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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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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.

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. 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. METHODOLOGY

2.1.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 1.

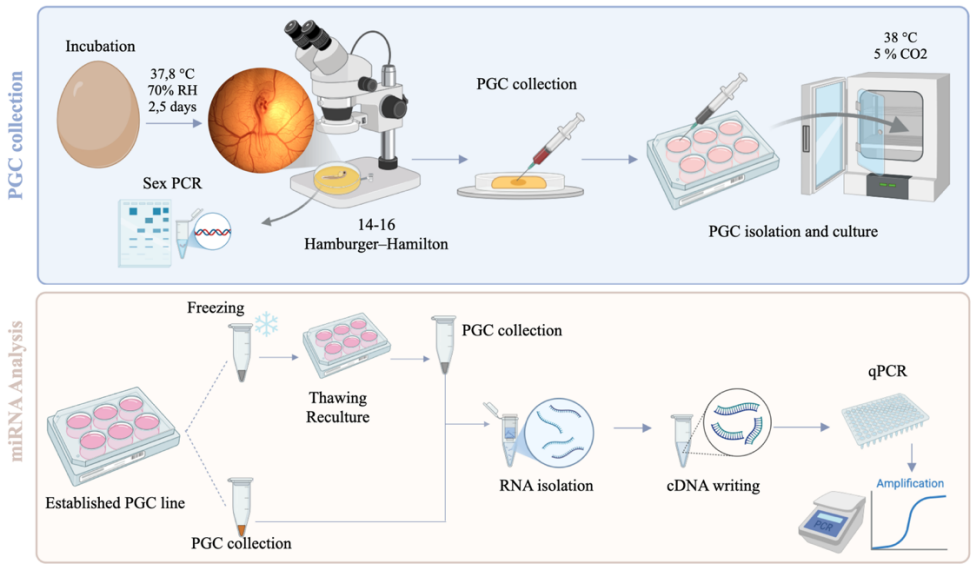


Figure 1 Schematic flowchart summarising the steps of the experimental process. Created with BioRender.com.

2.1.1.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). 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 2.1.3).

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.

2.1.2. 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 PCR and agarose gel electrophoresis. Sex-specific PCR amplification

was performed using primers targeting the CHD1-Z and CHD1-W. Reactions were carried out in a MyTaq-based master mix containing MyTaq DNA polymerase and other components. 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.

2.1.3. 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). The medium is enriched with a range of key components designed to optimise the growth and viability of PGCs. The medium was replaced every two days to support PGC growth and survival. Once the culture was established, aproximately 1-2 × 10^5 cells were taken for RNA isolation, while the rest of the cells were frozen.

2.1.4. Cell counting and proliferation assay

Cell viability and cell counting of the primordial germ cells were performed before freezing using the Arthur Novel Fluorescence Cell Counter (NanoEnTek, Pleasanton, USA).

2.1.5. PGC freezing

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. An equal volume (250 µL) of freshly prepared freezing medium was then added dropwise with gentle mixing to minimise osmotic shock.

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) .

2.1.6. 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.

2.1.7. RNA isolation

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 samples 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).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.

2.1.8. 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. 9 µL of RNA Sample 25 ng/mL was added to 11 µL of the High-capacity RNA-to-cDNA master mix in an individual PCR tube. The ProFlex PCR System (Applied Biosystems, Life Technologies) was used to perform

the reverse transcription reaction. The cDNA samples were stored at 2-8°C for the short term.

2.1.9. 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 with 0.5 **µL**10× Poly(A) buffer, 0.5 **µL** ATP, 0.3 **µL** Poly(A) enzyme, 1.7 **µL** RNase-free water. 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.

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 with 3.0 µL 5× DNA ligase buffer, 4.5 µL 50% PEG 8000, 0.6 µL 25× Ligation adaptor, 1.5 µL RNA ligase, and 0.4 µL RNase-free water.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.

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 with 6.0 µL 5× RT buffer, 1.2 µL dNTP mix (25 mM), 1.5 µL 20× universal RT primer, 3.0 µL 10× RT enzyme mix, 3.3 µL RNase-free water. 15 µL of the RT reaction mixture was added to the previous tube and incubated at 42 °C for 15 minutes.

miR-Amp reaction

miRNAs were amplified to increase the cDNA levels of low-abundance targets. The miR-Amp reaction mixture was prepared with 25 µL 0 2× miR-Amp master mix, 2.5 µL 20× miR-Amp primer mix, and 17.5 µL RNase-free water. 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 at 95°C for 5 minutes, 95°C for 3 seconds, 60°C for 30 seconds, and 99°C for 10 minutes. 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.

2.1.10. qPCR for markers

TaqMan™ universal PCR master mix (Applied Biosystems by Thermo Fisher Scientific) was utilised to study the PGCs marker expression. The Taq primers used were cGAPDH, cPOUV, cDDX4, and cDAZL. The master mix was prepared with 5.75 µL RNase free water (NFW), 7.5 µL Master Mix universal TaqMan™, 0.75 µL TaqMan ™ Primer. 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). The qPCR was performed in 40 cycles by Eppendorf MasterCycler Realplex thermal cycler with the following conditions: ezyme activation for 10 minutes at 95°C, denaturation for 20 seconds at 95°C and annealing at 60°C for 1minute and 5 seconds.

2.1.11.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 with 10 µL TaqMan® Fast Advanced Master mix, 1 µL TaqMan® Advanced miRNA Assay (primer), and 4 µL Free RNasa water. The primers were hsa-miR-92a-3p, hsa-miR-138-5P, and hsa-miR-302c-3P 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). The qPCR was performed for 45 cycles by Eppendorf MasterCycler Realplex thermal cycler with the following parameters: enzyme activation for 20 seconds at 95°C, denaturation for 3 seconds at 95°C, and annealing for 30 seconds at 60 °C.

2.1.12.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. 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 $2^{-\Delta\Delta Ct}$. T-test (type=2, tail=2) in GenEx 7.0 software was used to evaluate statistical differences between the groups.

2.2. 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 2.

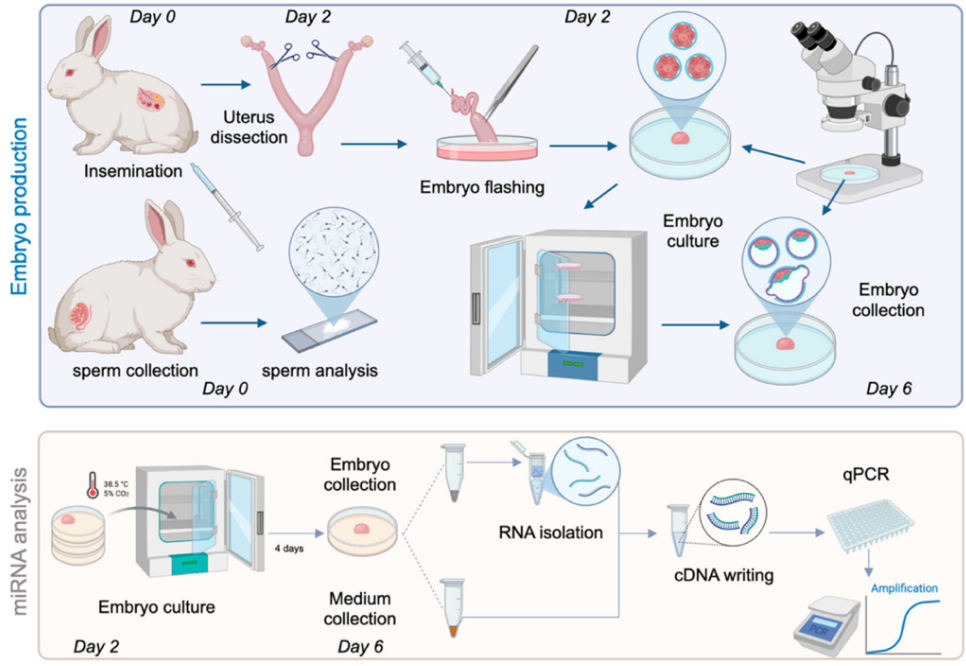


Figure 2: Schematic flowchart summarising the steps of the experimental process. Created with BioRender.com

2.2.1.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) (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.

2.2.2.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.

2.2.3.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. 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.

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. 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 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 2-4 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.

2.2.4.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.

2.2.5.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. All the components and procedure are detailed in section 2.1.7. The samples were kept at -70 °C.

2.2.6.cDNA writing for miRNA

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).

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 consisted of four reactions: Poly(A) tailing reaction, adaptor ligation reaction, reverse transcription (RT) reaction, and miR-Amp reaction. All the components and reaction condition are detailed in section 2.1.9. 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.

2.2.7. 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 proportions requested by the brand. 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. Reaction conditions are detailed in section 2.1.11 and primers are detailed in section 3.2.2.

2.2.8. 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.

3. RESULTS AND DISCUSSION

3.1. 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). 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 (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 (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 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.

3.1.1. 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. The proliferation rates of the 4 cell lines were compared in Figure 3. A t-test (tails=2, type=2) was applied to detect any significant differences.

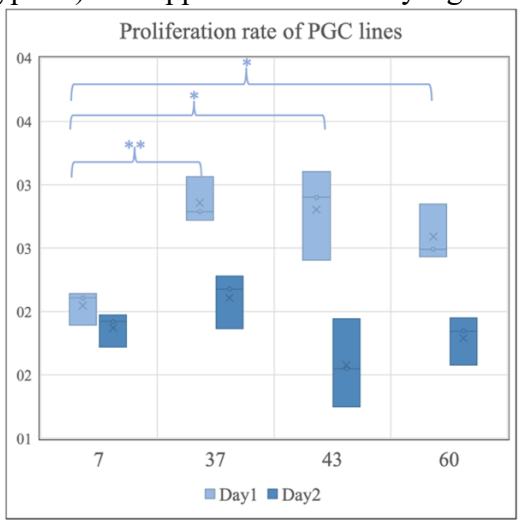


Figure 3: proliferation rate of PGC line 7 (female), 37 (female), 43 (male) and 60 (male)

The PGC lines showed higher proliferation rate on day 1 compared to second day, with considerable significance difference, 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 were analysed for both female and male PGC lines over the first two days following thawing. 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).

3.1.2. Gene expression analysis

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 and miR-92a-3p for miRNAs,

with sample 37A designated as the internal reference for all calculations. A boxplot and a t-test (tails=2, type=2) were applied to study gene expression.

3.1.2.1.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. 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).

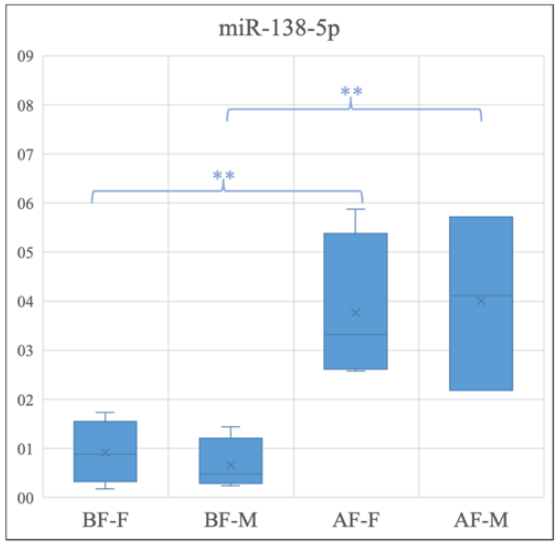


Figure 4: Changes in miR-138-5p expression in PGCs in response to cryopreservation.
BF: before freezing. AF: after freezing. F: female. M: male

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).

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 (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.

3.1.2.2.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 5. 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).

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.

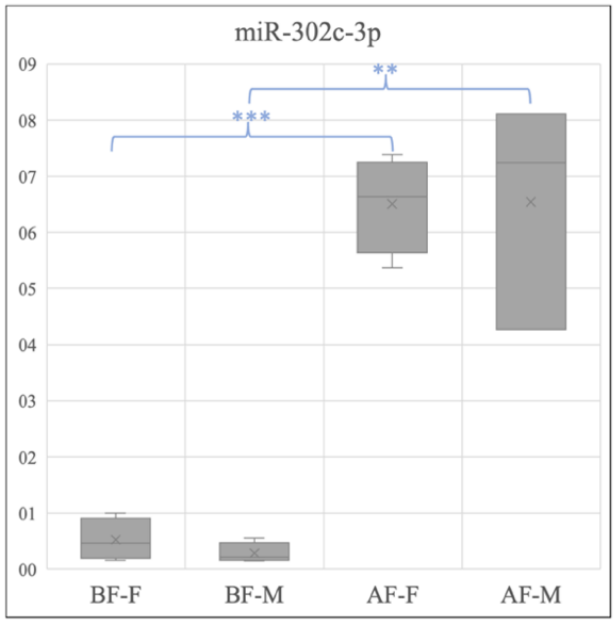


Figure 5: Relative expression of miR-302c-3p in female (F) and male (M) PGCs before freezing (BF) and after thawing (AF).

3.1.2.3.CVH or DDX4 expression

In Figure 6, the expression of CVH or DDX4 is shown. CVH or DDX4 expression differed significantly between females before and after freezing.. In Figure 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.

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).

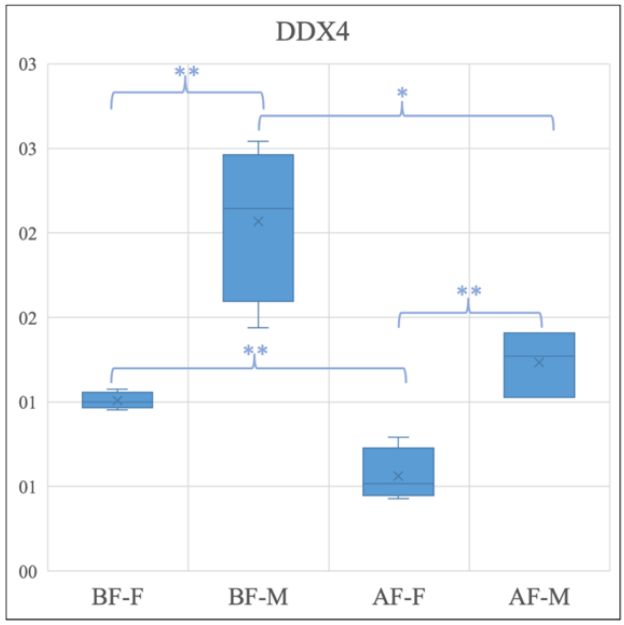


Figure 6: Relative expression of DDX4 in female (F) and male (M) PGCs before freezing (BF) and after thawing (AF).

3.1.2.4.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. 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.

3.2.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.

3.2.1.Embryo collection and culture

The collected embryos were characterised according to the rabbit embryo morphology explained by Sultana *et al., (*2009). 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.

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.

3.2.2.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. 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.

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

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. hsa-miR-302b-3p and hsa-miR-371a-5p were excluded from the present study due to their very high Ct value (no expression). According to SOLiD sequencing results and abundance analysis, 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 just in 7-day-old *in vivo* rabbit embryos. 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.

3.2.3.Relative miRNA expression calculation

Hsa-miR-92-3p was used as a reference gene, and the F3_m culture medium sample was used as the reference sample in the relative miRNA expression calculation.

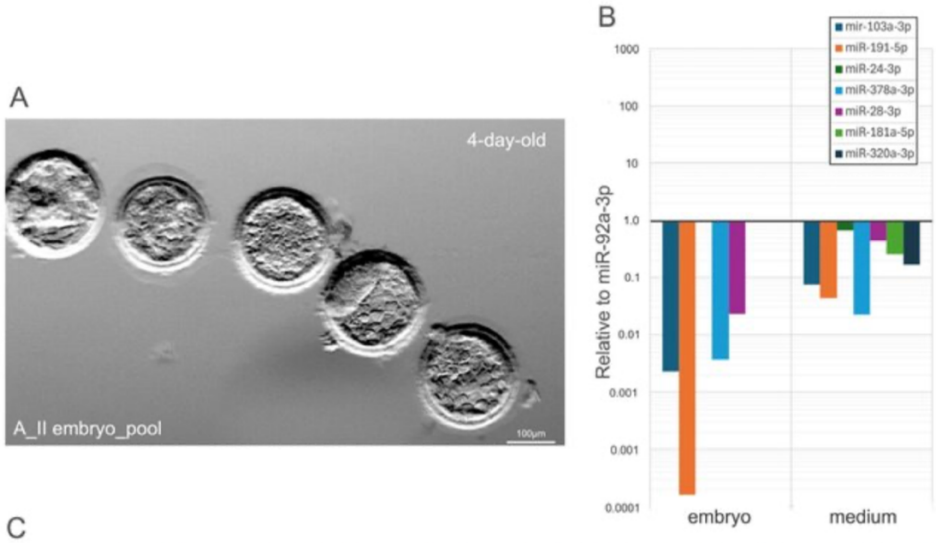
3.2.4.miRNA expression analysis

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

3.2.4.1.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 7 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 7 B and C.



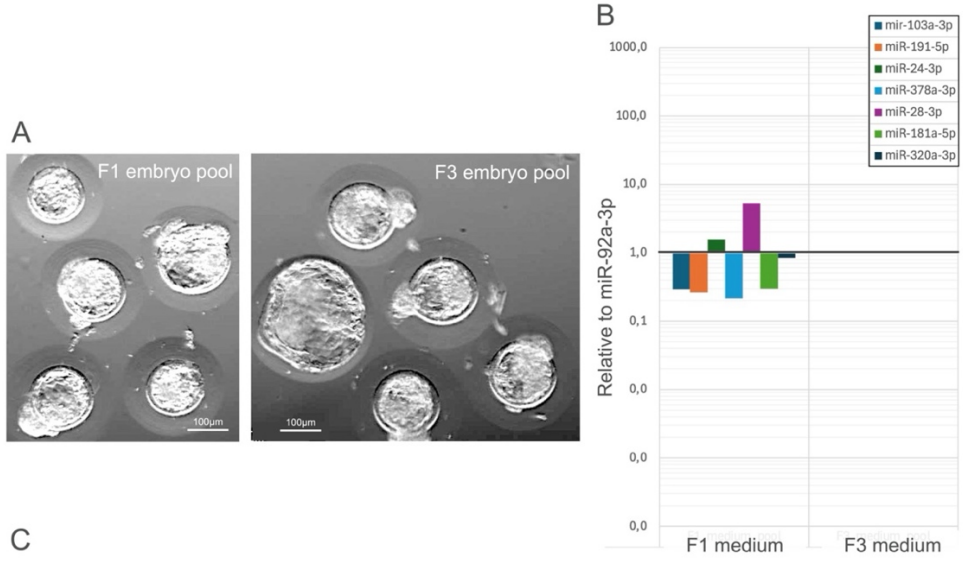
C

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 7: 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 8.



C

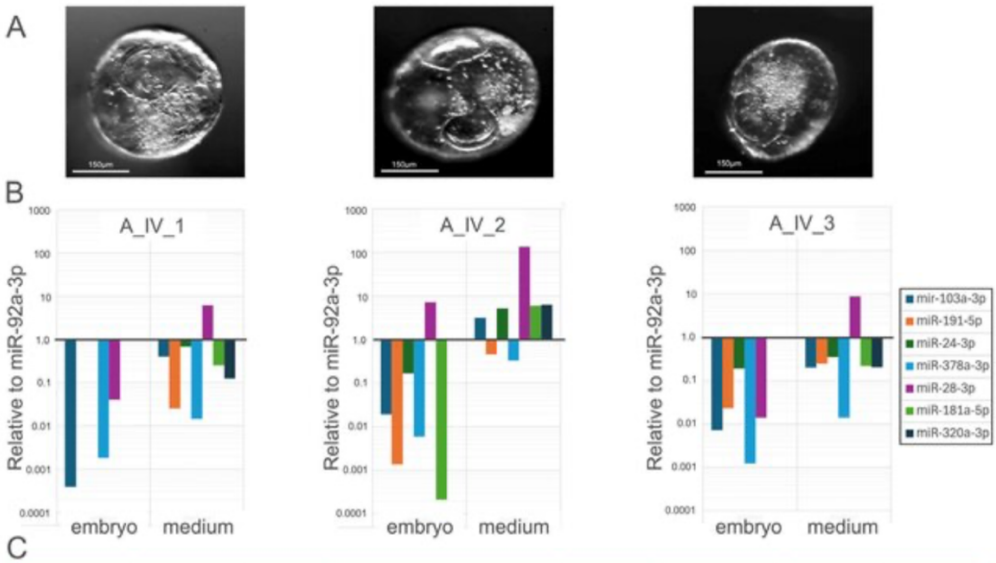
sample	mir-92-3p	mir-103a-3p	miR-191-5p	miR-24-3p	miR-378a-3p	miR-28-3p	miR-181a-5p	miR-320a-3p
F1_medium_pool	+++	++	+++	+++	+++	++	+++	+++
F3_medium_pool	+++	+++	+++	+++	+++	+	+++	+++

Figure 8: 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.

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

Three embryos categorised as hatched blastocysts (Figure 9 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.



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 9: 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

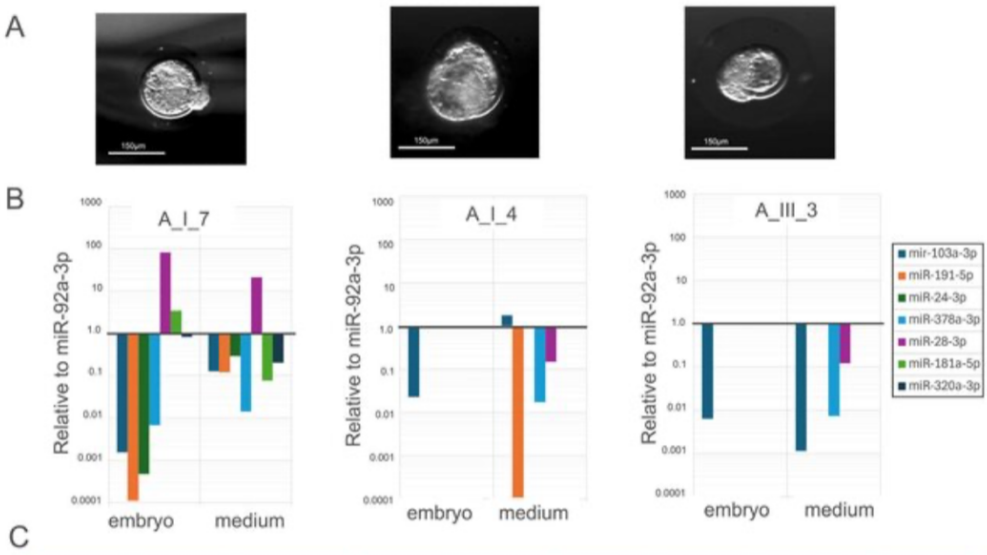
four levels based on cycle threshold (Ct) values from quantitative real-time PCR. Ct values (+++: Ct < 35; ++: 35 < Ct < 39; +: 39 < Ct < 45; no expression: Ct > 45).

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 down-regulated, 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.

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

Three embryos categorised as blastocysts (Figure 10 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 10 B,C.

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.



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 10: 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).

3.2.4.4.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 11). 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.

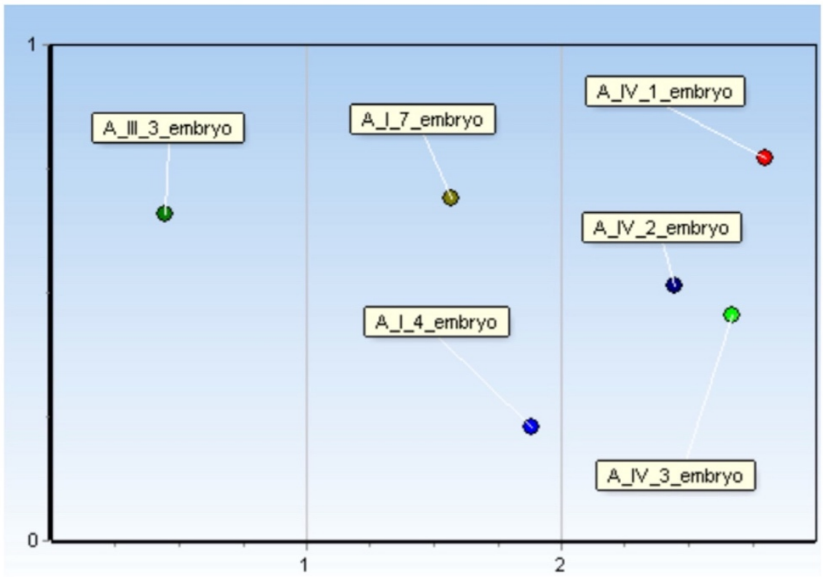


Figure 11: Kohonen Self-Organising Map Showing Clustering of Embryo Samples Based on miRNA Expression Profiles.

3.2.4.5.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 12). 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.

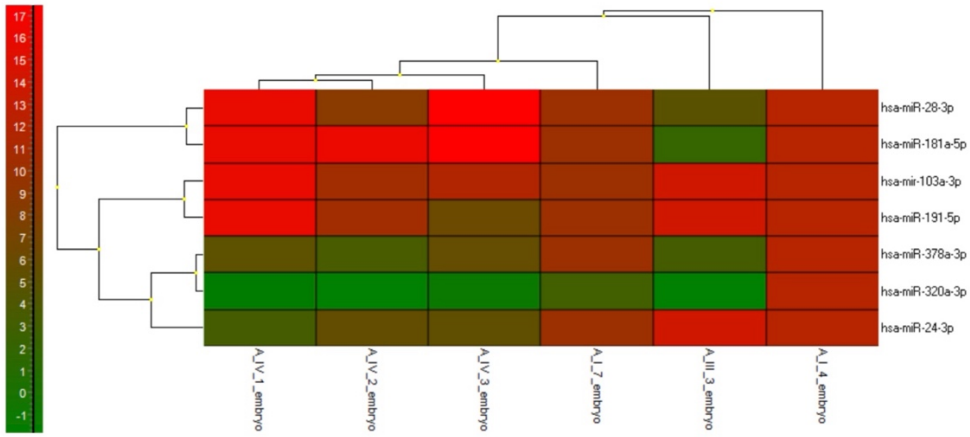


Figure 12: Heatmap and Clustering of miRNA Expression Across Rabbit Embryo Samples.

3.2.4.6.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 13A) cultured media were analysed to assess their miRNA expression profiles (Figure 13 B,C)

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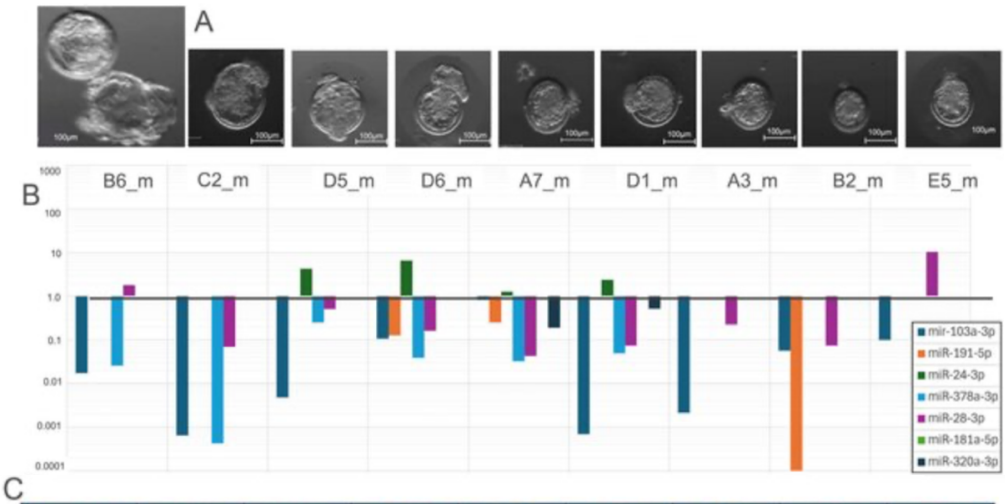

C

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 13: 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; 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).

4. 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.

5. 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.

6. 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

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