACU
hsa-miR-320a-3p	478594_mir	AAAAGCUGGGUUGAGAGGGCGA
hsa-miR-302c-3P	478509_mir	UAAGUGCUUCCAUGUUUCAGUGG
hsa-miR-28-3p	477999_mir	CACUAGAUUGUGAGCUCCUGGA
hsa-miR-24-3p	47932_mir	UGGCUCAGUUCAGCAGGAACAG
hsa-miR-191-5p	477952_mir	CAACGGAAUCCCAAAAGCAGCUG
hsa-miR-181a-5p	477857_mir	AACAUUCAACGCUGUCGGUGAGU
hsa-miR-103a-3p	478253_mir	AGCAGCAUUGUACAGGGCUAUGA

#endogenous control

3.2.2.Experimental animals and animal care

The New Zealand white rabbits (*Oryctolagus cuniculus*) were provided by Innovo Ltd, Isaszeg, Hungary. All procedures involving animals were conducted in accordance with the relevant European legislation on the protection of animals used for scientific purposes, specifically Directive 2010/63/EU of the European Parliament and of the Council. It was approved by the Pest County Government Office's Directorate of Food Chain Safety, under the permit number PE/EA/00741-7/2022.

The animals were housed at the Institute of Genetics and Biotechnology, Hungarian University of Agriculture and Life Sciences (MATE), in a climate-controlled animal facility maintained at a constant temperature of 19 ◦C. Continuous veterinary supervision was ensured throughout the study. Ad libitum access to feed (Purina Mills, LLC (Purina Animal Nutrition), Gray Summit, MO, USA) and drinking water, and environmental enrichment in the form of chew sticks was provided. A 24 h artificial light–dark cycle was maintained, consisting of 16 h of light and 8 h of darkness daily.

3.2.3.Embryo production

Female rabbits aged 16–20 weeks (mean body weight: 3.0–3.5 kg) were artificially inseminated using freshly collected sperm from male rabbits, aged ≥ 18–20 weeks.

Before use, sperm samples were examined microscopically to confirm sufficient motility. Ejaculate volume was measured using a precision pipette. Sperm motility was subjectively assessed under light microscopy (Leica Microsystems GmbH, Wetzlar, Germany). Sperm concentration was determined using a Makler counting chamber after a 1:4 dilution in distilled water, which immobilised sperm through hypotonic lysis. Concentrations were calculated from counts in 30 grid squares.

Semen from four males was collected during each experiment, and only samples of acceptable quality were pooled before insemination.

Before embryo collection, all donor females were euthanized with 0.25 mL/kg BW Euthanimal (Alfasan Nederland B.V., Utrecht, Netherlands) and dissected under sterile conditions.

The abdominal area of the female donor was sterilised with 70% ethanol, and the ovaries, the ipsilateral fallopian tube, and the cranial part of the uterine horn from both sides were removed carefully, and placed in Petri dishes with preheated DPBS (Gibco, catalogue number: 14190144) at 38.5 °C. The number of blood corpuscles present on the surface of the ovary (corpus hemorrhagicum) was counted to estimate the number of embryos that could be recovered from the female.

Eight-cell stage embryos were retrieved 38–40 h (day2) post-insemination.

To collect the embryos, the uterine horn or oviduct was flushed with a pre-heated medium by injecting the medium into the reproductive tract using a 20 ml syringe fitted with a 19G injection needle, effectively flushing the embryos out with the medium containing them. For each reproductive organ, three 35mm Petri dishes were filled with the flowing medium, which contained the embryos and the Petri dishes were kept at 38.5 °C.

The components of flushing medium are detailed in the following Table 3-24:

Table 3-24: Components of flushing medium.

Components	Catalogue number
79% DPBS (Dulbecco's Phosphate-Buffered Saline)	Gibco, Cat. No. 14190144
20% heat-inactivated FBS (fetal bovine serum)	Gibco, Cat. No. 10500-064; Lot: 07Q2130K
1% Penicillin-Streptomycin	Gibco, Cat. No. 15140122

The solution was sterilized by filtration through a 0.22 µm membrane (Merk Millipore Express PLUS membrane, catalogue number GPWP01300) and preheated to 38.5°C.

The effluent was collected in three individual 35 mm Petri dishes maintained at the same temperature, 38.5 °C.

Recovered embryos were washed through three successive drops of G-TL human embryo culture medium (Cat. No. 10145, Vitrolife, Göteborg, Sweden) supplemented with hyaluronan and human serum albumin with a mouth pipette.

The embryos were morphologically analysed with a stereomicroscope (Leica M205FCA supplied with DFC7000-T Leica camera, Wetzlar, Germany).

Embryos were subsequently transferred to fresh G-TL medium droplets, pre-equilibrated under sterile mineral oil (OVOIL, Cat. No. 100290, Vitrolife, Göteborg, Sweden) and cultured in an incubator set at 38.5 °C in a humidified atmosphere of 5% CO_2 for 6 days or until reaching the expanded blastocyst stage on day 6.

Embryos were washed out on day 2 and individually cultured in a separate drop of GT-L medium for time-lapse imaging. Time-lapse microscopy analysis began in the afternoon on Day

2. Embryonic development was monitored using the CytoSMART Lux2 system (Lonza Group Ltd., Basel, Switzer- land). Images were acquired at five-minute intervals under standard culture conditions (Appendix A2: Supplementary Movie S1).

3.2.4.Sample collection

Two types of samples were collected on day 6: the embryos themselves and the medium in which they were cultured.

For medium samples, 9 µL of medium was taken into sterile 1.5 mL tubes. For embryo samples, the embryos were transferred into 1.5 mL tubes containing 100 µL of lysis buffer (guanidinium thiocyanate). Both sample types were stored at -80°C.

3.2.5.RNA isolation from the embryo samples

RNA isolation was performed only on embryo samples with RNAqueous™-Micro total RNA Isolation Kit (catalogue number: AM1931).

Embryo tissue samples were initially treated with 100 µL of lysis buffer. Subsequently, 50 µL of ethanol was added, and the mixture was vortexed briefly. A total of 150 µL of the resulting solution was transferred into a spin filter column placed inside a collection tube.

The column was centrifuged at maximum speed for 10 seconds to allow RNA to bind to the filter membrane. Next, 180 µL of Wash Solution 1 was added to the column, followed by centrifugation at maximum speed (13,000 x g) for 10 seconds at 4 °C.

The column was then washed twice with 180 µL of Wash Solution 2. Each wash was followed by centrifugation at maximum speed (13,000 x g) for 10 seconds 4 °C, ensuring that RNA remained bound to the filter.

To remove any residual wash buffer, the empty column was centrifuged at maximum speed (13,000 x g) for 60 seconds at 4 °C with a clean collection tube.

The spin filter was then transferred to a new 1.5 mL microcentrifuge tube. For RNA elution, 10 µL of preheated (75 °C) Elution Solution was applied directly to the centre of the membrane. After a 1-minute incubation at room temperature, the tube was centrifuged for 30 seconds (13,000 x g) at 4 °C to collect the eluted RNA. The isolated RNA was quality checked using a NanoDrop (ND-1000, Thermo Fisher Scientific, Waltham, MA, UV-Vis). The samples were kept at -70 °C.

3.2.6.cDNA writing

Medium samples were taken directly to cDNA writing without RNA isolation, expecting that miRNAs would already be in the medium, while RNA from embryo samples were taken from isolation. Both sample types (embryo and medium) were written into cDNA using the Applied Biosystems TaqMan Advanced miRNA cDNA Synthesis Kit (catalogue number: A28007).

Since miRNAs are small (~22 nt), the traditional reverse transcription methods are not suitable nor efficient due to miRNA size and absence of poly(A) tails. The cDNA writing with the mentioned kit allows the addition of poly(A) tails and 5' adapters, which helps to robust and uniform cDNA synthesis. Additionally, for all the steps the ProFlex PCR System (Applied Biosystems, Life Technologies) was used. The cDNA writing with the mentioned kit consisted of four reactions: Poly(A) tailing reaction, adaptor ligation reaction, reverse transcription (RT) reaction, and miR-Amp reaction.

Poly(A) tailing reaction

The 3′ end of all mature miRNAs was enzymatically polyadenylated, where a string of adenosine nucleotides was added. The polyadenylation reaction mixture was prepared according to Table 3-25. A 2 µL sample was transferred to a PCR tube, and 3 µL of the reaction mix was added to each sample. After brief vortexing, the tubes were incubated for 45 minutes at 37 °C in and the reaction was stopped at 65 °C for 10 minutes. The thermocycler conditions are described in Table 3-26.

Table 3-25: Poly(A) tailing reaction components

Component	Volume (µL)
10× Poly(A) buffer	0.5
ATP	0.5
Poly(A) enzyme	0.3
RNase-free water	1.7
Total	3.0

Table 3-26: Thermal conditions for Poly(A) tailing reaction.

Step	°C	Time
Polyadenylation	37	45 min
Stop reaction	65	10 min
Hold	4	Hold

Adaptor ligation reaction

Following polyadenylation, a universal RNA adaptor was ligated to the 5′ end of the miRNAs. The adaptor ligation reaction mixture was prepared according to Table 3-27. 10 µL of the adaptor ligation reaction mix was added to the previous preparation. After thorough vortexing, the ligation reaction was incubated at 16 °C for 60 minutes. The thermocycler conditions are described in Table 3-28.

Table 3-27: Adaptor ligation reaction components

Component	Volume (µL)
5× DNA ligase buffer	3.0
50% PEG 8000	4.5
25× Ligation adaptor	0.6
RNA ligase	1.5
RNase-free water	0.4
Total	10.0

Table 3-28: Thermal cycling conditions for adaptor ligation

Step	°C	Time
Ligation	16	60 min
Hold	4	Hold

Reverse transcription (RT) reaction

Due to the addition of the RNA adaptor, a universal RT primer could bind to the poly(A) tail and adaptor-ligated RNA. Reverse transcriptase synthesised cDNA from the mature miRNA templates. The reverse transcription mixture was prepared according to Table 3-29. 15 µL of the RT reaction mixture was added to the previous tube and incubated at 42 °C for 15 minutes. The thermocycler conditions are described in Table 3-30.

Table 3-29: Reverse transcription reaction components.

Component	Volume (µL)
5× RT buffer	6.0
dNTP mix (25 mM)	1.2
20× universal RT primer	1.5
10× RT enzyme mix	3.0
RNase-free water	3.3
Total	15.0

Table 3-30: Thermal cycling conditions for reverse transcription.

Step	°C	Time
Reverse transcription	42	15 min
Stop reaction	85	5 min
Hold	4	Hold

miR-Amp reaction

miRNAs were amplified to increase the cDNA levels of low-abundance targets. The miR-Amp reaction mixture was prepared according to Table 3-31. 45 µL of the miR-Amp reaction mix was transferred into clean Eppendorf tubes, and 5 µL of the reverse transcription product was added. The mixture was incubated under the conditions detailed in Table 3-32.

Table 3-31: miR-Amp reaction components

Component	Volume (µL)
2× miR-Amp master mix	25.0
20× miR-Amp primer mix	2.5
RNase-free water	17.5
Total	45.0

Table 3-32: miR-Amp thermal conditions.

Cycle	°C	Time	Step
1×	95	5 min	Enzyme activation
14×	95	3 sec	Denaturation
1×	60	30 sec	Annealing/extension
1×	99	10 min	Stop Reaction
1×	4	hold	Hold

Storage

The cDNA samples were ready for real-time amplification and were stored at -20 °C. The remaining reverse transcription product was also stored at -20 °C.

3.2.7. qRT-PCR

The cDNA samples were diluted 1:10 by adding RNase-free water. TaqMan® Fast advanced master mix (Applied Biosystems by Thermo Fisher Scientific, catalogue number: 4444557) was prepared with the following proportions for each primer detailed in Table 3-33:

Table 3-33: Preparation of TaqMan Fast Advanced Master mix.

TaqMan® Fast Advanced Master mixture	**1x/sample**
TaqMan® Fast Advanced Master mix	10 µL
TaqMan® Advanced miRNA Assay (primer)	1 µL
Free RNasa water	4 µL
Total	15 µL

Table 3-34: PCR Thermal Cycling Conditions

Cycle	°C	Time	Step
1x	95	20 sec	Enzyme activation
45x	95	3 sec	Denaturation
	60	30 sec	Annealing

The TaqMan® Advanced miRNA Assay primers were detailed in Table 3-23 in miRNA selection section.

15 µL TaqMan® Fast Advanced Master mix containing the corresponding miRNA primer was added to each position of the qPCR plate, and then 5 µL of the samples (1:10) were added; additionally, 5 µL of RNase-free water was added, as well as a negative control.

For each sample, the reaction was performed in 3 parallel reactions for the tested miRNAs.

The qPCR plate was covered and centrifuged for 1 minute (300 x g), mixed for 10-20 seconds with a thermomixer (100 x g) and centrifuged for 1 minute (300 x g). The qPCR was performed by Eppendorf MasterCycler Realplex thermal cycler with the conditions detailed in Table 3-34.

3.2.8. Data analysis – MultiD GenEx

The 2 best RT-qPCR results were processed using the Eppendorf RealPlex program. The obtained data were analysed with the MultiD GenEx qPCR data analysis software (http://www.multid.se, version 7.0). Norm Finder tool, integrated within the GenEx software (version 7.0), was used to identify the most stable reference genes for normalisation in qPCR. According to this analysis we used the miR-92a-3p as an internal control. F3_medium pool was used as a reference sample during the data analysis. For comparing multiple groups, one-way ANOVA analysis of variance was used. When ANOVA was significant, Tukey's post hoc test identified which specific group pairs differed. The Kohonen and SOM (self-organizing map) hierarchical clustering function was used to create a heatmap and groups which illustrates the difference between expressions and the correlations between samples.

4. RESULTS AND DISCUSSION

4.1. Investigation of chicken PGCs: miRNA expression before and after freezing

The importance of primordial germ cells (PGCs) in this study is based on two key considerations.

First, PGCs are the precursors of gametes and play a central role in the transmission of genetic information across generations. PGCs provide a practical and effective approach for the cryopreservation of genetic material from both female and male individuals(Doddamani et al., 2025). In avian species, sperm cryopreservation has been successfully established; however, the cryopreservation of eggs remains challenging due to their large size, high yolk content and complex anatomy. In birds, males are homogametic (ZZ) and females are heterogametic (ZW). Consequently, the inability to efficiently cryopreserve eggs limits the preservation of female-specific genetic material, including the W chromosome. The use of PGCs overcomes this limitation by enabling the conservation of genetic information from both sexes(Ecker et al., 2023).

Secondly, PGC cryopreservation is a fundamental strategy for the long-term preservation of genetic resources. Cryopreservation is a preservation technique that uses very low temperatures to maintain biological material for long periods. However, freezing cells below 0°C can be lethal. Cells contain approximately 80% water, and when this water freezes, it can form harmful ice crystals inside the cell. These ice crystals can mechanically damage cellular structures, including membranes and organelles. In addition, as ice forms, the remaining unfrozen solution becomes more concentrated, increasing the solute concentration inside and outside the cell. This leads to hyperosmotic stress, causing excessive cell shrinkage and further structural damage(Whaley et al., 2021). Cryopreservation requires the use of cryoprotective agents such as dimethyl sulfoxide (DMSO), glycerol (GLY), ethylene glycol (EG), or propylene glycol (PG) to prevent ice-induced cellular damage during freezing. In this study, DMSO was used to protect cellular integrity from intracellular ice crystal formation. DMSO is a small, highly polar molecule that easily crosses cell membranes. Inside the cell, it binds to water molecules, reducing the amount of free water available to form ice crystals. It also lowers the freezing point and increases the viscosity of the solution, which helps slow ice formation and reduce crystal growth(Awan et al., 2020).

However, during thawing, ice melts, extracellular osmolarity rapidly decreases, DMSO diffuses out of the cell, and water quickly moves back in. This rapid influx of water can cause cell swelling, membrane stress, oxidative stress due to mitochondrial reactivation, and cytoskeletal reorganization. DMSO can not protect the cell from this stress that happens during thawing(Awan et al., 2020; Best, 2015).

In addition, DMSO can become toxic, as it can disturb membrane structure, alter protein shape, affect the cytoskeleton, and interfere with mitochondrial function. DMSO toxicity increases with higher concentrations, longer exposure times, and higher temperatures, which is why it is added at low temperature and removed promptly after thawing to minimize cellular damage. While extensive efforts have been made to improve cryopreservation protocols and mitigate these effects, cryogenic stress remains (Best, 2015; Cao et al., 2022; Chatterjee et al., 2017; Mazur, 1984, 2004).

In this context, cryopreservation can be viewed not only as a preservation tool but also as a biologically informative stress model. In the present study, cryopreservation-induced stress was used to measure the resilience and recovery capacity of PGCs, measuring changes in gene expression markers and miRNA related to pluripotency, proliferation and stemness. (Best, 2015; Cao et al., 2022; Chatterjee et al., 2017; Mazur, 1984, 2004).

Four PGC lines had been isolated, established and then *in vitro* cultured for approximately one month before the experiments. Simultaneously, sex PCR was performed. The initial concentration and after establishment and sex of the cell lines are shown in the following table 4-1.

Table 4-1: Cell densities and corresponding sex of the PGC lines included in the experimental analyses.

Cell line	Cells/mL	Sex
7	22x10^5	Female
37	42x10^5	Female
43	32x10^4	Male
60	10x10^5	Male

The four cell lines were divided into two groups; one group was frozen for minimum one week and thawed, while the other group had RNA isolated directly from the initial culture.
After the cryopreservation, the proliferation rate of the 4 cell lines (7, 37, 43, and 60) was measured as well.

4.1.1. Calculation of proliferation rate in cell lines after cryopreservation

Proliferation rate measurements were conducted to compare the recovery and growth dynamics of the PGC lines during the first two days after thawing, as shown in Table 4-2. The proliferation rates of the 4 cell lines were compared in Figure 4-1. A t-test (tails=2, type=2) was applied to detect any significant differences in the proliferation comparison among the cell lines on days 1 and 2 after freezing, shown in Table 4-2 and Table 4-3.

Table 4-2: Proliferation rate on day 1 and day 2 after freezing and thawing of the 4 PGC line and statistical comparison.

Cell line	Day1	Day2	p-value d1vs d2
7 Female	2.11	1.72	0.18420 ns
7 Female	2.14	1.92	
7 Female	1.89	1.97	
37 Female	2.72	1.87	0.01015 *
37 Female	3.06	2.18	
37 Female	2.79	2.28	
43 Male	2.90	1.25	0.01350 *
43 Male	2.40	1.94	
43 Male	3.11	1.55	
60 Male	2.43	1.58	0.00949 **
60 Male	2.85	1.84	
60 Male	2.49	1.95	

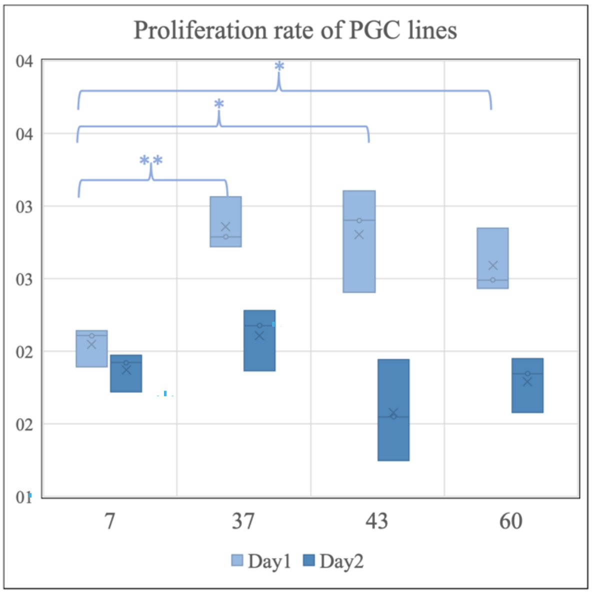


Figure 4-1: proliferation rate of PGC line 7 (female), 37 (female), 43 (male) and 60 (male)

Table 4-3: P-value of proliferation rate comparison among cell lines on day 1 and day 2.

Comparison	Day1	Significance	Day2	Significance
7 vs 37	0.00350	**	0.18216	ns
7 vs 43	0.02730	*	0.24710	ns
7 vs 60	0.02330	*	0.57957	ns
37 vs 43	0.83142	ns	0.08988	ns
37 vs 60	0.18664	ns	0.12983	ns
43 vs 60	0.43341	ns	0.41026	ns

The PGC lines showed higher proliferation rate on day 1 compared to second day, with considerable significance differences shown in Table 4-2, p-value d1/d2, except on PGC 7 which didn't show any important difference between day 1 and day 2. However, on the first day after thawing, the 7 PGC line exhibited a significantly lower proliferation rate compared with the other lines (PGC 37 p = 0.0035, **; PGC 43 p = 0.027, *; PGC 60 p = 0.023, *). This distinction disappeared by the second day.

The observed proliferation differences on Day 1 suggest that PGC 7 exhibit a distinct initial growth response compared with other cell lines. This may reflect developmental differences in the embryo where the PGC were taken from. In contrast, the absence of significant differences among the other PGC lines indicates broadly similar proliferative capacities at this time point. Natio M. (1994) demonstrated that PGC shows variable growth characteristics *in vitro*, according to the embryonic stages, suggesting that proliferation potential depends on the developmental embryonic stage (Naito et al., 1994). In addition, PGC proliferation has been reported that is influenced by developmental origin and that early-stage PGCs respond differently to culture conditions than more developmentally advanced lines (Whyte et al., 2015). By Day 2, the loss of statistically significant differences across all comparisons suggests a rapid normalization of proliferation dynamics, likely reflecting cellular adaptation to the culture environment. The difference of proliferation between day 1 and day 2 after cryopreservation has been seen in other studies: the rapid PGC adaptation *in vitro* conditions has been seen in around 24-48 hours (Macdonald et al., 2010; Nakamura et al., 2011). Moreover, there is strong evidence which proves that germ cells rapidly normalize proliferation after environmental adjustment once attachment and metabolic equilibrium are achieved (Freshney et al., 2016).

Proliferation rate after freezing analysis by gender

To evaluate post-thaw recovery, proliferation rates from the previous Table 4-2 were analysed for both female and male PGC lines over the first two days following thawing.

Figure 4-2 illustrates the distribution of proliferation rates one day after thawing (AF), showing similar growth dynamics between sexes. Figure 4-3 compares the proliferation of female (AF-F) and male (AF-M) PGC lines on Day 2 post-thaw (AF), any significant differences was found. To determine whether proliferation rates differed significantly between genders during the first and second day, a T-test (tails=2, type=2) was applied to data from days 1 and 2 post-freezing. The p-value and significance are provided in Table 4-4.

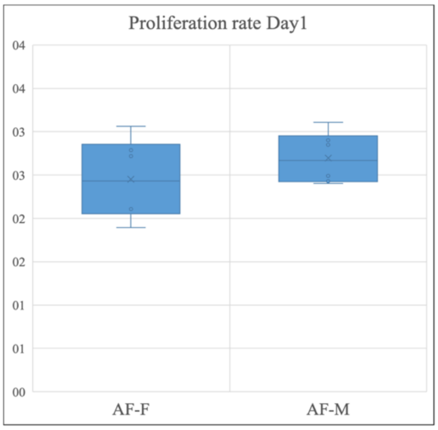


Figure 4-2: Comparison of Day 1 proliferation rates in female and male PGCs after freezing. AF: after freezing. F: female. M: male

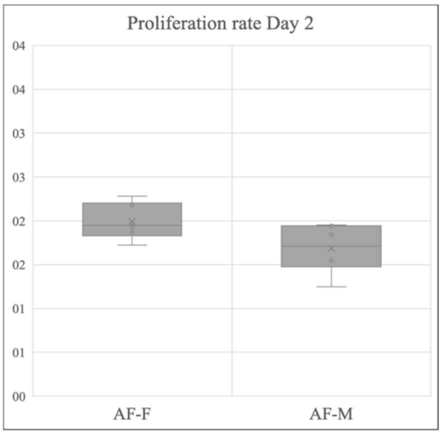


Figure 4-3: Proliferation rate of female and male PGCs on Day 2 after thawing. AF: after freezing. F: female. M: mal

Table 4-4: comparison of proliferation rate between genders on day 1 and day 2.

Comparison	p-value	Significance
AF_F vs AF_M (day 1)	0.30202158	ns
AF_F vs AF_M (day 2)	0.05608854	ns

No significant difference in proliferation rate was observed between male and female cell lines after thawing. Previous studies have reported that sex-specific differences in avian PGCs are more evident in differentiation, migration, or epigenetic regulation, rather than in short-term proliferation under standard culture conditions. Cryopreservation produced stress responses and dominate the cellular behaviour after thawing. During this short time, cells prioritize membrane repair, restoration of mitochondrial function, and management of oxidative stress. These processes are highly conserved and largely sex-independent(Macdonald et al., 2010; Whyte et al., 2015).

4.1.2. Gene expression analysis

RNA was isolated from the previous 2 groups of samples (before and after freezing of the cell lines 7,37,43, and 60). The quality of the isolated RNA is shown in the following Table 4-5 where RNA concentration (ng/µL) and absorbance ratios (A260/A280, A260/A230) were measured to evaluate RNA integrity and suitability for downstream cDNA synthesis and RT-qPCR.

Table 4-5: Quantification and purity assessment of RNA extracted from PGC lines.

Cell line	RNA ng/uL	A260/A280	A260/A230	A260	A280
7	960.925	2.142	2.116	24.023	11.214
37	1204.063	2.171	2.383	30.102	13.868
43	293.251	2.133	0.674	7.331	3.437
60	123.505	2.181	0.143	3.088	1.415
7 AF	1140.489	2.161	2.264	28.512	13.193
37 AF	1422.378	2.181	1.931	35.559	16.306
60 AF	4510.658	2.196	2.041	112.766	51.355
43 AF	244.505	2.176	0.262	6.113	2.81

AF: after freezing.

All RNA samples were prepared in parallel duplicates (A and B) to enhance data robustness as detailed in the following table 4-6. This duplication was maintained for both pre-freeze and post-freeze (AF) conditions.

Table 4-6: Duplication scheme for PGC lines RNA before and after freezing.

RNA	RNA parallelled
7A	7B
37A	37B
43A	43B
60A	60B

RNA	RNA parallelled
7A AF	7B AF
37A AF	37B AF
43A AF	43B AF
60A AF	60B AF

AF: After freezing

After cDNA synthesis and RT-qPCR, relative expression levels of the germ cell markers DDX4, POU, and DAZL, as well as the miRNAs miR-138-5p and miR-302c-3p, were quantified from the resulting CT values. Normalisation was performed using GAPDH for gene markers and miR-92a-3p for microRNAs, with sample 37A designated as the internal reference for all calculations. The relative expression is detailed in the following Table 4-7.

Table 4-7: Normalised expression levels of DDX4, POU, DAZL, miR-138-5p, and miR-302c-3p.

Cell lines	Gender	Cryopreservation Status	DDX4	POUV	DAZL	miR-138-5p	miR-302c-3p
7A	Female	Before Freezing	1.00	1.12	0.99	1.74	0.30
7B	Female	Before Freezing	1.08	0.82	0.94	0.18	0.16
37A	Female	Before Freezing	1.00	1.00	1.00	1.00	1.00
37B	Female	Before Freezing	0.95	1.23	1.64	0.77	0.63
43A	Male	Before Freezing	2.23	1.27	1.25	1.44	0.23
43B	Male	Before Freezing	1.44	1.21	1.07	0.52	0.55
60A	Male	Before Freezing	2.06	0.89	1.50	0.43	0.15
60B	Male	Before Freezing	2.54	0.94	1.43	0.24	0.20
7AFT	Female	After Freezing	0.50	0.72	0.60	3.89	6.84
7BFT	Female	After Freezing	0.53	0.70	0.79	2.58	6.43
37AFT	Female	After Freezing	0.43	1.06	0.69	5.88	5.37
37BFT	Female	After Freezing	0.79	1.19	1.23	2.74	7.39
43AFT	Male	After Freezing	1.03	1.05	0.84	14.32	20.82
43BFT	Male	After Freezing	1.03	0.99	0.90	5.72	8.11
60AFT	Male	After Freezing	1.41	0.84	0.84	4.11	7.24
60BFT	Male	After Freezing	1.27	1.05	1.18	2.18	4.27

A boxplot and a t-test (tails=2, type=2) were applied to study gene expression and detect any significant differences in the expression comparison.

4.1.2.1.miR-138-5p expression

A significant difference was observed in the expression levels of miR-138-5p after freezing with a p-value of 0.01359 (**) in females and a p-value of 0.01449 (**) in males, as shown in Figure 4-4 and Table 4-8. miR-138-5p levels are shown for female (F) and male (M) PGCs before freezing (BF) and after thawing (AF), demonstrating increased expression in both sexes after the freezing process (figure 4-4).

miR-138-5p targets SIRT1, a deacetylase dependent of NAD^+, which contributes to epigenetic regulation and metabolic homeostasis in PGCs by deacetylating histones and transcription factors, thereby supporting genomic stability and regulating stress responses. Importantly, SIRT1 has been shown to negatively regulate HIF-1α transcriptional activity through deacetylation, linking cellular energy status to hypoxia signalling (Lim et al., 2010; Sharma et al., 2023; Q. Yu et al., 2018).

miR-138-5p functions is a post-transcriptional regulator that can suppress SIRT1 expression by binding to its 3′-UTR, thereby indirectly enhancing HIF-1α activity (Heo et al., 2017). miR-138-5p targets SIRT1 and HIF-1α, both essential for metabolic and oxidative stress resistance (Yu et al., 2023).

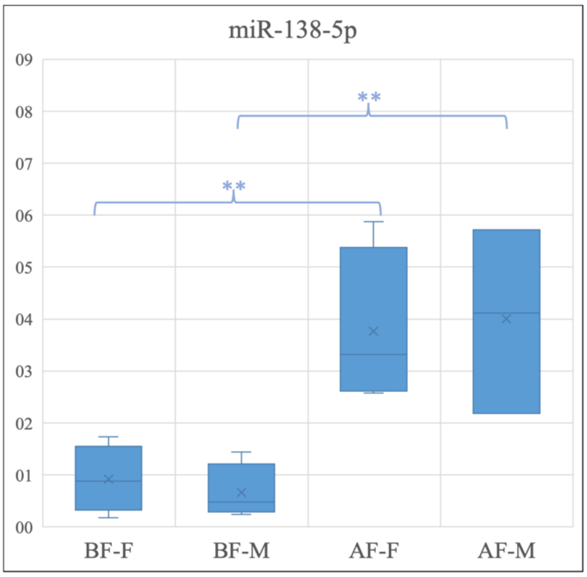


Figure 4-4: Changes in miR-138-5p expression in PGCs in response to cryopreservation. BF: before freezing. AF: after freezing. F: female. M: male

Table 4-8: Statistical significance of miR-138-5p expression changes after freezing.

Comparison	p-value	Significance
BF-F vs AF-F	0.01358836	**
BF-M vs AF-M	0.01449115	**

In chicken PGCs, this relation between miR-138-5p, SIRT1 and HIF-1α is considered as a regulatory axis which builds a stress-adaptive mechanism, particularly under hypoxic or freeze-thaw conditions, allowing the suppression of SIRT1 to facilitate the activation of HIF-1α.

HIF-1α is a key regulatory protein that becomes active when oxygen is limited and helps PGCs survive by shifting their metabolism toward glycolysis and reducing oxidative damage. The cell stress after cryopreservation might be the reason for the increment of miR-138-5p expression and the decrease of cell proliferation during the 2 days after thawing. During the freezing and thawing process, several reactions related to cell stress occur due to water crystal presence, cryo-preservative toxicity, dehydration, and cell damage, during thawing the cells faces mitochondrial dysfunction and altered redox balance similar when the oxygen level is low (Bojic et al., 2021; Heo et al., 2017; Kim et al., 2006; Semenza, 2012).

While direct evidence of this axis in avian PGCs remains limited, its components and interactions are conserved across species, especially miR-138-5p that shows conserved sequence among species (humans, rodents, birds), supporting its relevance to chicken germline biology(Ichikawa & Horiuchi, 2023; Park, 2014; Tian et al., 2017).

Other studies have related miR-138-5p to apoptosis, cancer and fertility potential. Yu (2023) reported that in chicken granulosa cells, BCL2 (anti-apoptotic gene) showed an expression increment due to the miR-138-5p suppression, resulting in SIRT1 mRNA increment. Yu (2023) suggested that miR-138-5p promotes apoptosis in chicken granulosa cells through downregulating SIRT1 (C. Yu et al., 2023). According to Li et al. (2023), overexpression of miR-138 significantly decreases cervical cancer cell proliferation, migration, and invasion, and induces pronounced apoptotic responses, indicating a strong tumour-suppressive role (Y. Li et al., 2023). A study found miR-138 expression changes in cryopreserved bovine sperm, showing that its levels were associated with fertility potential after freezing, suggesting cryo-related changes can occur for miR-138 family members in gametes. However, this research focused on sperm function and fertility, not on stress responses or recovery markers per se(Salas-Huetos et al., 2023).

The results of this study support the statement that miR-138-5p is a cryo-responsive miRNA in PGCs, likely involved in stress response, cell cycle regulation, or survival pathways activated during the recovery after thawing. The upregulation was found in both female and male

PGCs suggesting that miR-138-5p may serve as a potential molecular marker of cellular stress (induced by cryopreservation method) and recovery in germ cells.

4.1.2.2.miR-302c-30p expression

A highly significant difference was observed in the expression levels of miR-302c-30p after freezing, with a p-value of 0.00001 (***) in females and a p-value of 0.00138 (**) in males, as shown in the figure 4-5 and Table 4-9.

Expression is low in both groups before freezing and increases substantially following thawing, indicating a strong upregulation of miR-302c-3p associated to cryopreservation. miR-302c-3p is a component of the miR-302/367 cluster, one of the most highly conserved pluripotency-associated microRNA clusters across vertebrates. This cluster comprises miR-302a, miR-302b, miR-302c, miR-302d, and miR-367 (Rahimi et al., 2021). The expression analysis of miR-302c-3p revealed a marked increase in transcript abundance following cryopreservation, with both female (AF-F) and male (AF-M) samples exhibiting substantially higher levels compared with their respective pre-freezing groups (BF-F and BF-M). This upregulation is consistent with the established role of the miR-302/367 cluster in maintaining pluripotency and promoting cellular reprogramming, as described by Rahimi et al. (2021), who demonstrated that miR-302 family members facilitate genomic DNA demethylation, support the activation of core pluripotency factors, and suppress pathways of differentiation (Maraghechi et al., 2023; Rahimi et al., 2021).

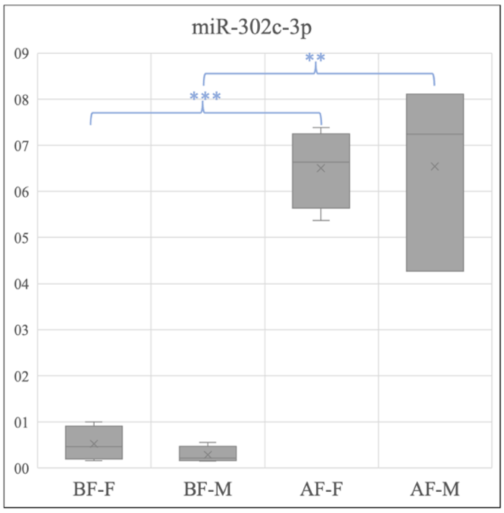


Figure 4-5: Relative expression of miR-302c-3p in female (F) and male (M) PGCs before freezing (BF) and after thawing (AF).

Table 4-9: Statistical significance of miR-302c-3p expression changes after freezing.

Comparison	**p-value**	**Significance**
BF-F vs AF-F	0.000014	***
BF-M vs AF-M	0.001382	**

The elevated levels observed after freezing may therefore reflect a compensatory response to cryo-induced cellular stress, whereby germ cells activate pluripotency-related miRNA circuits to stabilise cell identity and promote post-thaw recovery. Notably, female samples exhibited a more uniform and robust increase in miR-302c-3p expression relative to males, suggesting potential sex-specific differences in the activation of pluripotency or stress-response pathways. In contrast, the greater variability observed in AF-M samples may indicate heterogeneous responses among male germ cells to freezing and thawing stress (Lázár et al., 2018; Maraghechi et al., 2023; Rahimi et al., 2021).

These findings support the conclusion that miR-302c-3p is a key cryo-responsive miRNA in PGCs, likely contributing to the restoration of pluripotency and cellular homeostasis following freezing and thawing. Its strong and reproducible induction positions miR-302c-3p as a potential molecular indicator of post-cryopreservation recovery capacity in germline stem cells.

4.1.2.3.CVH or DDX4 expression

In Figure 4-6, the expression of CVH or DDX4 is shown. Significant differences are detailed in Table 4-10. CVH or DDX4 expression differed significantly between females before and after freezing, with a p-value of 0.00181 (**).

CVH or DDX4 expression differed significantly between males before and after freezing, with a p-value of 0.03491 (*).

CVH or DDX4 expression differed significantly between females and males before freezing, with a p-value of 0.00389 (**).

CVH or DDX4 expression differed significantly between females and males after freezing, with a p-value of 0.00385 (**).

In Figure 4-6, before freezing, DDX4 expression is higher in male than in female PGCs. After thawing, expression declines in female cells but persists at intermediate levels in male cells.

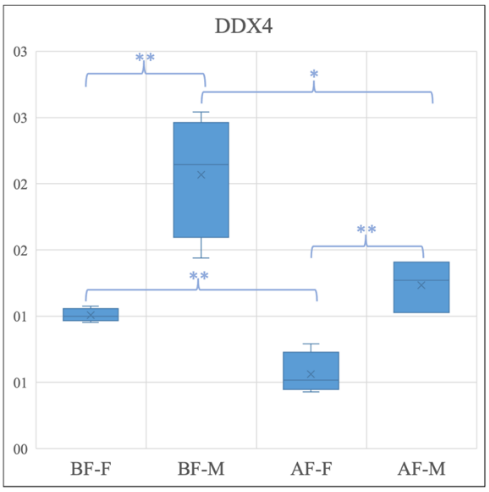


Figure 4-6: Relative expression of DDX4 in female (F) and male (M) PGCs before freezing (BF) and after thawing (AF).

Table 4-10: Statistical significance of DDX4 expression changes after freezing.

Comparison	p-value	Significance
BF-F vs AF-F	0.001810573	**
BF-M vs AF-M	0.034912755	*
BF-F vs BF-M	0.003893309	**
AF-F vs AF-M	0.003846875	**

In this study, DDX4 expression showed statistically significant differences between female and male primordial germ cells both before freezing and after thawing, with male PGCs displaying higher expression levels in both conditions. These sex-dependent differences were maintained despite the significant changes in DDX4 expression induced by cryopreservation in each sex.

The persistence of higher DDX4 expression in male PGCs is consistent with the localization of the DDX4 gene on the Z chromosome in chickens. As male PGCs carry two Z chromosomes (ZZ) compared with one Z chromosome in females (ZW), differences in gene copy number may be associated with the observed expression patterns.

Although the present data do not directly assess dosage compensation mechanisms, the results indicate that sex chromosome composition remains associated with DDX4 expression levels before and after cryopreservation. In previous studies, DDX4 or CVH has shown similar behaviour, demonstrating that freezing treatment might affect its expression (Ecker et al., 2023).

4.1.2.4.POU and DAZL expression

No significant change in POU and DAZL expression was detected when comparing pre- and post-freezing samples within the same gender as shown in Figures 4-7 and 4-8.

DAZL and POU expression remain consistent across both genders and treatments, indicating that in this study, cryopreservation has minimal impact on this germ cell marker.

The consistent expression of POU and DAZL suggests that these genes represent robust markers of PGC identity, remaining unaffected by both sex and cryopreservation. This contrasts with the changes observed for specific miRNAs and highlights that while cryopreservation induces selective molecular responses, it does not globally disrupt key germ cell regulatory pathways.

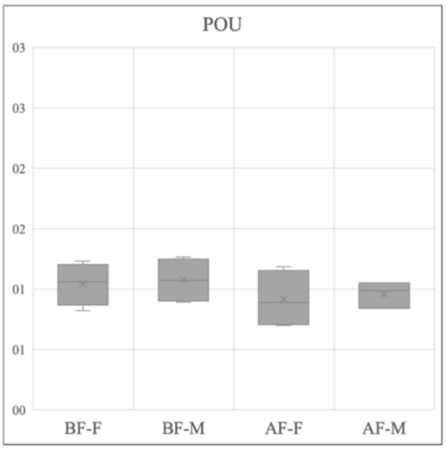


Figure 4-7: Relative expression of POU in female (F) and male (M) PGCs before freezing (BF) and after thawing (AF).

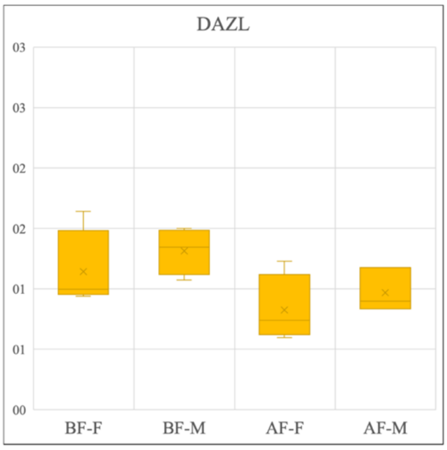


Figure 4-8: DAZL expression in in female (F) and male (M) PGCs before freezing (BF) and after thawing (AF).

4.2.Investigation of rabbit embryos: From studying developmental embryonic stages to verifying the presence of miRNAs in culture media.

This study aimed to examine the association between embryo quality and the presence of specific microRNAs (miRNAs) in the embryo culture medium. By analysing miRNA expression profiles in embryos and their corresponding culture media, the study sought to identify correlations between miRNA signatures, embryonic developmental stage, and morphological quality. The objective was to assess whether extracellular miRNAs in the culture medium reflect intrinsic developmental status and could serve as reliable, non-invasive molecular indicators of embryo competence and viability.

4.2.1.Embryo collection and culture

The collected embryos were characterised according to the rabbit embryo morphology explained by Sultana *et al., (*2009). According to the comparison with the literature, the time the embryos were in the blastocyst stage and hatched blastocyst stage (Sultana et al., 2009) as shown in Table 4-11 .

Table 4-11: Classification of the cultured embryos.

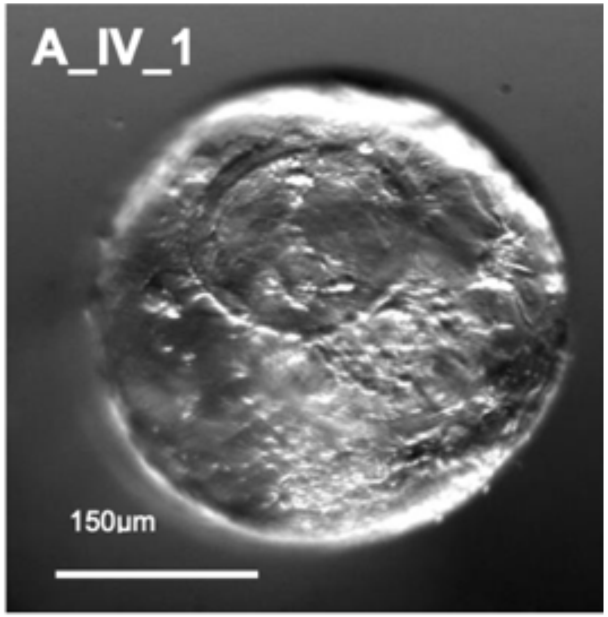
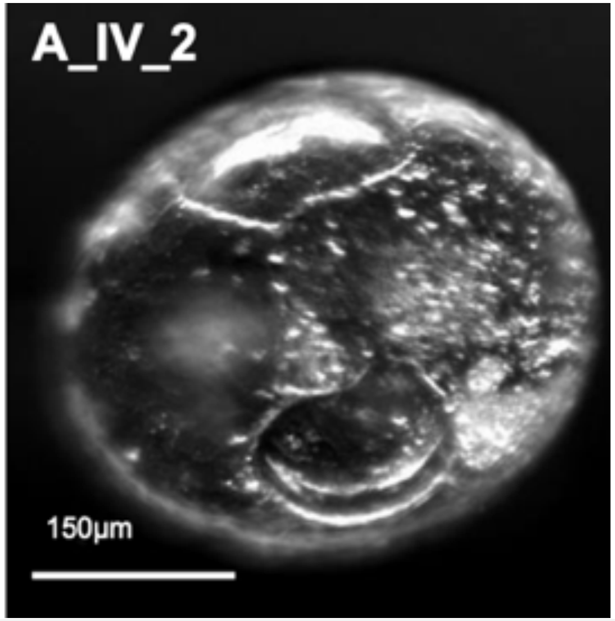
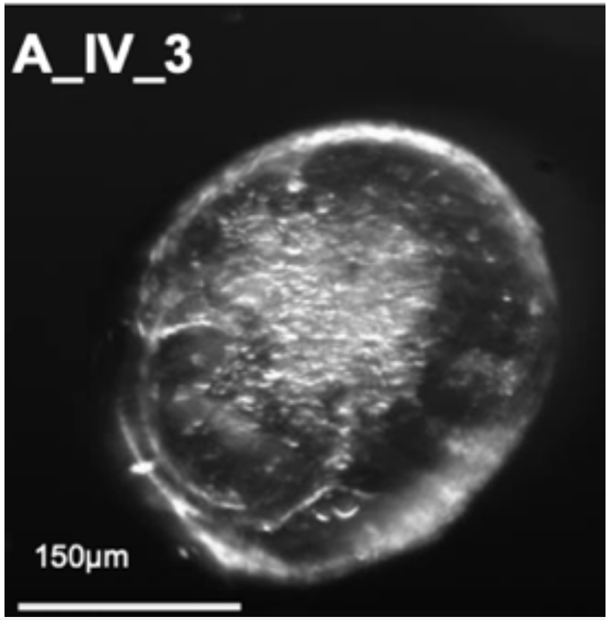
Embryo			
Class	A_IV_1 Hatched Blastocyst-3	A_IV_2 HatchedBlastocyst-3	A_IV_3 Hatched Blastocyst-3

Table 4-11: Classification of the cultured embryos (continued).

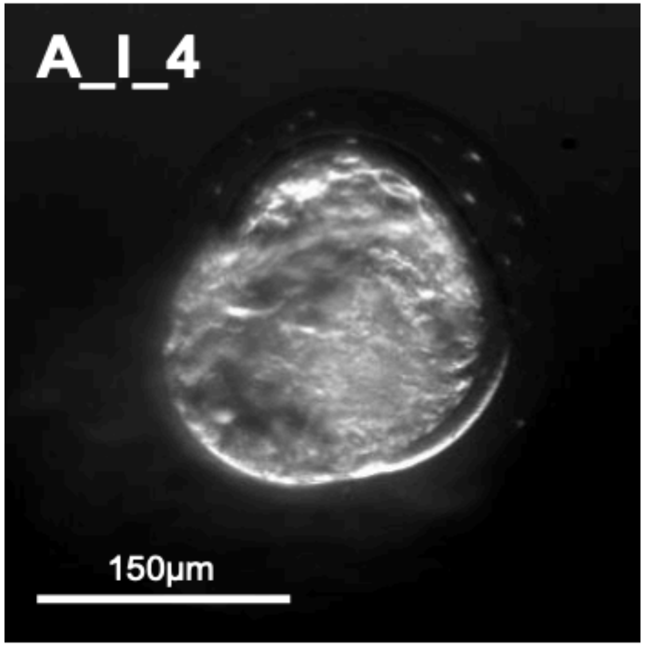
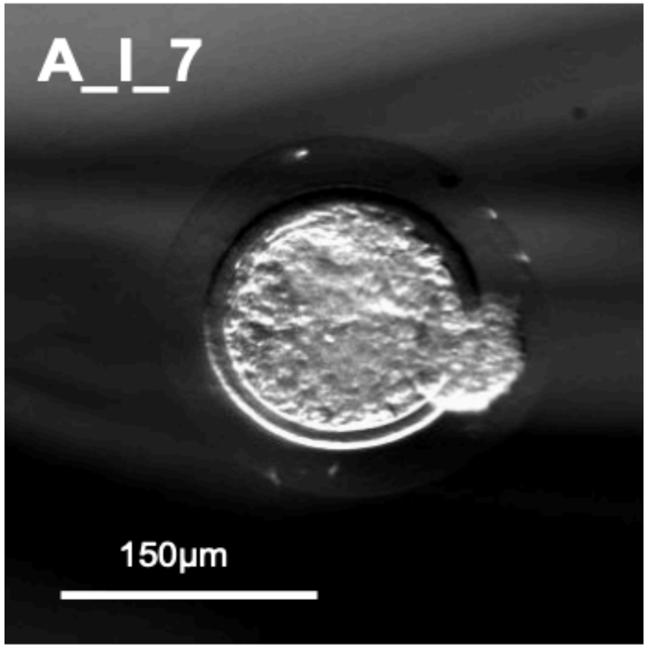
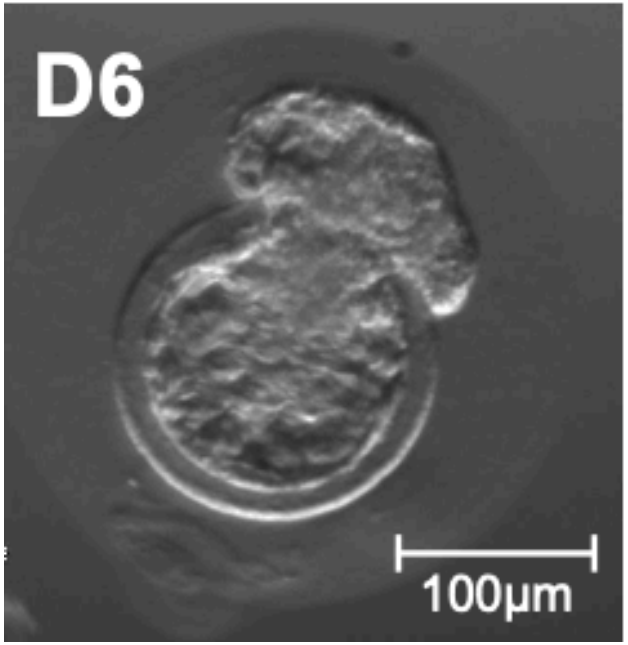
Embryo			
Class	A_I_4 Blastocyst-2	A_I_7 Blastocyst-2	D6 Blastocyst-2

Table 4-11: Classification of the cultured embryos (continued).

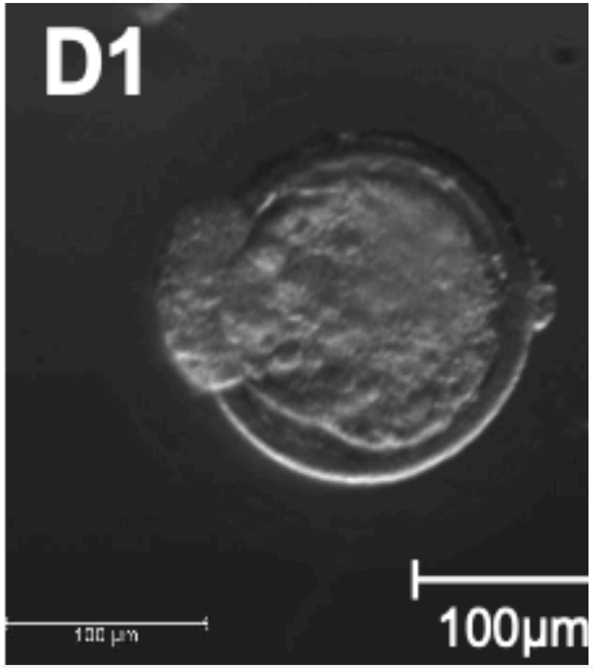
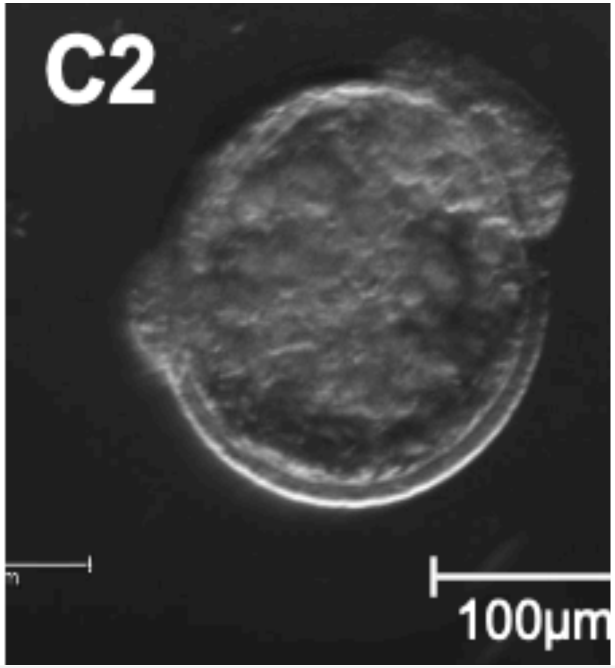
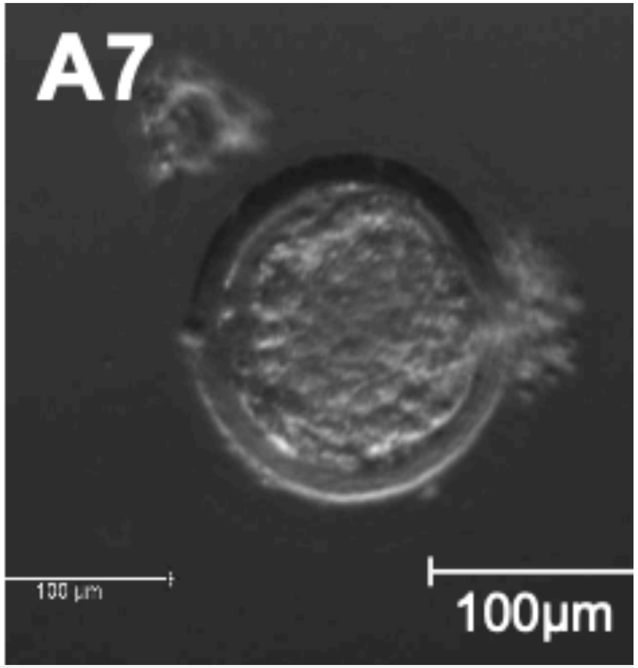
Embryo			
Class	D1 Blastocyst-2	C2 Blastocyst-2	A7 Blastocyst-2

Table 4-11: Classification of the cultured embryos (continued).

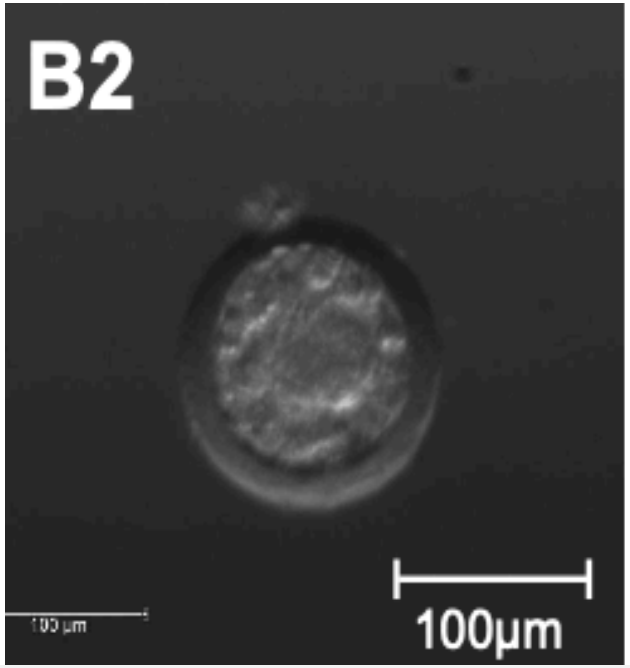
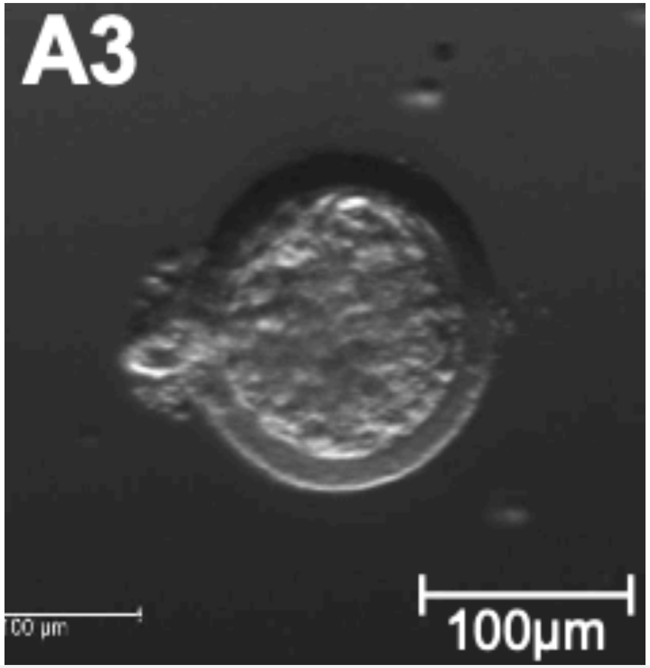
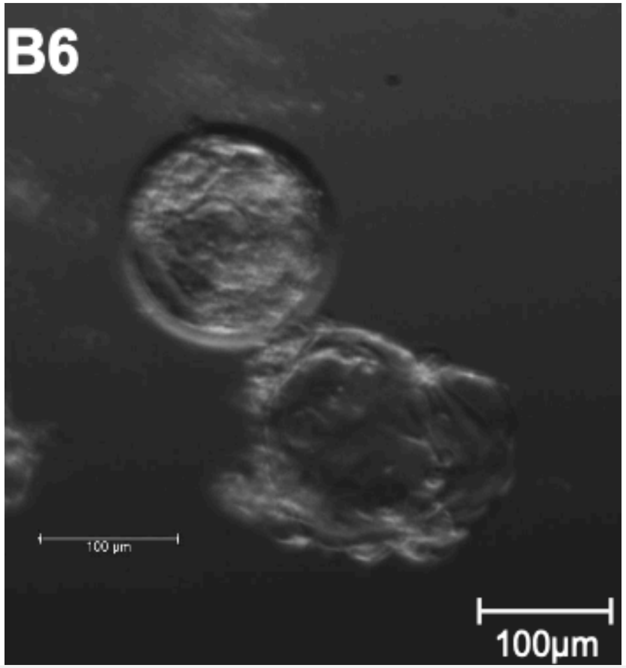
Embryo			
Class	B2 m Blastocyst-1	A3 m Blastocyst-1	B6 Hatched Blastocyst-2

Table 4-11: Classification of the cultured embryos (continued).

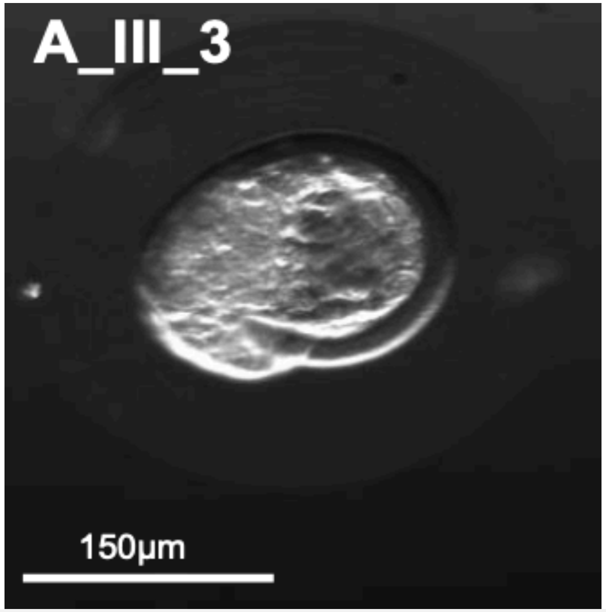
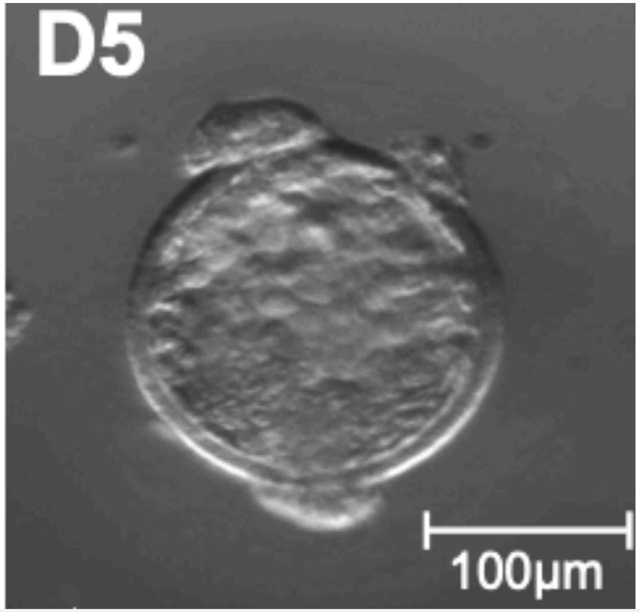
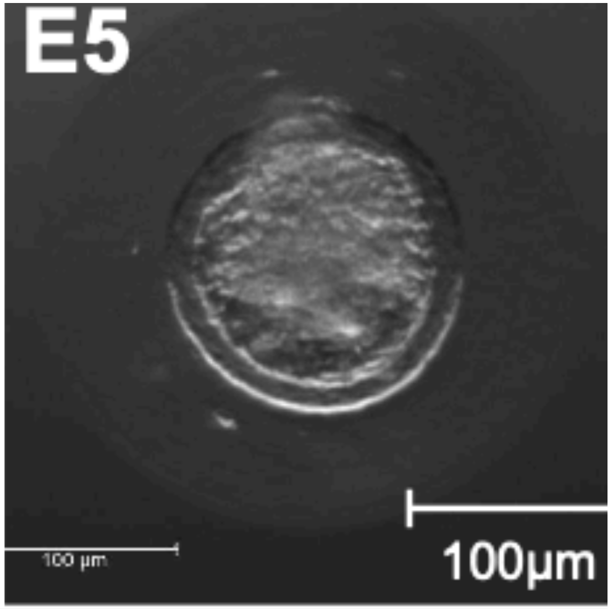
Embryo			
Class	A_III_3 Blastocyst-2	D5 Blastocyst-2	E5 Blastocyst-1

Table 4-11: Classification of the cultured embryos (continued).

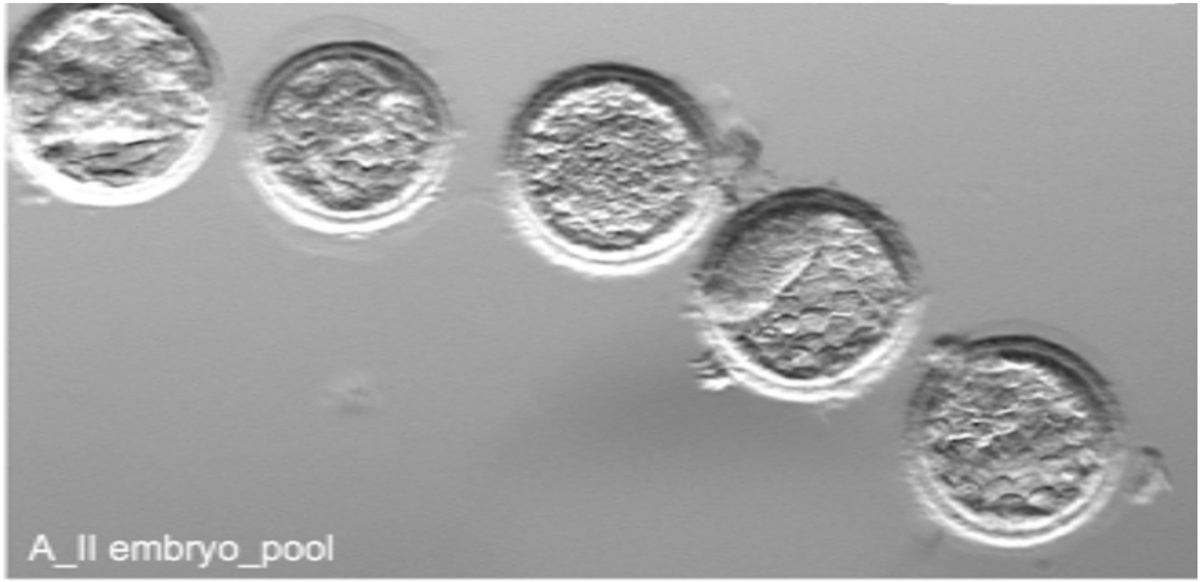
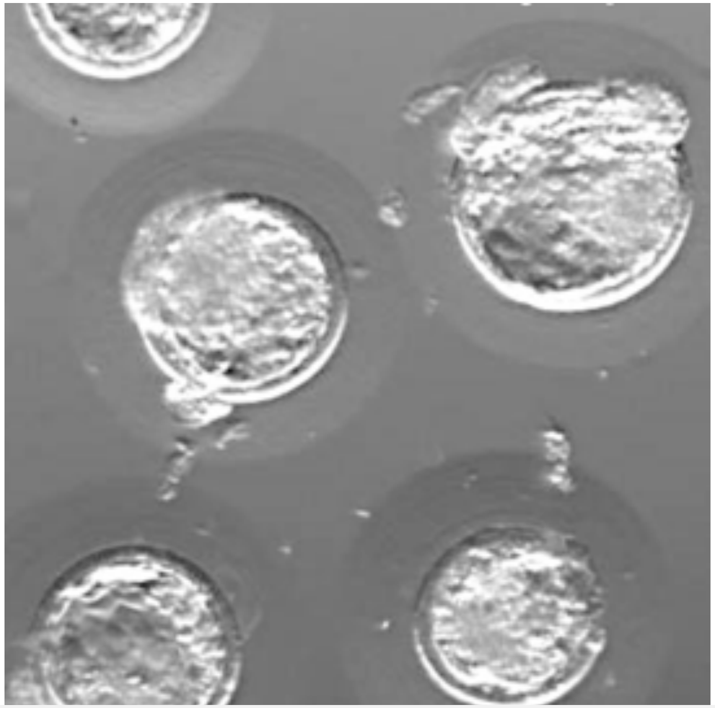
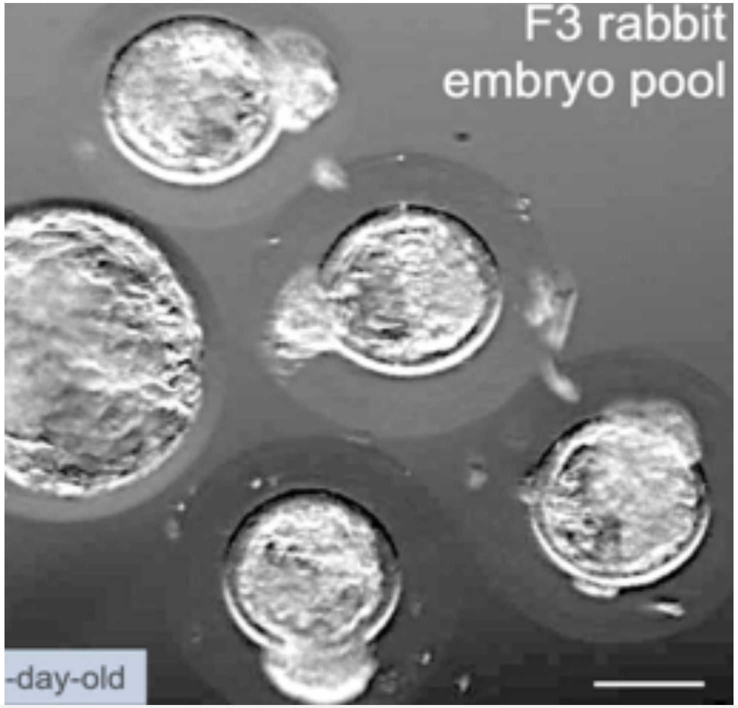
Embryo Pool	
Class	F1 Hatched Blastocyst -1

Table 4-11: Classification of the cultured embryos (continued)

Embryo Pool		
Class	F1 Hatched Blastocyst -1	F3 Hatched Blastocyst -2

The embryos were cultured in groups and individually. The samples taken from the embryos cultured in groups consisted of the five embryos themselves and their corresponding culture media, designated Pool A_II_e and A_II_m, respectively. In addition, the samples referred to as F1_m and F3_m were derived from the culture media of embryo drops, each containing five embryos cultivated together. The developmental stages and morphological classifications at the time of RNA isolation for each sample are detailed in Table 4-12.

Table 4-12: Description of samples used for miRNA taken from embryo culture.

Sample Identification	Sample type	Number of cultured embryos	Age	Developmental stage
A_II_e	embryo	5	4-day-old	blastocyst - stage 2
A_II_m	medium	5	4-day-old	blastocyst - stage 2
F1_m	medium	5	6-day-old	hatched blastocyst - stage 1
F3_m*	medium	5	6-day-old	hatched blastocyst - stage 2

*Reference sample

In total, fifteen embryos were individually cultured, and the culture media were collected separately.

In the case of six embryos, both the embryos and the six related culture media were used for subsequent miRNA analysis. Additionally, nine culture media collected from individually cultivated embryos were taken for miRNA expression analysis. The developmental stages and morphological classifications of each sample are provided in Table 4-13.

Table 4-13: Lists of individually cultured embryos and collected culture medium.

`Sample ID	Sample type	Age	Class
A_I_4_e	Individual embryo	6-day-old	blastocyst - stage 2
A_I_7_e	Individual embryo	6-day-old	blastocyst - stage 2
A_IV_1_e	Individual embryo	6-day-old	hatched blastocyst - stage 3
A_IV_2_e	Individual embryo	6-day-old	hatched blastocyst - stage 3
A_IV_3_e	Individual embryo	6-day-old	hatched blastocyst - stage 3
A_III_3_e	Individual embryo	6-day-old	blastocyst - stage 2
A_I_4_m	Individual medium	6-day-old	blastocyst - stage 2
A_I_7_m	Individual medium	6-day-old	blastocyst - stage 2
A_IV_1_m	Individual medium	6-day-old	hatched blastocyst - stage 3
A_IV_2_m	Individual medium	6-day-old	hatched blastocyst - stage 3
A_IV_3_m	Individual medium	6-day-old	hatched blastocyst - stage 3
A_III_3_m	Individual medium	6-day-old	blastocyst - stage 2
B6_m	Individual medium	6-day-old	hatched blastocyst - stage 2
D6_m	Individual medium	6-day-old	blastocyst - stage 2
D5_m	Individual medium	6-day-old	blastocyst - stage 2
D1_m	Individual medium	6-day-old	blastocyst - stage 2
C2_m	Individual medium	6-day-old	blastocyst - stage 2
A7_m	Individual medium	6-day-old	blastocyst - stage 2
E5_m	Individual medium	6-day-old	blastocyst - stage 1
B2_m	Individual medium	6-day-old	blastocyst - stage 1
A3_m	Individual medium	6-day-old	blastocyst - stage 1

4.2.2.miRNA expression profile

The image-based categories were correlated with the related miRNA's expression profile.The most relevant miRNAs were selected based on the previously constructed rabbit SOLiD miRNA-based sequencing database, abundance analysis and a literature review, listed in Table 3-23 (methodology). The expression of ten miRNAs was analysed from isolated RNA samples collected from rabbit embryos and medium drops, and the read numbers in the previous experiment. The quantitative miRNA expression values, represented as cycle threshold (Ct) measurements (qPCR Ct values), were grouped into four qualitative categories to facilitate visualisation of expression differences. The categories used were:

High expression (+++): Ct<35
Medium expression (++): 35<Ct<39
Low expression (+): 39<Ct<45
No expression (no): Ct>45

The categorised expression of the samples is listed in the following Table 4-14. Although the Ct values were also used to calculate the miRNA relative expression in further analysis

Table 4-14: Expression (Ct) of seven miRNAs in embryo samples and in their corresponding medium.

N	**Sample**	**Age**	**miR-92-3p**	**miR-103a-3p**	**miR-191-5p**	**miR-24-3p**	**miR-378a-3p**	**miR-28-3p**	**miR-181a-5p**	**miR-320a-3p**
1	A_II_embryo_pool	4d	+++	+	no	++	+++	no	no	+++
2	A_II_medium_pool	4d	+++	++	+++	+++	+++	+	++	+++
3	F1_medium_pool	6d	+++	++	+++	+++	+++	++	+++	+++
4	F3_medium_pool	6d	+++	+++	+++	+++	+++	+	+++	+++
5	A_I_4_embryo	6d	+++	no	no	no	no	no	no	no
6	A_I_4_medium	6d	+++	no	no	++	+++	no	no	++
7	A_I_7_embryo	6d	++	no	no	no	no	no	no	++
8	A_I_7_medium	6d	+++	++	no	+++	+++	no	no	+++
9	A_III_3_embryo	6d	+++	no	no	no	++	++	+++	+++
10	A_III_3_medium	6d	+++	+++	+++	+++	+++	+++	+++	+++
11	A_IV_1_embryo	6d	+++	no	no	+++	++	no	no	+++
12	A_IV_1_medium	6d	+++	+++	+++	+++	+++	++	+++	+++
13	A_IV_2_embryo	6d	+++	+	+	++	+++	++	no	+++
14	A_IV_2_medium	6d	+++	++	++	+++	+++	++	+++	+++
15	A_IV_3_embryo	6d	+++	+	+++	+++	+++	no	no	+++
16	A_IV_3_medium	6d	+++	+++	+++	+++	+++	+++	+++	+++
17	B6_medium	6d	++	no	no	++	++	no	no	no
18	C2_medium	6d	+++	no	no	+++	++	no	no	+++
19	D5_medium	6d	+++	no	++	+++	+++	no	no	+++
20	D6_medium	6d	+++	++	++	+++	+++	no	no	+++
21	A3_medium	6d	+++	no	no	no	+++	no	no	++
22	A7_medium	6d	+++	++	+++	+++	+++	no	+	+++
23	B2_medium	6d	+++	++	no	no	++	no	no	+++
24	D1_medium	6d	+++	no	+++	+++	+++	no	++	+++
25	E5_medium	6d	++	no	no	no	+++	no	no	+++

(d: days)

Hsa-miR-92a-3p and hsa-miR-320a-3p exhibited robust expression levels across nearly all embryo and medium samples analysed, indicating their potential roles in embryonic development and signalling.

In contrast, the expression of mmu-miR-302b-3p and hsa-miR-371a-5p was undetectable throughout the analyses. According to SOLiD sequencing results and abundance analysis in Table 4-15, mmu-miR-302b-3p had low read numbers in the sequence library of 6-day-old and 7-day-old *in vivo* rabbit embryos, while hsa-miR-371a-5p obtained high read numbers in 7-day-old *in vivo* rabbit embryos. hsa-miR-302b-3p and hsa-miR-371a-5p were excluded from the present study. Furthermore, abundance analysis revealed that hsa-miR-302c-3p and hsa-miR-372-3p were expressed at low levels and did not demonstrate meaningful abundance; therefore, these miRNAs were also excluded from subsequent analyses.

Abundance analysis showed in Table 4-15 demonstrates the counts numbers of miRNA found in mouse embryonic fibroblast (MEF), rabbit embryonic fibroblast (REF), rabbit embryonic stem cells (rabESC), rabbit embryo and rabbit PGC (Maraghechi et al., 2013). miR-28-3p was hardly detected in the study, the abundance analysis showed that miR-28-3p was present only in later embryonic stages, as indicated in Table 4-15.

Table 4-15: hsa-miRNA count numbers in different sample.

miRNAs	**MEF (p2)**	**REF (p2)**	**rabESC (p2)**	**rabbit embryo (dpc 6)**	**rabbit embryo (dpc 7)**	**rabbit PGCs (dpc 14)**
mmu-miR-302b-3p	0	0	1	1	4	0
hsa-miR-92a-3p	125	350	95	61	471	1540
hsa-miR-378a-3p	90	48	21	0	13	16
hsa-miR-372-3P	-	-	-	-	-	-
hsa-miR-371a-5p	0	0	0	7	362	0
hsa-miR-320a-3p	70	41	29	6	15	34
hsa-miR-302c-3p	0	0	0	2	7	0
hsa-miR-28-3p	3	8	1	0	0	2
hsa-miR-24-3p	2410	2130	471	3	34	87
hsa-miR-191-5p	462	555	56	7	187	82
hsa-miR-181a-5p	248	96	57	1	3	51
hsa-miR-103a-3p	675	662	55	7	90	77

(p2: passage 2) (dpc: days post coitum)

Finally, following the evaluation of miRNA expression profiles, seven miRNAs were selected for subsequent analysis of their relative expression levels and one for control and normalisation.

4.2.3.Relative miRNA expression calculation

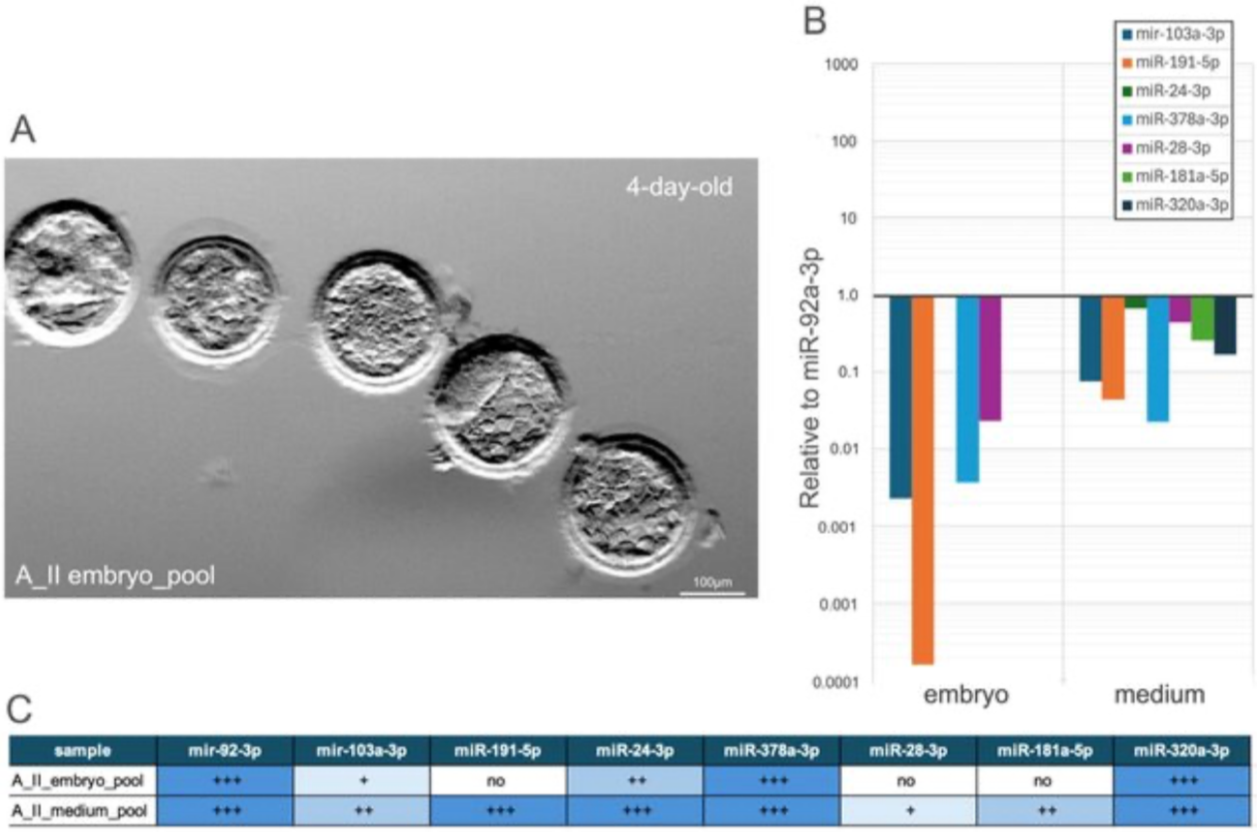
Hsa-miR-92-3p was used as a reference gene, as it was found to have constant and high expression in all examined samples. In addition, the F3_m culture medium sample was used as the reference sample in the relative miRNA expression calculation.

4.2.4.miRNA expression analysis

For the succeeding analysis, the embryos were organised into distinct groups.

4.2.4.1.Comparative analysis of miRNA expression profiles in the blastocysts Pool and their culture medium

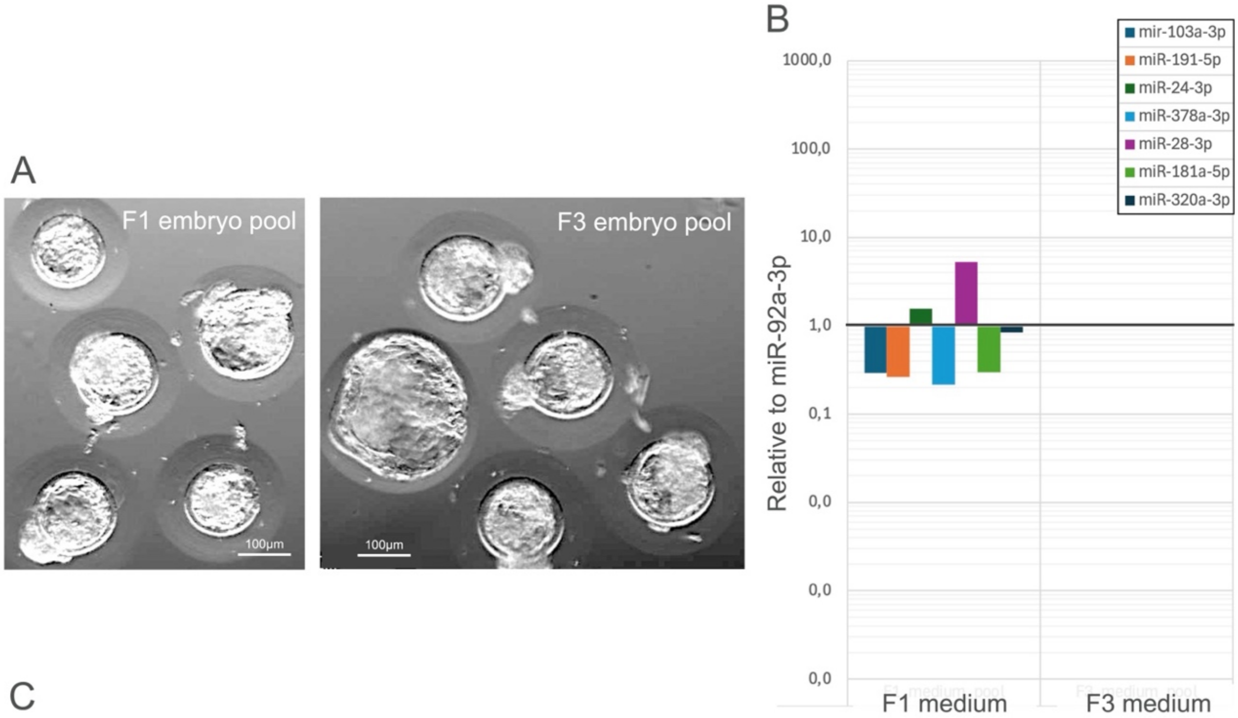
Sample A_II consisted in a pooled collection of five 4-day-old embryos (blastocyst stage 2) (Figure 4-9 A), along with the corresponding culture medium. The expression of seven miRNAs was analysed separately for both the embryos and the medium to detect differences in miRNA expression between the embryonic tissue and its surrounding environment. The comparison of both (embryo pool and medium) miRNA expressions is shown in Figure 4-9 B and C.



sample	mir-92-3p	mir-103a-3p	miR-191-5p	miR-24-3p	miR-378a-3p	miR-28-3p	miR-181a-5p	miR-320a-3p
A_II_embryo_pool	+++	+	no	++	+++	no	no	+++
A_II_medium_pool	+++	++	+++	+++	+++	+	++	+++

Figure 4-9: Analysis of miRNA Expression in embryo pool and medium pool.
A) Image-based categories of A_II embryo pool. B) Relative expression levels of the analysed miRNAs, normalised to miR-92a-3p as the reference gene, and used the F3_m culture medium sample as the reference sample, as all investigated miRNAs were detected in this pooled medium sample. Data are presented on a logarithmic scale for the interpretation of variation in expression levels. C)Summary table of miRNA expression in different samples, categorised into four levels based on cycle threshold (Ct) values from quantitative +++: Ct<35; ++: 35<Ct<39; +: 39<Ct<45; no expression: Ct>45.

The samples F1 and F3 are culture media taken from a medium, each with 5 embryos. The expression and relative expression were studied and compared in Figure 4-10.



C

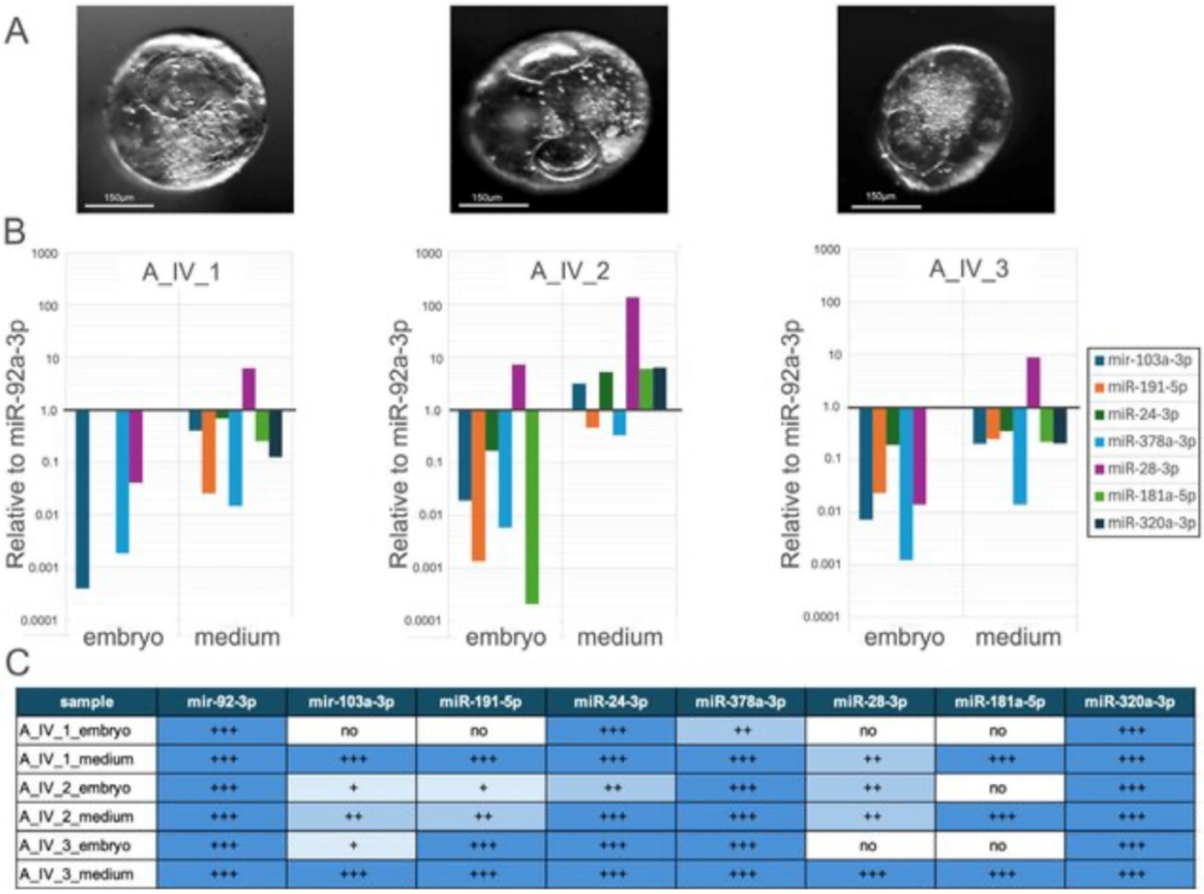
sample	mir-92-3p	mir-103a-3p	miR-191-5p	miR-24-3p	miR-378a-3p	miR-28-3p	miR-181a-5p	miR-320a-3p
F1_medium_pool	+++	++	+++	+++	+++	++	+++	+++
F3_medium_pool	+++	+++	+++	+++	+++	+	+++	+++

Figure 4-10: Analysis of miRNA Expression of medium pools.
A) Image-based categories of F1 and F3 embryo pool. B) Relative expression levels of the analysed miRNAs, normalised to miR-92a-3p as the reference gene, and used the F3_m culture medium sample as the reference sample, as all investigated miRNAs were detected in this pooled medium sample. Data are presented on a logarithmic scale for the interpretation of variation in expression levels. C)Summary table of miRNA expression in different samples, categorised into four levels based on cycle threshold (Ct) values from quantitative +++: Ct<35; ++: 35<Ct<39; +: 39<Ct<45; no expression: Ct>45.

The expression of miR-191-5p, miR-28-3p and miR-181a-5p was detected only in the medium, indicating that these miRNAs are either secreted or less retained within the embryos. In contrast, the downregulated expression of hsa-miR-103a-3p, hsa- miR-378a-3p, hsa-miR-191-5p, and hsa-miR-28-3p in the medium was significantly higher compared to their expression levels in the embryos. MiR-92a-3p was used as an internal control, and the F3_medium pool served as a reference sample during data analysis, as all examined miRNAs were present in this sample.

4.2.4.2.Comparative analysis of miRNA expression profiles in hatched blastocysts and their culture medium

Three embryos categorised as hatched blastocysts (Figure 4-11 A) were cultured individually and designated as A_IV_1, A_IV_2, A_IV_3. The expression levels of seven miRNAs were analysed independently in both the embryos and their corresponding culture media, as presented in Figure 4-11 B and C.



sample	mir-92-3p	mir-103a-3p	miR-191-5p	miR-24-3p	miR-378a-3p	miR-28-3p	miR-181a-5p	miR-320a-3p
A_IV_1_embryo	+++	no	no	+++	++	no	no	+++
A_IV_1_medium	+++	+++	+++	+++	+++	++	+++	+++
A_IV_2_embryo	+++	+	+	++	+++	++	no	+++
A_IV_2_medium	+++	++	++	+++	+++	++	+++	+++
A_IV_3_embryo	+++	+	+++	+++	+++	no	no	+++
A_IV_3_medium	+++	+++	+++	+++	+++	+++	+++	+++

Figure 4-11: Expression Profiles in Hatched Blastocysts and Their Culture Medium.
(A) Morphology of a rabbit hatched blastocyst. (B) Relative expression levels of miRNA profile in embryo and culture medium, normalised to miR-92a-3p. (C) Summary table of miRNA expression in different samples, categorised into four levels based on cycle threshold (Ct) values from quantitative real-time PCR. Ct values (+++: Ct < 35; ++: 35 < Ct < 39; +: 39 < Ct < 45; no expression: Ct > 45).

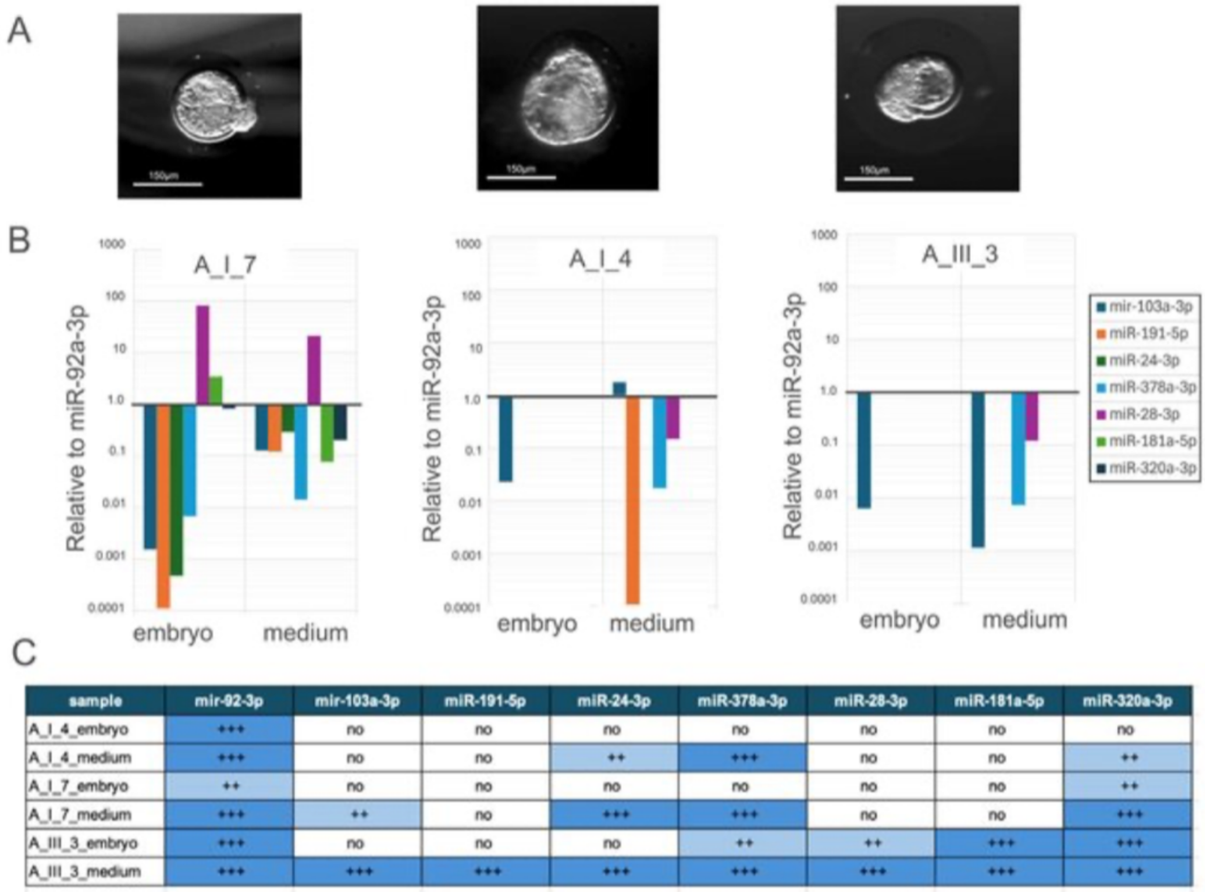
In sample A_IV_1, seven miRNAs were detected as downregulated relative to miR-92, with the exception of hsa-miR-28-3p, whose expression was upregulated in the medium. Hsa-miR-191-5p, hsa-miR-24-3p, hsa-miR-181-5p, and hsa-miR-320a-3p were not detected in A_IV_1 embryo. Hsa-miR-103a-3p, hsa-miR-378a-3p, and hsa-miR-28-3p were detected in both samples; nevertheless, those miRNA expressions were higher in the medium than in the embryo sample.

In sample A_IV_2_embryo, all miRNAs were downregulated relative to the reference miRNA except for hsa-miR-28-3p, which was upregulated; additionally, hsa-miR-320a-3p was not detected. In A_IV_2_medium, the expression of all miRNAs was upregulated except for hsa-miR-191-5p and hsa-miR-378a-3p, whose expression levels were higher than those observed in A_IV_2_embryo. Hsa-miR-24-3p high expression has been seen in both samples, and hsa-miR-181-5p was strongly downregulated in A_IV_2_embryo. Hsa-miR-28-3p expression was found strongly upregulated in the medium.

In sample A_IV_3_embryo and A_IV_3_medium, miRNA expression remained downregulated, except hsa-miR-28-3p, which was upregulated in medium. Hsa-miR-181-5p and hsa-miR-320a-3p were not detected in the embryo. Hsa-miR-24-3p in both samples keeps balanced expression, while hsa-miR-378a-3p was strongly downregulated in the embryo.

4.2.4.3. Comparative analysis of miRNA expression profiles in individually cultured blastocysts and their culture medium

Three embryos categorised as blastocysts (Figure 4-12 A) were cultured individually and designated as A_III_3, A_I_4 and A_I_7. The expression levels of seven miRNAs were determined separately for both the embryos and their corresponding culture medium, as presented in Figure 4-12 B,C.



sample	mir-92-3p	mir-103a-3p	miR-191-5p	miR-24-3p	miR-378a-3p	miR-28-3p	miR-181a-5p	miR-320a-3p
A_I_4_embryo	+++	no	no	no	no	no	no	no
A_I_4_medium	+++	no	no	++	+++	no	no	++
A_I_7_embryo	++	no	no	no	no	no	no	++
A_I_7_medium	+++	++	no	+++	+++	no	no	+++
A_III_3_embryo	+++	no	no	no	++	++	+++	+++
A_III_3_medium	+++	+++	+++	+++	+++	+++	+++	+++

Figure 4-12: miRNA Expression Profiles in Individually Cultured Blastocysts and Their Culture Medium. (A) Morphology of a rabbit blastocyst. (B) Relative expression levels of miRNA profile in embryo and culture medium, normalised to miR-92a-3p. (C) Summary table of miRNA expression in different samples, categorised into four levels based on cycle threshold (Ct) values from quantitative real-time PCR. Ct values (+++: Ct < 35; ++: 35 < Ct < 39; no expression: Ct > 45).

In sample A_I_7_embryo, the expression of hsa-miR-103a-3p, hsa-miR-191-5p, has-miR-24-3p, hsa-miR-378a- 3p, and hsa-miR-320a-3p was downregulated in the embryo and in medium; nevertheless, their expression in the medium is higher. The expression of hsa-miR-28-3p and hsa-miR-181-5p was upregulated in the embryo, and their expression was higher than that found in the medium.

In sample A_I_4_embryo just hsa-miR-103a-3p was detected and strongly found down-regulated. In A_I_4_medium, the expression of hsa-miR-103a-3p was found upregulated, while just hsa-miR-191-5p, hsa-miR-378a-3p and hsa-miR-28-3p were detected slightly.

In sample A_III_3, hsa-miR-103a-3p was found downregulated in both samples. In the medium sample, only hsa-miR-181a-5p and hsa-miR-320a-3p were found downregulated.

4.2.4.4.miRNAs expression profile—Kohonen's Self-Organising Map (SOM)

Additionally, Kohonen's Self-Organising Map (SOM) was applied to identify and visualise groups of samples exhibiting similar expression patterns.

The six samples (A_IV_1, A_IV_2, A_IV_3, A_I_4, A_I_7, and A_III_3) were grouped using Kohonen's self-organising grouping SOM (Figure 4-13).

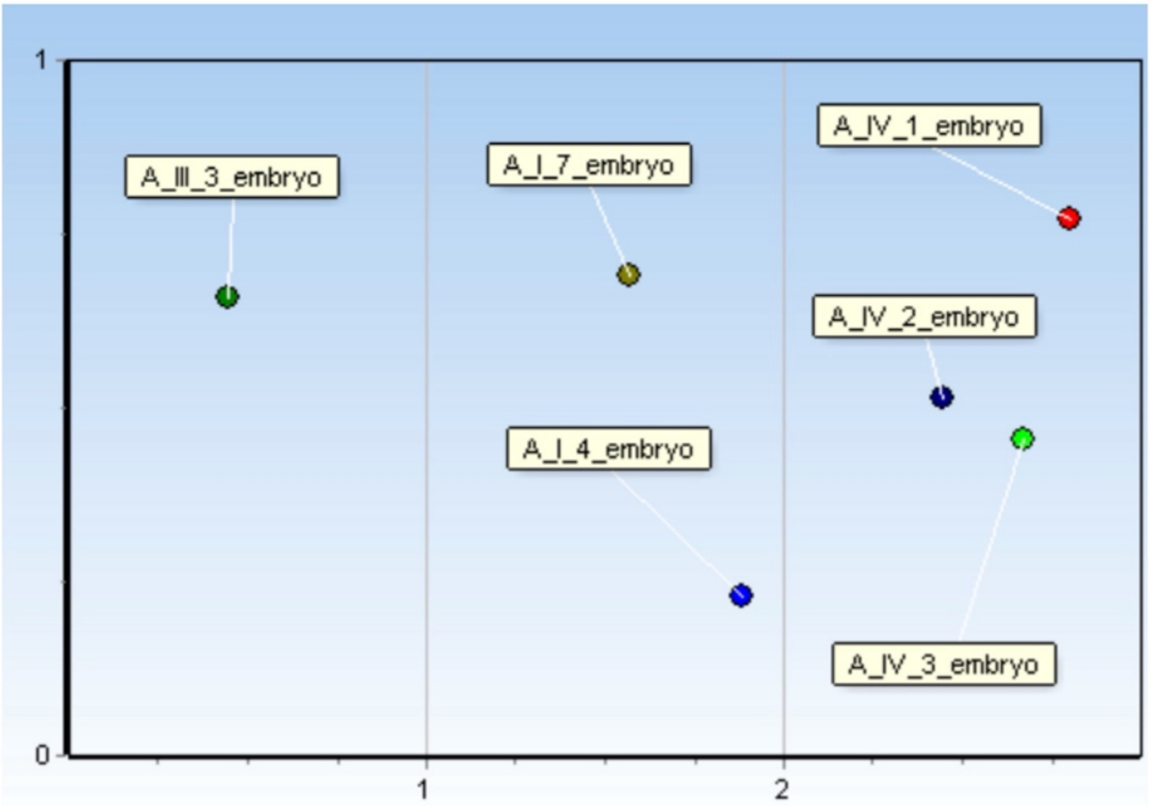


Figure 4-13: Kohonen Self-Organising Map Showing Clustering of Embryo Samples Based on miRNA Expression Profiles.

A_III_3 sample presented a unique miRNA profile, suggesting that it is develop- mentally distinct profile. A_I_7_embryo and A_I_4_embryo shared similar miRNA pattern. A_IV_1_embryo, A_IV_2_embryo and A_IV_3_embryo shared strong miRNA profile similarities.

4.2.4.5.miRNAs expression profile—Heatmap

Six samples (A_IV_1, A_IV_2, A_IV_3, A_I_4, A_I_7, and A_III_3) were analysed using the ΔCt value to generate a heatmap visualisation (Figure 4-14).

The expression levels of hsa-miR-28-3p, hsa-miR-103a-3p, miR-181a-5p and hsa-miR- 191-5p remained consistently high across most samples, suggesting that these miRNAs may have stable or essential functions in embryonic development. In contrast, hsa-miR- 320a-3p and hsa-miR-378a-3p exhibited relatively low expression levels in most samples. Hsa-miR-24-3p has shown moderate expression.

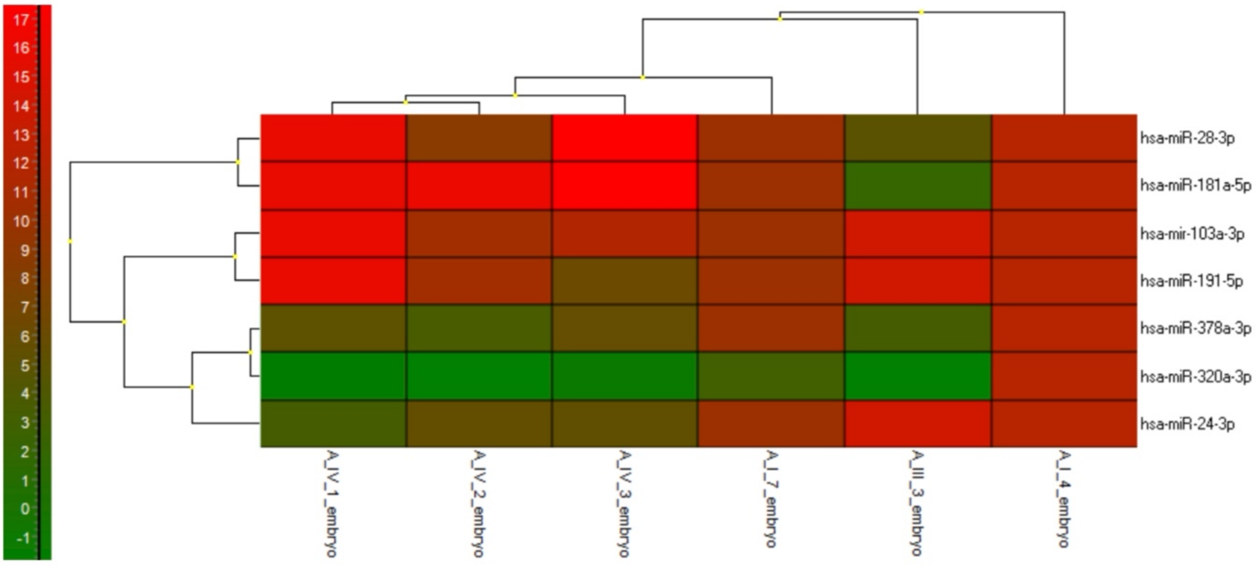
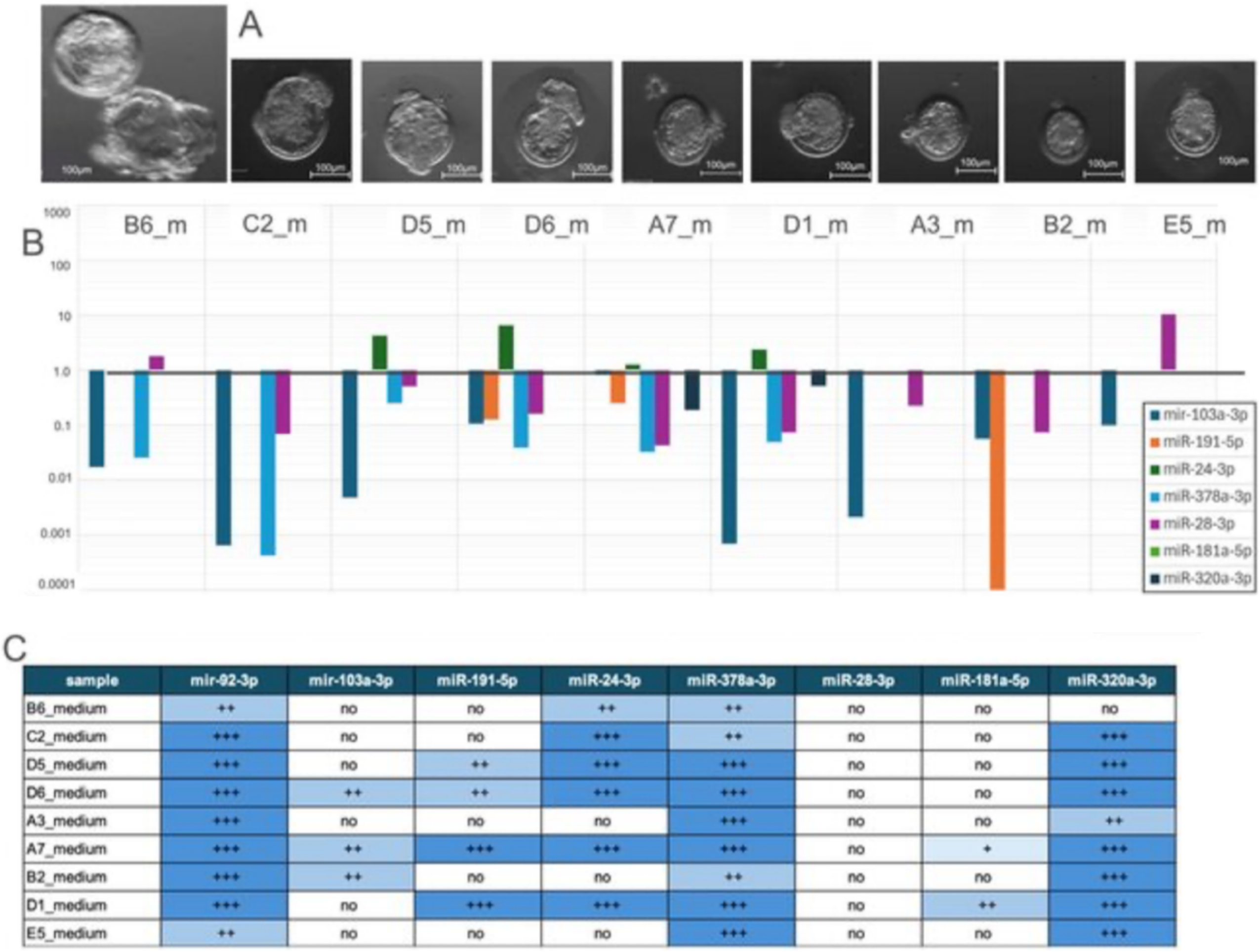


Figure 4-14: Heatmap and Clustering of miRNA Expression Across Rabbit Embryo Samples.

4.2.4.6. Comparative analysis of miRNA expression profiles in culture medium of embryos cultured individually

A total of 9 samples corresponding to hatched blastocysts stage 1 and stage 2 (Figure 4-15A) cultured media were analysed to assess their miRNA expression profiles (Figure 4-15 B,C) .



sample	mir-92-3p	mir-103a-3p	miR-191-5p	miR-24-3p	miR-378a-3p	miR-28-3p	miR-181a-5p	miR-320a-3p
B6_medium	++	no	no	++	++	no	no	no
C2_medium	+++	no	no	+++	++	no	no	+++
D5_medium	+++	no	++	+++	+++	no	no	+++
D6_medium	+++	++	++	+++	+++	no	no	+++
A3_medium	+++	no	no	no	+++	no	no	++
A7_medium	+++	++	+++	+++	+++	no	+	+++
B2_medium	+++	++	no	no	++	no	no	+++
D1_medium	+++	no	+++	+++	+++	no	++	+++
E5_medium	++	no	no	no	+++	no	no	+++

Figure 4-15: miRNA Expression Profiles in Samples and Their Culture Medium.

(A) Morphology of a rabbit blastocyst. (B) Relative expression levels of miRNA profile in culture medium, normalised to miR-92a-3p. (C) Summary table of miRNA expression in different samples, categorised into four levels based on cycle threshold (Ct) values from quantitative real-time PCR. Ct values (+++: Ct < 35; ++: 35 < Ct < 39; +: 39 < Ct < 45; no expression: Ct > 45).

The expression of hsa-miR-103a-3p was detected in 3 medium samples, hsa-miR-28-3p was not detected in medium, hsa-191-5p was detected in 4 samples and hsa-miR-181a-5p was predominantly downregulated found in just 2 medium samples. In contrast, hsa-miR-24-3p was found in 5 media. Notably, and hsa-miR-320a-3p was detected in all samples except in one sample and hsa-miR-378a-3p was strongly present in all medium samples.

MiRNAs have been implicated in key reproductive processes, including fertilisation, gametogenesis, embryo development, and implantation. Given the well-established roles of various miRNAs in embryonic development, research has increasingly focused on exploring their potential applications and practical uses in this research area. Our results are consistent with other researchers demonstrating that several miRNAs are detectable in the culture medium of embryos, offering a promising approach for assessing embryo viability without invasive techniques (Khan et al., 2021; Nadri et al., 2024; S. Wang et al., 2021).

MiR-103a-3p moderate-high expression has been observed in the culture medium during our comparative analysis compared to its moderate expression in embryo samples. MiR-103a-3p and members of in this group of miRNAs have been associated with cell proliferation (Rodrigues et al., 2016), contributing to the understanding of embryonic stem cell regulation and function (Zhu et al., 2025). Furthermore, miR-103a-3p shows stability in porcine liver and uterus, making it suitable for normalisation in expression analysis (Mahdipour et al., 2015).

Low-to-moderate miR-191-5p expression has been detected in the embryo and high expression in most of the medium samples. Several sources confirmed the expression of miR-191-5p in failed IVF, in aneuploid embryos and patients with cancer, suggesting its tumour involvement(Kamijo et al., 2022; Mahdipour et al., 2015; Sromek et al., 2025). MiR-191-5p is correlated with successful pregnancy states, suggesting its role in supporting the developmental processes necessary for implantation and early embryogenesis, making it a good embryo viability and predicting implantation marker (Hawke et al., 2021).

Mutia, (2023) describes the miR-24-3p role as a regulator of differentiation of ESCs by pluripotent markers OCT4, NANOG, KLF4, and c-MYC (Mutia et al., 2023). MiR-24-3p was highly detected in most of the medium samples and in half of the embryo samples. Additionally, miR-24-3p contributes to the cell survival of porcine granulosa and overall follicular health, with regulatory effects on proliferation and apoptosis through its interaction with the target gene (Shi et al., 2024).

MiR-378a-3p was strongly present in most medium and embryo samples. Bta-miR- 378a-3p has been investigated and is known to enhance blastocyst quality, promote cell survival, and regulate embryo hatching. Similarly, hsa-miR-378a-3p has been associated with the regulation of oocyte meiosis, which is a vital process for the development of viable eggs and successful fertilisation. Pavani, (2022) linked the higher levels of hsa-miR-378a-3p to successful implantation outcomes P27 (Kamijo et al., 2022; Pavani et al., 2022).

MiR-28-3p showed moderate expression levels in medium samples and in few embryo samples, suggesting a significant role in intracellular and extracellular regulation. Higher levels of hsa-miR-28-3p may be linked to successful implantation results and embryo development by various signalling pathways. In rabbits, the expression of miR-28-3p is high in good-quality blastocysts. Additionally, miR-28 may influence gene expression linked to cell proliferation, apoptosis, and metastasis (Kamijo et al., 2022; Shi et al., 2024).

High-moderate miR-181a-5p expression has been found in medium, suggesting that there is an active participation of miR-181a-5p in embryo regulation. During embryonic development, hsa-miR-181a-5p is involved in the oestrogen signalling pathway that influences embryo viability and implantation success. It is considered a key regulator of factors that affect embryo viability. MiR-181a-5p is known to act suppressing or promoting (EMT) epithelial–mesenchymal transition by regulating molecules within pathways such as TGF-β, Wnt/β-catenin, and NF-κB, thereby influencing cancer cell invasion, migration, and stemness properties(Kamijo et al., 2022; Yang et al., 2025).

MiR-92a-3p was used as an inner control gene. According to Caporali (2011) the overexpression of miR-92a induced severe defects in intersegmental vessel formation in zebrafish (Caporali & Emanueli, 2011).

High miR-320a-3p expression was found in rabbit blastocysts and the culture media. It was published that miR-320 was in the top 5% most highly expressed miRNAs in the initial 9 euploid embryos; moreover, miR-320 target has been identified ITGB5, which plays a role in cell–matrix interaction (Caporali & Emanueli, 2011; Rosenbluth et al., 2013) . MiR-320a-3p has been found in amniotic fluid and plays a significant role in preventing epithelial–mesenchymal transition (EMT) in lung epithelial cells by targeting connective tissue growth factor (CTGF) and ATG5-associated autophagy (Timofeeva et al., 2025). miR-320a-3p has been detected in bovine oocytes, embryos, and conditioned culture media, and differential expression has been associated with embryo developmental competence. It is functionally linked to pathways regulating proliferation, metabolism, and stress response(Rio et al., 2024).

A summary of the most significant effects of the miRNAs we investigated, related to embryo quality, abnormalities, and pregnancy success as reported in earlier studies, is provided in Table 4-16 (Caporali & Emanueli, 2011; Hawke et al., 2021; Kamijo et al., 2022; Kotarski et al., 2025; Lázár et al., 2018; Mahdipour et al., 2015; Mutia et al., 2023; Pavani et al., 2022; Pendzialek et al., 2019; Rosenbluth et al., 2013; Shi et al., 2024; Timofeeva et al., 2025; Turri et al., 2021; Yang et al., 2025; Zhou & Dimitriadis, 2020; Zhu et al., 2025).

Table 4-16: Reported Functions and Clinical Associations of Selected miRNAs in Reproduction and Early Development.

miRNA	Literature review
miR-103	miR-103: Stable in porcine liver and uterus, used for normalization.
	miR-103a-3p: Regulates embryonic stem cell function, potential therapeutic target.
miR-181	miR-181: Modulates EMT through TGF-β, Wnt/β-catenin, and NF-κB pathways, linked to cancer cell behavior.
	miR-181a-5p: Higher in non-developing embryos; associated with estrogen signaling and implantation; regulates MMP14 affecting cell adhesion and proliferation.
miR-191	miR-191-5p: Elevated in aneuploid embryos and failed IVF; linked to cell adhesion and successful implantation.
	miR-191: Expression varies with embryo viability and pathology (e.g., preeclampsia, diabetes); insulin-regulated; lineage-specific effects in embryoblast vs. trophoblast cells.
miR-24	miR-24-3p: Regulates ESC differentiation; inhibits apoptosis via target gene P27; supports follicular health.
	miR-24: Highly expressed in euploid embryos and polycystic ovaries.

Table 4-16: Reported Functions and Clinical Associations of Selected miRNAs in Reproduction and Early Development (Continued).

miRNA	Literature review
miR-28	miR-28-3p: High in good-quality rabbit blastocysts; supports implantation; influences cancer-related genes.
miR-320	miR-320: Highly expressed in early euploid embryos; target: ITGB5; linked to vascular development defects when overexpressed.
	miR-320a: Higher in non-pregnancy groups; may negatively affect implantation; associated with CDH and lung fibrosis.
miR-378	miR-378a-5p: Influences trophoblast cell invasion and migration in placentation.
	miR-378-3p: Linked to oocyte meiosis, blastocyst quality, embryo hatching, and implantation success.
	miR-378: Highly expressed in euploid embryos; associated with sperm motility and quality.
miR-92	miR-92: Found in polycystic ovaries.
	miR-92a-3p: Regulates genes involved in cell adhesion, important for implantation; may serve as a biomarker for embryo viability.
	miR-92a: Highly expressed in euploid embryos; overexpression causes vascular defects.

5. CONCLUSIONS AND RECOMMENDATIONS

This study characterised developmentally relevant miRNA in stem cells in chicken primordial germ cells and rabbit preimplantation embryos. The aim of identify conserved miRNA regulatory mechanisms, species-specific features, and potential biomarkers for germ cell function and embryo quality.

Cryopreservation experiment provided stress conditions to the cell lines, which significantly alter the miRNA expression in chicken PGCs**,** with both miR-138-5p and miR-302c-3p exhibiting strong post-thaw upregulation in males and females. These miRNAs are known regulators of stress responses, pluripotency, and apoptosis, and their induction coincided with reduced cellular proliferation during the initial recovery period. This indicates that PGCs activate conserved stress-responsive and stem-cell-associated miRNA pathways to restore cellular homeostasis following an injury produced by cryopreservation**.**

The strong activation of stem cell-related miRNA clusters especially shows that key pluripotency mechanisms are shared between birds and mammals. The increase of miR-302c-3p in chicken PGCs, along with the presence of similar miRNAs in rabbit embryos, indicates that these miRNA networks are evolutionarily conserved and play an important role in maintaining stem cell characteristics. In contrast, the expression of miR-371a-5p and miR-302b-3p (miR-302/367 family members) in rabbit was not detected in this study most probably due to the age of the embryo. It is therefore advisable to evaluate their expression across additional rabbit preimplantation stages to confirm their temporal regulation and potential involvement in early embryogenesis.

The analysis of rabbit embryos revealed that preimplantation embryos (blastocyst and hatched blastocyst stages) secrete several extracellular miRNA profile into the culture medium, involving the eight studied miRNAs. The presence and relative abundance of these miRNAs in the medium often exceeded their intracellular levels, indicating active release or selective retention mechanisms. These findings confirm that embryo culture medium carries a molecular signature reflective of embryonic physiology and developmental competence, supporting its use for non-invasive embryo assessment.

This study identified several miRNAs that play important roles in early embryo development, including the regulation of cell growth, survival, implantation, and interaction with the surrounding environment. In particular, miR-378a-3p and miR-320a-3p were strongly associated with good-quality blastocysts and pathways related to implantation, suggesting their involvement in supporting normal embryo development. In addition, miR-191-5p and miR-181a-5p showed expression patterns consistent with previous reports linking these miRNAs to embryo viability and successful implantation. In contrast, members of the miR-302 family were not detected *in vitro* cultured embryos, highlighting differences between *in vitro* and *in vivo* developmental regulation. Overall, these findings demonstrate that miRNA expression profiles provide biologically meaningful information and can be used to assess embryo competence.

Kohonen's Self-Organising Maps and hierarchical clustering analyses proved effective in validating the discriminatory power of miRNA expression signatures to classify embryo developmental states and distinguish among embryo types. Clear differences in miRNA profiles were observed between blastocysts and hatched blastocysts, as well as among embryos belonging to different morphological classes. Hatched blastocysts displayed more homogeneous miRNA expression patterns, whereas blastocysts showed greater variability, reflecting developmental heterogeneity at earlier stages. Multivariate analyses further demonstrated that embryos with similar developmental stages clustered together based on their miRNA expression profiles, independently of culture conditions. These findings indicate that miRNA signatures function as molecular fingerprints that reflect intrinsic developmental status, embryo maturity, stress adaptation, and developmental trajectory, extending beyond the classification based on morphology alone.

Collectively, these findings demonstrate that stem cell-specific and developmentally regulated miRNAs constitute a conserved regulatory axis across birds and mammals, responsive both to intrinsic developmental signals and to external interventions such as cryopreservation.

6. NEW SCIENTIFIC RESULTS

I. This study provides the first evidence that miR-138-5p is regulated in primordial germ cells under cryopreservation conditions, indicating its involvement in molecular pathways associated with post-thaw cellular stress and recovery.

II. miR-138-5p expression was significantly increased in both female and male chicken primordial germ cells following cryopreservation, demonstrating a sex-independent transcriptional response to freezing–thawing–induced stress.

III. miR-302c-3p showed a consistent and significant upregulation in cryopreserved chicken primordial germ cells of both sexes, indicating its sensitivity to cryogenic stress and its potential role in post-thaw cellular adjustment processes. As a member of the pluripotency-associated miR-302/367 cluster, miR-302c-3p may serve as a molecular indicator of post-thaw cellular status in germline stem cells.

IV. Rabbit preimplantation embryos were demonstrated, for the first time, to actively and developmentally stage-dependently secrete a defined set of microRNAs (miR-24-3p, miR-103a-3p, miR-191-5p, miR-378a-3p, miR-28-3p, and miR-320a-3p) into the culture medium.

7. KEYNOTES OF THE STUDY AND FUTURE PERSPECTIVES

Since miRNAs have demonstrated to be a powerful biomarkers and regulatory molecules, demonstrated strong associations with developmental competence, cellular stress responses, and pluripotency regulation, future studies should aim to study deeply the mechanism and how this knowledge can be used in different fields.

Future work should include gain- and loss-of-function experiments using CRISPR/Cas9, antagomiRs, or miRNA mimics to elucidate the causal roles of these miRNAs in PGC survival, post-thaw recovery, blastocyst formation, and trophoblast differentiation.

In order to develop miRNA non-invasive diagnostic tools for embryo selection in assisted reproduction is important to include larger embryo cohorts, and correlations with implantation success and embryo development, reducing invasive embryo biopsy and improving selection accuracy in both human and ART animal systems.

Several miRNAs identified in this study, including miR-302 family members, miR-138-5p, miR-24-3p, miR-191-5p, and miR-378a-3p, have documented roles in both stem cell maintenance and cancer progression. Future work should investigate how these miRNAs coordinate cell-cycle, apoptosis, and metabolic regulation in germ cells and embryos, and compare these functions with their known activity in cancer stem cells. Such comparative studies may clarify how developmentally essential pathways become dysregulated during tumorigenesis.

The discovery that rabbit embryos secrete distinct miRNAs into culture media parallels findings in oncology, where circulating miRNAs serve as biomarkers for tumour presence and progression. Further analysis of extracellular miRNA packaging, release mechanisms, and target-cell signalling could reveal conserved communication strategies shared between early embryos and cancer cells.

8. SUMMARY

The research investigates the expression and regulatory relevance of microRNAs (miRNAs) during early developmental processes, with particular emphasis on chicken primordial germ cells (PGCs) and rabbit preimplantation embryos. The objective was to characterise developmentally relevant and stress-responsive miRNAs, and evaluate their potential as molecular indicators of cell and embryo developmental status.

The first part of the study focused on chicken PGCs subjected to cryopreservation, a procedure known to induce cellular stress. MiRNA expression profiling revealed that cryopreservation led to consistent changes in miRNA expression, most notably the upregulation of miR-138-5p and miR-302c-3p after thawing. These miRNAs are associated with pathways involved in stress response, pluripotency regulation, and apoptosis. Their increased expression coincided with a temporary reduction in cellular proliferation during the early post-thaw recovery phase, suggesting the activation of adaptive regulatory mechanisms aimed at restoring cellular homeostasis. Statistical analyses indicated no significant differences between male and female PGCs in proliferation rates or pluripotency-associated gene expression, demonstrating that both sexes exhibited comparable responses to cryopreservation under the experimental conditions applied.

The second part of the thesis examined miRNA expression in rabbit preimplantation embryos and their corresponding culture media, with the aim of determining whether extracellular miRNAs reflect embryo quality and developmental progression. Several miRNAs, including miR-28-3p, miR-320a-3p, miR-378a-3p, miR-24-3p, miR-191a-5p, miR-103a-3p and miR-181a-5p, were consistently detected in culture media across developmental stages. Distinct differences in miRNA expression profiles were observed between blastocysts and hatched blastocysts, as well as among embryos belonging to different morphological classes. Hatched blastocysts exhibited more homogeneous miRNA profiles, whereas blastocysts showed greater variability, consistent with increased developmental heterogeneity at earlier stages.

To further explore relationships among samples, multivariate analyses were performed using Kohonen's Self-Organising Maps and hierarchical clustering. These approaches demonstrated that embryos clustered according to developmental stage based on miRNA expression patterns, largely independent of culture conditions. This finding supports the interpretation that miRNA signatures are closely linked to intrinsic developmental status rather than morphology alone. The identified miRNA profiles therefore function as molecular fingerprints reflecting embryo maturity, developmental trajectory, and potential stress adaptation.

In summary, this research provides a characterisation of miRNA expression dynamics in germline cells and early embryos under both stress and non-stress conditions. The results support the biological relevance of miRNA profiling as a complementary molecular approach for assessing developmental status and embryo competence. Although direct clinical application is beyond the scope of this work, the findings establish a foundation for future research into non-invasive embryo assessment strategies in both animal and human assisted reproductive technologies.

9. ÖSSZEFOGLALÓ

A kutatás a mikroRNS-ek (miRNS-ek) expresszióját és szabályozó szerepét vizsgálja a korai fejlődési folyamatok során, különös tekintettel a csirke primordiális csírasejtekre (PGC-k) és a nyúl preimplantációs embrióira. A munka célja a fejlődés szempontjából releváns és stresszre reagáló miRNS-ek azonosítása és jellemzése, valamint annak értékelése volt, hogy ezek alkalmazhatók-e a sejtek és embriók fejlődési állapotának molekuláris indikátoraiként.

A vizsgálat első része a krioprezerváción átesett csirke PGC-kre fókuszált, mivel ez az eljárás ismerten celluláris stresszt idéz elő. A miRNS-expressziós profilozás kimutatta, hogy a fagyasztás–olvasztás következetes változásokat eredményezett a miRNS-ek expressziójában, különösen a miR-138-5p és a miR-302c-3p szintjének emelkedését az olvasztást követően. Ezek a miRNS-ek a stresszválaszban, a pluripotencia szabályozásában és az apoptózisban szerepet játszó jelátviteli útvonalakhoz kapcsolódnak. Megemelkedett expressziójuk egybeesett a sejtek proliferációjának átmeneti csökkenésével a korai posztolvasztási regenerációs fázisban, ami adaptív szabályozó mechanizmusok aktiválódására utal, a sejtes homeosztázis helyreállítása érdekében. A statisztikai elemzések nem mutattak szignifikáns különbséget a hím és nőstény PGC-k proliferációs rátája vagy a pluripotenciához kapcsolódó génexpresszió tekintetében, ami arra utal, hogy mindkét nem hasonló módon reagált a krioprezervációra az alkalmazott kísérleti feltételek mellett.

A dolgozat második része a nyúl preimplantációs embrióinak és a hozzájuk tartozó tenyésztőközegnek a miRNS-expresszióját vizsgálta annak meghatározására, hogy az extracelluláris miRNS-ek tükrözik-e az embriók minőségét és fejlődési előrehaladását. Számos miRNS – köztük a miR-28-3p, miR-320a-3p, miR-378a-3p, miR-24-3p, miR-191a-5p, miR-103a-3p és miR-181a-5p – következetesen kimutatható volt a tenyésztőközegben a különböző fejlődési stádiumok során. Jelentős különbségek mutatkoztak a blasztociszták és a kikelő blasztociszták miRNS-profiljai között, valamint az eltérő morfológiai osztályba sorolt embriók között is. A kikelő blasztociszták homogénebb miRNS-profilt mutattak, míg a blasztociszták nagyobb variabilitást, ami összhangban áll a korábbi fejlődési stádiumokra jellemző fokozott heterogenitással.

A minták közötti kapcsolatok további feltárása érdekében többváltozós elemzéseket végeztünk Kohonen-féle önszerveződő térképek és hierarchikus klaszteranalízis alkalmazásával. Az eredmények azt mutatták, hogy az embriók a miRNS-expressziós mintázatok alapján elsősorban fejlődési stádium szerint csoportosultak, nagyrészt függetlenül a tenyésztési körülményektől. Ez alátámasztja azt az értelmezést, hogy a miRNS-alapú jelátviteli mintázatok szorosabban kapcsolódnak a belső fejlődési állapothoz, mint a morfológiai jellemzők önmagukban. Az azonosított miRNS-profilok így molekuláris ujjlenyomatként értelmezhetők, amelyek tükrözik az embrió érettségét, fejlődési irányát és potenciális stresszadaptációját.

Összefoglalva, a kutatás átfogó képet ad a miRNS-expresszió dinamikájáról csírasejtekben és korai embriókban stresszes és nem stresszes körülmények között egyaránt. Az eredmények alátámasztják a miRNS-profilozás biológiai relevanciáját mint a fejlődési állapot és az embriókompetencia értékelésére szolgáló kiegészítő molekuláris megközelítést. Bár a közvetlen klinikai alkalmazás túlmutat a jelen munka keretein, az eredmények megalapozzák a jövőbeli kutatásokat a nem invazív embrióértékelési stratégiák fejlesztése irányában, mind állati, mind humán asszisztált reprodukciós technológiák területén.

10. PUBLICATIONS

1. Salinas, María ; Tokodyné Szabadi, Nikolett* ; Dévai, Gréta ; Urbán, Martin ; Tóth, Arnold ; Lázár, Bence ; Pintér, Timea ; Nemes, Annamária ; Fancsovits, Péter ; Bodrogi, Lilla et al. *Detection of Development-Specific MicroRNAs in Rabbit Embryos and Culture Media: A Potential Biomarker Approach for Embryo Quality Assessment.*
   GENES 16 : 9 Paper: 1042 , 16 p. (2025)
   DOI WoS Scopus PubMed
   Article (Journal Article) | Scientific[36320809] [Validated]

2. Tóth, Arnold ; Ecker, András ; Tokodyné, Szabadi Nikolett ; María, Salinas ; Bence, Lázár ; Gócza, Elen ; Tóth, Roland. *Investigating the effect of heat treatment and monitoring the molecular changes in primordial germ cells (pgc) before and after freezing*
   In: Benczúr, Kinga; Gócza, Elen; Pál, Magda; Pusztahelyi, Tünde (eds.) FIBOK 2024 6th National Conference of Young Biotechnologists
   Bp, Hungary : MTA Agrártudományok Osztálya, Mezőgazdasági Biotechnológiai Tudományos Bizottság (2024) 111 p. p. 100
   Abstract (Conference paper) | Scientific[35575098] [Approved]

3. Tóth, Arnold ; Tóth, Roland ; Maria, Teresa Salinas ; Ecker, András ; Gócza, Elen
   Hőkezelés hatásának vizsgálata a primordiális őssejtek fagyasztására és a felolvasztás utáni markerek mintázatára
   In: VI. Sejt-, Fejlődés- és Őssejtbiológia Konferencia - Absztraktfüzet
   (2024) p. 49
   Abstract (Conference paper) | Scientific[35575160] [Approved]

4. Maraghechi, Pouneh ; Aponte, Maria Teresa Salinas ; Ecker, András ; Lázár, Bence ; Tóth, Roland ; Szabadi, Nikolett Tokodyné ; Gócza, Elen
   Pluripotency-Associated microRNAs in Early Vertebrate Embryos and Stem Cells
   GENES 14 : 7 Paper: 1434 , 20 p. (2023)
   DOI WoS Scopus PubMed Other URL
   Article (Journal Article) | Scientific[34063802] [Validated]
   All citations+mentions: 3, External citations: 2, Self citations: 1, Unhandled citations:0

5. Lázár, Bence ; Tokodyné Szabadi, Nikolett* ; Anand, Mahek ; Tóth, Roland ; Ecker, András ; Urbán, Martin ; Aponte, Maria Teresa Salinas ; Stepanova, Ganna ; Hegyi, Zoltán ; Homolya, László et al.
   Effect of miR-302b MicroRNA Inhibition on Chicken Primordial Germ Cell Proliferation and Apoptosis Rate
   GENES 13 : 1 Paper: 82 , 16 p. (2022)
   DOI WoS Scopus PubMed Other URL
   Article (Journal Article) | Scientific[32557627] [Validated]
   All citations+mentions: 11, External citations: 10, Self citations: 1, Unhandled citations: 0

11. APPENDIX

Appendix A1: Bibliography

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Appendix A2: Supplementary Movie S1

https://drive.google.com/file/d/1oGmZgnPF9u1QJrmyy4JnjeMTB2A0jsXl/view?usp=share_link

Appendix A3:

STUDENT DECLARATION

This dissertation is my original work and has not been presented for a degree in any other university. No part of this dissertation may be reproduced without prior permission of the author and/or Hungarian University of Agriculture and Life Sciences.

____________________________________ Date______________________

Appendix A4:

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