THESIS

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MSc. ANIMAL BIOTECHNOLOGY

GÖDÖLLŐ

2023

Hungarian University of Agriculture and Life Sciences

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ANALYSIS OF THE MICRORNA EXPRESSION PROFILES OF CHICKEN PRIMORDIAL GERM CELLS BEFORE AND AFTER FREEZING

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Gödöllő

2023

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ABBREVIATION AND ACRONYMS

AGO2	Argonaute 2
cDAZL	Chicken Deleted in Azoospermia-like
cDNA	Complementary Deoxyribonucleic Acid
CHD1	Chromodomain helicase DNA binding protein 1
CVH	Chicken Vasa Homologue
CXCR4	C-X-C chemokine receptor type 4
DAZL	Deleted in azoospermia-like
DAZL	Deleted in Azoospermia-like (gene)
DGCR8	Drosha and DiGeorge Syndrome Critical Region gene 8
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EG&K, EGK	Eyal-Giladi & Kochav scale
EMA-1	Embryonic Mouse Antigen-1
ESC	Embryonic stem cells
FAM1	Freezing Avian Medium-1
FAM2	Freezing Avian Medium-2
FBS	Fetal Bovine Serum
FGF2	Fibroblast growth factor 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GV	Germinal Vesicle
H&H, HH	Hamburger and Hamilton stage of chick embryos
IgG	Immunoglobulin G
IgM	Immunoglobulin M
miRNAs	microRNAs
mRNA	Messenger RNA
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGCs	Primordial Germ Cells
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary microRNA
qPCR	Quantitative Real-Time Polymerase Chain Reaction
Ran-GTP	Ras-related nuclear protein -guanosine-5'-triphosphate
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNA pol-II/III	RNA polymerase II/III
rpm	Revolutions per minute
RT	Room Temperature
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SDF1	Stomatal Cell Derived Factor 1
SSEA-1	Stage-specific Embryonic Antigen1
TRBP	TAR-RNA binding protein

1. INTRODUCTION

Avian embryos are useful models for studying higher vertebrate development. The ease of access to growing avian embryos allows for a wide range of experimental applications (Sato & Lansford, 2013). Primordial germ cells (PGCs), the precursors of functional gametes, offer significant potential for the use of genetic resources and transgenesis in chickens. PGCs can be extracted from the developing embryo at diverse stages and expanded in vitro (Han & Lee, 2017). Germ cells are the only type of cell capable of passing on genetic information to future generations. They separate from the embryo's somatic cells during development. Primordial germ cells (PGCs) are the only cells in growing embryos capable of transmitting genetic information to future generations. PGCs in avian embryos are transported to the eventual gonadal area through blood circulation, unlike in other species. This peculiar accessibility of avian PGCs during early development allows for the collection and transplantation of PGCs. The latest development of methods for producing germline chimeras by PGC transfer, as well as long-term cultivation methods of chicken PGCs without losing their germline transmission ability, has provided significant breakthroughs for germplasm preservation, transgenic bird production, and germ cell system research (Nakamura et al., 2013).

Initially, chicken PGCs colonize the anterior extraembryonic region known as the "germinal crescent," and after blood vascular circulation is established, they temporally circulate across the embryonic blood vascular system with embryonic blood cells. They continue to colonize microcapillary networks in both the right and left future gonadal regions. They then travel interstitially to the germinal ridge, where they start to differentiate and finally mature into future eggs or sperm (Szczerba et al., 2021).

Germ cells are completely separated from somatic cell types in many vertebrate and invertebrate species during the early stages of development. Germ cells in these organisms arise from sites other than the gonads. They then migrate to the developing gonads. Through gametogenesis, germ cells transmit genetic information to the next generation. Primordial germ cells (PGCs) are the first germ-cell population established during early embryonic development and are the progenitor of both oocytes and spermatogonia. PGCs in birds undergo blood circulation to migrate toward the genital ridge, which is one of the key biological features of avian PGCs. Female germ cells undergo meiosis and arrest at prophase I during embryogenesis, but male germ cells enter mitotic arrest during embryogenesis and begin meiosis only after birth (Tagami et al., 2017).

Primordial germ cells (PGCs) have the potential to be utilized in cell-based research, in the preservation of the avian germline as well as for genetic manipulation of the avian genome. In vitro cultures of germline competent avian PGCs provide a unique system for studying early germ cell differentiation as well as a comparative system for mammalian germ cell development. Primary PGC lines will serve as the foundation for an alternate strategy for avian germline preservation, as well as a vital tool for transgenic technology with both research and industrial uses (Macdonald et al., 2010).

PGCs from chickens can be separated, cultivated, and genetically modified while remaining committed to the germ line. Furthermore, in vitro differentiation of chicken PGCs into embryonic germ cells that contribute to the development of somatic tissues is possible. The ability of PGCs to retain their commitment to the germ line following long periods in culture and genetic change, together with their ability to gain somatic competence in vitro, gives a new model for developmental biology. The avian embryo, which allows access to the very early phases of development and provides a simple route for reintroducing PGCs into the embryonic vasculature. Furthermore, these characteristics open up new avenues for manipulating chicken genomes for agricultural and medicinal uses (Van De Lavoir et al., 2006).

1.1. OBJECTIVES OF THE RESEARCH

General objectives

1. To determine the expression of CVH, DAZL, miR-92, and miR-302b-5P before and after freezing in order to test two freezing media for long PGC conservation.

Specific objectives

1. Establish a PGC line from a blood sample taken from a chicken embryo.
2. Perform PCR-sexing with W chromosome-specific primers, CHD1 forward and reverse primers, in order to determine the gender of the isolated PGCs.
3. Freeze PGC lines in 2 freezing avian media.
4. Examine the overexpression and downregulation of CVH, DAZL, miR-302b-5p, and miR-92 in both male and female PGC cell lines before freezing.
5. Examine the overexpression and downregulation of CVH, DAZL, miR-302b-5P, and miR-92 in both male and female PGC cell lines after freezing with FAM1 and FAM2.

2. LITERATURE REVIEW

2.1 CHICK EMBRYO DEVELOPMENT

The infundibulum, magnum, isthmus, uterus, and vagina are the five functional areas of the avian oviduct. A mature chicken oocyte gets fertilized at the infundibulum after ovulation. Before oviposition, the fertilized oocyte passes through the magnum, which secretes egg white; the isthmus, which produces a soft shell; and the uterus, which produces a hard shell. The complete procedure takes about 25 hours. The first cleavage occurs about 5 hours after fertilization (Gipson, 1974), and the remaining of pre ovipositional development (20 hours) occurs in the uterus. The Hamburger and Hamilton (HH) and Eyal-Giladi and Kochav (EGK) staging systems have typically been used to characterize chick development (Fig. 2.1) (Kochav et al., 1980).

Eyal-Giladi and Kochav classified pre-streak development (HH stage 1) into 14 stages (EGK-I to -XIV) (Kochav et al., 1976; Kochav et al., 1980). The intrauterine phase includes EGK-I through -X. EGK-I to -VI are known as "cleavage" phases and require about 10 hours to complete. The phases EGKVII to -X are generally known as "formation of the area pellucida" (a.p. formation) and take another 10 hours. Early divisions are meroblastic, dividing only a portion of the egg. Cellularization and the development of subgerminal cavities begin between EGK-II and EGK-III. From EGK-III, the blastoderm becomes multilayered in the center, reaching to 5-6 cell layer thick at EGKVI, after which the number of cell layers in the area pellucida gradually decreases to 1-2 at EGK-X, with an upper continuous, one cell-layered epiblast and a lower non - continuous layer of hypoblast cells. The peripheral cells, which are precursors of the region opaca, continue to be multilayered and in direct contact with the underlying yolk cell.

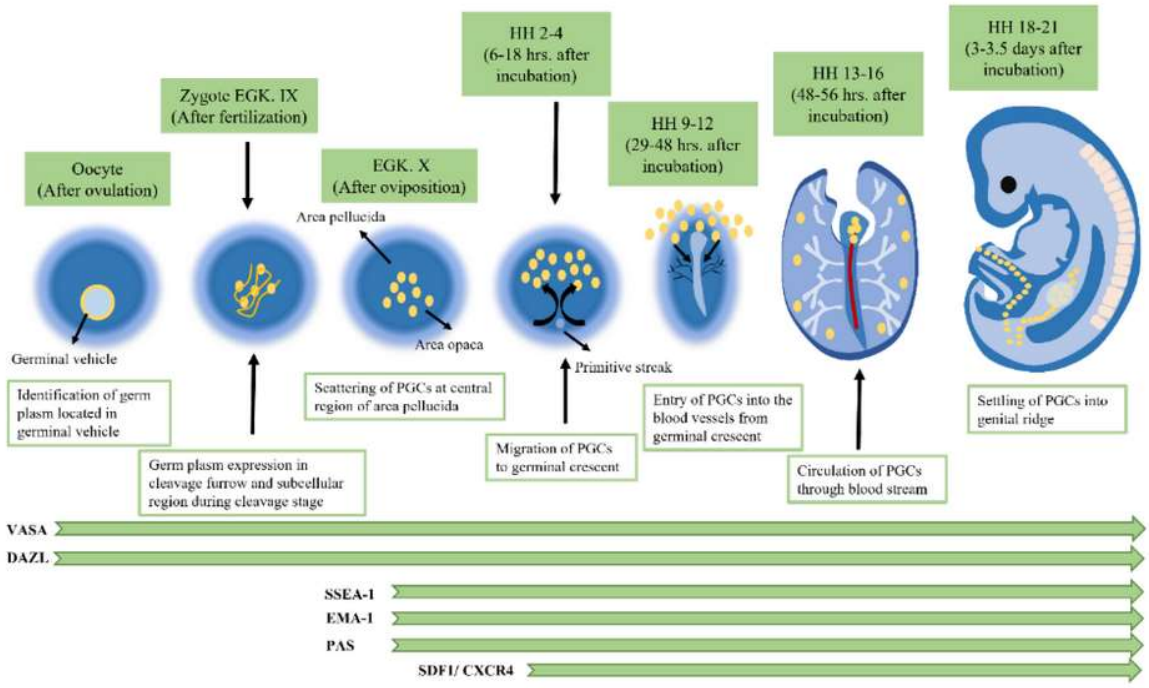

Figure 2.1 Schematic depiction of the development of PGCs and the expression of markers. VASA and DAZL germplasms are maternally inherited and expressed in a chicken oocyte's germinal vesicle (GV) and in the cleavage furrow and subcellular area after cleavage phases, indicating a predefined precursor PGC. PGCs expressing VASA and DAZL are found in the central zone of the zona pellucida at stage X (after oviposition). At Stage X, PGCs are positive for SSEA-1 and EMA-1 antibodies and PAS staining. After epiblast and hypoblast layer migration, they travel to the anterior marginal zone and are eventually localized in the germinal crescent. And germinal crescent PGCs reach extraembryonic blood vessels and circulate through the circulation. Lastly, PGCs attracted by SDF1 link with the chemokine receptor CXCR4 and enter the coelomic epithelium of the genitalia (Mathan et al., 2023).

2.1.1 The Cycle of Germ Cells

The germ line must be preserved if the whole genetic composition of an endangered species is to be preserved. Fertilization initiates the germ cell cycle. It is required to target germ cells between fertilization and the start of sexual differentiation for cryopreservation. The oviposition of a hard-shelled egg with a big yolky ovum surrounded by layers of albumen is a key feature of bird reproduction. Following ovulation, the ovum is trapped by the oviduct's infundibulum, where fertilization occurs. Fertilization in birds is polyspermic, with several sperm nuclei identified after vitelline membrane penetration. However, only one sperm will fertilize the egg. Following that, the fertilized egg enters the magnum, where it is encased in a firm albumen

capsule. Later, as it crosses the isthmus, the outer and inner shell membranes are put down in preparation for eggshell deposition. When the ovum enters the shell gland, the first cleavage division occurs. The egg spends the most time in the shell gland, spending 20 to 22 hours to form the eggshell. During eggshell production, fast cell division occurs, and the embryo acquires polarity, e.g., anterior/posterior, while remaining visually and radially symmetric. When an egg is laid, the disk-shaped embryo, or blastoderm, includes approximately 50,000 to 60,000 cells that lie on the surface of the yolk. At this stage, the blastoderm can be separated into a peripheral ring of cells linked to the yolk and a central, more translucent region, the area pellucida. The pellucida is floating above a non-yolky fluid that the embryo has developed. This structure is favourable because it allows for easy manipulation of the embryo. In general, the opaca area will only contribute to extra-embryonic structures. The area pellucida divides into two layers during incubation: an upper epiblast and a lower hypoblast. Only the epiblast gives rise to the embryo, but the hypoblast contributes to several extraembryonic tissues (Petitte, 2006).

Eyal-Giladi and Kochav (Kochav et al., 1976) classified the period of domestic hen development from fertilization to hypoblast formation into 14 stages, which are indicated by Roman numerals. Subsequent phases are labelled with Arabic numerals according to Hamburger and Hamilton's (Hamburger & Hamilton, 1951) staging scheme. Establishing the germ line is the next stage of embryo development, which is important for preserving poultry germplasm. PGC in the germinal crescent, an extraembryonic area. The germinal crescent is located in an anterior region generated during gastrulation as the endoderm displaces the hypoblast. The germinal crescent is identified based on the physical properties of PGCs (Swift, 1914). The germ cells must go from the germinal crescent to the growing gonadal ridge. The embryonic circulation transports the PGC to the germinal ridge as blood islands form and intra- and extraembryonic vasculature develops (Swift, 1914).

Following that, blood-borne PGC actively exits the vessels and moves to the germinal epithelium via the dorsal mesentery. Sexual differentiation begins when germ cells enter the primitive gonad. Like all other vertebrates, the avian germ line is formed in an extragonadal site early in embryonic development. Following that, during the first week of embryonic development, germ cells travel to the germinal ridge and proliferate. Male germ cells do not proliferate significantly until sexual maturity, when the process of spermatogenesis begins, leading to the development of mature sperm. Oocytes in females undergo meiotic arrest shortly

before or after hatching and stay inactive until sexual maturity when oocyte development and maturation occur. Following fertilization, the germ cell cycle is repeated (Petitte, 2006).

2.1.2 Development of germ cells in chickens

In chickens, the inherited mode determines the precursor cells of oocytes, known as PGCs (also referred to as the preformation mode). When the zygote is cleaved and cellularized, a collection of RNAs, proteins, and selected energy-rich mitochondria are stored in the germ plasm of mature oocytes and are solely allocated to prospective PGCs (Rengaraj & Han, 2022).

During chicken intrauterine embryonic development, PGCs are primarily found in the central area of the embryo after specification. PGCs polarize first and travel passively toward the anterior region by morphogenetic migration of the embryo during ovarian embryonic development. PGCs are absorbed into the semi-circular-shaped germinal crescent region in the anterior section. PGCs enter the lumina of blood vessels around embryonic day 2 and remain in blood circulation. Circulating PGCs enter the genital ridge (i.e., the eventual gonadal area) between embryonic days 3 and 3.5 (Fig.2.2) (Rengaraj & Han, 2022).

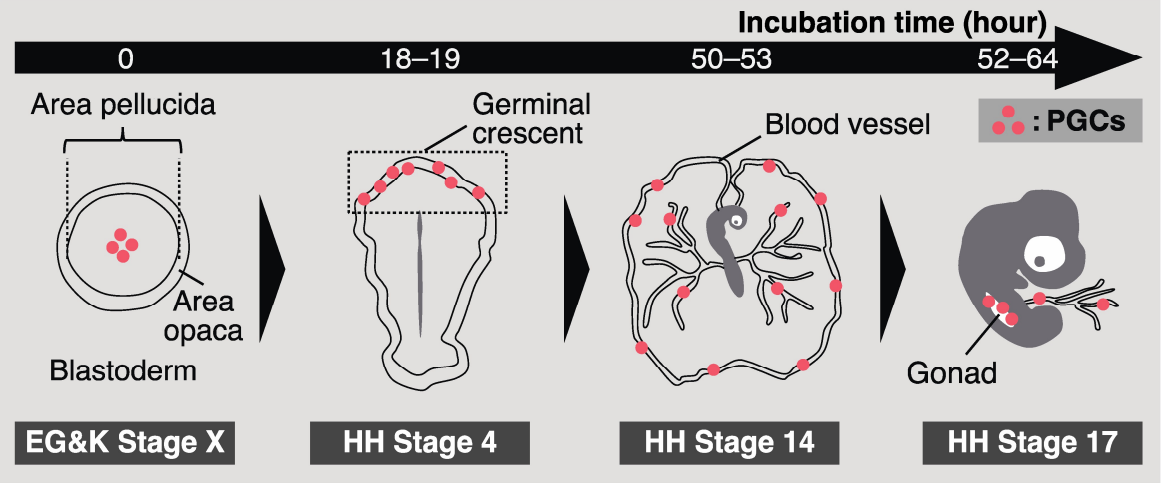

Figure 2.2 A diagram illustrating the development of chicken primordial germ cells (PGCs) (Ichikawa & Horiuchi, 2023).

PGCs are the precursor cells for mature gametes, which are the vehicles through which individuals pass genetic and epigenetic information to future generations (Kim & Han, 2018). PGCs were initially detected in the germinal crescent region of Hamburger and Hamilton (HH) stage 4-10 embryos after the establishment of the primitive streak and were assumed to come from the hypoblast based on their location. PGCs in the germinal crescent were identified based on physical criteria such as large cell size in comparison to surrounding somatic cells and a high

concentration of glycogen granules in the cytoplasm. Because PGCs contain a large number of glycogen granules, they can be stained with Periodic Acid Schiff (PAS) solution (Lee et al., 2016).

Further studies have focused at Eyal-Giladi and Kochav stage X, PGCs separate from the epiblast in the blastoderm, which consists of 40,000 to 60,000 undifferentiated embryonic cells, and translocate into the hypoblast area of the pellucida. They circulate through the vascular system and eventually settle in the gonadal anlagen during gastrulation. These cells continue to proliferate after the arrival of PGCs until they begin meiosis (Jin et al., 2017). Because no evidence of germ plasm or PGCs was identified during the cleavage stages, even in quail, chicken PGCs were widely assumed to emerge around EGK stage X in response to inductive signals. Except for Periodic Acid Schiff, no other marker was available to detect PGCs or germ plasm (PAS). Chicken PGCs could be traced back to the early stages of development after a chicken vasa homolog (CVH) was identified (Lee et al., 2016).

2.1.3 Importance of chicken PGCs

PGC storage in gene banks is very important in avian species because it has lately become the most practicable technique to preserve the genetic material found in the female W chromosome and mitochondrial DNA. Unlike mammals, male birds have homogametic ZZ chromosomes, whereas females have heterogamous ZW chromosomes (Lázár et al., 2021a).

Semen cryopreservation is the most practical way for managing genetic resources in birds ex-situ. However, only the male genome is conserved because only the female birds are heterogametic, having two different sex chromosomes (ZW). As a result, after semen cryopreservation, backcrossing would take one to six generations (depending on the genotype and necessity) to return to the desired genotype. Cryopreservation of embryos and oocytes would allow the W chromosome to be retained, but such approaches are not practical due to the megalecithal egg's features (Blesbois, 2007). Because of all these disadvantages of semen and embryo preservation, PGC storage could become the most important in avian species.

PGCs are potential tools in gene editing as well as genetic preservation, mainly in avian species. It has been demonstrated that the original breed could well be regenerated from primordial germ cells preserved in a gene bank. Male and female PGC lines from indigenous chicken breeds can be kept in a gene bank to conserve the gene and regenerate the original breed by donor-derived cryopreserved PGC injection into the recipient embryo (Fig.2.3) (Lázár et al., 2021a).

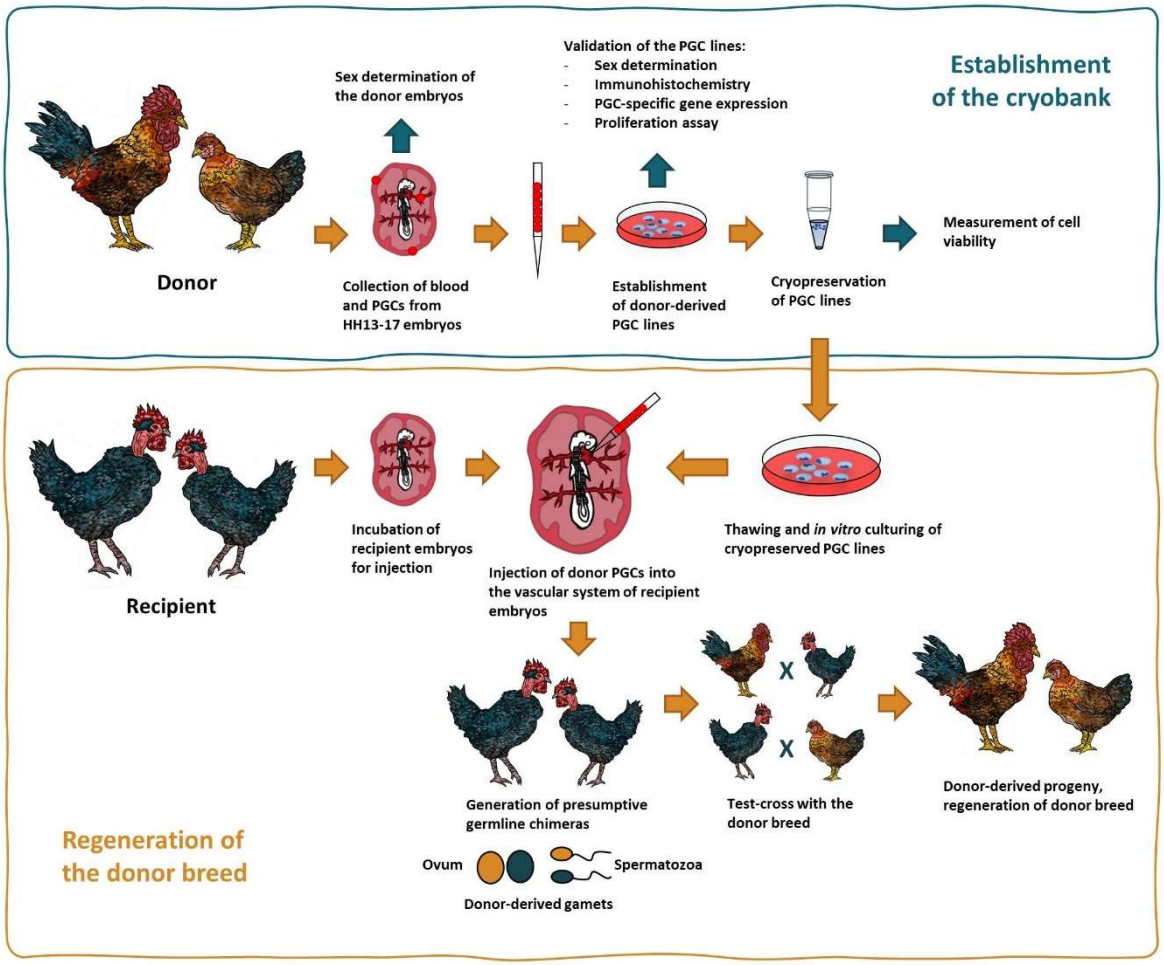

Figure 2.3 An overview of creating donor-derived hatchlings using cryopreserved indigenous PGC lines (Lázár et al., 2021a).

PGCs are the lineage-restricted cells that create sperm and eggs in the early embryonic stages of birds. According to Nandi and his colleagues, flocks of partially inbred, lower-fertility-restricted line chicken could be efficiently derived for cryopreservation. Cryopreserving a sufficient number of distinct lines of male and female PGCs could be used to recreate a poultry breed. The study demonstrated that germ-line transfer from these PGCs can be achieved using a commercial layer line of chickens as a surrogate host. PGCs that have been cryopreserved can be utilized to biobank specialized flocks of birds (Nandi et al., 2016a).

Avian primordial germ cells are important because of their ability to create functional gametes, and they are also bipotent (capable of developing into two types of cells) for either male or female gametogenesis. Avian PGCs are not sexually constrained in their ability to create functional gametes, which opens up new possibilities for cryopreservation of poultry and other bird species utilizing diploid stage germ cells. Males are the homogametic sex (ZZ) in birds, while females are the heterogametic sex (ZW) (Ballantyne et al., 2021).

2.2 GERMLINE MARKER IN ANIMALS (VASA GENE/SPECIFIC PROTEIN)

2.2.1 VASA'S ROLE IN GERM LINE DEVELOPMENT

Vasa homologs are common in animals, and vasa has emerged as the gene of choice for identifying germ cells. Vasa is a DEAD box helicase that is found in the germline of Drosophila at all stages of development (Renault, 2012). Drosophila genetic screens for maternal-effect genes first showed the vasa function in oocyte formation. Vasa function in posterior patterning and germ cell specification in the embryo was discovered through mutation and gene inactivation investigations (Gustafson & Wessel, 2010).

In chickens, the vasa homologue gene has been shown to have germline-specific expression during development. Considering vasa is one of the very few germline-specific markers in chickens, the majority of the study on chicken PGCs has concentrated on the vasa gene (Nakamura et al., 2013).

2.2.2 MECHANISMS OF GERMLINE SPECIFICATION

PGCs travel to the somatically generated gonads and proliferate to become germ line stem cells capable of self-renewal and differentiation into gametes. Within the animal kingdom, at least three general processes are utilized to specify PGCs. In flies and nematodes, the germ line can form early in embryogenesis as a result of the inheritance of maternal components (maternally derived, also known as preformation). Preformation refers to the acquisition of germ cell fate via localized, inherited cytoplasmic determinants that are subsequently both required and sufficient to confer germ cell fate on the cell containing them. The molecules that make up these determinants are both mRNA and protein products of genes that are found in all metazoans. Dipterans and nematodes are well-known and long-standing examples of animals that display this manner of PGC determination (C. G. M. Extavour, 2007). The second way is through cell-cell interactions early in embryogenesis (inductive, also known as epigenetic), as demonstrated in mice (Rosner et al., 2009). The third is at any stage of the animal's life, even adulthood, from a multipotent stem cell precursor (permanent multipotent cell-derived germ cells), as seen in planaria and (C. G. Extavour & Akam, 2003).

Thus, the integrity of PGC specification and development is intimately connected to sexually reproducing animals' survival and fitness. PGC differentiation can be divided into two categories: induction and preformation (C. G. Extavour & Akam, 2003). However, Tsunekawa reported that, in chickens, PGCs are thought to originate through the preformation mode via maternally inherited determinants such as CVH (Tsunekawa et al., 2000).

2.3 MOLECULAR MARKERS EXPRESSED BY CHICKEN PGCS (IMPORTANT MARKERS IN PGCS)

2.3.1 Expression of marker genes in chicken germ cells

Several marker genes are expressed particularly in avian germ cells during distinct developmental stages to maintain germ cell characteristics. Meiosis-related genes, Pluripotency-related genes, methylation-related genes, migration-related genes, and germline-related genes are such markers. PGCs can thus express specific pluripotency-related genes such as the "POU domain class 5 transcription factor 1" (POUV), "Nanog homeobox" (NANOG), and "SRY (sex determining region Y)-box 2" (SOX2). PGCs express these genes until they settle in the gonads, implying that they are essential for the pluripotency and self-capacity of PGCs until they develop into germ cells. Germline-related gene expression can be observed in germ cells at all stages of development (Lavial et al., 2007).

In avian species, several germline-related genes have been identified, including the "chicken vasa homolog" (CVH), "deleted in azoospermia-like" (DAZL), and "chicken dead end homolog" (CDH) genes. An anti-CVH antibody was used in immunohistochemistry to detect CVH-positive cells from fertilization to the formation of the adult testis or ovary, indicating that CVH is essential for the normal development and maintenance of germline cells (Tsunekawa et al., 2000).

2.3.1.1 *Chicken vasa homolog gene (CVH)*

The majority of research on chicken PGCs has concentrated on the vasa gene because vasa is among the few germline-specific markers in chickens (Nakamura et al., 2013).

Tsunekawa et al., (2000) identified the vasa homologue gene in chickens in order to establish a valid molecular probe for tracing the origin of germ cell lineages and demonstrating germline-specific expression of CVH protein throughout all stages of development. CVH protein was found in the cytoplasm of germ cells extending from presumptive primordial germ cells (PGCs) in uterine-stage embryos through spermatids and oocytes in adult gonads, according to immunohistochemical investigations utilizing specific antibodies produced against CVH protein. CVH protein was restricted to the basal region of the cleavage furrow during the early cleavages. At stage X, around 30 CVH-expressing cells were discovered in the central zone of the region pellucida; later, 45–60 cells were discovered in the hypoblast layer; and finally, 200–250 positive cells were discovered anteriorly in the germinal crescent due to morphogenetic migration. Furthermore, CVH protein was found in granulofibrillar structures surrounding the

mitochondrial cloud and in a spectrin protein-enriched structure in the oocytes. The CVH-containing cytoplasmic structure is the chicken's precursory germ plasm.

A maternally transmitted factor can specify chicken germ cells (preformation mode). Chicken PGCs could be traced back to the early stages of development after a chicken vasa homolog (CVH) was identified. In developing oocytes, CVH protein co-localized with spectrin and mitochondrial clouds, indicating the presence of germplasm in chickens (Tsunekawa et al., 2000).

PGCs undergo a series of developmental events in early embryogenesis, such as specification, migration, and differentiation, to form a new organism in the next generation. Certain genes are efficiently suppressed at the transcriptional level, whereas other genes are exclusively expressed to maintain the levels of germline-expressed gene products. In comparison to somatic cells, PGCs have distinct characteristics and use multiple distinct molecular processes to maintain pluripotency and stemness. They modulate tissue-specific cis- and trans-regulatory elements to express germ cell-specific RNA binding proteins (RBPs). CVH is a chicken RNA-binding protein that is engaged in the temporal and spatial regulation of the molecular pathways that regulate germ cell fate (Jin et al., 2017).

2.3.1.2 *Deleted in* AZOOSPERMIA *Like gene (DAZL)*

The Deleted in Azoospermia-Like (DAZL) protein, coded by the Dazl gene, is a germline-specific RNA-binding protein required for vertebrate gametogenesis, and the chicken Dazl gene has also been found in PGCs. cDAZL was found in the nucleus and cytoplasm of circulating PGCs using fluorescent immunohistochemistry (Kito et al., 2010).

A previous study in chickens demonstrated germ cell-specific expression of chicken DAZL from embryonic to adult stages. DAZL is a functional gene that encodes an RNA-binding protein that is expressed in germline cells. Its roles in germ cells have been widely investigated in vertebrate species, and these include meiotic progression and germ cell pluripotency. According to the analysis of the expression of the chicken DAZL gene during germ cell specification, the origin of PGCs, and germplasm dynamics, PGCs are produced in the middle of oocytes due to the asymmetric localization of germ plasm from preovulatory follicle stages. The DAZL expression pattern varies from subcellular localization to diffuse before and after zygotic genome activation during cleavage phases. PGCs are transcriptionally active during the specification process. The knockdown studies of cDAZL that result in decreased proliferation, abnormal gene expression patterns, and PGC death in vitro show that

it may function in chickens' PGC development. DAZL expression in chickens reveals the formation and early positioning of PGCs (Lee et al., 2016).

2.3.1.3 *Glyceraldehyde 3 – phosphate dehydrogenase (GAPDH)*

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a housekeeping gene. GAPDH is commonly utilized as an internal control in protein, mRNA, and DNA assays(Zhang et al., 2015). Quantitative real-time PCR (qPCR) measures gene expression levels in real time under a variety of experimental circumstances. Selecting appropriate reference genes is critical for effective normalization and correct data interpretation. The GAPDH gene is often used as a reference gene for RT-qPCR analysis. (Olias et al., 2014). For quantitative gene expression investigations, GAPDH is a valid reference gene. GAPDH is commonly utilized as an endogenous control for quantitative RT-PCR analysis since its expression is constant across time points and experimental manipulations. The messenger RNA (mRNA) expression levels of reference genes are consistent among cells and under diverse research conditions (Zainuddin et al., 2010).

2.4 MICRORNA

MicroRNAs (miRNAs) are small noncoding RNAs, about 22 nucleotides long, that target mRNAs for translational repression or degradation. MiRNAs target mRNAs by base-pairing with the 3' untranslated regions (3' UTRs). The MiRNAs are present in a variety of species, including both plants and animals (Hicks et al., 2008).

2.4.1 BIOGENESIS OF MIRNAS

MicroRNAs are non-coding RNAs of about 20–25 nucleotides that bind to target mRNAs' 3′ UTR site in an imperfect match to suppress translation and stability. MiRNAs are produced in animals by miRNA genes found in intergenic regions or introns of protein-coding genes. This biogenesis process is a canonical pathway of miRNA processing (Fig. 2.4). RNA polymerase II transcribes non-protein-coding miRNA genes to produce primary miRNAs (pri-miRNAs). MiRNAs are derived from primary miRNAs (pri-miRNAs), and are processed in the nucleus with the help of RNase III Drosha to form 70-nucleotide precursor miRNAs (pre-miRNAs) with a stem-loop structure. The Microprocessor protein complex, which includes the DGCR8 (also known as Pasha) proteins, processes primary miRNAs into intermediate forms (pre-miRNAs). The shuttle protein Exportin-5 transports pre-miRNAs from the nucleus to the cytoplasm with the help of the G-protein Ran. Pre-miRNAs are detected and cleaved in the cytoplasm by the RNase III Dicer to generate approximately 22 nucleotide double-stranded

RNAs. When the double strands unwind, one strand enters the RNA-induced silencing complex (RISC), maturing into a mature miRNA capable of gene silencing (Gangaraju & Lin, 2009). Non-canonical miRNA biogenesis (Fig.2.5) arises independently of Drosha/DGCR8 and dicers. Introns can also generate guide miRNAs (e.g., Mirtrons). Mirtrons are produced from the introns of mRNA during splicing (Okamura et al., 2007; Ruby et al., 2007), as well as other additional small RNAs like endo-siRNA, shRNAs, tRNase Z, snoRNAs, and tRNAs (Ender et al., 2008; Miyoshi et al., 2010). Moreover, short hairpin RNAs generated in a Dicer-independent mechanism can be loaded directly into AGO2, where the process of miRNA maturation happens (Yang et al., 2010; Cheloufi et al., 2010).

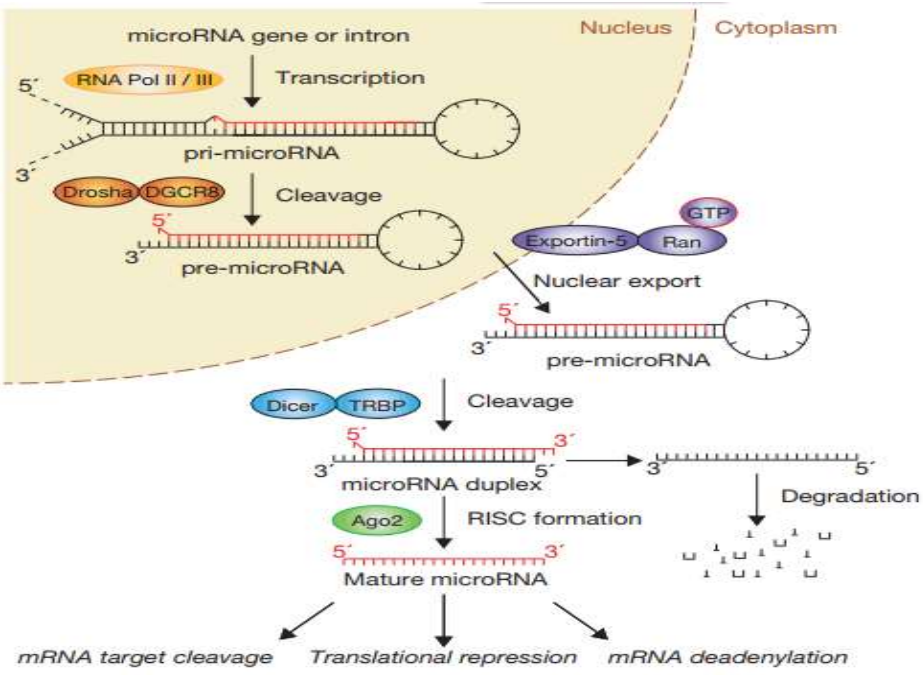

Figure 2. 4 Canonical pathway of miRNA processing (Khawar et al., 2017)**.**

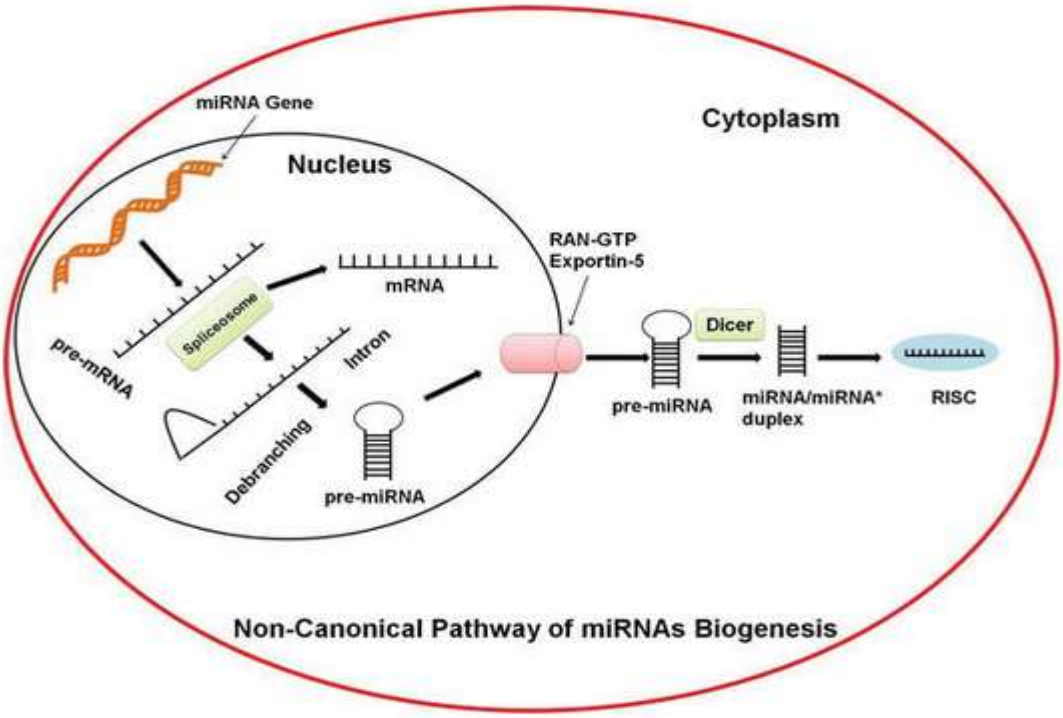

Figure 2. 5 Non-canonical pathway of miRNAs biogenesis (Khawar et al., 2017).

2.4.2 Mechanism of miRNA action

There are three ways that miRNA regulates the target mRNA, depending on their degree of complementarity (Fig.2.5). Partial complementarity inhibits translation, while entire or nearly perfect matching cleaves target mRNA. Since animal miRNAs are frequently poorly complementary to their target sites, the much more likely method miRNAs suppress mRNA transcription in animal cells is by translational repression (Lee et al., 1993).

Plant miRNAs generally have perfect or almost perfect complementarity to their target sites. As a result, cleavage of target mRNAs is the primary effect of miRNA-mRNA interaction in plants. Despite imperfect complementarity, several animal miRNAs can trigger cleavage of their target mRNAs. The third way miRNA regulates mRNA expression is by mRNA deadenylation. When miRNA binds to an imperfectly complementary target site on mRNA, the deadenylation of the poly (A) tail increases. Although complementarity between a miRNA and its target mRNA does not need to be perfect, nucleotides 2–8 from the miRNA's 5' end, often known as the seed sequence, must be precisely complementary to the target mRNA (Wang et al., 2013).

2.4.3 The function of miRNA in stem cells

The potential of stem cells to self-renew and produce a large number of differentiated cells distinguishes them. This kind of characteristic is governed by dynamic interactions between

extrinsic signalling, epigenetic, transcriptional, and post-transcriptional regulatory mechanisms. MicroRNAs (miRNAs) play a key role in stem cell self-renewal and differentiation by inhibiting the translation of specific mRNAs in stem cells and differentiating daughter cells. The role of miRNAs has been shown in embryonic stem cells, germline stem cells, and other somatic tissue stem cells. These findings shed light on a new aspect of gene regulation in the control of stem cell fate and behaviour.(Gangaraju & Lin, 2009).

The miR-302 cluster is evolutionarily conserved and is localized on chromosome 4 (Barroso-deljesus et al., 2008). Several miRNA studies have been conducted to describe the most critical miRNAs responsible for regulating the pluripotency and self-renewal of PGCs. The evolutionary conserved and vertebrate-specific miRNA cluster (Table 2.1), gga-miR-302, is stem cell-specific and plays a crucial role in cell cycle control (Lázár et al., 2018).

Table 2.1. miRNA cluster, gga-miR-302 in chicken PGCs

gga-miR-302 cluster of miRNA
miR-367
miR-302d
miR-302a
miR-302c-3p
miR-302b-3p
miR-302c-5p
miR-302b-5p

(Lázár et al., 2018).

2.4.4 MIR- 302 IN CHICKEN

The miR-302 family, which is found in the gene La-related protein 7 (LARP7), was discovered to be generated particularly in undifferentiated human embryonic stem cells (hESCs) (Chen et al., 2019).

Cell-specific miRNAs in embryonic stem cells, particularly miR-302, can enhance the G1 to S transition and repair the proliferation deficit. miR-302 has the ability to target both positive and negative cell cycle-related genes, thereby increasing the number of cells in the S phase and forming an ESC-like cell cycle (Li et al., 2016).

MicroRNAs from the miR-302 family are key players in the regulation of cell proliferation. By suppressing gga-miR-302b-5P (5P) and gga-miR-302b-3P (3P), the relevance of gga-miR-302b

on chicken PGC proliferation was discovered. It has been demonstrated that inhibiting gga-miR-302b-5P and gga-miR-302b-3P reduces PGC proliferation(Lázár et al., 2022).

2.5 CHICKEN GERM CELL IDENTIFICATION

PGCs are distinguished by their remarkably large size, large spherical nuclei, and refractive cytoplasmic lipids, which are combined with a histochemical marker such as periodic acid-Schiff (PAS), which stains glycogen, or immunohistochemical staining of cell surface antigens like embryonic mouse antigen-1 (EMA-1) and stage-specific embryonic antigen-1 (SSEA-1). Periodic acid-Schiff staining and immunohistochemical staining of such cell surface antigens, however, are insufficiently specific to evaluate germline development in chickens. Because periodic acid-Schiff staining efficiently detects primordial germ cells after stage 4 of Hamburger & Hamilton (HH), and EMA-1 and SSEA-1 are expressed not only by PGCs but also by undifferentiated cells such as chicken embryonic stem cells (Nakamura et al., 2013).

Stem cell markers are genes that produce a protein that is used to identify undifferentiated stem cells. Following primary cultivation, PGCs can be characterized using marker reagents. PGCs express immunological markers such as stage-specific embryonic antigens (SSEA-1, SSEA-3, and SSEA-4), epithelial membrane antigen (EMA-1), integrin alpha-6, and integrin beta-1. Antibodies can identify markers on chicken PGCs. The cytochemical reagents employed in the study comprised periodic acid-Schiff (PAS) stain, antibodies to stage-specific embryonic antigens (SSEA-1, SSEA-3, and SSEA-4), antibodies to epithelial membrane antigen (EMA)-1, and antibodies to integrins alpha 6 and beta1. The cell-surface glycoprotein marker SSEA-1 is frequently used to trace and identify avian PGCs at various phases of embryonic development. (Jung et al., 2005).

SSEA-1 immunostaining and the RT-PCR amplification of the pluripotency markers (cPOUV and cNANOG) and PGC stem cell markers (cDAZL and CVH) indicate the cultured PGCs' stem-cell character (Altgilbers et al., 2021).

2.5.1 Reverse transcription polymerase chain reaction (RT-PCR)

The most common approach for validating gene expression and comparing mRNA levels in different samples is reverse transcription polymerase chain reaction (RT-PCR). Moreover, real-time RT-PCR is a sensitive and accurate method for quantifying mRNA. To assess gene expression, the transcript must first be reverse-transcribed to cDNA. Primers used in reverse

transcription (RT) experiments for cDNA synthesis are either oligo (dT), random oligonucleotides, or sequence specific oligonucleotides (Adrover et al., 2010).

2.5.2 Real – time quantitative PCR (RT-qPCR)

Thermal cyclers designed for qPCR applications include a fluorometer to detect fluorescence. As the heat cycler runs, the fluorometer detects the fluorescence and provides readings throughout the PCR amplification process. As a result, quantitative PCR is often referred to as "real-time PCR" (RT-PCR). Nucleic acid quantification using real-time quantitative PCR is sensitive, specific, and reproducible. Real-time quantitative PCR has transformed the field of molecular diagnostics since its introduction, and the method is now being applied in a large range of applications. One of the most important advantages of qPCR is its capacity to assess gene expression. Gene expression, also known as mRNA synthesis, is an essential step in protein synthesis. For molecular biologists, gene expression is an active area of study that contributes to the understanding of several biological pathways and disorders. The researcher uses reverse transcriptase to make complementary DNA (cDNA) copies of the RNA in their sample, and then uses that cDNA to perform qPCR. This is known as a reverse transcription polymerase chain reaction. The type of data provided by a qPCR run is determined by the primers and dyes used (Arya et al., 2005).

2.5.2.1 *TaqMan® assay*

A fluorogenic, nonextendable "TaqMan" probe is used in the real-time quantitative TaqMan® assay. A fluorescent reporter dye is linked to the probe's 5′ end, while a quencher dye is attached to its 3′ end. If the target sequence is present, the fluorogenic probe anneals downstream to one of the primer regions and is cut by the Taq polymerase enzyme's 5′ nuclease activity during the PCR extension phase. Fluorescence resonance energy transfer (FRET) occurs while the probe remains intact, and the reporter dye's fluorescence emission is absorbed by the quenching dye. Taq polymerase cleavage of the probe during PCR separates the reporter and quencher dyes, enhancing the fluorescence from the former. Furthermore, cleavage separates the probe from the target strand, allows primer extension to continue to the end of the template strand without interfering with the exponential accumulation of PCR product. With each cycle, additional reporter dye molecules are cut from their respective probes, resulting in a rise in fluorescence intensity proportional to the amount of amplicon produced (Arya et al., 2005).

A redesigned thermocycler can detect the rise in fluorescence emission during the PCR reaction in real-time. With the fluorescence emission data signal obtained during PCR amplification, the computer software generates amplification curves. The number of cycles needed for the fluorescent signal to cross the threshold is defined as the Ct (cycle threshold) (i.e., exceeds the background level). The sample's quantity of target nucleic acid has an inverse relationship with Ct values. The lower the Ct level, the more target nucleic acid is in the sample. The presence of more templates at the beginning of the reaction results in fewer cycles reaching the point where the fluorescent signal is statistically significant above the background (Arya et al., 2005).

2.6 PGC CULTURE AND CRYOPRESERVATION

2.6.1 SOURCES OF CELLS FOR CRYOPRESERVATION

Much refining in all parts of the preceding methods is required to fully exploit the potential for preserving avian genetic diversity using blastodermal cells, ESC, or PGC. Currently, the best strategy is to use blastodermal cells or PGC. However, if PGC could be cultivated to increase the quantity of germ cells for cryopreservation, it would be a significant step in preserving the remaining poultry stocks, which are constantly threatened with extinction.

Primordial germ cells: the germinal crescent, germ cells circulating in embryonic blood, and germ cells extracted upon arrival in the primitive gonad have all been used to create germ line chimeras. PGC from the germinal crescent could be transferred into recipient embryo blood vessels and settle in the host gonad. The germinal crescent PGC of quail could be exploited to create germ-line chimeras. However, due to the low quantity of germ cells during germ cell development (Stages 4 to 8), the germinal crescent is the least useful source of PGC for cryopreservation. PGC from circulating chicks could be used as donors and move to the gonad. Following the transfer of blood PGC to recipients at the same stage of development, germ line chimeras successfully hatched. Germ line chimeras can also be generated via gonadal PGC. PGC derived from the gonad of 5- to 7-day-old embryos is currently accepted to actively move to the gonadal ridge in embryos throughout the time of blood circulation, i.e., Stages 14 to 17 (Petitte, 2006).

Blastodermal cells: Freshly separated cells from the Stage X embryo can be used to create germ line chimeras. Injecting stage X blastodermal cells into the subgerminal cavity of unincubated embryos resulted in a somatic and germ cell chimera rooster. Despite the fact that germ line transmission efficiency was only approximately 0.3%, this stimulated a significant

amount of research into the production of chimeras utilizing fresh blastodermal cells (Petitte, 2006).

Embryonic stem cells: Avian ESC has been cultivated successfully from Stage X embryos (Zhu et al., 2005). Embryonic stem cells (ESC) are pluripotent cells that can give rise to any form of tissue, including the germ line. Avian embryonic stem cells (ESC) can be cultivated, frozen, and used to create somatic and germ-line chimeras. Embryonic stem cells can even be used to create high-grade somatic chimeras. However, the limited efficiency of germ line transmission is the principal hurdle to their use for banking avian germplasm. After culture for more than a few weeks, the capacity of the cells to give rise to the germ line is lost; this property alone makes avian ESC inappropriate (Petitte, 2006).

2.6.2 PGC Culture

According to the first research by Van De Lavoir and colleagues, PGCs can be cultured in vitro indefinitely and genetically altered while remaining committed to the germ line. To culture PGCs, blood from stage 14–17 HH chicken embryos can be collected and cultured in knockout (KO)–DMEM medium (Van De Lavoir et al., 2006).

Previous research has shown that the precise self-renewal of the germ cell lineage is crucial to fertility and reproductive success. By using the modified basal medium (avian KO-DMEM) comprising Activin A, chicken serum (FAIcs medium), FGF2, and IGF-1, both male and female PGC cell lines may possibly be derived from the chicken embryo by taking a blood sample, and PGCs could be propagated and proliferated indefinitely in suspension devoid of feeder cells (Whyte et al., 2015).

PGCs can be extracted and cultured to show that germline transmission is possible when reconstituting a poultry breed. According to Nandi and his colleagues, fertile eggs were incubated for 2.5 days (stage 16 Hamburger & Hamilton (HH)), and 1 µL of blood was extracted and transferred to culture media from the dorsal aorta. For two to three weeks, the blood sample was cultured in suspension. During this time, the blood cells in the sample were lysed, and the PGCs in the well grew as single, distributed cells. After three weeks, cultures containing more than 100,000 cells were scored as a positive cell line derivation, and these cells were cultivated for another week in increasing volume (100,000 cells per 0.5 mL media) and then frozen in aliquots of 50,000 to 100,000 cells per vial (Nandi et al., 2016b).

In vitro propagation of PGCs, on the other hand, is technically challenging and costly, requiring a complicated cell culture media as well as specialized cell culture equipment. PGC in vitro propagation results in epigenetic alterations and reduced germline transmission as the in vitro culture period increases. PGCs cultured for a shorter period of the time competed better for the niche of stem cells and produced more offspring. Because diploid PGCs have the potential to be utilized for the cryopreservation of avian species, cryopreserved PGC was used for the cryopreservation of chicken breeds to overcome the limitations. Thus, for poultry conservation, a biobanking technology that does not depend on germ cell culture would indeed be desirable. PGCs obtained from several commercial chicken species could be maintained for several weeks prior to cryopreservation, and then when thawed, they migrate to the developing gonads of a surrogate host embryo and contribute to their offspring's genome (Woodcock et al., 2019).

2.6.3 Cryopreservation and thawing of PGCs

Most cryopreservation procedures for stem cells use 10% (v/v) DMSO, frequently combined with 10% to 90% (v/v) FBS. DMSO is a standard cryoprotectant to stabilize cell proteins and membranes and prevent intracellular ice formation. FBS is frequently added to freezing solutions because of its ability to maintain the cell membrane and adjust cell osmotic pressure (Di Bella et al., 2021). The freezing of chicken PGCs in a serum-containing medium supplemented with 10% DMSO as a cryoprotectant is the most extensively utilized freezing protocol (Nakamura, 2016). In the cryopreservation of chicken PGCs in a 5 % DMSO and 1 % chicken serum solution, the chicken serum improves the survival and recovery rates of post-thawing PGCs (Hamai et al., 2023). Previous research has shown the cryopreservation and thawing of PGCs. PGCs can be cryopreserved in a solution comprising 10% dimethylsulfoxide (DMSO) (Gibco) and 90% fetal bovine serum (FBS) (Gibco), utilizing a cell number of 2×10^6 – 1×10^7 cells in each cryogenic vial put in a freezing container and frozen overnight in a -80°C freezer (Kong et al., 2018).

3 MATERIAL AND METHODS

3.1 WORKFLOW OF THE EXPERIMENT

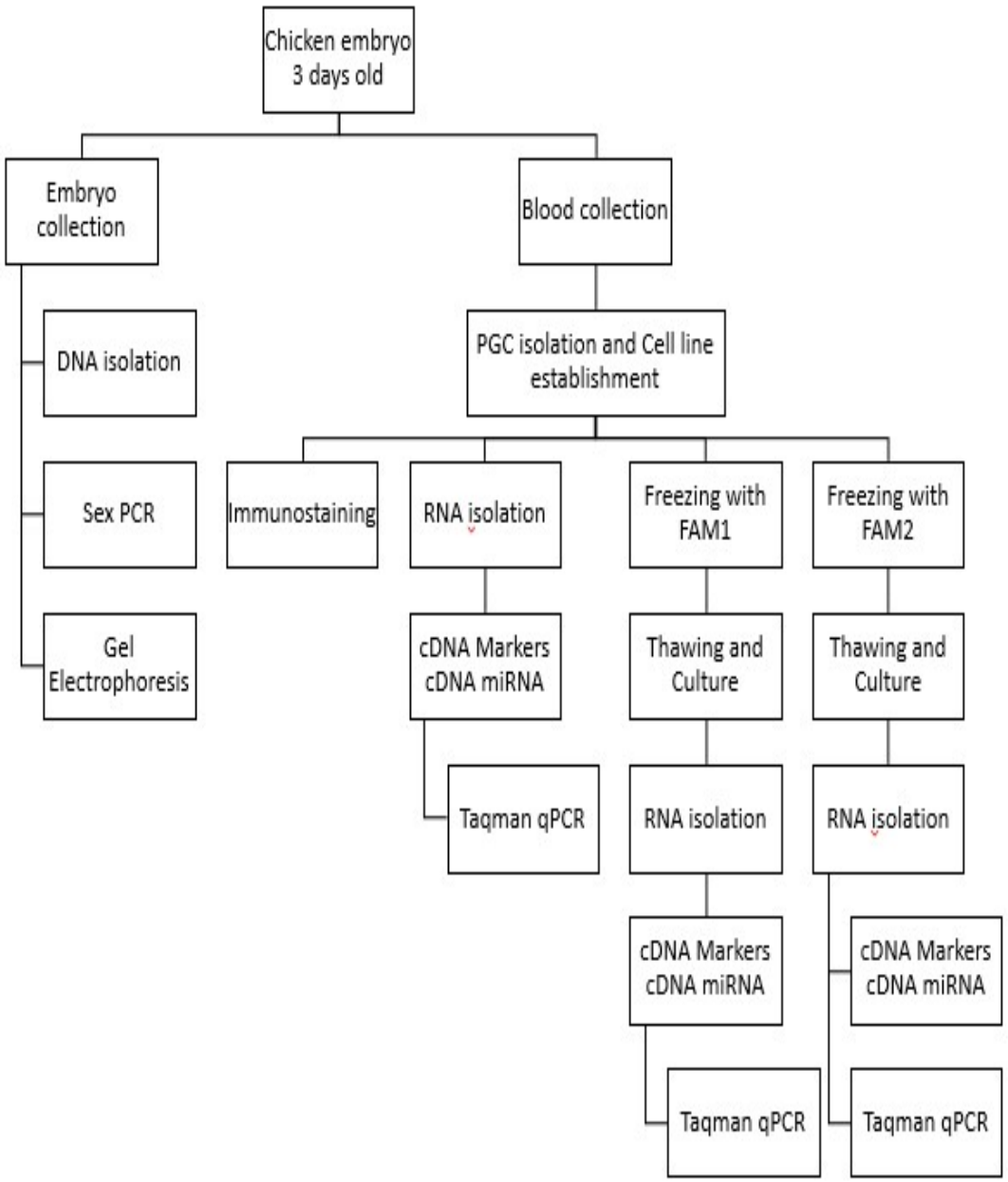

Figure 3.1 workflow of the experiment before freezing

3.2 CHICKEN BREEDS, PERMISSIONS

The research experiment was conducted at MATE, GBI Animal Biotechnology Department, Applied Embryology and Stem Cell Biology Group. The department provided all research materials, including eggs, kit protocols, and all biotechnological laboratory equipment throughout the research.

Following the Hungarian Animal Protection Law (1998. XXVIII), the animals were provided. The National Food Chain Safety Office, Animal Health and Animal Welfare Directorate (Budapest, Hungary) granted experimental animal research at the National Centre for Biodiversity and Gene Conservation, Institute for Farm Animal Gene Conservation (Gödöllo, Hungary). The National Centre for Biodiversity and Gene Conservation, Institute for Farm Animal Gene Conservation (Gödöllo, Hungary) donated fertilized eggs from White Hungarian chickens.

3.3 PGC ISOLATION FROM CIRCULATING BLOOD OF CHICKEN EMBRYO

The white Hungarian chicken eggs were incubated at 38°C with 60-80% humidity. The eggs were removed from the incubator on the third day of incubation and poked to pour over into a petri dish. Before cracking the eggshell, the eggs from the incubator were placed horizontally on the egg plate and wiped with sanitized tissues soaked in alcohol (70% ethanol).

The embryos were gently placed on a petri dish after they had been cracked. The chicken PGCs were isolated from 3-day-old embryos between the 14-17 HH (Hamburger & Hamilton, 1951) stages. A sterile glass microcapillary and a mouth-controlled pipette were used to collect 1 to 2 µl blood from the dorsal aorta under a stereomicroscope. The isolated chicken PGCs were then transferred to a medium-containing 48-well plate containing 300 μl of medium without feeder cells (Fig.3.3).

The isolated blood was added to the selective PGC culture medium developed by McGrew and colleagues (Whyte et al., 2015) to completely remove the blood cells while supporting the growth and division of PGCs. After one to two weeks, the red blood cells vanished, leaving only the chicken PGCs to be seen. Half of the medium was substituted in every two days. By the end of the third week, a PGC line is considered successful if the cell number has reached 1×10^5 (Whyte et al., 2015).

Following the successful establishment, the cells were divided into two wells and propagated at 2- 4.0 x 10^5 cells/ml medium in a 24-well plate. The PGCs were harvested for RNA isolation and further study when the cell number achieved confluence.

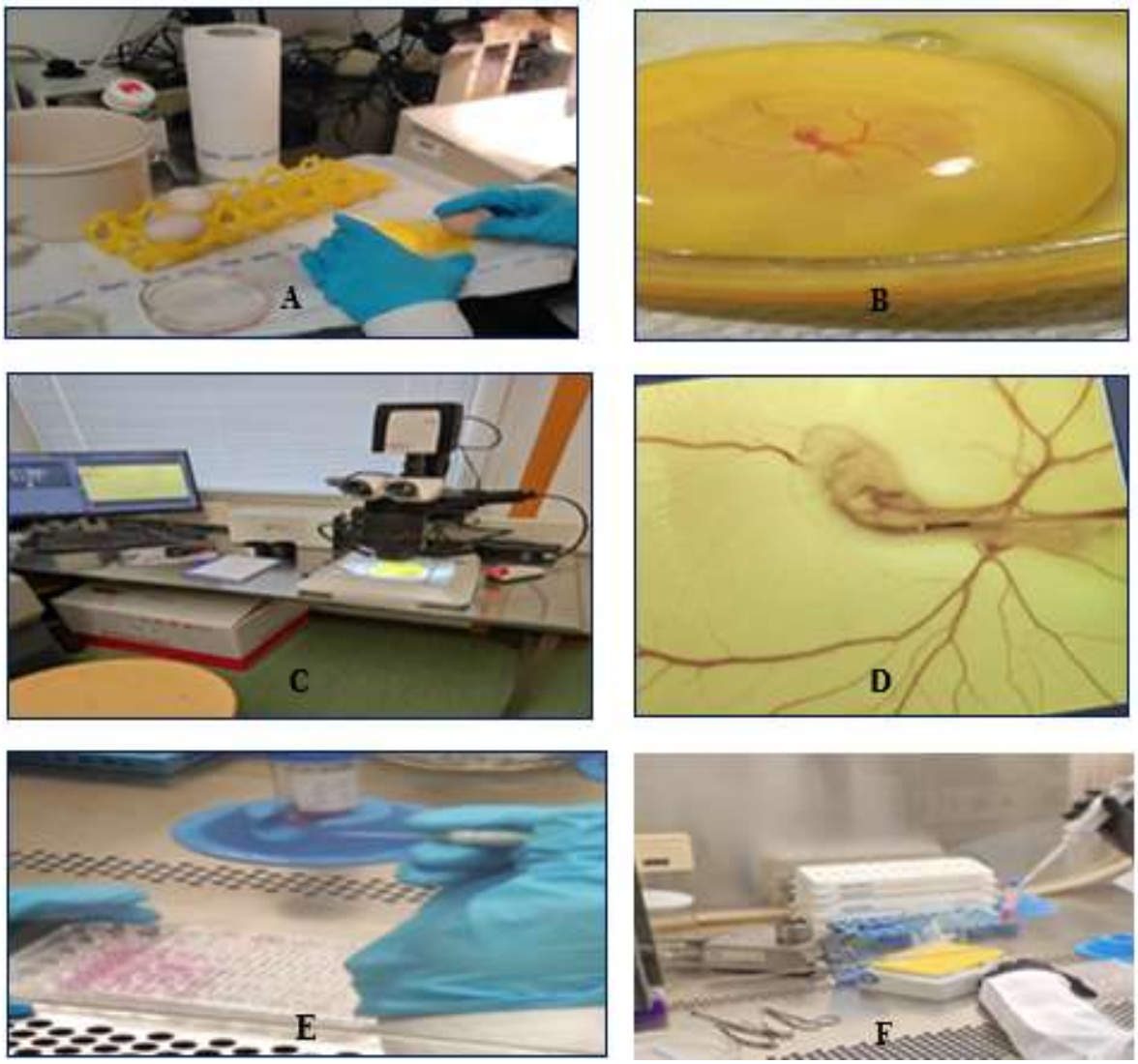

Figure 3.2 shows the isolation of PGCs from a chicken embryo (own pictures). A) 3-day-old embryo. B) 16 HH chicken embryo stage. C) Embryo under the stereomicroscope. D) Blood isolation from the dorsal aorta. E) PGC culture medium. F) Medium change.

3.4 DNA ISOLATION FROM TISSUE

The tissue samples from the embryo were digested using a 0.1 % Proteinase K Lysis Buffer solution and incubated at 56 °C for 3 hours. After the incubation, the tubes were vortexed and centrifuged for 1 minute at 13000 rpm, and then the Proteinase-K was inactivated at 100 °C for 15 min. After the centrifugation (13000 for 5 minutes), 50 µl phenol and 50 µl chloroform were added to the tube under sterile conditions. The samples were thoroughly shaken and put into a high-standard centrifuge. The tubes were centrifuged at 4 °C for 10 minutes at 13000 rpm. After centrifugation, the supernatant was removed and placed in another tube. 100 µl of Isopropanol (stored at -20 °C) was added to the tube and mixed thoroughly. Then 0.75 µl GlycoBlue stain (stored at -20 °C) was added to the samples and incubated for 30 minutes at -

20 °C. Right after incubation, the tube was centrifuged at 13000 rpm for 5 minutes at 4 °C. After the blue-coloured pellet (DNA) was formed, the supernatant was thoroughly removed, and 500 µl (75% ethanol) was added. The samples were suspended slightly without breaking the DNA strands and then centrifuged at 13000 rpm for 5 minutes at 4 °C. After centrifugation, the supernatants were removed thoroughly with a pipette, and the pellets were dried up under the sterile chamber for 5 minutes (until the ethanol smell vanished). 30 µl nuclease-free water was added, and the samples were put into the preheated water bath for 5 minutes to dissolve the DNA. Before the storage of the DNA samples, the concentration of the samples was measured by a Nanodrop machine. The samples were stored at -20 °C.

3.5 SEX PCR

The following method was used for DNA amplification during PCR (Table 3.2). PCR Mix was prepared to measure 13 µl reaction mix into each tube. The content of the reaction mix for one sample is detailed in the below table (Table 3.1).

Table 3.1 Preparation of a PCR mix for sex determination

1x PCR mix preparation	
Components	Amount (µl)
MyTaq Ready Mix	6.75
Forward Primer (CHD1)	0.5
Reverse Primer (CHD1)	0.5
Nuclease Free Water	4.25
DNA sample (25 ng/µl).	1.0
Total	**13**

The evaluation requires two sorts of controls: positive and negative. Positive controls included samples of male and female tissues, whereas negative controls included water. The values were multiplied by the number of samples and controls.

The established PGC lines' sex was identified using the:

CHD1 primer set (FW:5'-TATCGTCAGTTTCCTTTTCAGGT-3'; RV: 5'-CCTTTTATTGATCCATCAAGCCT-3'), as previously described by (Lee et al., 2010). For the PCR process and gel electrophoresis, the isolated DNA was diluted to a concentration of 25 ng/l.

Sequences used for PCR products are identified as specific and can be found with the following information:

NC_006127.4 in chromosome Z, Gallus_gallus, Ref number: 74492662. Product length = 461.

NC_006126.4 in chromosome W Gallus_gallus, Ref number: 74492663. Product length = 322.

The PCR program was running with cycles and changes of temperature as next:

Table 3.2 PCR running program with cycles and changes in temperature

Step	Temperature	Time	No. of cycles
Initial denaturation	95ºC	60 s	1
Denaturation	95ºC	15s	28
Primer annealing	48ºC	30s	
Extension	72ºC	10s	
Final extension	72ºC	5 min	1
Storage	4ºC	∞	-

3.6 GEL ELECTROPHORESIS

The PCR product was tested using gel electrophoresis. It was made out of 1.5% agarose gel stained with ethidium bromide. The ladder (3 µl) from the company "Thermo Scientific" was used. The gel was inserted with 7 µl of PCR product in each pocket. Then the samples were run in the gel at 100 V for 40 min. The finding was validated by identifying the band's number. Male samples (ZZ) were identified by one band, whereas two bands identified female samples (ZW).

3.7 PGC CULTURING

The blood was isolated (approximately 1µl) from HH stage 14-17 embryos and added to the selective PGC culture medium developed by McGrew and colleagues (Whyte et al., 2015) to completely remove the blood cells while supporting the growth and division of PGCs. Half of the medium was replaced every two days. By the end of the third week, a PGC line is considered successful if the cell number reaches 1×10^5. (Whyte et al., 2015). The number of cells reached 5.0×10^4 before the end of the third week, indicating that the line establishment was successful. Following the successful establishment, the cells were divided into two wells

and propagated at 2- 4.0 x 105 cells/ml medium in a 24-well plate. The PGCs were harvested for RNA isolation and further study when the cell number achieved confluence.

3.8 CULTURE MEDIUM FOR PRIMORDIAL GERM CELLS

The medium protocol formulated by McGrew and his colleagues (Whyte et al., 2015) was used for PGC culture. The component of the PGC culture medium is a special AVIAN DMEM (Dulbecco's Modified Eagle Medium) containing B27, ovalbumin, and heparin. The medium contains the growth factors h-Activin A (25 ng/µl) and h-FGF2 (10 ng/µl). A 0.22-µl syringe filter was used to filter the stock medium. The filtered medium was held at 4 degrees Celsius (Anand et al., 2018).

The PGC medium used was the Avian-KO-DMEM basal media (FACs medium), a modification of the Knock-out DMEM (250 mOsm/kg, 12.0 mM glucose, and calcium chloride free) manufactured by Life Technologies. FAcs medium and its variants contained avian KO-DMEM basal medium, B27 supplement (50x, Gibco 17504044), Glutamax (100 x Gibco 35050038), NEAA (100 x Gibco 11140035), Nucleosides (100 x, Embryo Max ES-008 - D), Pyruvate (100 x, Gibco 11360070), Beta- mercaptoethanol (50 mM, Gibco 31350010), $CaCl_2$ (1000 mM, C4901- 1000G)**, Ovalbumin (20%, Sigma A5503)* Na Heparin (50 mg /ml, Sigma H3149 – 25 Ku)*, and Pen /strep (100x, 15070-063) along with chicken serum (0.2% final conc.,C5405), h- ActivinA (25ng/ml, PHC9564), h- FGF2(10 ng/µl, 13256-029), and ovatransferin (10mg/ml, C7786). The stocks were prepared according to the instruction of the devised protocol (Whyte et al., 2015). The stock medium was filtered using a 0.22-µl syringe filter. These tubes were wrapped with aluminum foil to protect them from light. The filtered medium was kept at 4 °C.

3.9 CELL COUNTING

The Arthur Novel Fluorescence Cell Counter (NanoEnTek, Pleasanton, USA) was used to count the cells. This cell counter is a 3-channel desktop image analyzer that allows users to run experiments for cells in suspension, including GFP and RFP expression, apoptosis, cell viability, cell cycle, and cell counting.

3.10 IMMUNOSTAINING

The chicken PGCs were put for immunostaining under a confocal microscope using primary antibodies, secondary antibodies, and nuclear staining with TopRo3. The chicken PGCs were collected from the wells and then transferred to an Eppendorf tube for centrifugation at 1000 rpm for 3 minutes. Right after centrifugation, the supernatant was removed, and the pellet was re-suspended in 0.1 % BSA – PBS for 15 minutes at room temperature. The cell suspension was added, 10 µl dropped into the cell culture slide, and dried out on the heating surface (38°C). Fixation was done with 4% PFA for 10 minutes at room temperature. 20 µl drop of 4% PFA was added to the plate. After washing three times with 0.1% BSA- PBS for 5 minutes at room temperature, the washing solution was removed, and 50 µl drops of 0.01 % BSA –PBS were added to the plate. The fixed cells were washed with PBS before being blocked, using one solution for the cell surface and another for the cell nuclei. The blocking solution (50 µl) was added and left for 45 minutes. The first antibody staining was added for one night at 4 °C in 0.1% BSA-PBS (30 µl drops per sample), and it was incubated using each of the primary antibodies, comprising mouse anti-SSEA-1 (1:10; Developmental Studies Hybridoma Bank, USA) and rabbit anti-DAZL (1:100). Following the incubation that lasted the night in the primary antibody solution in a moist chamber at 4°C, the cells were washed 3 times with 0.01% BSA-PBS. A washing solution of 50 µl per wash was used, with each wash lasting 5 minutes at room temperature. The cells were then incubated later with the secondary antibodies, mouse immunoglobulin D 549 (m-Igm D 549) and rabbit -IgG-A 488, respectively. Donkey anti-mouse IgM FITC Cy3 (1: 400, Jackson ImmunoResearch, USA), donkey anti-rabbit IgG FITC (1: 400, Jackson ImmunoResearch, USA), and donkey anti-rabbit IgG conjugated to Alexa 555 (1: 400, Molecular Probes Inc., USA) were then incubated for 1 hour at room temperature in a dark, humid chamber. The nucleus staining was done with TO-PRO®-3 stain (1 µl Topro-3 in 500 µl 0.01% BSA-PBS intended for 15 minutes at room temperature in the dark with 30 µl drops, Molecular Probes Inc., Eugene, OR, USA) after washing with 0.01% BSA-PBS in a 50 µl drop in the dark at room temperature (5 minutes). After washing three times with 0.01% BSA-PBS in a 30 µl drop per wash for 5 minutes at room temperature in the dark, coverslips were put on the slide with 10 µl VECTASHIELD® Mounting Media (Vector Laboratories Inc., US) and examined by confocal microscopy (TCS SP8, Leica). Only the secondary antibody was used to stain the negative controls.

3.11 FREEZING OF PGC LINES

Male and female PGCs cell lines were treated with two freezing media, freezing avian medium 1 (FAM1) and freezing avian medium 2 (FAM2) (Table 3.3). The freezing protocol was carried out after the freezing media had been prepared. The cells were suspended gently, and then all cells were transferred from the well to a 1.5ml Eppendorf tube for centrifugation at 2000rpm for 3 minutes. The supernatant was removed with careful attention to the pellet. The pellet was re-suspended in 250µl Avian-KO-DMEM (AKODMEM), and 250µl freezing medium (FAM1 or FAM2) was added slowly. Then the cell suspension was pipetted to the labelled cryovial tube and placed into the freezer at -70°C. After one night, the cells should be transported to -150 °C or liquid nitrogen for ongoing long-term cultivation.

The components created a Freezing Avian Medium -1 (FAM1) mix: 800 µL of DMSO, 500 µL of chicken serum, and 15 µL of $CaCl_2$ and 20 µL of sodium pyruvate were added to 3665 AKODMEM. A syringe was used to transfer the mix for filtering into an Eppendorf tube.

Freezing Avian Medium-2 (FAM2) mix was prepared with the following components: 1000 µL DMSO, and 4000 µL FBS (fetal bovine serum). DMSO was combined with fetal bovine serum (FBS) in a small glass and filtered into an Eppendorf tube with a syringe. The medium was kept at 4 °C.

Table 3. 3 PGC Freezing culture medium protocol

FAM1 components 2x (16%DMSO)	**FAM2 components 2x (final 20%DMSO)**
800 µL DMSO 500 µL chicken serum 20 µL sodium pyruvate 15 µL $CaCl_2$ 3665 µL AKODMEM	1000 µL DMSO 4000 µL fetal bovine serum (FBS)
Add 250 µL of FAM1 into each of the two cryovial tubes containing PGC lines plus 250 µL AKODMEM	Add 250 µL of FAM2 into each of the two cryovial tubes containing PGC lines plus 250 µL AKODMEM
AKODMEM: 664 µL water 332 DMEM 4 µL sodium pyruvate	

3.12 THAWING OF PGC LINES

In the thawing process, a water bath at 37°C was used to thaw PGCs, and the cells were transferred into 1.5 mL of an Eppendorf tube containing 1 mL of PGC culture media. The supernatant was removed right after centrifugation at 1000 rpm for 3 minutes. The cells were re-suspended in fresh PGC culture media and placed into cell culture wells for continued growth for around 1 week until the viability became optimal. Samples were taken for RNA isolation and further analysis.

3.13 RNA ISOLATION FROM CHICKEN PGC CELL LINES

RNA was isolated from samples collected before and after freezing with avian medium -1 (FAM1) and Freezing avian medium – 2 (FAM2). We performed the RNA isolation by using the RNA Aqueous Lysis Buffer Micro Kit (Applied Biosystems). The solution was first mixed using a vortex mixer, and then 50µl ethanol was added to each Eppendorf tube. Then the samples were vortexed before the transfer of 150µl from the Eppendorf tube into the column tube. The samples were centrifuged to the maximum for 10 seconds, and then 180µl wash solution was added to each column tube and centrifuged to the maximum for 10 seconds. The same amount of wash solution (180µl) was added for the second round to each column tube and centrifuged at the maximum rpm for 10 seconds. After centrifugation, the column tube was emptied and centrifuged at the maximum rpm for 1 minute.

The filter was transferred into another labelled tube, and 10µl of elution solution was added, which was pre-heated in a thermomixer at 75°C before being added into the tube. Incubation for 1 minute at room temperature was done after the filter transfer and the elution solution's addition. Following the incubation, centrifugation was done for 30 seconds at a maximum rpm.

The concentration of the isolated RNAs was measured by the equipment NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The blanking of the NanoDrop One machine was done using a 1 µl elution solution. Then immediately after that, the concentration of extracted RNA from each sample was measured by taking 1µl of RNA. The isolated RNA samples were then stored at -70 ºC for further analysis. The isolated RNA was then used for cDNA writing and qRT-PCR analysis of the germ cell-specific markers (CVH, GAPDH, and DAZL) and miRNA: gga-miR-302b-5P and gga-miR-92.

3.14 CDNA WRITING FOR GENE EXPRESSION, DAZL, CVH, AND GAPDH

The isolated RNA concentration of each sample was first diluted to 25 ng/µl. The final concentration of each sample RNA was measured on the NanoDrop One Spectrophotometer

(Thermo Fisher Scientific, Waltham, MA, USA). Following the dilution, a cDNA master mix was prepared and the isolated RNA samples were reverse transcribed into cDNA with a high-capacity cDNA Reverse Transcription Kit, following the information provided by the manufacturer (Applied Bio Systems, Life Technologies, Carlsbad, CA, USA). The RT master mix was used for cDNA writing. The cDNA was stored at −20 ◦C.

3.14.1 Procedure for the preparation of cDNA writing

Master mix was prepared according to Table 3.4. 10 µl of the master mix was added to each PCR tube, and then after that, 10 µl of RNA was transferred into the particular labelled tube (Table 3.5). The samples run into the PCR machine (Thermo Applied Biosystem ProFlex 3x32-well PCR system). After two hours and fifteen minutes of running into the PCR machine, the cDNA was ready for qPCR.

Table 3.4 preparation of master mix for cDNA writing

RT mix 1 x (1.5 ml Eppendorf tube) for cDNA writing	
Components	Amount (µl)
Nuclease free water	3.2
10 X dNTP	0.8
10 X Buffer	2.0
10 X Random primer	2.0
RNase inhibitor, 20 u/µl	1.0
Multi scribe Reverse Transcriptase	1.0
Sum	**10.0**

Table 3.5 preparation of cDNA writing

PCR tube (200 µl)	
Components	Amount (µl)
RT master mix cDNS	10.00
RNA sample (25ng/ µl)	10.00
Sum	**20.00**

Table 3.6 Run method of reverse transcriptase (RT) PCR (1x cycle)

Stage	**Step**	**Temperature**	**Time**
1	Annealing	25 °C	10 min
	Elongation	37 °C	2 hours
2	Enzyme deactivation	85°C	5 min
3	Storage	4°C	∞

3.15 CDNA WRITING FOR MIRNA (MIR-92, MIR-302b-5P)

The isolated RNA concentration of each sample was first diluted to 5 ng/µl. The final concentration of each sample RNA was measured on the NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Following the dilution, a cDNA master mix was prepared. The isolated RNA samples were reverse transcribed into cDNA with a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems ™ made in USA) specifically designed to generate microRNA-specific cDNA.

3.15.1 Procedure for the preparation of *cDNA* writing

The RT master mix was prepared for the RNA samples from the cell culture, after treatment with freezing avian medium 1 (FAM1) and after freezing avian medium 2 (FAM2). The RNA samples were used for the cDNA writing. The total volume of the RT master mix was 2.3 µl for each sample (Table 3.7), and the components were: 0.05 µl of 100 mM dNTP (with DTTP), 0.3 µl Multi scribe Reverse Transcriptase, 0.5 µl 10x RT Buffer, 0.1 µl RNase inhibitor 20 u/µl, and 1.4 µl of Nuclease free water. The RT primers were designed for miRNA-92 and miRNA-302-5P . PCR tubes were prepared for each RT primer of the two miRNAs. 2.3 µl of RT master mix, 1 µl of RT primer for miRNA, and 1.7 µl of RNA sample (5 ng/ µl) were put together into each of six labelled tubes of two sets prepared for all RNA samples, and the two miRNA primers (Table 3.8, 3.9). The samples run into the PCR machine (Thermo Applied Biosystem ProFlex 3x32-well PCR system, 0.2 mL). After one hour and five minutes of running into the PCR machine (table 3.10), the cDNA was ready for qPCR.

Table 3.7 RT master mix preparation for the cDNA writing of miRNA

RT master mix 1 x for miRNA	
5 µl sum	Solution in µl
100 mM dNTP (with DTTP)	0.05
Multi scribe Reverse Transcriptase	0.3
10 X Buffer	0.5
RNase inhibitor , 20 u/µl	0.1
Nuclease free water	1.4
RT master mix	**2.3**

Table 3.8 components of cDNA writing for miRNA

cDNA writing components	**Amount (µl)**
RT master mix miRNA	2.3
RT Primer for miRNA	1.0
RNA sample (5ng/ µl)	1.7
Sum	**5**

Table 3.9 Sample preparation for cDNA writing of microRNA using RT Primer – miR-92 and miR-302b-5P

1x Sample preparation for cDNA writing of miRNA using RT primers			
Sample FAM1-miR-92	Amount (µl)	Sample FAM2-miR-302b-5P	Amount (µl)
RT master mix miRNA	2.3	RT master mix miRNA	2.3
RT Primer-miR-92	1.0	RT Primer-miR-302b-5P	1.0
Sample FAM1 A2 (5ng/ µl)	1.7	Sample FAM2 A2 (5ng/ µl)	1.7
Sum	**5** µl		**5** µl

Table 3.10 Run method of reverse transcriptase (RT) PCR (1x cycle)

Stage	**Step**	**Temperature**	**Time**
1	Annealing of the primer	16 °C	30 min
	Elongation	42 °C	30 min
2	Enzyme deactivation	85°C	5 min
3	Storage	4°C	∞

Table 3.11 RT primers for miRNAs

Name	**Gene**	**Accession Number**	**Assay ID**	**Sequence**
gga-miR-302b-5P	gga-miR-302b-5P	MI0003700	008131_mat	ACUUUAACAUGGAGGUGCUUUCU
miR-92	hsa-miR-92	MI0000719	000430	UAUUGCACUUGUCCCGGCCUG

3.16 QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) FOR GENE EXPRESSION, CVH, GAPDH, AND DAZL

The cDNA was used for qPCR. The reaction was carried out using the Eppendorf MasterCycler Realplex machine.

The qPCR master mix was prepared for CVH, DAZL, and GAPDH using TaqMan™ universal Master Mix II, with UNG (Thermo Fisher Scientific Baltics UAB, made in Lithuania). The qPCR master mix and cDNA sample were mixed to run the qPCR machine.

3.16.1 Procedure for the preparation of qPCR master mix

Each qPCR master mix contains the specific probe for CVH, DAZL, and GAPDH, respectively (Table 3.13). And other components are detailed in Table 3.11. 14 µl of qPCR master mix was pipetted from each master mix and transferred to the 96-well PCR plate (Table 3.12), labelled for each primer and sample. Each cDNA except the control was repeated three times to minimize errors. The method for qPCR is explained in table 3.14.

Table. 3.12 qPCR master mix preparation for three markers (CVH, DAZL, and GAPDH)

1x DAZL qPCR master mix (1.5ml Eppendorf tube)	**Amount (µl)**
RNase free water	5.75
Master Mix universal TaqMan	7.50
Taq TM Primer-DAZL	0.75
Sum	**14**

1x GAPDH qPCR master mix (1.5ml Eppendorf tube)	**Amount (µl)**
RNase free water	5.75
Master Mix universal TaqMan	7.50
Taq TM Primer-GAPDH	0.75
Sum	**14**

1x CVH qPCR master mix (1.5ml Eppendorf tube)	**Amount (µl)**
RNase free water	5.75
Master Mix universal TaqMan	7.50
Taq TM Primer-CVH	0.75
Sum	**14**

Table. 3.13 Sample preparation for qPCR

1x sample preparation for qPCR (CVH, DAZL, and GAPDH)	
Components	Amount (µl)
Master Mix (Taqman)/well	14
cDNA template /well	1
sum	**15.00**

Table 3.14 Primers used in qPCR TaqMan for GAPDH, CVH and DAZL.

Gene Symbol	Gene Full Name (Organism)	Catalog Number
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (Gallus gallus)	PN 4351372 Gg03346984-g1 GAPDH FAM
CVH	DEAD-box helicase 4 (DDX4) (Gallus gallus)	PN 4351372 Gg03338555-m1 DDX4 FAM
DAZL	Deleted in zoospermia-like	PN 4351372 Gg03363982-m1 DAZL FAM

Table 3.15 Run method of TaqMan qPCR

Stage	Step	Temperature	Time	Cycle
1	Denaturation	95 °C	10 min	1x
2	Annealing	95 °C	20 s	40x
3	Extension	60°C	65min	
3	Storage	4°C	-	3x

3.17 QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) FOR MIRNA

The synthesized cDNA (transcribed with TaqMan® MicroRNA Reverse Transcription Kit) was then used for qPCR. The reaction was carried out by using the Eppendorf MasterCycler Realplex machine. According to the manufacturer's recommendations, TaqMan™ universal Master Mix II, with UNG (Thermo Fisher Scientific Baltics UAB, made in Lithuania). TaqMan PCR master mix was used for the qPCR as a double-stranded fluorescent DNA-specific dye. The qPCR master mix and cDNA sample were mixed to run the qPCR machine.

3.17.1 Procedure for the preparation of qPCR master mix

The qPCR master mix was prepared for the miRNAs, miR-92, and miR-302-5P using TM primers. The details of primers are indicated in Table 3.17. The qPCR master mix was prepared for each miRNA. The same amount of RNase-free water, Master Mix universal TaqMan, and Taq TM Primer (in microliters) was measured for the two miRNAs to have a final volume master mix of qPCR TM primer mix (Table 3.15). 14 µl was pipetted from each master mix and transferred to the 96-well PCR plate, labelled for each sample and miR-92b, and miR-302b-5P, including the negative control. Each cDNA except the control was repeated three times to minimize errors. 14 µl of each master mix and 1.0 µl of cDNA were pipetted into the respective wells for the qPCR analysis (Table 3.16). From each tube of the cDNA sample

containing 5 µl, 1.0 µl was transferred into each well. The method for qPCR is explained in table 3.18.

Table 3.16 Preparation of Taq mix qPCR for miR-92 and miR-302b-5P

1x Taq mix qPCR Preparation			
mix TM primer for miR-92	Amount (µl)	mix TM primer for miR-302-5P	Amount (µl)
RNase-free water (NFW)	5.75	RNase-free water (NFW)	5.75
Master Mix universal TaqMan	7.5	Master Mix universal TaqMan	7.5
Taq TM Primer for miR-92	0.75	Taq TM Primer for miR-302-5P	0.75
Sum	**14.00**		**14.00**

Table 3. 17 Preparation of qPCR for miRNA into the qPCR plate

1x qPCR preparation for miRNA into the PCR plate	
Components	Amount (µl)
Mix miR qPCR special TM primer	14
cDNA template for special miRNA /special sample	1.0
Sum	**15.00**

Table 3.18 Primers used in the miRNA analysis.

Name	**Gene**	**Accession Number**	**Assay ID**	**Sequence**
gga-miR-302b-5P	gga-miR-302b-5P	MI0003700	008131_mat	ACUUUAA CAUGGAG GUGCUUU CU
miR-92	hsa-miR-92	MI0000719	000430	UAUUGCA CUUGUCC CGGCCUG

Table 3.19 Run method of TaqMan qPCR

Stage	**Step**	**Temperature**	**Time**	**Cycle**
1	Denaturation	95 °C	10 min	1x
2	Annealing	95 °C	20 s	40x
3	Extension	60°C	65min	
3	Storage	4°C	-	3x

3.18 STATISTICAL ANALYSIS

Each sample was evaluated in three parallel repetitions using qPCR. The data was analysed by the GenEx software (version 7.0). The data for the qPCR analysis of the germ cell-specific markers and stem cell-specific miRNAs (miR-302b-5p & miR-92) was done using the GenEx software.

GenEx calculates the relative expression $2^{\wedge(-\Delta\Delta Ct)}$ based on the gene of reference and sample of reference. In each qPCR run, the expression variations of the target genes were computed and compared to the level of the housekeeping gene using the standard formula:

2^ (-ΔΔCt) method, where Ct = cycle threshold; ΔCt = Ct (target gene) - Ct (housekeeping gene); and ΔΔCt = ΔCt (test sample) - ΔCt (control sample).

GAPDH was the reference gene for the CVH, DAZL, miR-302b-5P, and miR-92 expression assessments.

A t-test was applied using Excel to investigate statistical variations among the samples studied. Levels of significance were applied at $p<0.05$ (table 3.19).

Table 3.20 Accepted p values and symbols.

Symbol	Meaning
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$

4 RESULT AND DISCUSSION

4.1 ISOLATION AND ESTABLISHMENT OF PGC LINES

In this research, we collected blood samples from seven embryos. The blood sample was isolated from each embryo as PGCs circulate in the blood in the early embryonic stages at 14-17 HH stages (Fig. 4.1) because the highest total concentration and quantity of circulating PGCs are greater in embryonic stage 14 HH (Szczerba et al., 2021).

From fifteen isolations, three PGC lines were successfully established. The number of isolations is determined by the number of viable embryos at the proper stage of development and the fertility of the eggs. Three PGC cultures were successfully obtained, resulting in a cell line establishment rate of 20%.

The cells were propagated in *vitro* for 3 weeks before getting used for RNA isolation before and after freezing in further experiments. Since the number of circulating PGCs in avian embryonic blood cells is low compared to many erythrocytes, the cells were cultured to enhance their numbers through in vitro propagation. The number of isolated PGCs from circulating blood is low compared to gonadal PGCs (Park et al., 2003). The cells were cultured with chicken serum-selective media for chicken PGCs (Whyte et al., 2015). PGCs were grown in a thermostat at 38 °C with 5% CO2 concentration for three weeks. Sub-culturing or passage was done every other day to keep the cells alive, and half of the culture medium was substituted by the new medium. Within a week, the proliferating PGCs reached confluency and were extended into another well plate. This culture phase allows for the eradication of all cell types except PGCs. The isolated PGCs and blood cells were plated together, and the blood cells died after 8- 10 days of growth due to the selective media culture, which is PGC-specific. For 8 days, the circulating PGCs were grown and viewed under an inverted microscope. Their large size and bright cells identified PGCs compared with the surrounding cells. The cells were divided into two when the total cell number reached 1.0×10^5, showing that the line was successfully established. As indicated in the previous study, the line is considered successful if the cell number of PGCs from one embryo reaches 1.0×10^5 cells in three weeks (Whyte et al., 2015).

McGrew and his colleagues developed the protocol for preparing the PGC culture media (Whyte et al., 2015). Female and male PGCs could be maintained in this study in a basal medium with low osmolality and calcium levels. The female and male PGC cell lines were successfully generated and propagated in suspension without feeder cells using a modified basal medium (avian KO-DMEM), which contains Activin A, fibroblast growth factor 2 (FGF2), ovotransferrin, and chicken serum (FAcs medium). Growth factors are important for self-renewal, proliferation, and survival, all possible in low chicken serum levels (0.2%). Heparin

was also used to stimulate FGF2 signalling while decreasing cell adhesion. A previous study revealed that PGC cultivation without growth factors results in cell differentiation into distinct kinds over several days (Whyte et al., 2015). The seven isolated PGCS were propagated in *vitro* for 3 weeks before getting used for RNA isolation before and after freezing in further experiments.

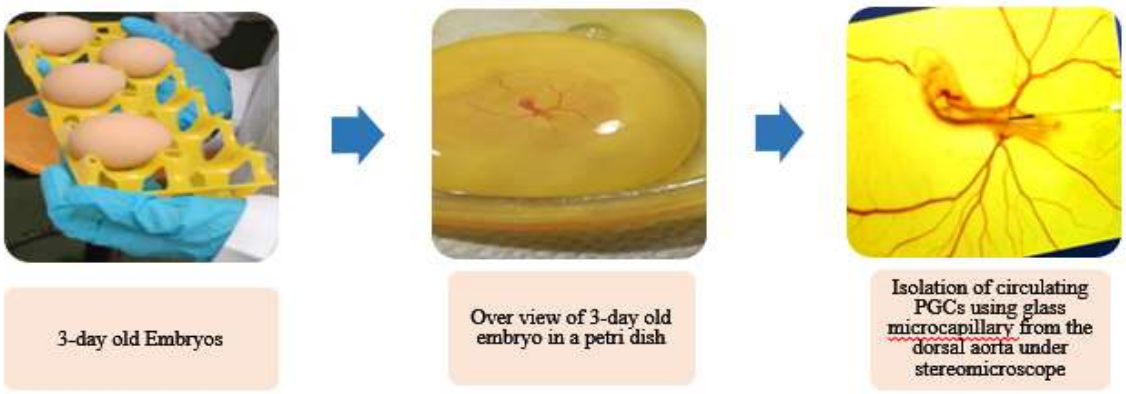

Figure 4.1 Isolation of circulating PGCs (own picture).

4.2 SEX DETERMINATION

The tissue samples of the embryo that were isolated right after the isolation of circulating PGCs from the same embryos were used to determine the sex of the embryo before PGC line establishment.

Fifteen embryos were sampled, and three were successfully established, providing male (Y7) and female (Y13) PGC lines (7 ZZ and 13 ZW) for the present experiment.

The sex PCR was performed to determine the sex of the embryos using the isolated DNA from each embryo. The PCR mix was prepared to measure 13 µl reaction mix into each tube. The PCR product was tested using gel electrophoresis to determine the sex of each embryo; the ZW female (F) cell line has two bands, but the ZZ male (M) cell line has only one band (Fig. 4.2). In avian species, the sex is determined by the females because of the heterogametic sex chromosomes (ZW).

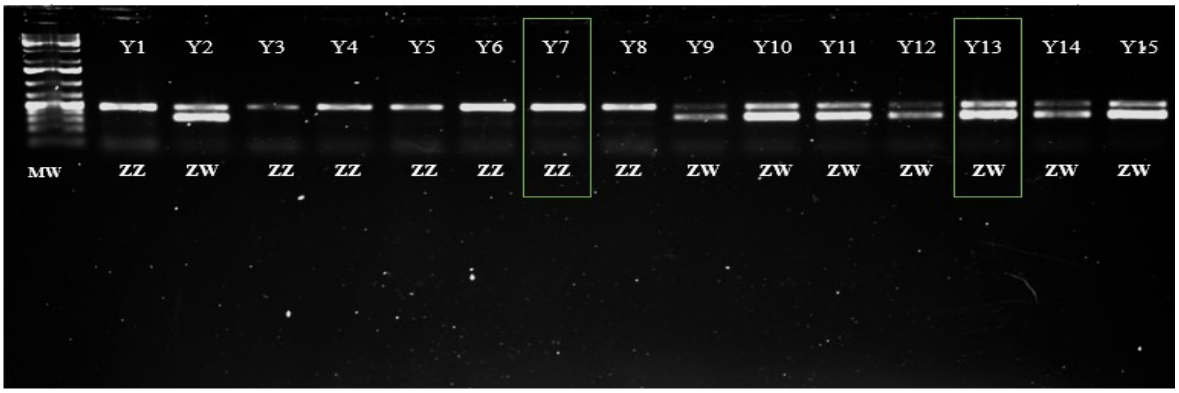

Figure 4.2 Sample Y13 and Y7 show the gender contrast between the two cell lines. 7 males were observed, and 8 females. The image demonstrates that the ZW female (F) cell line exhibits two bands, whereas the ZZ (M) male cell line displays only one band.

4.3 IMMUNOSTAINING ANALYSIS

PGC immunostaining was done on cell lines. Two cell lines were chosen at random, one male and one female. After the cell lines were cultured and then frozen and thawed, cell lines were obtained. Each cell line examined was stained for germ cell-specific DAZL, and stem cell-specific SSEA1. DAZL is cytoplasmic, whereas SSEA1 is on the cell surface. TO-PRO-3, a nuclear stain, was used (Fig.4.3).

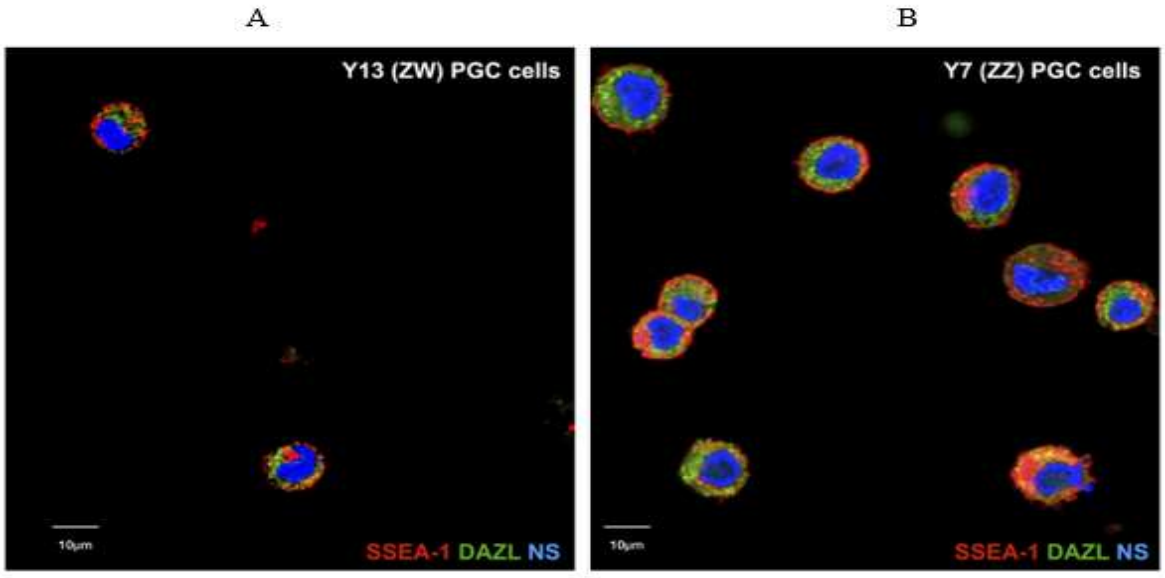

Figure 4.3 A) Y13 (ZW) PGC cells immunohistochemistry analyzed by confocal microscopy. B) Y7 (ZZ) PGC cells immunohistochemistry analyzed by confocal microscopy. The immunostaining of each cell line was conducted with SSEA-1 (in red) for cell surface, DAZL (in green) for cytoplasmic, and TO-PRO™-3 (in blue) for nuclear staining. The scale was 10 µm.

4.4 FREEZING AND THAWING OF PGCS

In this experiment, four samples were frozen (Table 4.1). In two distinct types of freezing medium, male and female PGC lines were examined. One male and one female PGC line were frozen in freezing medium-1 (FAM1), while the other two samples, one male and one female line, were frozen in freezing medium-2 (FAM2).

Table 4.1 Sample preparation for freezing media of PGC lines derived from two embryos.

Treatment of PGCs with two different media		
Embryo	Freezing Avian medium -1 (FAM 1)	Freezing Avian medium - 2 (FAM2)
Female (Y13)	Y13 FAM 1	Y13 FAM2
Male (Y7)	Y7 FAM1	Y7 FAM2

The reason for freezing PGC lines is to maintain their stemness and avoid differentiation because PGCs may lose their stemness when cultured over extended periods. In vitro cultivation of PGCs is technically challenging and costly, requiring complicated cell culture medium and specialized cell culture equipment. PGC in vitro propagation results in epigenetic changes and decreased germline transmission as the in vitro culture time increases (Woodcock et al., 2019; Ballantyne et al., 2021; Soler et al., 2021).

As a cryoprotectant, DMSO (Dimethyl Sulfoxide), a polar, aprotic organic solvent (Verheijen et al., 2019), was utilized in this investigation to freeze the PGC lines. As a result, the freeze-thawing process was carried out carefully and successfully. DMSO was added to the freezing medium due to its capability to penetrate the cell membrane and displace the water. It was included in the cell culture medium to avoid cell death during the freezing process by reducing ice formation.

The final concentrations of DMSO added to FAM1 and FAM2 were 8.0% and 10%, respectively. The cells were slowly frozen to -70 degrees in the medium of DMSO to avoid forming intracellular ice crystals. The cell lines were cultured slightly more after freeze-thawing to remove the DMSO. When used over the acceptable dosage, DMSO causes DNA toxicity, induces apoptosis, and causes pore formation in the cell membrane. When used at less than 10% (v/v), DMSO is usually considered nontoxic, and its effects are considered insignificant in practice (Verheijen et al., 2019).

4.5 RNA ISOLATION

Three cell lines were successfully established during this research, and two cell lines were used (Y13-ZW and Y7-ZZ) though; nevertheless, other PGC cell lines were used as controls (A1-5-ZZ) for relative expression calculation and to increase the reliability of the gene expression calculations (A1-ZZ and A2-ZW). The extra samples (A1-ZZ and A2-ZW) were treated with FAM1 and FAM2. The total of the used samples is described in Table 4.2.

Table 4.2 Summary of samples used for RNA Isolation.

Samples identification				
Cell Line	**Gender**	**Before freezing**	**FAM1**	**FAM2**
A2	ZW	A2 (ZW)	A2(ZW)-FAM1	A2(ZW)-FAM2
M1-5	ZZ	A1 (ZZ)	A1(ZZ)-FAM1	A1(ZZ)-FAM2
Y13	ZW	Y13 (ZW)	Y13(ZW)-FAM1	Y13(ZW)-FAM2
Y7	ZZ	Y7 (ZZ)	Y7(ZZ)-FAM1	Y7(ZZ)-FAM2
M1-5 cont.	ZZ	A1-5 (ZZ)	-	

The purity and concentrations of the isolated RNA samples were measured using NanoDrop. RNA was isolated from all samples, and the best quality ones were used for cDNA writing and qPCR.

Sample Y13 (ZW) FAM1, Y13 (ZW)-FAM2, Y7 (ZW) FAM1, and Y7 (ZW)-FAM2 were discarded due to bad quality of RNA.

4.6 CDNA WRITING FOR GENE EXPRESSION, DAZL, CVH, AND GAPDH

RNA samples from both male and female lines were used in the process of the cDNA synthesis. The isolated RNA concentration was first diluted to 25 ng/µl in sterile water. A cDNA master was prepared using random primers, and the RNA was reverse-transcribed into cDNA.

4.7 CDNA WRITING FOR MIRNA (MIR-92, MIR-302b-5P)

The isolated RNA concentration of each sample was first diluted to 5 ng/µl. A cDNA was prepared from the six RNA samples. The cDNA for miRNA was generated from an RNA template by reverse transcription with RT primers.

4.8 qPCR ANALYSIS FOR THE MARKERS CVH, GAPDH, AND DAZL

The qPCR master mix was prepared from cDNA samples derived from male and female PGC lines. It was prepared three times with CVH, DAZL, and GAPDH primers, respectively.

4.9 qPCR ANALYSIS FOR MIRNA (MIR-302B-5P AND MIR-92)

A 96-well PCR plate was used; each cDNA except the control was replicated three times to reduce errors. For the qPCR analysis, 14 µl of each master mix and 1.0 µl of cDNA were pipetted into the appropriate wells (Fig.4.4). From each tube of cDNA, which contains 5 µl, 1.0 µl of cDNA was pipetted into each well of repeats. The analysis was done using a qPCR machine.

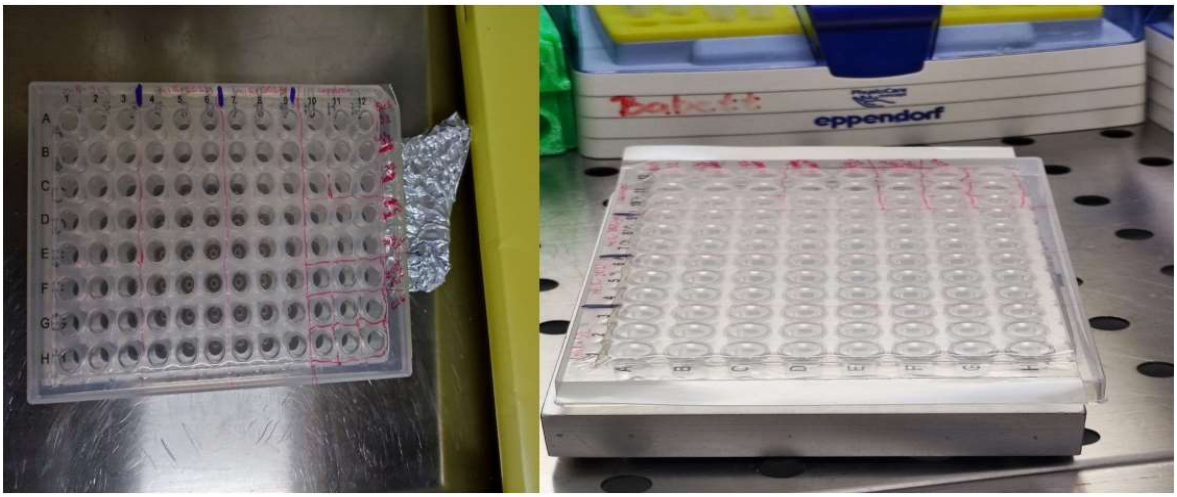

Figure 4.4 qPCR plate prepared for miRNA analysis

4.10 GENE EXPRESSION ANALYSIS OF GERM CELL-SPECIFIC MARKER AND STEM CELL-SPECIFIC MIRNAS

4.10.1 ANALYSIS OF GERM CELL-SPECIFIC MARKERS AND MIRNAS

The isolated RNA templates from male and female samples of PGC lines were reverse transcribed into cDNA by RT-PCR. The generated cDNA for marker genes and miRNAs was used in the qPCR analysis. A qPCR machine was used to examine the expression of germ cell-specific markers and stem cell-specific miRNAs. The germ cell-specific markers CVH and DAZL were used. Furthermore, GAPDH was used as a housekeeping gene (internal control). The stem cell-specific miR-302b-5P and miR-92 were used, as well as the housekeeping gene GAPDH. The mRNA expression of each marker gene and miRNA was calculated based on a

qPCR machine's cycle threshold (Ct) values (Table 4.3 a & b). The intensity of each fluorescence emission was recorded at each cycle during the PCR run.

Table 4.3 (a) Ct values for Y7 (ZZ), A1 (ZZ), Y13 (ZW), and A1-5 (ZZ)

Cell line	Sample	Ct GAPDH	Ct CVH	Ct DAZL	Ct miR92	Ct miR302-5p
Y7	Y7 (ZZ)	19.275	22.12	19.885	22.635	28.535
M1-5	A1 (ZZ)	18.76	21.49	19.1	19.56	30.245
Y 13	Y13 (ZW)	19.13	22.285	20.18	23.145	27.645
M1 5	A1-5 (ZZ)	18.85	21.325	19.995	23.405	29.895

Table 4.3 (b) Ct values for A1 (ZZ), A1 (ZZ) (FAM1), A1 (ZZ) (FAM2), A2 (ZW), A2 (ZW) (FAM1), A2 (ZW) (FAM2), and A1-5 (ZZ).

Cell line	Sample	Ct GAPDH	Ct CVH	Ct DAZL	Ct miR92	Ct miR302-5p
A2 3	A2 (ZW)	17.24	20.145	17.665	21.01	31.485
A2 5	A2 (ZW)	18.52	20.82	17.33	20.14	31.17
A2 FAM1 1	A2 (ZW)(FAM1)	16.73	19.725	17.42	19.64	31.59
A2 FAM1 2	A2 (ZW)(FAM1)	17.965	20.555	18.225	24.895	35.07
A2 FAM2 1	A2 (ZW)(FAM2)	16.945	20.205	17.63	19.875	32.23
A2 FAM2 2	A2 (ZW)(FAM2)	17.1	20.015	17.3	21.205	31.225
M1 4	A1 (ZZ)	18.005	19.315	17.43	21.365	32.105
M1 5	A1 (ZZ)	17.615	18.81	17.045	20.365	31.245
M1 FAM1 1	A1 (ZZ) (FAM1)	17.14	19.345	17.8	20.215	30.815
M1 FAM1 2	A1 (ZZ) (FAM1)	15.24	18.9	16.98	19.265	30.605
M1 FAM2 1	A1 (ZZ) (FAM2)	17.89	20.12	17.935	19.375	30.64
M1 FAM2 2	A1 (ZZ) (FAM2)	16.675	19.425	17.415	21.215	31.49
M1 5 cont.	A1 (ZZ)	17.765	18.925	17.32	20.57	30.955
M1 5 cont.	A1-5 (ZZ) – cont.	17.145	18.885	16.625	20.295	31.28

The higher the Ct value, the more cycles were required to observe the expression of the marker genes and miRNAs due to the lower abundance of loaded cDNA samples in the expression of the marker genes and miRNAs. Fewer cycles were required for the lower Ct values. And this is due to the abundance of loaded cDNA samples(Arya et al.. 2005).

ΔCt Relative expression to the reference gene:

In every qPCR run, the expression of the genes was calculated based on the reference gene, referring to levels of the housekeeping genes for markers and miRNAs.

The relative quantification was determined using the GenEx7 software in two steps. First, the ct values were used to calculate the relative expression to the reference gene GAPDH (Table 4.4 and Table 4.5). GenEX uses the following formula to calculate ΔCt.

$$\Delta Ct = Ct_{sample} - Ct_{GAPDH}$$

Table 4.4 Relative Expression to GAPDH for Y7 (ZZ). A1 (ZZ). Y13 (ZW). and A1-5 (ZZ)

Cell Line	sample	ΔCt CVH	ΔCt DAZL	ΔCt miR92	ΔCt miR302b-5p
Y7	Y7 (ZZ)	2.845	0.61	3.36	9.26
M1-5	A1 (ZZ)	2.73	0.34	0.8	11.485
Y 13	Y13 (ZW)	3.155	1.05	4.015	8.515
M1 5	A1-5 (ZZ)	2.475	1.145	4.555	11.045

Table 4.5 Relative Expression to GAPDH for A1 (ZZ). A1 (ZZ) (FAM1). A1 (ZZ) (FAM2). A2 (ZW). A2 (ZW)(FAM1). A2 (ZW)(FAM2). and A1-5 (ZZ).

Cell Line	sample	ΔCt CVH	ΔCt DAZL	ΔCt miR92	ΔCt miR302b-5p
A2 3	A2 (ZW)	2.905	0.425	3.77	14.245
A2 5	A2 (ZW)	2.3	-1.19	1.62	12.65
A2 FAM1 1	A2 (ZW)(FAM1)	2.995	0.69	2.91	14.86
A2 FAM1 2	A2 (ZW)(FAM1)	2.59	0.26	6.93	17.105
A2 FAM2 1	A2 (ZW)(FAM2)	3.26	0.685	2.93	15.285
A2 FAM2 2	A2 (ZW)(FAM2)	2.915	0.2	4.105	14.125
M1 4	A1 (ZZ)	1.31	-0.575	3.36	14.1
M1 5	A1 (ZZ)	1.195	-0.57	2.75	13.63
M1 FAM1 1	A1 (ZZ) (FAM1)	2.205	0.66	3.075	13.675
M1 FAM1 2	A1 (ZZ) (FAM1)	3.66	1.74	4.025	15.365
M1 FAM2 1	A1 (ZZ) (FAM2)	2.23	0.045	1.485	12.75
M1 FAM2 2	A1 (ZZ) (FAM2)	2.75	0.74	4.54	14.815
M1 5 kont	A1-5 (ZZ)	1.16	-0.445	2.805	13.19
M1 5 kont	A1-5 (ZZ) - cont	1.74	-0.52	3.15	14.135

$2^{\wedge-\Delta\Delta Ct}$ Relative expression to the reference sample

With the software GenEX. The calculated relative expressions to the reference gene (GAPDH) were used to calculate the relative expression to the reference Sample A1-5 (ZZ) (Table 4.6, Table 4.7. and Table 4.8).

GenEX uses the following formula to calculate $2^{\wedge-\Delta\Delta Ct}$.

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{A1\text{-}5}$$

$$\text{Relative expression} = 2^{\wedge-\Delta\Delta Ct}$$

Table 4.6 Relative Expression to reference sample for Y7 (ZZ), A1 (ZZ), Y13 (ZW), and A1-5 (ZZ)

Cell Line	sample	$2^{\wedge-\Delta\Delta Ct}$ CVH	$2^{\wedge-\Delta\Delta Ct}$ DAZL	$2^{\wedge-\Delta\Delta Ct}$ miR92	$2^{\wedge-\Delta\Delta Ct}$ miR302b-5p
Y7	Y7 (ZZ)	0.773782	1.448942	2.289448	3.446185
M1-5	A1 (ZZ)	0.837987	1.747146	13.501053	0.737135
Y 13	Y13 (ZW)	0.624165	1.068065	1.453973	5.775717
M1 5	A1-5 (ZZ)	1	1	1	1

Table 4.7 Relative Expression to reference sample for A1 (ZZ), A1 (ZZ) (FAM1), A1 (ZZ) (FAM2), A2 (ZW), A2 (ZW) (FAM1), A2 (ZW) (FAM2), and A1-5 (ZZ).

Cell Line	sample	$2^{\wedge-\Delta\Delta Ct}$ CVH	$2^{\wedge-\Delta\Delta Ct}$ DAZL	$2^{\wedge-\Delta\Delta Ct}$ miR92	$2^{\wedge-\Delta\Delta Ct}$ miR302b-5p
A2 3	A2 (ZW)	0.445964	0.519430	0.650671	0.926588
A2 5	A2 (ZW)	0.678302	1.591073	2.887858	2.799172
A2 FAM1 1	A2 (ZW)(FAM1)	0.418994	0.432269	1.180993	0.604997
A2 FAM1 2	A2 (ZW)(FAM1)	0.554785	0.582367	0.072796	0.127627
A2 FAM2 1	A2 (ZW)(FAM2)	0.348686	0.433769	1.164734	0.450625
A2 FAM2 2	A2 (ZW)(FAM2)	0.442884	0.607097	0.515842	1.006956
M1 4	A1 (ZZ)	1.347234	1.038859	0.864537	1.024557
M1 5	A1 (ZZ)	1.459020	1.035265	1.319508	1.419123
M1 FAM1 1	A1(ZZ) FAM1)	0.724471	0.441351	1.053361	1.375542
M1 FAM1 2	A1(ZZ) FAM1)	0.264255	0.208772	0.545254	0.426317
M1 FAM2 1	A1(ZZ) FAM2)	0.712025	0.675955	3.171137	2.611720
M1 FAM2 2	A1(ZZ) FAM2)	0.496546	0.417544	0.381565	0.624165
M1 5 kont	A1-5 (ZZ)	1.494849	0.949342	1.270151	1.925189
M1 5 kont	A1-5(ZZ) - cont	1	1	1	1

Table 4.8 Compilation of relative expression values to reference sample:

Cell Line	sample	$2^{-\Delta\Delta Ct}$ CVH	$2^{-\Delta\Delta Ct}$ DAZL	$2^{-\Delta\Delta Ct}$ miR92	$2^{-\Delta\Delta Ct}$ miR302b-5p
A2 (ZW)	ZW	0.45	0.52	0.65	0.93
A2 (ZW)	ZW	0.68	1.59	2.89	2.80
A2 (ZW) (FAM1)	ZW-FAM1	0.42	0.43	1.18	0.60
A2 (ZW) (FAM1)	ZW-FAM1	0.55	0.58	0.07	0.13
A2 (ZW) (FAM2)	ZW-FAM2	0.35	0.43	1.16	0.45
A2 (ZW) (FAM2)	ZW-FAM2	0.44	0.61	0.52	1.01
A1 (ZZ)	ZZ	1.35	1.04	0.86	1.02
A1 (ZZ)	ZZ	1.46	1.04	1.32	1.42
A1 (ZZ)	ZZ	1.49	0.95	1.27	1.93
A1 (ZZ) FAM1)	ZZ-FAM1	0.72	0.44	1.05	1.38
A1 (ZZ) FAM1)	ZZ-FAM1	0.26	0.21	0.55	0.43
A1 (ZZ) FAM2)	ZZ-FAM2	0.71	0.68	3.17	2.61
A (ZZ) (FAM2)	ZZ-FAM2	0.50	0.42	0.38	0.62
Y13 (ZW)	ZW	0.62	1.07	1.45	5.78
Y7 (ZZ)	ZZ	0.77	1.45	2.29	3.45

Analysis of gene expression comparisons

Excel was used to analyze comparisons. The t-test was used to investigate the statistical differences by comparing before and after treatment of PGC lines derived from male and female lines.

A comparison of females and males was done between CVH. DAZL. miR-92. and miR-302b-5p expressions. The expression of CVH shows a significant difference between males and females (*) (Fig. 4.5) (Table 4.9). In males, CVH expression is higher than in females. There are no significant differences in the DAZL. miR-92. and miR-302-5p expression in females and males.

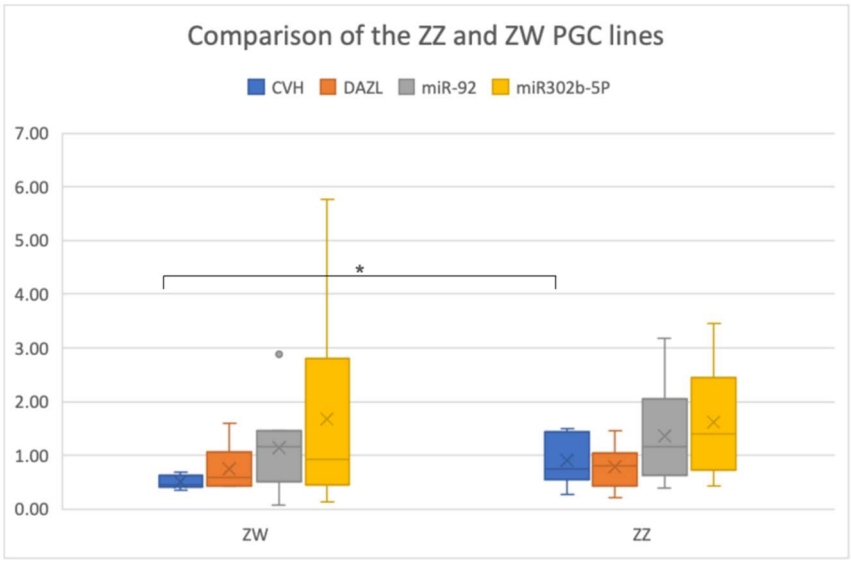

Figure 4.5 CVH, DAZL, miR-92, and miR-302b-5p expression in females and males (before freezing).

Table 4.9 P-value for gene expression (CVH. DAZL. miR-92, and miR-302b-5p) comparison between females and males.

T-probe (2.2): p-values	CVH	DAZL	miR-92	miR-302b-5P
ZW-ZZ	**0.04307899**	0.89512672	0.63840289	0.938147396

The germ cell-specific marker CVH was expressed in all the PGC lines examined. Nevertheless, the expression of CVH is higher in males than in females. CVH expression is higher in males than females because males are homogametic and females are heterogametic in chickens. Males have two ZZ chromosomes, whereas females have one Z and one W chromosome. Since the CVH gene is located on the Z chromosome, male PGCs have greater CVH gene expression than females (Lázár et al.. 2021b).

The expressions of CVH, DAZL, miR-92, and miR-302-5p were compared between; before freezing-FAM1, before freezing-FAM2, and FAM1-FAM2 (without considering gender). In Figure 4.6. DAZL expression shows a significant difference before freezing and after FAM1 treatment (***) and before freezing and after FAM2 treatment (**) (Table 4.10). The DAZL expression is decreased after FAM1 and FAM2. No significant differences exist in the

expression of CVH, miR-92, and miR-302-5p before and after freezing media. There is no significant difference in the expression of DAZL, CVH, miR-92, and miR-302-5p between FAM1 and FAM2.

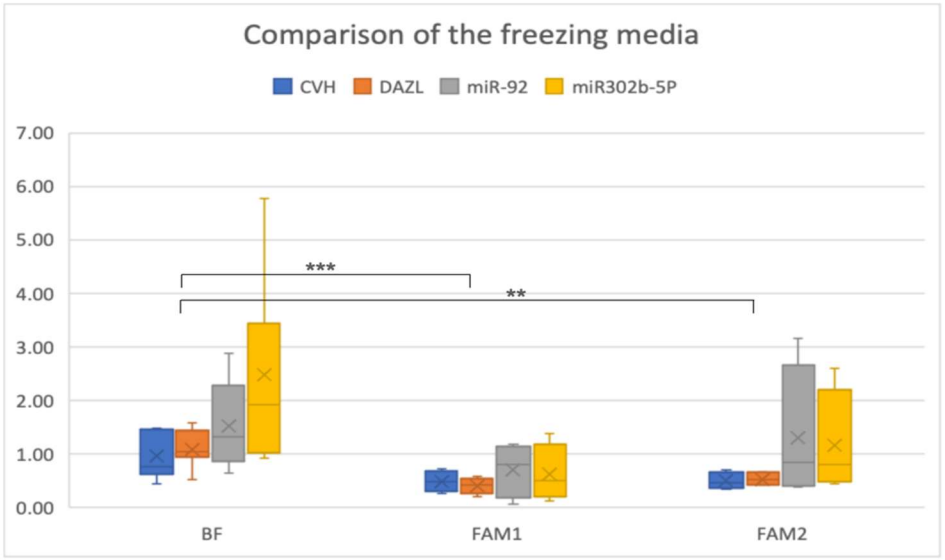

Figure 4.6 CVH, DAZL, miR-92, and miR-302b-5p expression before and after freezing in PGCs.

Table 4.10 P-value for gene expression (CVH, DAZL, miR-92, and miR-302b-5p) comparison between PGCs before and after freezing (FAM1 and FAM2).

T-probe (2.2): p-values	CVH	DAZL	miR-92	miR-302b-5P
Before freezing -FAM1	0.072	**0.006**	0.098	0.072
Before freezing -FAM2	0.072	**0.014**	0.723	0.205
FAM1-FAM2	0.942	0.286	0.423	0.373

The expression of CVH, miR-92, and miR-302-5P in both females and males didn't show any significant differences after treatment with FAM1 and FAM2. This suggests that both FAM1 and FAM2 are effective in maintaining the integrity of the cells during freezing stress. On the other hand, DAZL expression seems to be affected by the freezing procedure.

The expression of CVH, DAZL, miR-92, and miR-302-5p were compared among the following groups; Females before and after freezing with FAM1. Females before freezing and after freezing with FAM2. Males before freezing and after freezing with FAM1. Males before freezing and after freezing with FAM2.

In males, CVH expression shows a significant difference (*) (Table 4.12). CVH expression is higher before freezing than after treatment with FAM1 (Figure 4.7A). DAZL expression is higher before freezing than after the treatment with FAM1 (**) and FAM2 (*) (Figure 4.7B) (Table 4.12).

The significance level; no significant differences exist in the expression of miR-92 and miR-302-5p before freezing and FAM1 and FAM2 in males (Figure 4.7CD).

There was no significant difference in CVH, DAZL, miR-92, and miR-302-5p expression in females related to the freezing media treatments (Table 4.11).

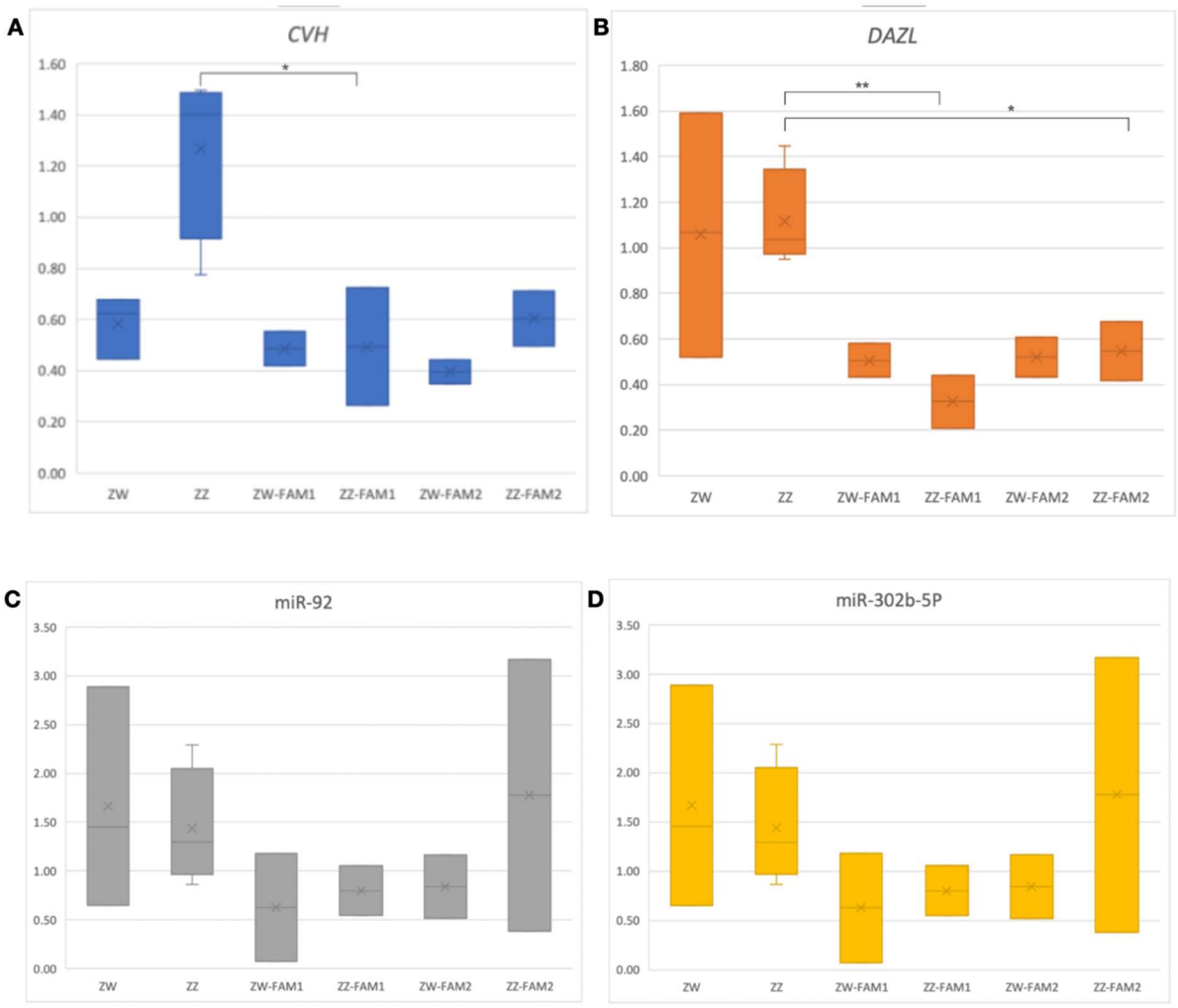

Figure 4.7 A. Comparison of CVH expression before and after freezing in females and males.

B. Comparison of DAZL expression before and after freezing in females and males.

C. Comparison of miR-92 expression before and after freezing in females and males.

D. Comparison of miR-302b-5P expression before and after freezing in females & males.

Table 4.11 P-value for gene expression (CVH, DAZL, miR-92, and miR-302b-5p) comparison before and after freezing in females.

T-probe (2.2): p-values	CVH	DAZL	miR-92	miR-302b-5P
ZW-ZW-FAM1	0.424	0.264	0.351	0.224
ZW-ZW-FAM2	0.150	0.275	0.418	0.276
ZW-FAM1--ZW-FAM2	0.385	0.919	0.771	0.427

Table 4.12 P-value for gene expression (CVH, DAZL, miR-92, and miR-302b-5p) comparison before and after freezing in males.

T-probe(2.2): p-values	CVH	DAZL	miR-92	miR-302b-5P
ZZ-ZZ-FAM1	**0.055**	**0.012**	0.255	0.282
ZZ-ZZ-FAM2	0.063	**0.037**	0.743	0.754
ZZ-FAM1--ZZ-FAM2	0.707	0.330	0.562	0.582

In the previous study with male and female PGC lines where freezing with 8% DMSO was used. The expression of CVH was detected after freeze-thawing using qPCR analysis (Lázár et al.. 2021b). Research on long-term PGC culture and cryopreservation found that germline-specific genes (markers) maintained the expression of genes like CVH and DAZL after long-term culture (151–540 days) and cryopreservation. This discovery is when they utilize two distinct combinations of DMSO concentrations of 5% (v/v) and 10% (v/v) and two Fetal Bovine Serum (FBS) concentrations of 20% (v/v) and 50% (v/v) (Tonus et al.. 2016). This suggests that cryopreservation media, including high and low DMSO media, could preserve germline competency.

This study focuses on the expression of miR-92 and miR-302b-5P because these miRNAs are indispensable stem cell-specific miRNAs. The miRNA cluster, miR-302, is an evolutionary conserved and vertebrate-specific (Gao et al.. 2015). While miR-92 is related to important functions such as cell development and aging in normal cells. Anormal expression is seen in cardiovascular and immune diseases. The miR-92 expression is present and somehow constant; due to its expression, miR-92 is used as a marker and sometimes as a housekeeping gene (Mogilyansky & Rigoutsos. 2013; Huang et al.. 2019).

It has been proved that miRNAs play a role in regulating stem cells and maintaining pluripotency (Lázár et al.. 2018). It was revealed that the higher expression of miR-302b-5p on high proliferating PGC lines compared to miR-302b-3p. And the low proliferating PGC lines showed higher expression of miR-302b-3p than miR-302b-5p (Lázár et al.. 2018).

According to Lazar Bence and his colleagues, the importance of miR-302 is proved by inhibiting the expression of gga-miR-302b-5P and gga-miR-302b-3P. The Inhibited PGC lines show a reduced proliferating rate (Lázár et al.. 2022).

4.11 FREEZING MEDIA

In this study, two freezing media with different concentrations of DMSO were used to freeze PGC lines. In FAM1, 800 µL (16%) DMSO, 500 µL chicken serum (8.5 %), 20 µL Sodium pyruvate, 15 µL $CaCl_2$, and 3665 µL AKODMEM were used. FAM2 included 1000 µL DMSO (20%) and 4000 µL (80%) FBS. The final concentration of FAM1 and FAM2 was 8% and 10%, respectively, when the freezing medium was added to the cells. Accordingly, a significant difference is found in the expression of DAZL before freezing and after freezing with FAM1 and before freezing- after freezing with FAM2. The two similar results of FAM1 and FAM2 could be due to freezing media components. Therefore, this could be the reason for the lower expression of DAZL in FAM1 and FAM2. Nevertheless, the other expressions seem to be protected by the freezing media. Abrahamsen and his colleagues reported post-thawing recovery of chicken PGCs in a medium containing 5% DMSO concentration. They suggested using more than 10% DMSO has a toxic effect in long-term culture PGCs (Abrahamsen et al.. 2002). Another study used different combinations of two DMSO concentrations (5% v/v and 10% v/v) and two FBS concentrations (20% v/v and 50% v/v). A medium containing low DMSO (5%) concentrations showed a significant result compared to high DMSO (10%) concentrations. And they reported that increased FBS concentrations in low DMSO media resulted in high PGC viability. The study suggested using 50 % FBS and 5 % DMSO for further use when they found 92% PGC viability rates after the freeze-thawing method (Tonus et al.. 2016).

Research demonstrated that cryopreserving PGCs using a solution containing 10% DMSO and 90% FBS resulted in the effective expression of genes related to gamete formation, including germline-associated markers (chicken DAZL) and PGC-specific markers (SSEA-1) (Kong et al.. 2018).

5 CONCLUSIONS AND RECOMMENDATIONS

This study analyzed miRNA (miR-92 and miR-302b-5P) expression and markers (CVH and DAZL) expression profiles of male and female chicken PGCs lines before and after the freezing treatments through RNA isolation, cDNA writing, and qPCR analysis.

Fifteen embryos (3 days old) were used to isolate circulating PGCs, of which 3 PGC lines, one male and two female, were successfully established after three weeks of culture with a culture medium. Sex determination was possible after the DNA isolation from the tissue of an embryo using W chromosome-specific primers.

Freezing of PGCs could be possible under the careful attention of the combination of freezing media components. This research experiment customized the existing freezing media protocol to recommend a proper freezing media by using a different combination of components and concentrations that can minimize the effect of freezing media on PGCs. As a result, that can reduce the expenses of freezing media components as it is costly. The research found viable PGCs after freeze-thawing.

The experiment compared male and female PGC lines for the expression of germline genes and stem cell-specific miRNAs before and after freezing treatments to find a proper freezing medium for PGC conservation. The downregulation generated by the FAM1 and FAM2 is acceptable. FAM2 includes the components DMSO and fetal bovine serum (FBS). It seems to be an optimal medium for PGC freezing, demonstrating that it can be used in PGC freezing in other avian species. Further research is needed to customize the freezing media approach by freezing PGCs in other chicken breeds.

The research describes how the two freezing treatments affect the expression of germ cell-specific markers (CVH and DAZL) compared to the expression of markers before freezing. The germ cell markers and stem cell-specific miRNAs maintain the stemness of PGCs. The GAPDH, the housekeeping gene, was used as a reference gene for the markers and miRNAs in the qPCR analysis. The expression of CVH is higher in males than in females before freezing. However, it showed a drastic decline in the expression of CVH after freezing treatment with FAM1 in males. Before freezing, DAZL is expressed highly compared to FAM1 and FAM2. In addition, in males, DAZL expression decreased after both treatments.

The analysis could be done by contrasting them with untreated PGC lines. Nevertheless, FAM1 is not suitable for other avian species.

Avian gene banks are important for several biotechnology applications; FAM1 is already known as a good freezing medium for the genetic cryopreservation of PGC lines in chickens. FAM2 freezing procedure could be an appealing answer to the problem of maintaining the stemness for the conservation of PGCs for genetic material in avian species.

6 SUMMARY

Primordial germ cells are the precursors of functional gametes. Semen cryopreservation is the most practical method for preserving genetic resources in birds, but it only conserves the male genome. Embryo and oocyte cryopreservation would retain the W chromosome but is impractical due to the nature of the egg (large yolk). Because of all these disadvantages of semen and embryo preservation, PGC storage could become the most important in avian species. PGCs from chickens can be isolated, cultured, and cryopreserved and remain committed to the germ line.

MicroRNAs (miRNAs) are small noncoding RNAs, about 22 nucleotides long, that target mRNAs for translational repression or degradation. Stem cell-specific miRNAs are important in chicken primordial germ cells (PGCs) because they regulate gene expression during PGC development. PGCs are the cells that give rise to the germ cells in the adult chicken, and they undergo a series of developmental changes as they differentiate and migrate to the gonads.

This study aims to determine the expression of CVH, DAZL, miR-92, and miR-302b-5P before and after freezing to test two freezing media for long PGC conservation. PGCs were established from a 3-day-old chicken embryo blood sample by isolating, culturing, and expanding PGCs in vitro. PCR sexing was used to determine the embryo's gender after extracting DNA from the isolated tissue. RNA samples were extracted from male and female PGC cell lines before and after freezing with two different avian-freezing media. cDNA was synthesized from the RNA samples to examine the expression levels of CVH, DAZL, miR-302b-5p, and miR-92 via qPCR. GenEx software analyzed the Ct (cycle threshold) values obtained from the qPCR machine. By examining gene expression levels, the study aimed to determine the effects of freezing on PGCs' gene expression and identify the most suitable freezing medium for PGC storage.

The study compared the expression of marker genes and miRNAs in male and female samples. The results showed a significant difference (P = **0.043)** in the expression of CVH between males and females, with higher expression in males. Conversely, there were no significant variances in the expression of DAZL, miR-92, and miR-302b-5p between the two genders.

In addition, a Comparison was done on the expression of four genes in three groups of samples without considering gender. The groups were "Before freezing-FAM1", "Before freezing-FAM2", and "FAM1-FAM2". The result showed significant differences in DAZL expression

before and after FAM1 treatment (**p = 0.006**) and before and after FAM2 treatment (**p = 0.014**). DAZL expression was significantly decreased after FAM1 and FAM2 treatments. However, there were no significant variances in the expression of CVH, miR-92, and miR-302-5p among the three groups.

Furthermore, the study compared the expression of CVH, DAZL, miR-92, and miR-302-5p among four groups: females before freezing and after freezing with FAM1, females before freezing and after freezing with FAM2, males before freezing and after freezing with FAM1, and males before freezing and after freezing with FAM2. The study found that in males, CVH expression was significantly higher before freezing than after treatment with FAM1 (**P = 0.055**). Meanwhile, DAZL expression was higher before freezing than after treatment with FAM1 (**P = 0.012**) and FAM2 (**P = 0.037**). However, no significant difference was detected in the expression of miR-92 and miR-302-5p before freezing and after treatment with FAM1 and FAM2 in males. In females, no significant differences related to the freezing media treatments were observed in the expression of CVH, DAZL, miR-92, and miR-302-5p.

FAM2, including DMSO and fetal bovine serum, was the optimal medium for PGC freezing. The expression of CVH is higher in males than in females before freezing. DAZL expression was higher before freezing than after FAM1 and FAM2 treatments in males. The study suggests that the FAM2 freezing procedure could be an appealing answer to the problem of maintaining the stemness for conserving PGCs for genetic material in avian species.

7 ACKNOWLEDGMENT

I thank the almighty **God** for his unwavering grace, blessings, and guidance throughout my academic journey. I am profoundly thankful to my **parents** for instilling a love for learning and making countless sacrifices to provide me with the best education possible.

First and foremost, my heartfelt gratitude and appreciation to my supervisor, **Dr.Elen Gocza**, leader of the Applied Embryology and Stem Cell Biology Group, for her invaluable guidance, support, and feedback throughout the research process. Her skill and knowledge in the field have been invaluable in shaping the direction of this work; I am forever grateful. Her dedication to fostering a positive and productive research environment and the collaborative spirit she has cultivated has been an inspiration for me and significantly impacted my overall experience. Her kindness and generosity will remain everlasting, and I will apply the lessons I have learned from her throughout my academic and professional journey.

My heartfelt gratitude goes to my independent consultant, **Maria Teresa Salinas Aponte**, who provided invaluable assistance and guidance in the laboratory throughout my MSc thesis. Her expertise and guidance were invaluable in helping me to refine my research methodology, analyze my data, and interpret my findings. Her generosity and support mean so much to me, and I am deeply grateful for all she has done. I feel fortunate to have had the golden opportunity to work with such a brilliant and supportive consultant.

I am grateful to **András Ecker** for his priceless help in isolating, culturing, establishing, and immunostaining the PGC lines utilized in this study. His skills and commitment played a significant role in securing the successful completion of these critical aspects of the project.

I acknowledge the **Project: RRF-2.3.1-21-2022-00007; 2019-2.1.11-TÉT-2019-00036** for providing the funding and resources that made this research possible.

I am deeply grateful to the Embryology and Stem Cell Biology Research Group members for their support and contributions to this thesis. Their dedication, skill, and collaborative spirit have been helpful in the completion of this research. The research work would not have been possible without the collective effort of the Embryology and Stem Cell Biology Research Group.

I would like to thank the department coordinator **Dr. Varga László** and all the Department of Agricultural Biotechnology staff members for their invaluable support and dedication in giving a lecture during my studies. Additionally, I am thankful to the **Stipendium Hungaricum**

Scholarship Program (SH) for providing me with a wonderful opportunity to pursue my education in Hungary.

Non-educational coordinator **Mrs. Judit Tallárомné Czingili** and faculty's Registrar coordinator **Nagy Andrea**, and **Kinga Püspök-Szabados** (Former), International Student Coordinator MATE Dormitory Gödöllő **Ms. Bea Karpati** deserve great appreciation.

Last, I would like to extend my deepest gratitude to my friends who have supported me throughout this journey.

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STUDENT DECLARATION

Signed below, **Afewerki Yosief Alem**, student of the Szent István Campus of the Hungarian University of Agriculture and Life Science, at the MSc Course of **Animal Biotechnology** declare that the present Thesis is my own work and I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page-summary of my thesis will be uploaded on the website of the Campus/Institute/Course and my Thesis will be available at the Host Department/Institute and in the repository of the University in accordance with the relevant legal and ethical rules.

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