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The Effect of mycotoxin treatment in domestic chicken PGC Cultures

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Abbreviations and Acronyms

ALP	Alkaline Phosphatase
AMPK	AMP-Activated Protein Kinase
Arthur	Arthur Fluorescence Cell Analyzer
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
bFGF	Basic Fibroblast Growth Factor
CAFs	Cancer-Associated Fibroblasts
cDNA	Complementary DNA
CNS	Central Nervous System
CO_2	Carbon Dioxide
CVH	Chicken Vasa Homolog
DAPI	4′,6-Diamidino-2-Phenylindole
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FCF5	Female Chicken Cell Line
FCM5	Male Chicken Cell Line
FITC	Fluorescein Isothiocyanate
FM/ZZ	Male Genetic Marker in Birds
FM/ZW	Female Genetic Marker in Birds
FUCCI	Fluorescent Ubiquitination-based Cell Cycle Indicator
G1/G2/S	Phases of the Cell Cycle
GFP	Green Fluorescent Protein
GGT	Gamma-glutamyltransferase
GSH	Glutathione
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIF-1α	Hypoxia-Inducible Factor 1 Alpha
HPLC	High-Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-6	Interleukin-6
LC-MS	Liquid Chromatography-Mass Spectrometry
LDH	Lactate Dehydrogenase
LPO	Lipid Peroxidation
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MEM	Minimum Essential Medium
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
mAG1	Monomeric Azami Green 1
mKO2	Monomeric Kusabira Orange 2
MMP	Matrix Metalloproteinase
MTT	Methyl Thiazolyl Tetrazolium
NADH	Nicotinamide Adenine Dinucleotide
NGS	Next-Generation Sequencing

OD	Optical Density
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PGC	Primordial Germ Cell
Pico	ImageXpress Pico Cell Imaging System
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR-Restriction Fragment Length Polymorphism
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-qPCR	Real-Time Quantitative PCR
SBRI	Stem Cell and Brain Research Institute
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
SSEA-1	Stage-Specific Embryonic Antigen 1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBST	Tris Buffered Saline with Tween
TEM	Transmission Electron Microscopy
TGF-β	Transforming Growth Factor Beta
T2 Toxin	Mycotoxin Type 2
TO-PRO™-3	Fluorescent Dye
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UPR	Unfolded Protein Response
UV	Ultraviolet Light
UV-Vis	Ultraviolet-Visible Spectroscopy
VPA	Valproic Acid
ZZ/ZW	Male and Female Sex Chromosomes in Birds
β-ME	Beta-mercaptoethanol

1. Introduction

The progenitor cells that give birth to gametes, or sperm and eggs, are known as primordial germ cells (PGCs), and they are essential to the reproductive biology of creatures that reproduce sexually. The capacity of these cells to pass genetic information from one generation to the next, maintaining the continuation of the species, makes them special. During the early phases of embryogenesis, PGCs are initially detectable in avian species such as domestic chickens. They can go to the developing gonads, where they will eventually undergo differentiation to become adult gametes. Understanding the development, differentiation, and genetic inheritance mechanisms of germ cells require a thorough understanding of PGCs.

PGCs are distinguished from somatic cells throughout the early stages of development by unique genetic and epigenetic programming that directs their migration and differentiation. Wylie (1999) states that PGCs go through carefully regulated procedures, including moving from the epiblast to the gonadal ridge, where they proliferate and, depending on the sex of the organism, mature into either sperm or eggs.

There are multiple reasons why understanding PGC development and function are important. PGCs serve as a paradigm for studying the mechanisms of cell fate decisions, cell-cell signalling, genetic and epigenetic regulation. They are used to explore some of the fundamental mechanisms of cell differentiation and development in developmental biology (Nakamura et al., 2007). Pathological studies of PGCs in reproductive medicine will contribute to the development of new therapeutic strategies for infertility treatment, and the transplantation and manipulation of germ cells to protect endangered species. The significance of PGCs is reflected on genetic engineering and breeding programs on agricultural biotechnology especially for poultry. These enable generation of transgenic lines with valuable characteristics such as faster growth rate, disease resistance and efficient egg production (Nakamura et al., 2007). In addition to their role in genetic structure, PGCs also provide a unique perspective on mechanisms of evolutionary change, as they play a central role in generating and maintaining genetic diversity in populations.

The Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) is a groundbreaking device that enables real-time imaging of cell cycle dynamics in living cells. This technique

makes use of two separate fluorescent proteins that are destroyed differently during the cell cycle, allowing cells to be tracked through several phases (G1, S, G2, and M) depending on their fluorescence manner. In FUCCI, cells in the G1 and S phases produce red fluorescence, while cells in the S/G2/M phases emit green fluorescence (Sakaue-Sawano et al. 2008).

The use of FUCCI to examine PGCs has numerous notable advantages. FUCCI enables continuous monitoring of PGCs in live cultures, offering dynamic insights into cell cycle development and the consequences of diverse therapies (Zielke and Edgar, 2015). It is a non-invasive technology that does not involve the killing of cells, making it excellent for long-term investigations of cell proliferation and differentiation. FUCCI allows researchers to precisely identify the timing and control of cell cycle phases in PGCs, which is especially important for understanding how external variables like toxins or environmental stressors affect cell cycle dynamics and overall cell health.

They are toxic secondary metabolites of fungi that are able to contaminate the feed of poultry, posing a serious risk to the health and productivity of laying hens. Aflatoxins, ochratoxins, fumonisins, etc., are some of the toxins causing immunosuppression, cancer, and reproductive toxicity (Zain, 2011). Nonetheless, mycotoxin effect research on PGCs has a massive importance due to the necessity of these cells in chicken reproduction and genetics.

Several important pathways are implicated in mycotoxin toxicity in PGCs. Mycotoxins induce oxidative stress resulting in increased production of reactive oxygen species (even ROS) by the cells, since they can damage cell components, and thus impairing PGC viability and functionality, leading to decreased fertility and transgenerational genetic defects (Richard, 2007; Voss et al., 2021). Additionally, mycotoxins [including aflatoxins] can produce DNA adducts, causing mutations and impairing DNA replication and DNA repair mechanisms, which in turn can cause PGC cell cycle arrest, apoptosis or oncogenic modification compromising the integrity and function of PGCs (Amezqueta et al., 2012).

In addition, exposure to mycotoxin may induce the apoptosis of PGCs by activating specific signalling pathways. This significantly reduces the number of functional germs cells and affects reproductive functions in hens (Bouhet & Oswald, 2005).

The use of FUCCI to study mycotoxins effects on PGCs provides a tool for the high-throughput analysis of cell cycle dynamics and cellular response to toxin exposure during real-time. When tagged with FUCCI, not only can researchers visualize changes in cell cycle progression, but

they can also determine the extent of cell cycle arrest and identify time windows when mycotoxins induce damage to PGCs.

Effects of mycotoxins on PGCs may serve a larger purpose in food safety research and the development of policies and interventions aimed at reducing mycotoxin exposure in the food chain. The adverse impacts of mycotoxins on animal health and risk to human health through consumption of contaminated poultry products (Zain, 2011)

Moreover, experience based on research of mycotoxin mediated damage in PGCs can be extrapolated to other species, including humans and hence this study will ultimately prove beneficial for the improvement of human health and animal health through development of further toxicology studies and public health campaigns to reduce the level of exposure to mycotoxins and other environmental toxins (Richard, 2007).

1.1. The objectives

The primary objective of my thesis is to investigate the effects of cell culture conditions on male (FCM5-ZZ) and female (FCF5-ZW) FUCCI reporter system-expressing PGC lines. Utilizing data derived from the Arthur cell counting machine, I aim to compare the cell cycle phases of FCF5 and FCM5 PGC lines, distinguishing between high-quality and low-quality PGC cultures based on their in vitro integration capacity.

In addition, I will analyse data from the Pico Cell Image Analyzer system, provided by my supervisor, to compare the doubling times of the male (FCM5-ZZ) and female (FCF5-ZW) PGC lines. This analysis will include an examination of cell cycle alterations induced by T2 mycotoxin treatment.

Ultimately, I will conduct a more detailed analysis using the most effective concentration of T2 mycotoxin identified through my experiments.

The FUCCI system offers important insights into the development and differentiation of cells, particularly primordial germ cells (PGCs). Overall, this study not only enhances our understanding of germ cell biology but also has broader implications for environmental safety, animal husbandry, and public health. By elucidating the cellular responses to mycotoxins, researchers can contribute to the development of safer agricultural practices, improved food security, and healthier livestock populations.

2. Literature Review

2.1. Chicken Embryo Development

2.1.1. Key Stages of Chicken Embryo Development: From Fertilization to Hatching

Embryogenesis is one of the most complicated and complex processes in chickens, which covers a cycle of 21 days, from fertilization to the period when a chick breaks an eggshell. During this period, the embryo is known to undergo several stages that are well outlined and very important for the development of various body parts and their respective functions. At this process, what amazes me most is the organogenesis, the development of organ systems, and the integration of some physiological processes such as thermoregulation and hatching coordination. Very interesting is the fact that the embryo does not only grow but is growing in an environment in which it integrates internally and in a much more fascinating manner, interfaces externally with the external environment. This ability to adapt is very important in ensuring that the chick will be able to survive after it hatches (Molenaar et al., 2010).

The development process begins immediately after fertilization with cleavage divisions that lead to the creation of the blastoderm. (Fig. 1.) By the end of the first day, this blastoderm is already very active. Around 44 hours into the process, the heart begins to beat, and the vascular system starts to work, circulating blood throughout the growing embryo (Sellier et al., 2006). The primordial stripe occurs by day two, which is an important development since it serves as the basis for the body's central axis. Soon after, somites begin to form. These somites are significant because they eventually produce the muscles and vertebrae that will support the chick's body after hatching (Hamburger and Hamilton, 1992).

Between days 4 and 10, the speed of development accelerates significantly. On day 5, limb buds for the wings and legs begin to grow, and other essential organs take form. Around day 7, they notice the development of feather buds, as well as the auditory pit, which is an early evidence of the chick's sensory system activation (Eyal-Giladi and Kochav, 1976). By day 10, the beak begins to stiffen, and the toes are fully formed, demonstrating how quickly the embryo develops from a simple cluster of cells to a recognizable bird (Rebillard and Pujol, 1983). This stage of organ and limb development is crucial not only for physical structure, but also for functions that the chick will require just after hatching.

From day 11 to 18, the embryo prepares to hatch. Around day 14, the embryo begins to arrange itself for hatching by moving its head toward the big end of the egg. This is an important phase

because the chick will use its head and beak to burst through the shell when the time comes (Mellor and Diesch, 2007). Interestingly, rapid-eye-movement (REM) sleep begins around day 15, indicating that the brain is not only developing but also becoming active in a way that shows a level of perception. By day 12, the cochlea—the auditory part of the inner ear—has matured enough for the embryo to perceive external noise. This is especially essential since it allows the embryos to interact with one another via sound, therefore synchronizing their hatching dates (Jones et al., 2006). It's intriguing to imagine that even before they hatch, these embryos are conversing with one another, arranging their entry into the world.

The final stage of development occurs between days 19 and 21. On day 19, the chick begins the process of internal pipping, which involves puncturing the air sac within the egg with its beak. This movement enables it to begin breathing using its lungs, a vital step from chorioallantoic membrane-dependent oxygenation to complete pulmonary respiration (Menna and Mortola, 2002).

After the chick starts breathing on its own, it goes into a resting phase until day 20, when it starts the more dramatic process of external pipping. On day 21, the chick ultimately emerges from the egg after breaking through the eggshell with the help of its beak and neck muscles (Tzschentke and Plagemann, 2006). It's difficult not to be surprised by the complexity of this process—the chick, which was only an embryo days ago, now possesses the power and coordination necessary to hatch by itself.

During this period, several significant functional milestones are reached. The development of thermoregulation, which begins on day 15, is one of the most significant. By day 20, the chick has developed the ability to control its body temperature in reaction to its surroundings, a skill that is essential to its survival when it is outside of the egg (Tzschentke, 2008). Approximately concurrently, the auditory system achieves complete functionality. The embryo can hear external sounds by day 12, which plays a function in helping it synchronize its hatching timing with other embryos in the clutch (Friauf and Lohmann, 1999). The chicks will have a higher chance of surviving if they hatch at around the same time thanks to this synchronization, which is essential.

Hormones have a major role in hatching, in addition to physical prowess and coordination. For instance, the thyroid hormones, more especially T3 and T4, are crucial in controlling the metabolic processes of the developing chick before hatching. According to (Reyns et al., 2003), these hormones reach their peak right before hatching and are crucial for the switch to lung

breathing. Another key hormone is corticosterone, which increases dramatically around day 20. According to shell (Decuypere et al., 1991), this hormone aids in the hatching process by coordinating the last development and movements the chick must make in order to escape its shell. The balance and timing of these hormone messages are critical for ensuring that the chick is robust enough and prepared to hatch properly.

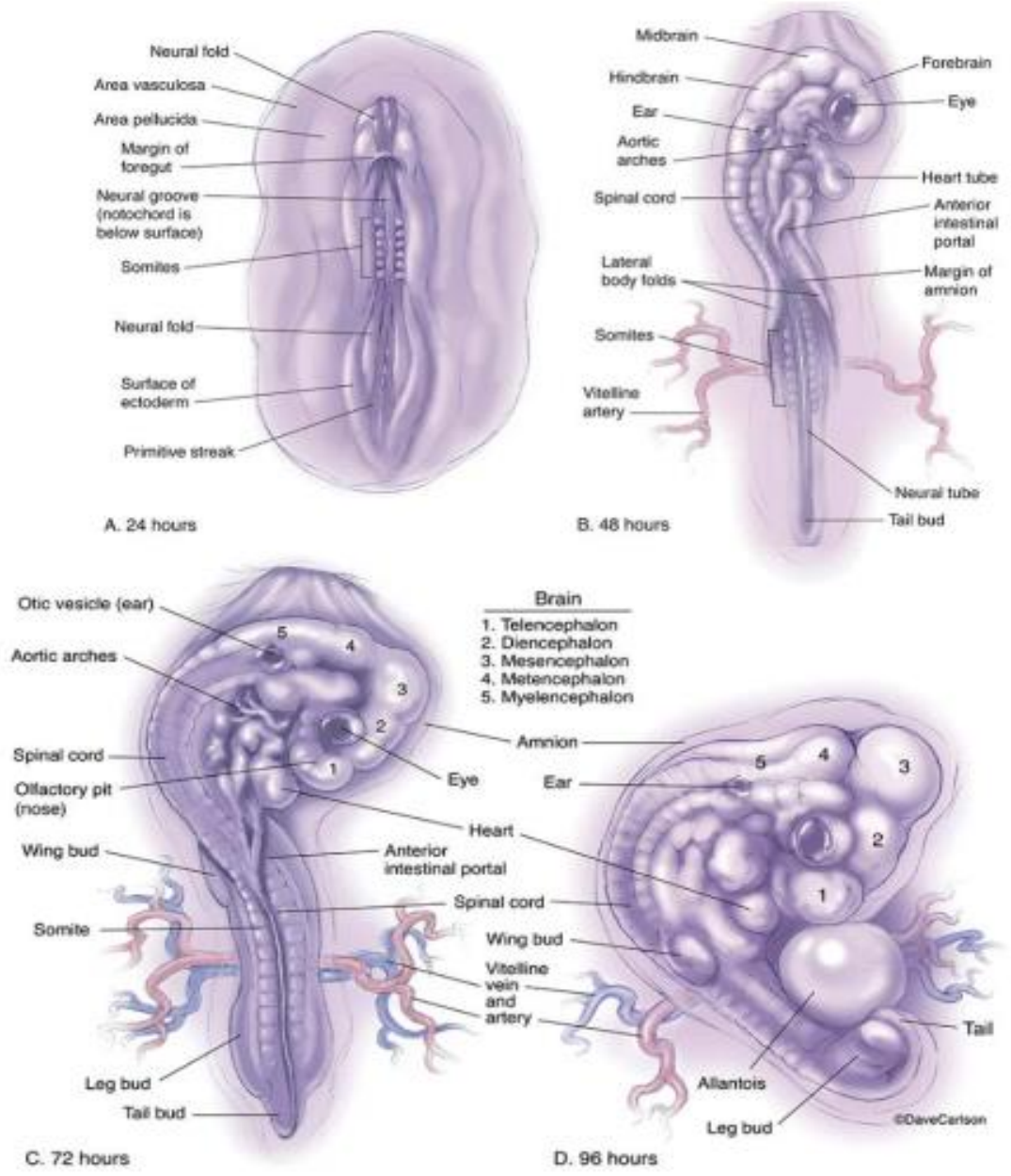

Figure 1. The Early Stages of Chicken Embryo Development

Carlson, Dave. Chicken Embryo Embryonic Development Illustration. Carlson Stock Art. Accessed 9 Sept. 2024. https://www.carlsonstockart.com/photo/chicken-embryo-embryonic-development-illustration/

2.2. An Overview of Primordial Germ Cells

2.2.1. The Importance of PGCs in Chickens

Primordial germ cells (PGCs) from chickens are essential for the genetic conservation and maintenance of avian species, especially when it comes to rare and native breeds. Their importance stems from their capacity to preserve and renew genetic material, making them indispensable for germline transmission, cryopreservation, and gene banking. PGCs are the ancestors of the germline cells that mature into adult chicken sperm and ova. Because of this, they are crucial for maintaining genetic variation, particularly in birds where sperm storage alone is unable to retain the mitochondrial DNA and the W chromosome, which is exclusive to females. PGCs provide a solution to this difficulty, guaranteeing that both male (ZZ chromosomes) and female (ZW chromosomes) genetic resources are preserved (Blesbois, 2007). Because of these two qualities, PGCs are essential for preserving the whole genetic range of rare or endangered breeds. This is especially true for native chicken breeds, which would otherwise lose their distinctive genetic characteristics because of interbreeding or extinction.

Cryopreserving PGCs has shown to be a significant advance in the field of bird conservation. PGCs from six Hungarian indigenous chicken breeds were effectively cryopreserved with derivation rates ranging from 28% to 50% (Figure 2). The genetic material of these breeds may be maintained eternally and used for future breed regeneration thanks to these frozen PGCs, which are kept in a gene bank. This technique is especially significant since it makes it possible to store genetic material for a long time without the need for ongoing breeding populations, which may be expensive and challenging to maintain. Thus, PGC cryopreservation is an effective, dependable, and long-term strategy for gene banking (Lazar et al., 2021).

The function of PGCs in breed regeneration may be its most important use. PGCs were cryopreserved from the Partridge-colored Hungarian chicken breed and subsequently implanted into host embryos that were Black Transylvanian Naked Neck. Through this method, germline chimeras were produced, which were then utilized in breeding to bring back the original Partridge-colored Hungarian breed. According to Taijima et al. (1993) and Naito et al. (1994), the study revealed a 5% recovery rate of pure-bred Partridge-colored chickens, proving that PGC technology may even bring back a breed that is on the verge of extinction. The ability of PGCs to regenerate a breed from stored genetic material highlights their importance in protecting biodiversity and maintaining uncommon and threatened chicken breeds. PGCs offer a potent tool for genetic study and modification in addition to their function in

conservation. Before reintroducing PGCs into host embryos, researchers can genetically alter them through in vitro culture. With this capacity, chickens may now be genetically modified to introduce features that increase production or resistance to illness. The work underlines the relevance of this technique for developing avian genetics and aiding future breeding efforts. PGCs are very helpful for research and real-world uses in chicken breeding and conservation since they may be altered in a lab before being reinjected into embryos (van de Lavoir et al., 2006).

PGCs play a critical role in maintaining the distinctive genetic characteristics of native chicken breeds. It was possible to effectively cryopreserve PGCs from six Hungarian breeds, including the Yellow, White, Speckled, and Partridge-colored Hungarian chickens. Because these breeds have spent decades adapting to their unique habitat, they have great genetic and national significance. By using PGC cryopreservation to preserve these native breeds, it is certain that

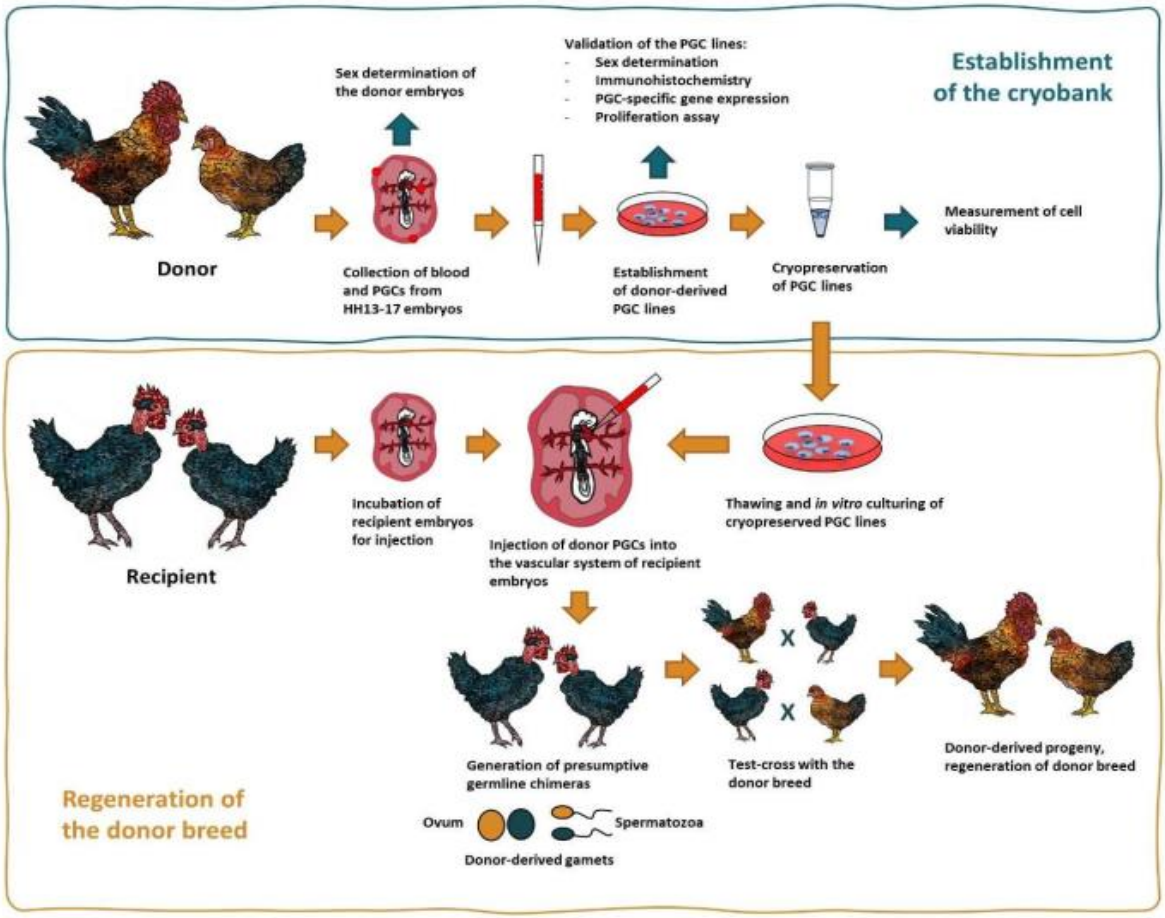

Figure 2. Illustrates the process of producing donor-derived hatchlings from cryopreserved indigenous PGC lines (Lázár et al., 2021a)

The larger context of biodiversity loss is often used to highlight the significance of PGCs. The utilization of PGCs presents a workable alternative to genetic variety conservation, as 28% of bird breed populations are now endangered or extinct worldwide. Endangered breeds can be preserved and regenerated even if their existing populations diminish or vanish by the storage and reinjection of PGCs. This technique protects unique breeds from genetic extinction and guarantees their survival for next generations (FAO, 2019; Naito et al., 1994).

2.2.2. Migration of Primordial Germ Cells (PGCs) During Embryo Development in Chickens

Primordial germ cells (PGCs) are one of the first wave poles of differentiation to occur in chickens that ultimately gives rise to an organism's sperm or egg gametes. The origin of PGCs in chickens has an unusual aspect compared to that of other animals. Originally, they are determined in the central portion of the blastoderm (area pellucida), not coming from any embryonic tissues during early stages of embryonic development (Niewkoop & Sutasurya, 1979. This first segregation is driven by molecular signals that identify these prospective germ cells from the surrounding somatic cells, notably the WNT signaling pathways and Bone Morphogenetic Proteins (BMPs) (Nakamura et al., 2007).

By the time the embryo reaches the late primitive streak stage, PGCs have moved from their site of specification to the germinal crescent, which is situated in the anterior area of the embryo (Ginsburg & Eyal-Giladi, 1987). Active cellular processes and signals that control their location inside the embryo are what propel this migration. PGCs enter the circulation early in the embryonic development of birds, in contrast to mammals, and travel through the growing vasculature of the embryo until they reach the genital ridges, which is where the future gonads would form. A complex interaction of chemotactic signals guides the passage of progenitor cells (PGCs) from the circulation to the vaginal ridges, ensuring that the PGCs are precisely steered to their eventual destination inside the embryo (Stebler et al., 2004).

2.2.3. PGC Migration

In chickens, migration of PGCs is a tightly controlled multi-step event. After being specified in the blastoderm and having migrated to the germinal crescent, PGCs must disperse across diverse embryonic tissues on their way to the nascent gonads (Nakamura et al. 2007).

PGCs enter the vascular plexus and move via the circulatory system. This is unique compared to other organisms as during bird development PGCs overcome the role of somatic tissue and disseminate throughout the developing body via the circulatory system (Raz, 2004). In mammals they migrate from their initial location, typically in the yolk sac endoderm, toward the developing gonads through a process of directed, active migration.

Chemotaxis, or the movement of cells in response to chemical gradients, is important to migration. One of the most prominent chemotactic signals involved in PGC migration is stromal cell-derived factor 1 (SDF-1), which binds to the receptor CXCR4 expressed on PGC surfaces (Stebler et al., 2004). This signaling system is critical for directing PGCs to the developing genital ridges. Failure of this signaling system can prevent PGCs from reaching the gonads, resulting in sterility or other developmental problems (Ginsburg & Eyal-Giladi, 1987). PGCs demonstrate extraordinary adaptability as they migrate through the bloodstream, adjusting to varied surroundings while keeping their unique identity by expressing germ cell markers such as CXCR4 and POU5F1 (Oct-4) (Saitou et al., 2002).

The primordial germ cells (PGCs) initiate extravasation upon reaching the gonadal ridge, migrating from the bloodstream into the developing gonadal tissue, where they arrest and proliferate (Niewkoop & Sutasurya, 1979). Gametogenesis is also tightly coordinated and depends on this movement. It is the first step towards generating gametes (sperm in males, ova in females) and thus maintenance of the species. In the absence of this highly regulated model, PGCs could not arrive at the gonads and proper gamete development would be lost (Ginsburg et al., 1990).

2.2.4. Process, Timing, and Importance

PGC migration occurs in a certain time sequence during chicken embryogenesis. The process begins early in development, around Hamburger and Hamilton stages 3 to 4 (the first 18-24 hours of development), when PGCs are first identified in the blastoderm (Hamburger and Hamilton, 1951). By stage 7, the PGCs had moved to the embryo's forward area, known as the germinal crescent (Nakamura et al., 2007). PGCs reach the circulation via the embryonic system of blood vessels during stages 9 to 10 (about 2 days of development).

PGCs exit the bloodstream and settle in the genital ridges, which are the sites where future gonads will form, by Hamburger and Hamilton stages 17 to 18, or around day 3 of development (Hamburger & Hamilton, 1951). The PGCs now cease their migration and start multiplying

inside the growing gonads. This exact time is crucial because migration that takes place too soon or too late may result in incorrect gonad colonization or perhaps the death of PGCs throughout the process (Ginsburg et al., 1990). Such mistakes may lead to abnormal germ cell growth or sterility. It is impossible to stress how important this procedure is. PGC migration is essential for gonad development as well as preserving genetic continuity between generations (Raz, 2004). Infertility, germ cell tumors, or even the total lack of gametogenesis can result from mistakes in the migration, specification, or proliferation of PGCs (Saitou et al., 2002). When PGCs reach the gonads, the surrounding somatic cells are prepared to facilitate their differentiation into mature gametes, thanks to the precise timing.

2.3. Gene Banking in Poultry Science

To preserve the genetic variety of different chicken species for use in future breeding and conservation initiatives, gene banking is an essential technique in poultry research. In the context of contemporary poultry breeding, where selective breeding has significantly reduced the gene pool, especially in commercial breeds created for qualities like high egg production or quick growth, this practice is especially crucial (Poultry Science Association, 2011). Poultry populations have lost genetic variety because of years of rigorous selection for a small number of characteristics, leaving them more susceptible to illnesses, alterations in their habitat, and other stresses.

In poultry gene banking, sperm, ova, embryos, and primordial germ cells (PGCs) are cryopreserved at ultra-low temperatures, often liquid nitrogen (-196°C), to be stored permanently. Cryopreservation aids in the conservation of important genetic material while maintaining its viability for future use (Seigneurin & Blesbois, 2015). PGCs are very important for gene banking because they can restore whole populations when transplanted into host embryos. This method is especially useful for protecting rare or endangered poultry breeds by preserving genetic material for future reconstitution (Nakamura et al., 2010).

2.3.1. Purpose of Gene Banking

The fundamental goal of gene banking is to conserve genetic material for later use in breeding, research, and conservation. Gene banking is an important technique for assuring the long-term survival of desirable features, given the fast loss of genetic diversity caused by selective

breeding and changing environmental variables. One of the primary functions of gene banks is to preserve genetic variation, which is critical for poultry populations to respond to unexpected challenges like disease outbreaks or climate change (FAO, 2007).

Furthermore, gene banks offer protection against the disappearance of uncommon or threatened chicken breeds. It is conceivable to reestablish these breeds using cutting-edge reproductive technologies like artificial insemination, in vitro fertilization, or germline transmission via PGC transplantation by preserving genetic material, including sperm, ova, embryos, and PGCs (Kemp, 2005). Additionally, gene banks provide as a storehouse for desirable qualities that selective breeding may have eliminated, enabling the restoration of traits like heat tolerance or disease resistance into contemporary chicken lines (chicken Science Association, 2011). Finally, gene banks are an invaluable resource for genetic research, helping the development of novel biotechnologies like gene editing as well as the study of genetic features, disease resistance, and the evolutionary history of species.

2.3.2. Importance of PGCs in Gene Banking

Primordial germ cells (PGCs) serve important functions for gene banking for poultry. This is because these cells are precursors of sperm and ova and can populate entire poultry stocks again in the future. This use of PGCs in gene banking encompasses possible preservation of both male and female gametes. The aim may be achieved as the frozen PGCs are implanted into the recipient embryos, where migration into developing gonads follows before ultimately developing into sperm or ova that would help restore the species (Nakamura et al., 2010).

PGCs' potential to become either male or female gametes is one clear advantage they have over the gene banking method of utilizing individually frozen sperm or ova which makes it possible to preserve only one sex. This is feasible through the transplantation of PGCs which ensures that an entire genetic line is preserved which will enhance flexibility in the restoration of chicken population (Kemp 2005). PGCs are also critical in restoring species or breeds that are in danger of extinction because they can be harvested during early development and stored for a long time without losing their effectiveness. This enables the genetic reconstruction of populations from various genetic material extracts derived from a single organism (Seigneurin & Blesbois, 2015).

In addition, PGCs are also useful in the studies related to gene-editing techniques. Some traits can be added or malformations repaired by genetically engineering PGCs prior to their transfer

into host embryos. This implies that PGCs are not only useful for conservation but also for further optimization of breeding practices by enhancing desirable features such as resistance to diseases, productivity and adaptability of poultry (Nakamura et al., 2010).

PGCs are especially useful for gene banking because they may grow into either male or female gametes, unlike cryopreservation of sperm or ova, which restricts conservation to one sex. PGC transplantation offers flexibility in poultry population regeneration by preserving a whole genetic lineage (Kemp, 2005). Because PGCs may be harvested at an early stage of development and preserved for extended periods of time without losing their viability, they are also crucial for the preservation of endangered or extinct breeds. According to Seigneurin and Blesbois (2015), this makes it possible to reconstruct genetic material from a single individual and reassemble large populations.

Furthermore, PGCs play an important role in gene editing studies. Scientists can introduce desired features or rectify genetic abnormalities into host embryos by changing the genetic composition of PGCs prior to transplantation. This makes PGCs useful not just for conservation, but also for developing breeding programs and enhancing attributes like disease resistance, productivity, and adaptability in chicken (Nakamura et al., 2010).

2.3.3. Preservation of Complete Genetic Material

PGCs serve an important function in genetic material preservation since they are undifferentiated germ cells capable of producing both male and female gametes. This dual feature makes them particularly useful for gene banking, since they enable the regeneration of a species or breed from a single donor. Studies, such as the successful cryopreservation and regeneration of a partridge-colored Hungarian native chicken breed, show that PGC preservation is efficient. In this work, cryopreserved PGCs were effectively transplanted into recipient embryos, enabling breed regeneration (Lázár et al., 2021). This achievement demonstrates the crucial significance of PGCs in preserving intact genetic material and conserving breeds at risk of extinction or genetic degradation.

2.3.4. Long-Term Storage and Regeneration

Long-term preservation of genetic material is a critical component of gene banking. Cryopreservation, which includes preserving biological material at extremely low temperatures,

often in liquid nitrogen (-196°C), preserves genetic material without the possibility of deterioration over time (Seigneurin & Blesbois, 2015). The capacity to retain PGCs, embryos, sperm, or eggs for extended periods guarantees that genetic material is available when needed for breeding operations, research, or conservation activities.

One significant use of PGCs for long-term preservation and regeneration is the restoration of chicken breeds. Cryopreserved pluripotent stem cells (PGCs) were utilized to restore the partridge-colored native chicken breed in Hungary, for instance, following extended preservation. Following the cryopreservation procedure, recipient embryos were implanted with PGCs, which moved to the developing gonads, developed, and eventually helped to produce viable children (Lázár et al., 2021). This indicates that PGCs have the potential to be used as a long-term storage option as well to fully regenerate chicken populations in the future. By providing flexibility and accuracy in the restoration of endangered or extinct breeds, this technology greatly aids in the conservation of biodiversity.

2.3.5. Techniques and Methodology of Storage and Utilization of PGCs

PGCs are stored and used in poultry using extremely specific methods that involve isolation, cryopreservation, and transplanting, among other stages. PGCs are usually isolated during the early stages of development, when the cells are taken out prior to differentiation into distinct gametes. After isolation, cells are subjected to cryopreservation procedures utilizing cryoprotectants such dimethyl sulfoxide (DMSO) to stop the development of ice crystals, which might harm the cells during the freezing process (Seigneurin & Blesbois, 2015).

The PGCs can be kept in liquid nitrogen for as long as necessary once they are frozen. Research has demonstrated that even after prolonged cryopreservation times, PGCs' long-term viability is unaffected, as evidenced by studies conducted on the native chicken breed of Hungary. For breeding or research reasons, this enables researchers to access genetic material years or even decades after storage (Lázár et al., 2021). After thawing, recipient embryos receive PGC transplants via microinjection into their bloodstreams. Following transplantation, the PGCs go to the recipient's gonads, where they integrate into the reproductive system and aid in the development of gametes. This makes it possible for a population to fully regenerate using the original donor's genetic makeup (Nakamura et al., 2010).

2.3.6. Successful Regeneration

Numerous research has shown that cryopreserved PGCs may successfully regenerate different poultry breeds, the most notable of which being the cryopreservation and regeneration of the partridge-colored Hungarian native chicken breed (Lázár et al., 2021). PGCs were effectively extracted from early-stage embryos in this work, cryopreserved, and then inserted into recipient embryos. The PGCs that were transplanted moved to the recipient's gonads, where they matured into viable germ cells that could bear fruit. The fact that an entire population was successfully regenerated from cryopreserved PGCs demonstrates how effective this method is for gene banking.

The cryopreserved PGCs are first thawed before being microinjected into the bloodstream of recipient embryos at appropriate developmental stages. These PGCs move to the developing gonads, integrate with the reproductive system, and eventually produce viable sperm or eggs in the recipient birds. The progeny of these birds carries the original donor's genetic material, renewing the breed (Lázár et al., 2021). This procedure assures that significant genetic features are retained and handed down to future generations, even after extended periods of cryopreservation.

2.3.7. Proposed General Protocol for Establishing and Testing PGC Lines for Gene Banking Purposes

The suggested procedure for creating and evaluating PGC lines is intended to preserve genetic resources while ensuring the survival and usefulness of the cell lines. The steps involved are (Table 1):

Table 1. General Protocol for Establishing & Testing PGC Lines (Lázár et al. 2021)

General Protocol for Establishing & Testing PGC Lines		
Step	**Description**	**References**
Isolation of Blood from Donor Embryos	A minimum of 50 isolations is required to gather a sufficient number of PGCs.	(Lázár et al. 2021)
Establishing PGC Cultures	The isolated cells are cultured in feeder-free conditions and the establishment of a PGC line is considered successful if 1.0 × 10^5 cells or more are produced within three weeks.	
Sex Determination of Cell Lines	At least 4-6 female cell lines are established ensuring that both male and female genetic lines are preserved.	
Proliferation Test	Cell lines must demonstrate a doubling time of 3-3.5 days or less indicating their ability to proliferate efficiently.	
Immunohistochemistry	The selected cell lines undergo immunohistochemical tests to confirm the presence of key germ cell markers (CVH and SSEA1) ensuring that the cells retain their identity as germ cells.	
Gene Expression Analysis	Selected cell lines are analyzed for the expression of germ cell-specific genes such as CVH and cPOUV further validating their authenticity as PGCs.	
In Vivo Migration Assay	To test the functionality of the PGC lines selected fluorescently labeled cells are injected into recipient embryos with a colonization rate of 60% or higher considered a positive outcome.	
Cryopreservation	Established and tested PGC lines are frozen for long-term storage. A minimum of 6 duplicate vials per line each containing at least 5.0 × 10^4 cells is required	

2.4. Advancements in Transgenesis and Genome Editing through Primordial Germ Cells in Avian Species

Gordon and Ruddle (1981) were the first to successfully integrate genetic material into the mouse genome by introducing foreign DNA into fertilized eggs' pronuclei. Eight years later, Salter and Crittenden (1989) achieved a big advance in poultry science by creating the first transgenic chicken that was resistant to Avian Leukosis virus subgroup A. This was done with an avian retrovirus proviral insert. Later, lentiviral vectors were employed to create chimeric chickens and quails from eggs, although this procedure resulted in random viral DNA integration into the chicken genome (McGrew et al., 2004).

In 2006, a significant milestone was achieved with the ability to grow and maintain Primordial Germ Cells (PGCs) in laboratory cultures. This success opened the door to injecting these cells into embryos, allowing them to migrate to the gonads and mature into fully functional gametes (van de Lavoir et al., 2006). Fast forward to 2012, when Macdonald et al. (2012) and Park and Han (2012) made notable strides by using vectors equipped with transposable elements and a transposase enzyme to introduce transgenes. Shortly after, Schusser et al. (2013) advanced the field further by applying gene targeting through homologous recombination in cultured PGCs,

which led to the production of birds with specific gene knockouts. Park et al. (2014) later demonstrated the use of TALENs to enable precise genome modifications, including gene knockouts and edits in PGCs. These breakthroughs have led to impressive innovations, such as the creation of chickens resistant to avian influenza (Lyall et al., 2011) and advancements in biopharming (Herron et al., 2018), marking the forefront of current research in this field.

2.4.1. Genome Editing Technology in Birds: A Focus on Primordial Germ Cell-Mediated Transgenesis

Genome editing tools have transformed genetic research by enabling precise adjustments in the DNA of numerous creatures, including birds. Birds, particularly poultry, play a significant role in agriculture, biomedicine, and evolutionary study. Recent advances in genome editing technologies, particularly the use of programmable nucleases like zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR-Cas systems, have allowed for more precise and efficient genetic modifications in avian species. These methods have altered the research of gene function and the creation of transgenic lines in birds, revealing genetics and disease resistance (Geurts et al., 2009; Carroll, 2011) (Figure 3).

ZFNs, one of the first programmable nucleases used for genome editing, are designed proteins that consist of a DNA-binding zinc-finger domain and a DNA-cleaving nuclease domain, commonly FokI. The zinc-finger domain identifies certain DNA sequences, whereas the FokI nuclease domain causes double-strand breaks (DSBs) at the target site. Repairing these breaks by non-homologous end joining (NHEJ) or homology-directed repair (HDR) allows for targeted gene disruption or insertion (Kim et al., 1996).

In avian species have helped to generate transgenic birds, notably in functional genomics and agricultural breeding. The capacity to create exact mutations in certain genes has been established in a variety of bird species, including chickens and quails. ZFNs have been utilized to investigate immunological function, muscular development, and feather color in birds. However, the complexity of engineering specialized zinc-finger domains, as well as the high production cost, have hampered their general acceptance in comparison to newer technologies such as TALENs and CRISPR-Cas systems (Sander et al., 2011; Carroll, 2011).

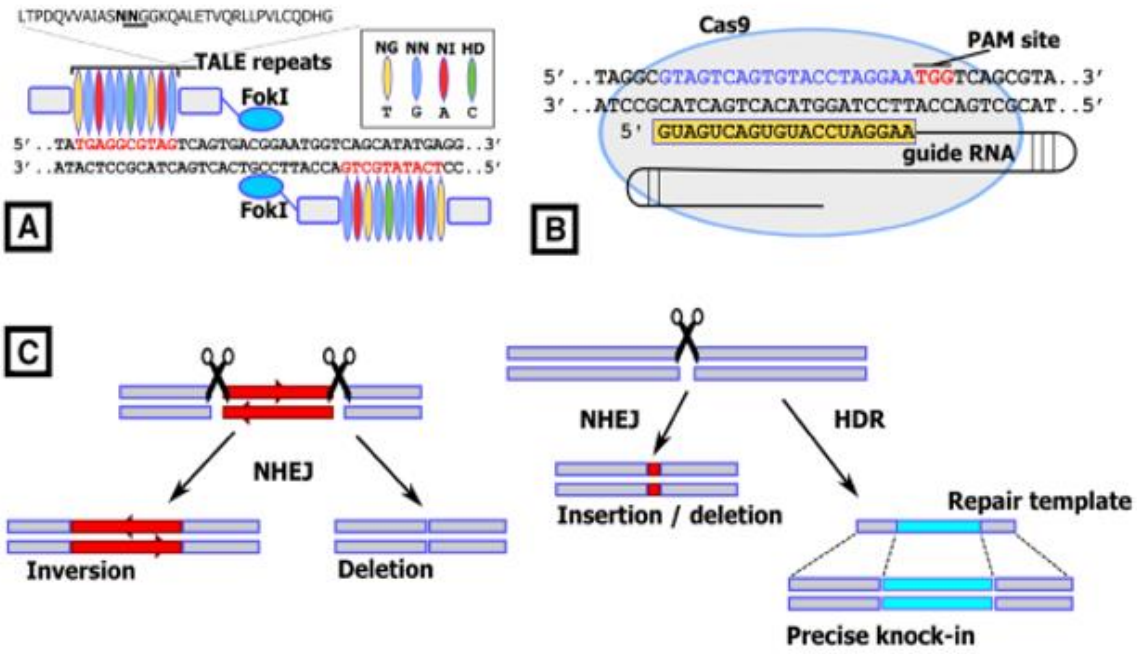

Figure 3. TALENs and CRISPR/Cas9 produce genomic alterations by targeting DNA via NHEJ and HDR repair pathways. **A:** TALE proteins are made up of repetitive modules joined to non-specific FokI cleavage domains that, when dimerized, cause double-stranded DNA breaks. Each module differs at amino acids 12 and 13, with these dipeptides affecting nucleotide binding selectivity. **B:** The CRISPR/Cas9 system uses a 20-nucleotide guide RNA (gRNA) to lead Cas9 to the target DNA. To activate the Cas9 nuclease, a PAM sequence (NGG) must be located directly downstream of the target site. The RNA-bound complex initiates double-strand cleavage via two active Cas9 domains at the PAM site. **C:** DNA breaks are then repaired using the NHEJ or HDR pathways

TALENs are simpler to create and more specialized than ZFNs. TALENs are composed of DNA-binding regions derived from transcription activator-like effectors (TALEs) and linked to the FokI nuclease domain. TALENs, unlike ZFNs, possess a modular DNA-binding domain that can be readily tailored to recognize specific DNA sequences. The modular structure of this trait increases their adaptability, making TALENs an appealing option for modifying avian genomes (Sun et al. 2015). Recent progress in utilizing TALENs in birds has allowed for precise gene disruption, specific insertions at desired locations, and the fixing of mutations. TALENs have shown great success in creating gene knockouts in chickens, with uses in researching developmental biology, disease resistance, and enhancing traits. For instance, disrupting the

gene responsible for the avian leukosis virus receptor through TALEN technology has resulted in chickens that are now immune to this viral infection, demonstrating the ability to improve disease resistance in poultry breeding (Bi et al., 2016). Even with their improved precision and effectiveness, TALENs encounter difficulties like off-target impacts and the necessity for expertise in protein engineering. However, advancements in high-fidelity TALENs and improvements in delivery methods, such as electroporation of primary germ cells, have greatly improved their utilization in birds (Carlson et al., 2012).

CRISPR-Cas systems have changed genome editing by simplifying it, increasing efficiency, and being versatile. The most frequently utilized form of CRISPR-Cas9 involves a guide RNA (gRNA) that guides the Cas9 nuclease to a particular DNA sequence, causing a double-strand break (DSB) at the designated location. Fixing these breaks enables accurate gene editing using NHEJ or HDR. CRISPR-Cas systems have overtaken ZFNs and TALENs because they are easy to design, cost-effective, and can be adapted for use in a variety of species, including birds (Jinek et al., 2012). CRISPR-Cas systems have been applied in birds to produce specific gene knockouts, induce accurate mutations, and cultivate disease-resistant strains. Important uses include developing chickens that are able to resist avian influenza by aiming at the ANP32A gene, which is essential for the replication of the virus. Moreover, CRISPR-Cas9 has been used to modify genes related to growth, reproduction, and plumage color, allowing for in-depth investigations of gene function in bird biology (Bi et al., 2016).

Recent developments have concentrated on improving the effectiveness and precision of CRISPR-Cas systems in avian species. This involves creating advanced Cas9 variants, base editors, and prime editing systems that provide more accurate genome modifications while minimizing off-target effects. Delivery methods, like in vivo electroporation and viral vectors, have been improved to enhance the effectiveness of gene editing in bird embryos (Liu et al., 2020).

2.4.2. Primordial Germ Cell (PGC)-Mediated Method for Genome Editing in Chickens

The PGC-mediated technique is commonly utilized for editing genomes in chickens because of the difficulties involved in directly manipulating genes at the zygote stage in bird species. This technique includes extracting primordial germ cells (PGCs), which are precursors of sperm and eggs, from bird embryos (Figure 4). The PGCs are grown in a laboratory setting, enabling scientists to make specific genetic changes using genome editing techniques such as CRISPR-

Cas9, TALENs, or ZFNs. After being altered, these PGCs are reinserted into recipient embryos, where they become part of the growing reproductive organs and help create edited offspring through the germline (van de Lavoir et al., 2006).

The method is dependent on several important stages.

- Extraction of PGCs: PGCs can be retrieved from embryonic blood or gonadal tissues at different developmental phases using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) paired with PGC-specific antibodies (Kim et al., 2010)
- In Vitro Culture and Genome Editing: Isolated PGCs are nurtured in a way that preserves their germ cell traits. Genome editing involves the use of CRISPR-Cas9 and other site-specific nucleases (SSNs) to create double-strand breaks (DSBs) at specific locations in the DNA. The breaks can be fixed by either non-homologous end joining (NHEJ) for gene knockouts or homology-directed repair (HDR) for accurate insertions or corrections (Macdonald et al., 2010).
- Injection of edited PGCs into recipient embryos involves them being injected into the blood vessels, moving to the genital ridge, and becoming part of the germline. Progenies produced from these PGCs are examined for the intended genetic changes (Park et al., 2003).

This technique enables screening of genetically altered cells prior to injection, enhancing the ability to breed offspring with specific genetic modifications. It is especially beneficial in creating knock-in birds by inserting specific genes at specified sites. The method is demanding in terms of effort and relies on knowledge in PGC isolation, culture, and manipulation, restricting its use primarily to chickens (Schusser et al., 2013).

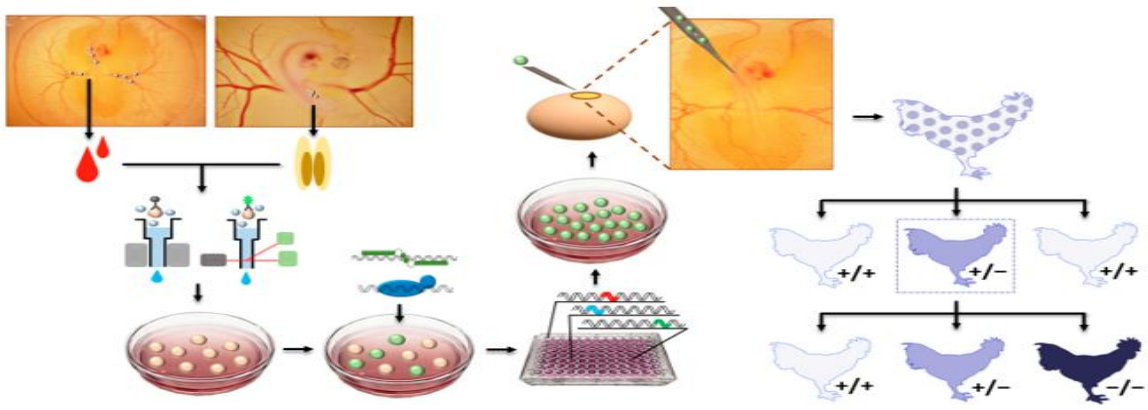

Figure 4. Schematic of PGC-Mediated Genome Editing Process in Chickens

2.4.3. Application of genome editing technology in birds

Recent advances in genome editing technology using primordial germ cells (PGCs) in birds have opened up exciting possibilities for manipulating the avian genome to create valuable avian models (Figure 5). One of the primary expectations in chickens is to develop an efficient bioreactor system for producing essential proteins through gene editing. Chickens are ideal bioreactors because egg white proteins are easy to purify, and chickens produce large quantities of these proteins on a daily basis (Han, 2009; Lillico et al., 2005).

While the main focus has been on using the ovalbumin promoter to produce target proteins in chickens, an alternative method involves inserting the target protein sequence directly into the ovalbumin locus using HDR-mediated gene editing. The HDR-induced incorporation of the target protein into the ovalbumin site may create an optimal bioreactor setup, potentially yielding more than one gram of target protein from one egg inexpensively. Genome editing in chickens could also lead to changes in or improvements to particular nutrients found in chicken meat and eggs. One method to produce allergen-free chicken meat and eggs is by inactivating genes associated with allergens like ovalbumin and ovomucoid (Park et al., 2014; Oishi et al., 2016). Furthermore, it is possible to create chickens with double muscles or muscle hypertrophy by modifying muscle-related genes such as myostatin, a method that has already been extensively researched in other animals (Lv et al., 2016; Wang et al., 2015).

In contrast to typical GMOs, genome-edited livestock like chickens are created using accurate genome-editing techniques instead of introducing foreign genes or causing uncontrolled mutations. These modifications reproduce natural changes present in the organism's genetic code, instead of depending on the introduction of external genes like conventional GMOs. Common worries about GMOs typically center on possible safety problems, like allergic responses or the insertion of antibiotic resistance genes; Promoting discussions among the public and reaching agreement within society may lead to genome-edited animals becoming more widely accepted by consumers soon (Tizard et al., 2016).

Birds are more prone to developing ovarian cancer compared to other animal models. This is primarily because they lay a large number of eggs throughout their lifespan and have a relatively short ovulation cycle, making them one of the most suitable animal models for studying human ovarian cancer (Johnson & Giles, 2013). With the application of precise gene editing targeting ovarian cancer-related genes, it may be possible to develop avian models that closely resemble human ovarian cancer. This would provide insights into the genetic mechanisms underlying

ovarian cancer pathogenesis using gene editing technology. Although most avian genome editing research has focused on chickens, it is expected that similar research will be extended to various other bird species in the near future. Notably, zebra finches have emerged as a unique non-human model organism, primarily for studying the biological basis of speech learning. They have been widely employed in neurobehavioral research (Petkov & Jarvis, 2012).

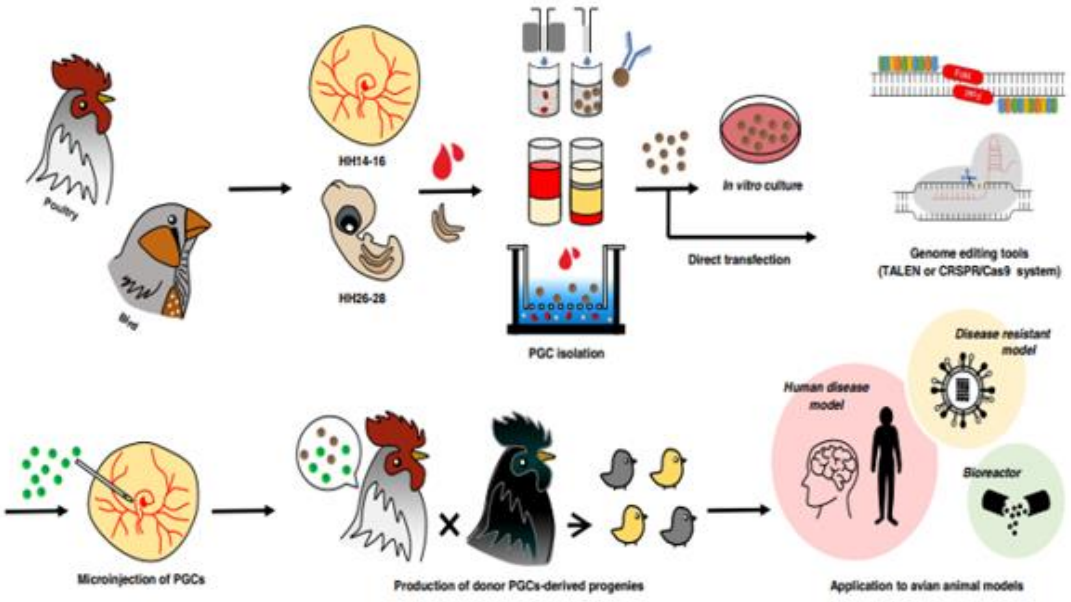

Figure 5. Strategies for Genome Editing in Avian Models Using Primordial Germ Cells

Furthermore, zebra finches are emerging as novel bird models for studying difficult-to-study human disorders such as Huntington's disease, voice learning, and neurological behavior, which are not achievable with existing animal models (Spierings & Ten Cate, 2016; Abe et al., 2015). Until recently, transgenic systems in zebra finches relied heavily on virus-mediated approaches, which involved injecting viruses directly into embryos (Velho & Lois, 2014).

Gene editing technology has enormous potential for discovering the function and processes of key genes in zebra finches. This can be accomplished by developing effective germline transmission mechanisms, such as PGC-mediated or sperm-mediated delivery, as well as other dependable methods. However, before proceeding, it is critical to understand the disease processes and host factors of avian viruses (Biggs & Nair, 2012; Long et al., 2016). Once these are better understood, avian gene editing is likely to open the door for the development of

disease-resistant birds by targeting and removing host components or receptors used by avian viruses.

2.4.4. Production of germline chimeras via primordial germ cells for avian transgenesis

Utilizing primordial germ cells (PGCs) in the creation of germline chimeras is a crucial method in avian transgenesis as it enables the introduction of genetic modifications into the avian germline.

Recent research has been heavily concentrated on enhancing the effectiveness of germline transmission, which pertains to the capacity of these altered PGCs to pass on to the succeeding generation. Efforts have aimed to improve PGC culture conditions, increase the accuracy of genetic modifications with tools like CRISPR/Cas9, and perfect PGC transplantation techniques for better integration rates into the recipient's germline (Macdon-ald et al., 2010). Furthermore, researchers have investigated how host embryo factors and compatibility between donors and recipients can enhance the efficiency of germline transmission, tackling issues such as immune rejection and the low survival rates of germ cells (Nakamura et al., 2010). These developments are intended to improve the accuracy and effectiveness of transgenic bird models, making it easier to investigate gene function and create avian models for studying human diseases (van de Lavoir et al., 2006).

2.4.5. Harnessing the piggyBac Vector: Enhancing Germline Modification and Transgenesis in Avian Models

The piggyBac transposon system is well-known in transgenesis for its distinct features, which increase its effectiveness in genetic modification, particularly in avian species such as chickens. PiggyBac is preferred because it can integrate long DNA sequences into the genome without restrictions on sequence size, making it ideal for inserting multiple or complex genes simultaneously (Li et al., 2013). In contrast to viral vectors, piggyBac targets TTAA genomic sites for integration, decreasing the chances of insertional mutagenesis and providing a safer choice for transgenesis. Furthermore, piggyBac provides a consistent, extended presence of the transgene, which is essential for experiments needing continual gene expression (Wilson et al., 2007).

2.4.5.1. Method of Using piggyBac for Transgenesis

The piggyBac system consists of two components: (1) a donor plasmid containing the transgene between piggyBac-specific inverted terminal repeats (ITRs), and (2) a transposase enzyme that enables the transgene's insertion into the host genome. The technique normally begins with cloning the desired gene into the donor plasmid. The plasmid is coupled with the transposase and injected into zygotes using direct injection, electroporation, or other gene delivery techniques (Ding et al., 2005). In birds, PGCs may be harvested, transformed with piggyBac, and reinserted into host embryos to create transgenic germline chimeras that carry the transgene to future generations (Park et al., 2009).

One of the standout features of piggyBac is its re-mobilization capability; the transposon can be excised and re-integrated, which means that modifications can be reversed or adjusted if necessary. This flexibility is not commonly found in other transposon systems or viral vectors (Lu et al., 2009). The system's ability to leave no residual sequences upon excision, a process known as "footprint-free" excision, ensures that the genomic integrity remains largely unaltered except for the inserted gene, which is particularly beneficial in precise genome editing applications (Li et al., 2013). Furthermore, piggyBac's non-viral nature minimizes immune responses, making it a gentler option for cells and embryos, and it has shown low cytotoxicity in many studies (Wilson et al., 2007).

2.4.5.2. Practical Applications of the PiggyBac Transposon System

The practical applications of piggyBac in avian models extend to creating genetically modified chickens that can serve as bioreactors for pharmaceutical production, studying gene functions relevant to human diseases, and generating disease-resistant poultry. These capabilities are crucial in biotechnology, agriculture, and medical research, highlighting piggyBac's versatility and efficiency as a transgenic tool (Luo et al., 2012). The ease of handling, combined with high transposition efficiency, makes piggyBac particularly appealing for large-scale genetic studies and developing new lines of transgenic birds.

2.5. Overview of the cell cycle and FUCCI Imaging

The cell cycle is a crucial process where cells increase in size, copy their DNA, and split to generate more cells. This process is vital for typical growth, healing of tissues, and regeneration of cells. The primary stages of the cell cycle consist of G1 (Gap 1), S (Synthesis), G2 (Gap 2),

and M (Mitosis). Every stage has specific responsibilities, and their correct control is crucial for preserving genomic integrity. Mistakes in these processes may result in conditions like cancer, characterized by uncontrolled cell division (Figure 6).

FUCCI, developed in 2008 by Sakaue-Sawano et al., is an innovative method for observing cell cycle dynamics in real time using live-cell imaging technology. This new system was developed to meet the demand for a way to keep track of cell cycle changes without being invasive, giving scientists the chance to see how cells move through different stages in a lively setting (Sakaue-Sawano et al., 2008). FUCCI has now become a crucial instrument in various sectors including cell biology, cancer research, developmental biology, and drug discovery.

The FUCCI system is based on two crucial fluorescent markers: Cdt1 labeled with mCherry, which builds up in the G1 phase, and geminin labeled with GFP, which builds up in S, G2, and M phases. The proteins are selectively degraded during specific cell cycle stages, distinguishing cells based on their current phase: G1 is indicated by red, S/G2/M by green, and the G1/S transition by yellow where both proteins overlap. This innovative visualization method enables the direct monitoring of cell cycle progression without using conventional invasive techniques like cell fixation or labeling (Koh et al., 2017).

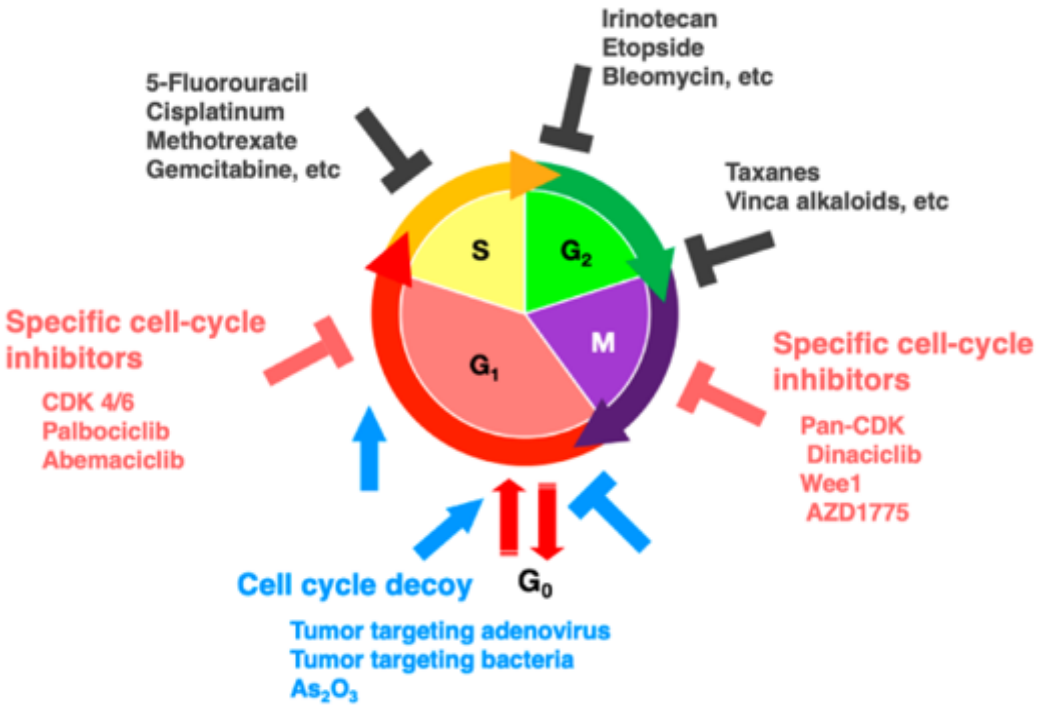

Figure 6 . Illustration of Cell Cycle Phases Targeted by Therapeutic Agents and Visualized Using FUCCI Imaging (Yano et al., 2020)

In the fields of developmental biology and stem cell research, FUCCI has played a key role in revealing the impact of cell cycle dynamics on tissue growth, regeneration, and differentiation. FUCCI helps clarify the intricate connection between cell cycle status and cell fate decisions by offering a visual depiction of cell transitions, shedding light on key processes in development and repair. This makes FUCCI a valuable tool for examining both disease states and regular cell behavior in developmental stages (Sakaue-Sawano et al., 2011).

2.5.1. Applications of FUCCI in Research and Therapeutics

The continuous development of FUCCI technology has greatly expanded its uses, turning it into a versatile instrument in various research fields, from fundamental cell biology to therapeutic medicine. FUCCI plays a significant role in cancer research by revealing the intricate processes of cell growth and cell cycle irregularities, which are key characteristics of cancer. FUCCI's capacity to offer immediate, non-intrusive visualization of cell cycle phases enables researchers to pinpoint dormant (G0/G1) cancer cells that frequently show resistance to traditional chemotherapy. These dormant cells can withstand treatments that usually focus on actively dividing cells in the S/G2/M phases, resulting in cancer coming back. FUCCI imaging helps in creating therapeutic plans to manipulate resistant cells and make them more vulnerable to cytotoxic drugs by getting them back into the cell cycle (Yano et al., 2020; Sakaue-Sawano et al., 2008). In the field of drug discovery, FUCCI is widely utilized for screening potential therapeutic compounds that can target specific stages of the cell cycle selectively. FUCCI offers a strong tool for assessing the effectiveness of new drugs and enhancing dosage schedules by enabling researchers to monitor the impact of drugs on cell cycle progression in real-time. This accuracy is especially important in cancer treatment, aiming to stop the uncontrollable growth of cancer cells with minimal damage to healthy cells. For example, FUCCI has been utilized to pinpoint cell cycle inhibitors capable of causing arrest at particular stages, effectively impeding or halting the proliferation of cancer cells (Koh et al., 2017).

FUCCI is not only useful for studying cancer, but also has applications in studying developmental biology and stem cell research. It is important to understand cell cycle dynamics in these fields to analyze tissue growth, differentiation, and regeneration. FUCCI has been used to show how the cell cycle status affects decisions concerning the fate of stem cells, like determining the trade-off between self-renewal and differentiation. In developmental biology, researchers use FUCCI imaging to monitor cell proliferation and differentiation in tissue

formation, offering knowledge on how cell cycle regulation affects developmental processes (Sakaue-Sawano et al., 2011; Coronado et al., 2013).

FUCCI is important in regenerative medicine because it helps researchers understand how cell cycle re-entry and exit effect tissue repair and regeneration. Researchers can improve therapeutic strategies that increase the body's natural healing processes by studying how different stages of the cell cycle contribute to regenerative results. This is especially relevant in situations when tissue regeneration is impeded, such as chronic wounds or degenerative illnesses, where encouraging normal cell cycle activity can greatly enhance recovery results (Masaki et al., 2015).

FUCCI is also utilized in neuroscience to investigate how neural stem cells control their cycle during brain development and in response to damage. Understanding these cells' cell cycle dynamics is critical for developing treatments for neurodegenerative diseases and brain injuries, as stimulating the appropriate balance of neural stem cell proliferation and differentiation can aid in neural repair and functional recovery (Pilaz et al., 2016).

2.5.2. T-2 toxin

T-2 toxin produced by specific types of Fusarium fungi such as Fusarium sporotrichioides and Fusarium poae, is frequently present in grains such as corn, wheat, and barley, especially in moist conditions. Famous for its harsh poisonous effects, T-2 toxin hinders crucial cellular functions, mainly by blocking protein production in cells, resulting in cell death and tissue harm. The protein synthesis is stopped by the toxin binding to ribosomes, preventing the elongation of protein chains crucial for cell function and survival, notably in fast-growing tissues like those in the immune and gastrointestinal systems (Zhang et al., 2020; Pestka, 2010). T-2 toxin also triggers oxidative stress by increasing the production of reactive oxygen species (ROS), resulting in additional cellular damage and inflammation, enhancing its toxicity. This oxidative stress plays a role in the immunosuppressive and hematotoxic effects of the toxin, which weaken the immune defenses and disrupt blood cell production (Li et al., 2011; He et al., 2017).

3. Material And Methods

3.1. Ethical Statement and Resource Support in Embryological Research

The research was conducted at the Hungarian University of Agriculture and Life Sciences (MATE), within the Institute of Genetics and Biotechnology (GBI), specifically in the Applied Embryology and Stem Cell Biology Group. The institute provided all necessary resources, including fertilized eggs, kit protocols, and cutting-edge biotechnological laboratory equipment essential for the study. In adherence to the Hungarian Animal Protection Law (1998. XXVIII), the animals were managed responsibly. The National Food Chain Safety Office, Animal Health and Animal Welfare Directorate (Budapest, Hungary) granted permission for the experimental procedures, which were carried out at the National Centre for Biodiversity and Gene Conservation, Institute for Farm Animal Gene Conservation (Gödöllő, Hungary). Fertilized eggs from White Hungarian chickens were generously provided by this institute for the project.

3.2. PGC Line Isolation, Development, and Maintenance

The eggs were incubated in a hatchery machine called MIDI F500S made by (PL Machine Ltd. in Tárnok, Hungary). They were rotated at 45° every half an hour, kept at 37.8°C temperature, and 70% relative humidity. At 2.5 days of growth, known as Hamburger and Hamilton stages 13-17 (Hamburger and Hamilton, 1951), the eggs were sanitized with 70% alcohol and carefully cracked open onto Petri dishes. Using a mouth pipette, blood samples of around 1 µL each were taken from the dorsal aorta of the embryos with a microcapillary. Furthermore, tiny tissue samples were obtained from every embryo to determine their sex later on.

The blood samples were moved into a culture medium that was placed in a 48-well plate. The medium contained calcium-free DMEM (Gibco, Billings, MT, 21068-028), tissue culture-grade water (Gibco, Billings, MT, A12873-01), pyruvate (Gibco, Billings, MT, 11360039), MEM vitamin solution (Gibco, Billings, MT, 11120052), MEM amino acids (Sigma, St. Louis, MO, M5550), B27 supplement (Gibco, Billings, MT, 17504044), Glutamax (Gibco, Billings, MT, 35050038), non-essential amino acids (Gibco, Billings, MT, 11140035), nucleosides (EmbryoMax, Munich, Germany, ES-008-D), β-Mercaptoethanol (Gibco, Billings, MT, 31350010), $CaCl_2$ (Sigma, St. Louis, MO, C4901-100G), ovalbumin (Sigma, St. Louis, MO, A5503), sodium heparin (Sigma, St. Louis, MO, H3149-25KU), a penicillin-streptomycin

mixture (Gibco, Billings, MT, 15070-063), chicken serum (Sigma, St. Louis, MO, C5405), human activin (Invitrogen, Waltham, MA, PHC9564), bFGF2 (Gibco, Billings, MT, 13256-029), and ovotransferrin (Sigma, St. Louis, MO, C7786), as detailed in Whyte et al.'s (2015) protocol. The medium was changed every second day. Cultures were kept in a Sanyo MCO-19AIC (UV) CO_2 Incubator (Sanyo, Osaka, Japan, 10040162) at 38°C with a 5% CO_2 environment.

3.3. Freezing and Thawing of PGC Lines

The PGC lines were stable and steady following four weeks of cultivation. The NanoEntek Arthur Fluorescence Cell Counter from Seoul, Korea was employed to evaluate both the number (80000 cells/300 µL medium) and the quality of cells, with morphology and size serving as criteria for verification. In the cryopreservation process, the freezing solution included fetal bovine serum (FBS; Gibco, Billings, MT, 10108-165) with an additional 10% DMSO (Sigma, St. Louis, MO, D2650) (Kong et al., 2018).

Cells were harvested, spun down, and suspended in 250 µL of a 2:1 DMEM-water mixture before mixing in 250 µL of 2x freezing solution. The specimens were put into a Mr. FrostyTM Freezing Container (Thermo Fisher, Waltham, MA) for a regulated freezing process and promptly moved to a -80°C freezer. The samples were transferred to a freezer at -150°C the next day for storage over the long term. The cell lines utilized in this research were included in the institution's genetic repository.

Thawing was conducted at a temperature of 38°C for a duration of 90 seconds, then promptly diluted with 900 microliters of culture medium at ambient temperature to decline the negative impacts of DMSO. The cells underwent centrifugation at 100 G to eliminate DMSO, were then resuspended in 300 µL of new culture medium, and finally moved to culture plates.

3.4. Plasmid Preparation and Electroporation Protocol for FUCCI Transgene Integration into Chicken PGCs

András Nagy from the Lunenfeld-Tanenbaum Research Institute, Toronto, Canada, generously provided our lab the FUCCI transgene containing hyperactive PiggyBac transposon, and hyPBase transposase plasmids. The plasmids were introduce to competent E. coli cells, which were then grown in culture to form single colonies. The colonies were exposed to ampicillin in

order to distinguish the ampicillin-resistant transfected colonies. Liquid bacterium cultures were developed and cultivated to achieve the necessary cell mass. These cell suspensions were utilized in the plasmid extraction process using the Qiagen EndoFree® Plasmid Maxi Kit (Qiagen, Hilden, Germany, 169026122). The plasmids that were separated were mixed with OptiMEM (Gibco, Billings, MT, 11058-021) until reaching a concentration of 1200 ng/µL.

The Neon Transfection System from Invitrogen was used for electroporation with a 1:1 FUCCI - hyPBase plasmid ratio. 100 µL of cell suspension with around 1 million cells was subjected to electroporation in every cell line. Altgilbers (Altgilbers et al., 2021) described conducting electroporation at 1300 V for 10 ms and with 4 repetitions. Following the electroporation process, the suspension was transferred onto a 12-well culture plate containing 1 mL of culture medium. Absolute controls, OptiMEM controls, and control cells electroporated in OptiMem not containing plasmids were utilized to assess the impacts of OptiMEM treatment and electroporation on the PGCs. The green/red fluorescence was confirmed the following day using analysis with the Leica DFC 7000T Stereo Microscope (Leica. Wetzlar, Germany). The detailed description of the transgenesis was documented by András Ecker (Ecker et al, 2024).

3.5. Establishing and Culturing Transgenic PGC Lines

The genetically modified cells were grown in culture for a period of fourteen days. Individual cells were isolated from cultures diluted at a 1:18 ratio. A microcapillary, about 40 µm diameter, was used for pipetting the individual cell, while the fluorescent cells were monitored under a stereo microscope. The separated cells were moved into a 50 µL medium drop and examined with a stereo microscope to confirm that only one fluorescence PG cell is in each drop, prior to being transferred to a 96-well plate with 200 µL of culture medium. After three weeks, six PGC lines were preserved in cryogenic storage for future use. The FCF5 and FCM5 one cell derived PGC lines were selected for the experiments because of their fast growth and marker presence.

3.6. Immunostaining and Confocal Microscopy Analysis of Transgenic PGC Cultures

The cells suspension were dried onto the surface of a microscope slide after being placed 10 µL of cell suspension in PBS (Gibco, Billings, MT, 14190-144) containing 0.1% BSA (Sigma, St. Louis, MO, A3311). Samples were fixed with 4% PFA (Fluka, Buchs, Switzerland, 30525-89-4), followed by the addition of blocking solution (PBS with 0.1% BSA, 0.1% Triton-X-100

(Fluka, Buchs, Switzerland, 93426), and 2.5% donkey serum) to enhance membrane permeability for nucleus and cytoplasm staining while preventing non-specific binding. Primary antibodies specific to stem cells, anti-SSEA-1 (MC480),(Millipore, Munich, Germany) and primordial germ cells, anti-CVH provided by Bertrand Pain from the Stem Cell and Brain Research Institute (SBRI) in Lyon, France). were utilized for immunostaining, The anti-SSEA-1 was combined with the aanti-Mouse-IgM-rD549® secondary antibody from Jackson ImmunoResearch, while the anti-CVH was labelled with green Alexa Fluor® 488 Anti-Rabbit-IgG (H+L) from Life Technologies/Molecular Probes. Far-red TO-PRO™-3 iodide (642-661) from Invitrogen in Waltham, MA (T3605) was utilized for nuclear staining. Next, the samples were coated with ProLong Diamond Antifade Mountant mounting medium containing DAPI (Invitrogen, Waltham, MA, P36962) and a glass cover.

Samples from the FCF5 and FCM5 PGC culture were additionally arranged in ProLong Diamond Antifade Mountant with DAPI without immunostaining under a cover glass for instant examination. The Leica TCS SP8 Confocal Microscope (Leica, Wetzlar, Germany) was used to examine them.

During the analysis of the FUCCI cultures, only the TO-PRO™-3 nucleus stain was utilized. As the peak emission occurs at 505 nm for mAG1 and 565 nm for mKO2, the TO-PRO™-3 (642-661) can be detected simultaneously. Still, the TO-PRO™-3 signal was coloured blue in the images to help in distinguishing it visually from the signal of mKO2.

3.7. Injection of FUCCI-Transgenic PGCs in Chicken Embryos

Regarding the injection of PGCs into gonads, the procedure begins with collecting the transgenic PGCs in a diluted DMEM-H2O mixture to ensure a precise cell concentration suitable for injection. For these injections, typically around 5000 cells/mL are prepared. The embryonic eggs are incubated for about 60 hours, reaching an optimal stage for injection. A small opening is created in the egg's air chamber, followed by a 1 cm window above the embryo, allowing access to its circulatory system. Using a microcapillary and mouth pipette, 1 µL of the cell suspension is injected directly into the embryo's heart to ensure the cells are efficiently delivered through the circulatory system. Post-injection, the eggs are sealed with sterilized parafilm and returned to incubation to foster further development.

For the dissection of gonads from 6-day-old embryos (Figure 7), the eggs are carefully opened after reaching the 6-day incubation mark. At this stage, the gonads have developed sufficiently

for identification and extraction. Dissection is performed under a stereo microscope, ensuring precision in isolating the gonads. Post-dissection, the gonads are examined under fluorescence microscopy to confirm the presence and proper integration of FUCCI-labeled PGCs, validating the successful migration and colonization of the gonadal tissue by the injected PGCs. This step is essential in analyzing the transgene's integration and cell cycle progression within the recipient's gonadal environment.

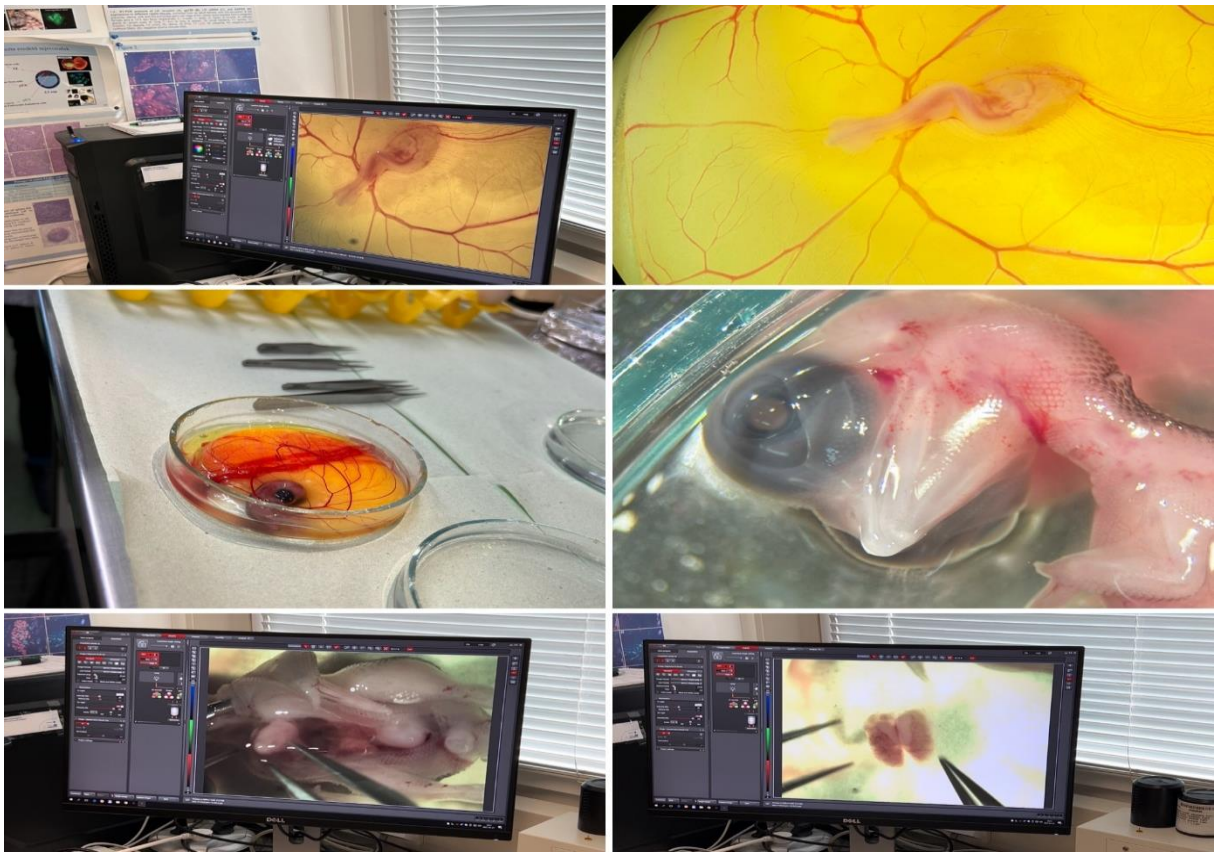

Figure 7. Isolation gonads from 6-day-old chicken embryos (own pictures)

3.8. ImageXpress Pico Automated Cell Imaging System

The ImageXpress Pico Automated Cell Imaging System (also known as the Pico machine) is a crucial instrument in cellular biology for live-cell imaging, providing strong capabilities for monitoring how cells react to treatments instantly.

This is essential for accurately detecting slight morphological changes and specific fluorescence in different phases. When conducting research with FUCCI, the Pico's capacity to distinguish cell cycle phases through fluorescent labels enables accurate monitoring of stages like G1, S,

and G2/M, proving essential for studying how environmental elements, including mycotoxins, impact every step of cell growth (ImageXpress Pico Automated Cell Imaging System, 2022; Molecular Devices, 2023).

The system's environmental regulation also promotes extended live-cell experiments by preserving consistent CO_2 levels, temperature, and humidity, enabling scientists to monitor cell reactions over time without disturbing the culture environment. This degree of environmental stability is particularly advantageous for researching toxicological impacts, as it allows for ongoing observation in carefully controlled conditions. The automated analysis protocols of the Pico machine, which consist of more than 25 pre-configured assays, simplify the workflow (Sirenko et al., 2022; Molecular Devices, 2022)

3.9. Arthur Image Based Cell Analyzer

The NanoEnTek Arthur Image Based Cell Analyzer is a small and flexible tool designed to simplify cellular analysis for tasks such as counting cells, testing viability, detecting apoptosis, and conducting cell cycle assays. This tool utilizes three types of imaging channels - brightfield, green fluorescence, and red fluorescence - which enable scientists to distinguish between live and dead cells, monitor cell cycle phases, and examine markers tagged with fluorescence like GFP (green fluorescent protein) and RFP (red fluorescent protein).

One of the main benefits of the Arthur Analyzer is its quick, automated cell counting, which can finish assays in 10 to 120 seconds based on complexity (MedicalExpo, 2024). The disposable slides of the device make it easier to manage, decreasing the chance of contamination and enabling it to be used for precise assays that require consistent results (NanoEnTek, 2023; MedicalExpo, 2024; GenBiotech, 2024).

3.10. Statistical analysis

A t-test was used for statistical analysis between groups using the GenEx 7.0 program. The presented data were averaged ± standard deviation, and a result below a p-value of 0.05 was treated as significant. Our significance levels were $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***

4. Results and Discussion

4 .1. Establishment, Characterization, and Functional Validation of Transgenic PGC Lines in Chicken Embryos

In this study, we aimed to establish and characterize transgenic Primordial Germ Cell (PGC) lines for in vitro and in vivo experiments. The initial phase involved the transgenesis process, where we introduced piggyBac plasmids into isolated PGCs to facilitate stable genomic integration. This process allowed us to develop PGC lines that were derived from a single cell, ensuring that each line contained the plasmid in an integrated and stable manner. We generated two lines: a female line, designated as FCF5, and a male line, FCM5. This step was crucial as deriving cell lines from a single genetically stable source minimizes variability, thus enhancing the reproducibility and reliability of future experiments using these lines.

Following the establishment of the FCF5 and FCM5 lines, we conducted a preliminary characterization to assess the quality and viability of these cell lines. For this, we employed immunostaining techniques to identify specific markers, which helped confirm the identity and pluripotency of the cells. Additionally, we measured the proliferation rates of these cell lines using the ImageXpress Pico machine, which allowed us to monitor cell division and assess cell health. The positive proliferation rates we observed indicated that both cell lines were of high quality, showing robust growth under in vitro conditions. This confirmed that FCF5 and FCM5 were suitable for further experimentation, as they exhibited both the necessary genetic stability and cellular viability.

To test the functionality of these PGC lines in a living organism, we injected the FUCCI-labeled PGCs into recipient embryos at the 2–3-day developmental stage. This approach allowed us to observe the ability of the cells to integrate within a developing embryo. Upon examination at 6 days post-injection, we observed successful integration of the PGCs into the embryonic gonads, indicating that these cells had retained their potential to incorporate into the germline of the host. This finding is particularly significant as it supports the potential application of the FCF5 and FCM5 lines in studies focused on germline transmission and gene inheritance.

Before and after injection, we utilized the Arthur machine to analyze the cell quality of the PGC lines. The Arthur machine allowed us to accurately calculate cell counts and assess the ratio of green to red cells, which reflects the distribution of cells in different cell cycle phases as indicated by FUCCI labeling. Establishing a baseline measurement with the Arthur machine

was important as it provided a standard for comparing pre- and post-injection data. This data comparison, especially for the non-integrating female line, helped us hypothesize possible reasons for integration failures, such as discrepancies in cell cycle phases or physiological incompatibilities with the host environment. Through this troubleshooting process, we identified areas for potential adjustments in cell culture or injection conditions, ensuring both male and female lines are equally viable for future experiments.

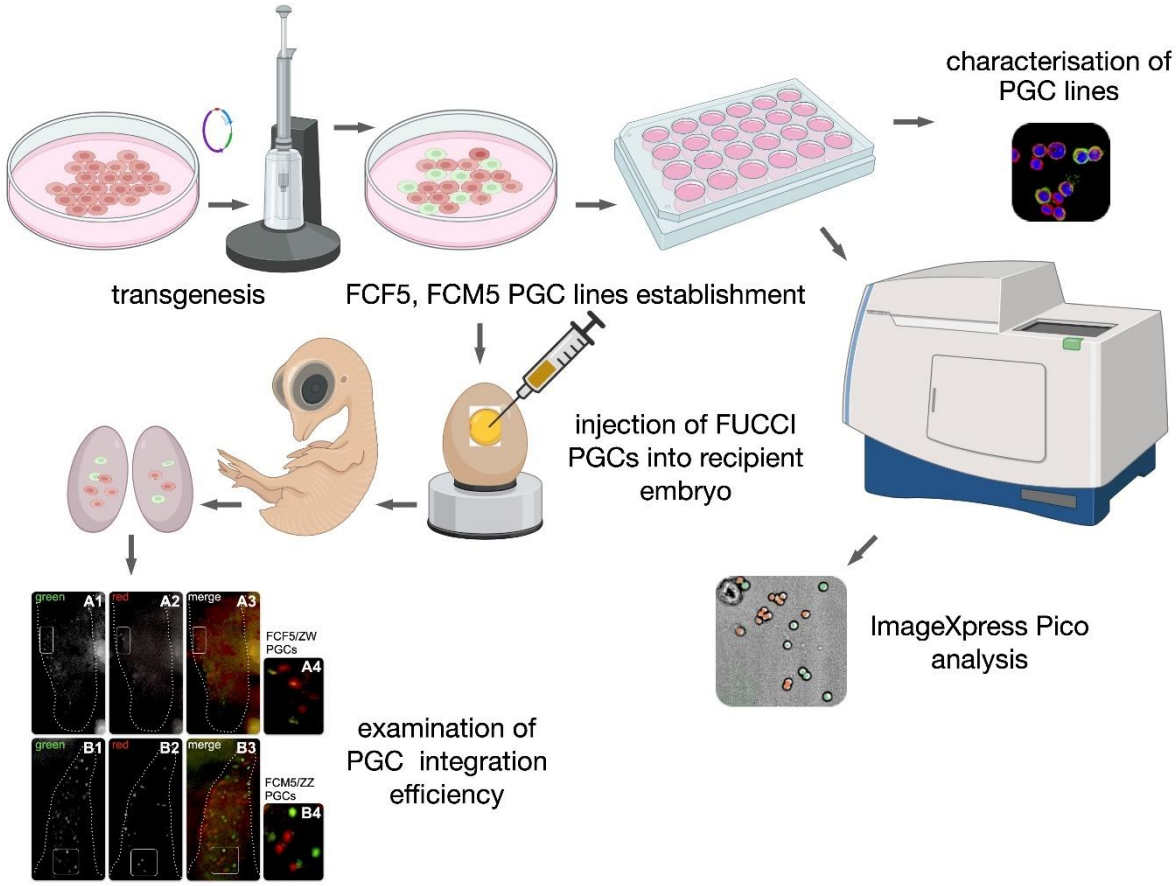

Figure 8. Establishment, Characterization, and Functional Validation of Transgenic PGC Lines in Chicken Embryos (This Figure was created using BioRender application)

Using the ImageXpress Pico machine opened possibilities for further experimentation and analysis. By conducting independent experiments with this machine, I could monitor various aspects of the PGCs, such as their proliferation patterns, morphology, and cell cycle progression under different experimental conditions. This capability not only allowed me to confirm the quality of the established PGC lines but also provided a basis for further investigation into their responses to environmental changes or specific treatments. The long-term implications of this setup are considerable, as it enhances our ability to establish protocols for consistent PGC

assessment, thus contributing valuable insights to embryology and genetic engineering research (Figure 8).

4.2. Evaluation of PGC Line Quality Through Stem and Germ Cell Marker Expression

The images presented two different lines of primordial germ cells (PGCs) stained with specific markers to assess their quality and characteristics prior to electroporation. The top image shows the 1116 FM/ZZ line, which, although originally classified as a female cell line, was used in this experiment as a "male cell line." The bottom image represents the 1111 FM/ZW line, confirming its identity as a female cell line. In avian species, "ZZ" designates male cells, while "ZW" designates female cells, providing a unique system for sex differentiation (Figure 9).

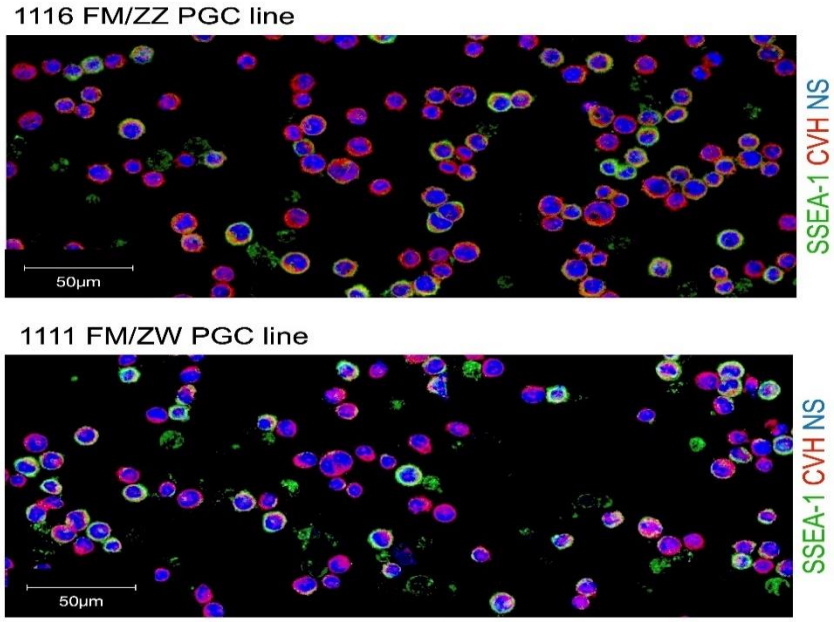

Figure 9. Assessment of Stem and Germ Cell Markers in FM/ZZ and FM/ZW PGC Lines Prior to Electroporation

Both lines were stained for three key markers that help determine their stem cell and germ cell status, each of which plays a specific role in assessing cell quality. The green staining represents SSEA-1, a stem cell-specific surface marker, indicating that these cells retain their undifferentiated state. This is crucial for transgenesis experiments, as only cells with stem-like characteristics can properly integrate genetic material and contribute to future generations. The red staining corresponds to CVH (Chicken Vasa Homolog), a marker specific to germ cells,

located primarily in the cytoplasm. CVH staining further confirms the identity of these cells as germ cells, ensuring that they are suitable for applications in germline transmission. Finally, the blue staining, likely DAPI, marks the nuclei, providing clear visualization of nuclear integrity. This nuclear staining is uniform and well-defined, indicating healthy cell structure and stable chromatin, both important for the cells' overall viability and their potential for genetic modification.

From these observations, we can conclude that both the 1116 FM/ZZ and 1111 FM/ZW PGC lines exhibit strong marker expression for SSEA-1 and CVH, alongside distinct nuclear staining, all of which are positive indicators of high cell quality. This suggests that the cells were well-prepared, maintaining their stem cell and germ cell identities with intact structural integrity. Such characteristics are essential for successful electroporation, as only cells in good health and with stable characteristics can efficiently incorporate foreign DNA. Based on this analysis, we can confidently state that the initial quality of these PGC lines was high, which sets a strong foundation for any subsequent transgenic work. Monitoring these markers after electroporation will be important to assess the impact of the procedure on cell viability and identity, ensuring that the cells maintain these crucial properties throughout the experiment.

4.3. Characterization and Quality Assessment of FCM5 and FCF5 Cell Lines Using FUCCI Cell Cycle Analysis

The characterization of FCM5 and FCF5 cell lines before injection, focusing on cell cycle distribution and quality assessment. The charts show how FCM5 and FCF5 cells are distributed across various cell cycle phases—G1, S, G2, and M/dead—under both "good quality" and "bad quality" conditions. Each color in the charts corresponds to a specific phase: red indicates the G1 phase, yellow represents the S phase, green stands for the G2 phase, and gray covers cells either in the M phase or already dead. In the good-quality samples, FCM5 cells show a higher proportion in the G1 phase (45%) compared to FCF5 (21%), which suggests more balanced cell cycle progression and fewer cells in the M or dead phase (22%) than in FCF5 (60%). This distribution reflects healthier cell culture conditions for FCM5, indicating robust cell cycle dynamics (Figure 10).

In contrast, the bad-quality FCF5 sample displays a significant increase in cells in the M/dead phase (74%) and a complete absence of cells in the G1 phase, signaling a breakdown in cell viability and cycle regulation. The absence of red (G1 phase) cells confirms that the cell line

loses its characteristic cycling pattern under poor conditions, which implies compromised quality, possibly due to suboptimal culture conditions or contamination (Figure 10).

The FUCCI vector system is used to track cell cycle phases using fluorescent markers. In this system, cells in the G1 phase are marked with red fluorescence, cells in the G2 phase have green fluorescence, and cells in the S phase display a yellow/orange hue, resulting from the overlap of the red and green markers. A microscopy image captured by the Pico Machine clearly shows these fluorescent markers, illustrating the effectiveness of the FUCCI system in differentiating between various cell cycle phases. This color-coded identification allows for precise recognition of cell phases and aids in assessing the quality of cell lines.

Figure 10. Cell Cycle Distribution and Quality Comparison of FCM5 and FCF5 Cell Lines with FUCCI Fluorescent Markers

This experiment emphasizes that, in low-quality cultures, like the bad-quality FCF5 sample, the typical structured cycling pattern of healthy cells deteriorates, leading to higher cell death or

exit from active cycling phases. These results highlight the importance of maintaining optimal culture conditions to preserve cell line integrity. If a cell line is found to be of poor quality, frozen stocks provide an opportunity to thaw a fresh sample and re-establish a healthy culture, ensuring continuity in research. This method guarantees that experiments can continue with cells that retain the desired transgenic characteristics and maintain high viability; this analysis underscores the usefulness of FUCCI technology in monitoring cell cycle dynamics, serving as a valuable tool for quality control in cell line management.

These figures were conducted on two cell lines, FCF5 (female) and FCM5 (male), to evaluate the impact of various toxin concentrations on cell proliferation over time. Cell numbers were measured across 15 time points, each representing four-hour intervals, allowing for a detailed view of cell growth patterns under both controlled and toxin-treated conditions. The control cells had six replicates, whereas the treated cells (with toxin concentrations of 1 ng/mL, 2.5 ng/mL, and 5 ng/mL) had three replicates each. This setup ensured statistically reliable data, enabling the calculation of averages and standard deviations. A T-test was performed to compare control and treated groups at each time point, with significant differences indicated in blue within the tables. The presence of blue highlights signifies statistically meaningful variations between the control and treated groups.

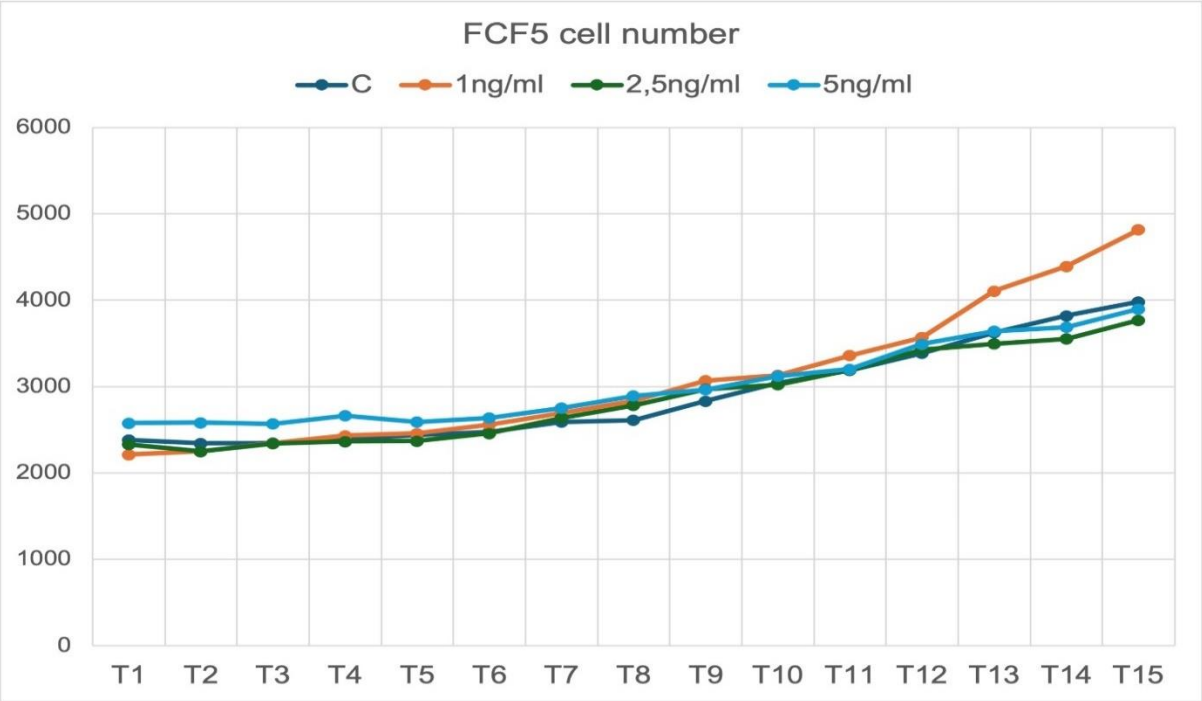

Figure 11. Proliferation of FCF5 (Female) Cell Line Under Different Toxin Concentrations Over 15 Time Points

For the FCF5 cell line, cell proliferation generally increased across all conditions over the 15 time points. Notably, the 1 ng/mL treatment group displayed a higher growth rate, especially evident in the later stages of the experiment. By time point T13, the cell counts in the 1 ng/mL group reached approximately 4,600 cells, compared to about 4,000 cells in the control group, highlighting a statistically significant increase in cell proliferation at this point ($p < 0.05$, as marked in blue) (Figure 11), (Table 2).

Table 2. T-test Table: Comparison of the Control and Experimental Groups (p values)

FCF5- cell number		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
C	**1ng/ml**	0,323	0,330	0,312	0,390	0,440	0,397	0,348	0,339	0,310	0,232	0,211	0,104	**0,051**
C	**2,5ng/ml**	0,280	0,453	0,459	0,516	0,552	0,614	0,514	0,366	0,228	0,157	0,132	0,071	**0,047**
C	**5ng/ml**	0,478	0,483	0,700	0,990	0,931	0,966	0,940	0,798	0,743	0,652	0,566	0,374	0,355

This effect aligns with a phenomenon known as hormesis, where low doses of certain toxins may stimulate biological activity rather than suppress it. In contrast, cells treated with the higher concentration of 5 ng/mL showed a less pronounced increase in cell number, with the count remaining close to the control group and slightly below the 1 ng/mL treatment. By the end of the experiment (T15), the 5 ng/mL group reached about 4,300 cells, compared to nearly 5,000 cells in the 1 ng/mL group. This suggests that while low doses of the toxin may temporarily promote cell growth, higher concentrations could inhibit proliferation due to cumulative toxic effects.

Table 3. T-test Table: Comparison of the Control and Experimental Groups (p values)

FCM5-cell number		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
C	**1ng/ml**	0,613	0,556	0,821	0,965	0,694	0,972	0,905	0,713	0,674	0,563	0,599	0,587	0,550
C	**2,5ng/ml**	0,770	0,807	0,707	0,493	0,647	0,334	0,290	0,242	0,281	0,206	0,182	0,202	0,185
C	**5ng/ml**	0,362	0,616	0,300	0,218	0,344	0,136	0,108	0,140	0,211	0,224	0,27853	0,36288	0,32373

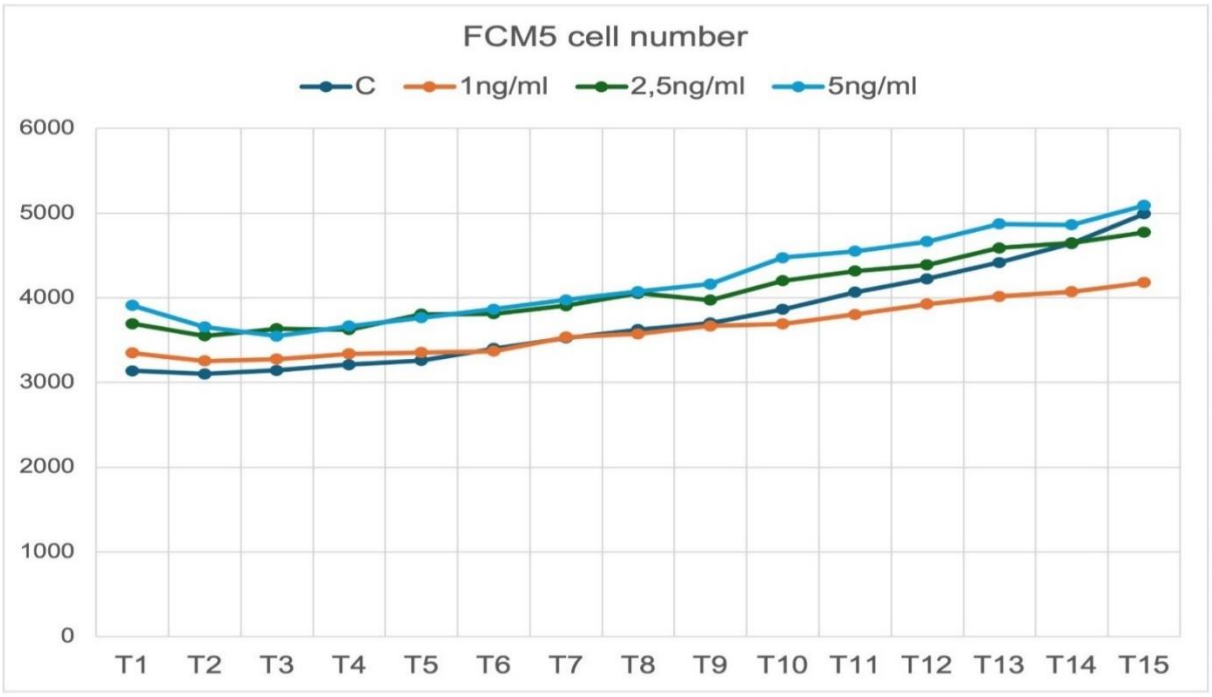

Figure 12. Proliferation of FCM5 (Male) Cell Line Under Different Toxin Concentrations Over 15 Time Points

In the FCM5 male cell line, however, the response to toxin exposure differed notably. Across all toxin concentrations, the cell numbers remained relatively stable, showing minimal deviation from the control values. For instance, at T13, the cell count for the 1 ng/mL treated group was around 3,800, close to the control group's cell count of approximately 3,600, with no statistically significant difference (Table 3). This pattern continued across all time points and concentrations, suggesting that the male cell line did not exhibit the same low-dose growth stimulation seen in the female cells. At the highest concentration (5 ng/mL), cell counts remained close to control levels, with a slight but steady increase in growth, indicating that the FCM5 line was largely unaffected by toxin exposure within this range. This lack of a significant response in the male line suggests a possible sex-specific resistance or insensitivity to the proliferative effects of low-dose toxins (Figure 12).

These results highlight several important points about cellular response to environmental toxins. The increase in proliferation in the female FCF5 cell line under low-dose toxin exposure suggests a hormetic response, where cells adapt or react to mild stressors by increasing their growth rate. This effect was only observed at the 1 ng/mL concentration, as higher toxin levels

seemed to counteract this stimulatory effect. Conversely, the male FCM5 cell line did not exhibit any statistically significant changes in proliferation across toxin levels, pointing to a possible inherent difference in how male and female cells respond to environmental stressors. These findings emphasize the importance of considering both dose-dependency and gender differences when assessing the biological impact of toxins. Further studies might investigate the molecular mechanisms behind these responses, particularly the pathways that allow female cells to react adaptively to low-level toxins, as well as explore the implications of these findings in environmental safety and toxicology research.

This experiment explores the effects of T-2 toxin on the proliferation rates of female (FCF5) and male (FCM5) chicken Primordial Germ Cell (PGC) lines, as measured by doubling time. Doubling time is an important metric in cell biology, indicating how long it takes for a cell population to double in number. A lower doubling time signifies a higher proliferation rate, meaning that the cells are dividing and growing more quickly. In contrast, a higher doubling time suggests slower cell division and lower proliferation. By analyzing how doubling times vary with different concentrations of T-2 toxin, we can gain insight into how this toxin influences cell proliferation in male and female PGC lines differently.

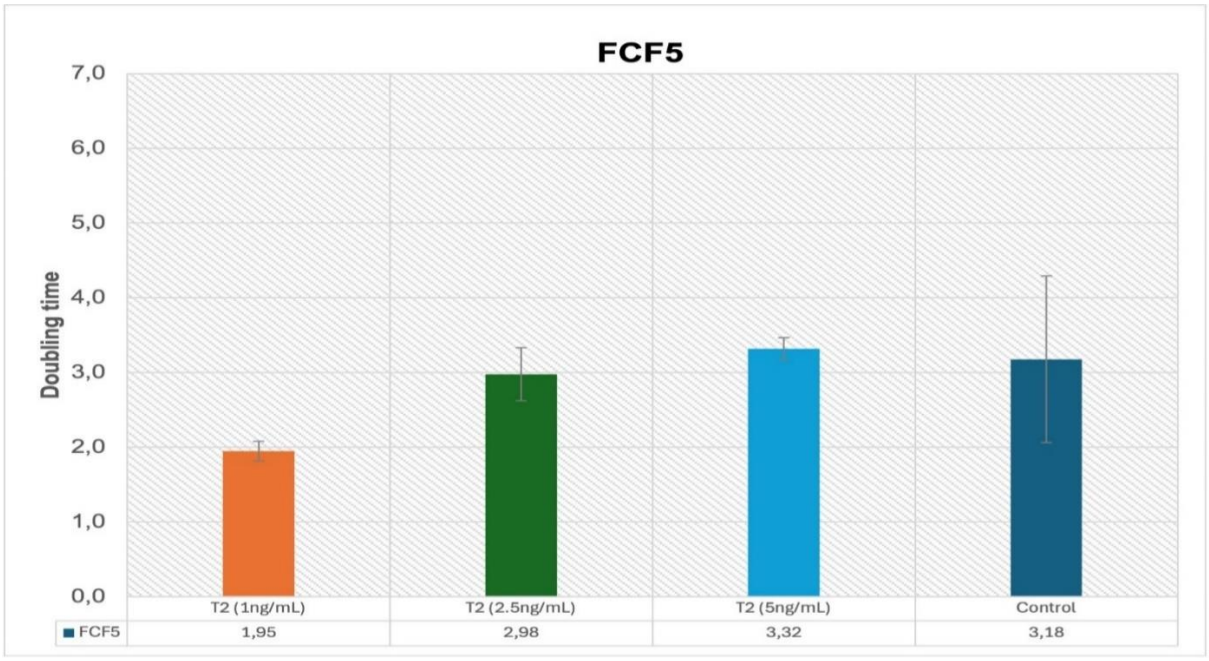

Figure 13. Effect of T-2 Toxin Concentration on Doubling Time of FCF5 PGC Line

In the female PGC line, FCF5, the doubling times reveal interesting patterns across the different concentrations of T-2 toxin. At the lowest concentration of 1 ng/mL, the doubling time is

significantly lower, at 1.95 hours, indicating that cell proliferation is notably high under this condition. This suggests that a low concentration of T-2 toxin may have a stimulatory effect on cell division in female PGCs. However, as the concentration of T-2 toxin increases to 2.5 ng/mL, the doubling time rises to 2.98 hours, and further to 3.32 hours at the highest concentration of 5 ng/mL. This trend shows a dose-dependent decrease in proliferation as the toxin concentration increases, with the doubling time for the control (no toxin) at 3.18 hours. The pattern suggests that while low-dose T-2 toxin may enhance proliferation in female cells, higher doses reduce cell division, possibly due to increased toxicity or stress response at elevated levels (Figure 13).

In contrast, the male PGC line, FCM5, shows a much different response to T-2 toxin. Across all concentrations tested, the doubling time remains relatively high, indicating a generally lower proliferation rate compared to the female line. At 1 ng/mL, the doubling time is 5.68 hours, signifying low proliferation. At 2.5 ng/mL, it decreases slightly to 5.10 hours, and at the highest concentration, 5 ng/mL, the doubling time drops to 3.85 hours. Although there is a reduction in doubling time at higher concentrations, the male cells do not reach the high proliferation rates seen in female cells, even under similar toxin conditions. The control doubling time is 3.87 hours, indicating that T-2 toxin appears to suppress cell division in male PGCs more consistently, especially at lower concentrations (Figure 14).

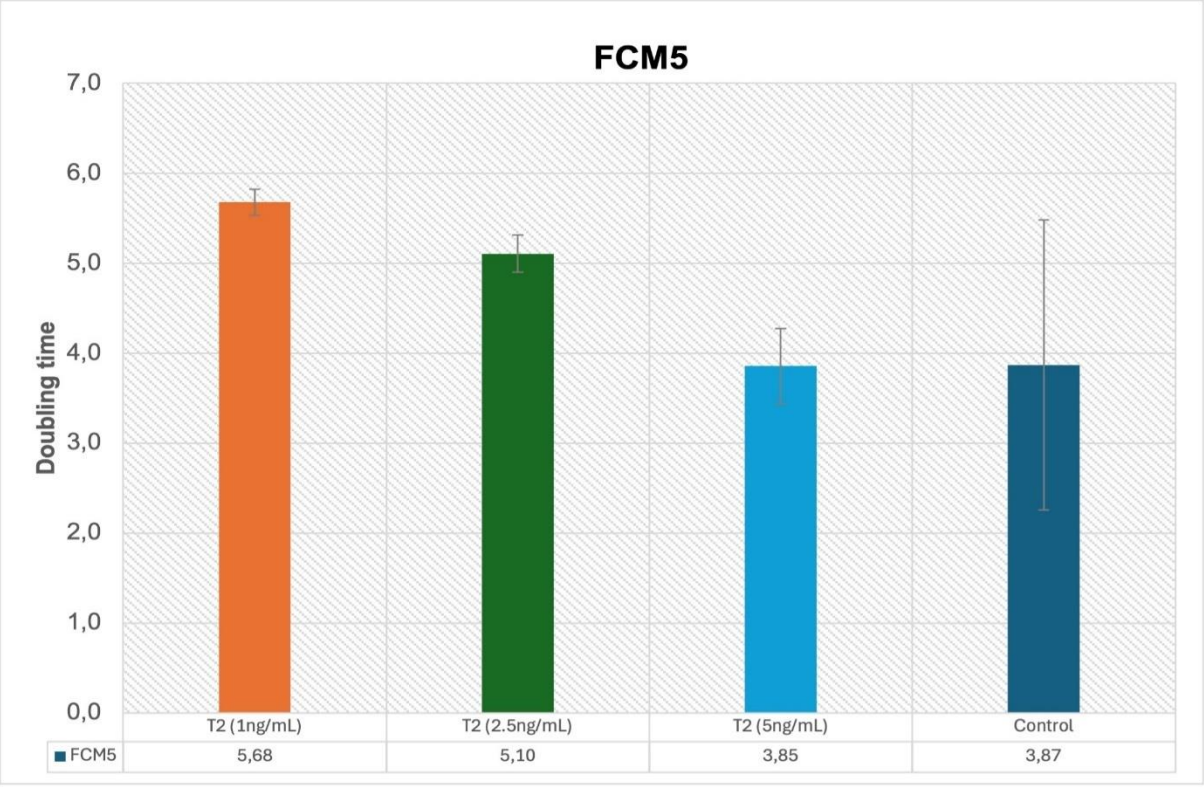

Figure 14. Effect of T-2 Toxin Concentration on Doubling Time of FCM5 PGC Line

Overall, the findings suggest that T2 toxin affects male and female PGC lines in distinct ways, demonstrating a possible sex-specific response to the toxin. The female cells show what appears to be a biphasic response, where low doses of T2 may enhance cell division, while higher doses inhibit it, approaching or exceeding the control level. The male cells, on the other hand, exhibit suppressed proliferation across all concentrations, with only a slight improvement at the highest concentration tested. These results could imply that male PGCs are more sensitive to T2 toxin's inhibitory effects on cell division, while female cells might experience an initial stimulatory effect at lower doses before inhibition sets in with higher toxin levels.

These observations highlight the need to consider sex differences in toxicological studies, as they can reveal unique responses to environmental toxins like T2. The differential impact of T2 on male and female PGC proliferation underscores the complex interactions between toxin dose, cell type, and sex. However, it's important to note that this experiment represents a single trial, and further studies are necessary to validate these results and understand the mechanisms behind these distinct responses in male and female PGCs.

4.4. Assessment of Toxin Sensitivity in Male and Female Chicken Primordial Germ Cells Using Fluorescence-Based Cell Analysis

In this experiment, I cultured two cell lines, FCFM5 (male) and FCF5 (female), derived from chicken Primordial Germ Cells (PGCs), and prepared them for treatment with varying concentrations of a toxin. Initially, I used the Arthur Fluorescence Cell Analyzer to measure the cell density before the toxin treatment. This machine provided an accurate count of live cells by detecting specific fluorescent markers, shown in the green and red fluorescence histograms at the bottom of (Figure 15).

These histograms indicate the distribution of different cell populations, which was essential for calculating the exact cell numbers in the PGC suspension. By using these measurements, I standardized each well by adding precisely 2050 cells, to ensure consistent experimental conditions. The plate setup was carefully organized using Biorender to visually arrange and mark each well. This layout shows that wells from B3 to B6 and G3 to G6 contain the FCF5 (female) cell line under various toxin concentrations. The remaining wells represent the male (FCFM5) cell line, also exposed to the same range of toxin concentrations, allowing for a direct comparison of both cell lines under similar treatment conditions. The next day, after seeding the cells, I introduced different concentrations of the toxin to the culture medium (Figure 15).

For the final step, I used the ImageXpress Pico Cell Imaging System to capture high-resolution images and conduct a quantitative analysis of cell viability and proliferation across the treatment conditions.

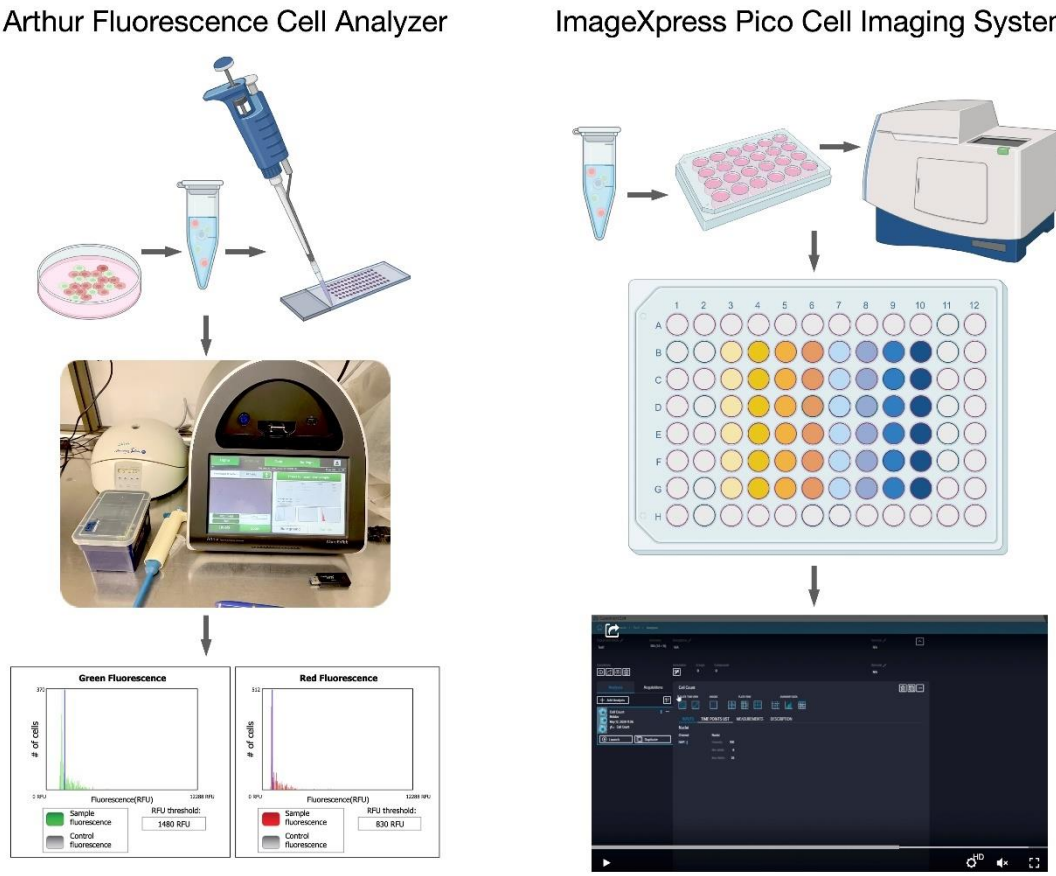

Figure 15. Workflow and Analysis of Chicken Primordial Germ Cells Using Arthur Fluorescence Cell Analyzer and ImageXpress Pico Cell Imaging System (This Figure was created using the BioRender application).

The Pico machine is equipped with multi-channel fluorescence capabilities, enabling it to identify and count cells based on green, red, blue, and orange fluorescence. This precise detection allowed me to monitor the health, proliferation rate, and population changes of the cells, providing insights into their response to the toxin. The data generated by the Pico machine, as displayed at the bottom of Figure 15, offers detailed information on cell viability across the treatments, highlighting any sex-specific responses in toxicity thresholds and cell health.

By assessing the differences in toxin sensitivity between male and female PGCs, this experiment contributes to our understanding of the potential impact of environmental toxins on reproductive cells. Monitoring the changes in fluorescence and cell populations under different conditions allows for a deeper analysis of cell health, providing valuable insights into sex-specific cellular responses and possible implications for environmental and biological research.

4.5. Optimization of Fluorescence Parameters for Accurate Cell Quantification

The top image shows a fluorescent picture of cells taken using the ImageXpress Pico Cell Imaging System. Cells are marked with different fluorescent signals, with colors such as green and red indicating specific fluorescence channels. Cells that express both markers appear in purple due to the overlap of red and green fluorescence. In the lower image, the system's software has analyzed and counted the cells based on predefined parameters, providing a color-coded output: red for green fluorescence, blue for red fluorescence, and purple for dual fluorescence (both green and red markers), (Figure 16).

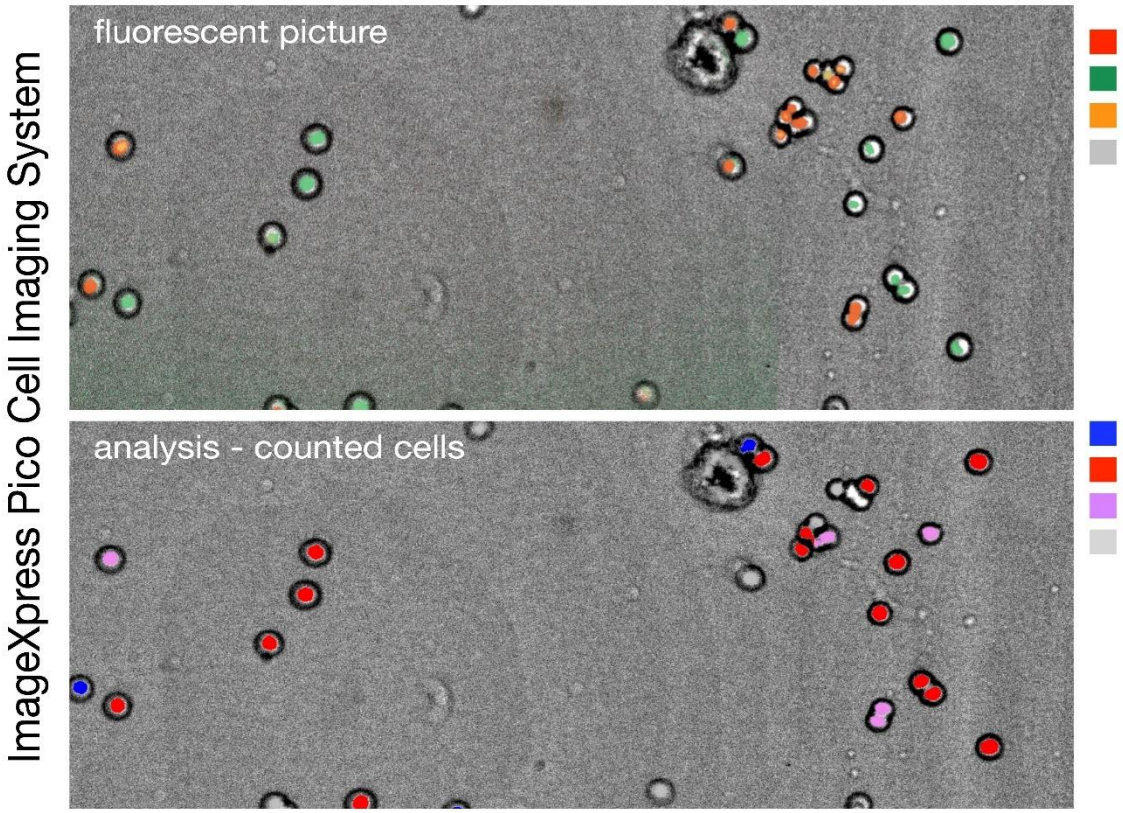

Figure 16. Fluorescent Imaging and Cell Counting Analysis Using ImageXpress Pico Cell Imaging System

This analysis image reflects how the system quantifies cell populations based on fluorescence. By adjusting the detection parameters, I can control which cells are counted and ensure the accuracy of the cell counts relative to the original fluorescent image. In this case, the parameters used were effective, as most cells from the fluorescent image are accurately counted and classified in the analysis. However, some cells, particularly those shown in gray, were not counted. These gray cells likely represent low-fluorescence cells where the intensity of the red or green signal was insufficient for detection by the system, as seen in the middle section with faint orange and blue cells. This level of parameter customization is valuable for optimizing detection accuracy, especially for low-expressing cells, allowing me to refine the settings to ensure comprehensive and accurate quantification.

4.6. Gender-Specific Responses in Cell Proliferation Under Varying Toxin Concentrations in FCF5 and FCM5 Cell Lines

The analysis of cell proliferation under different toxin concentrations in the FCF5 (female) and FCM5 (male) cell lines reveals notable differences in cellular response between the two cell types. In these experiments, the cells were exposed to varying concentrations of a toxin (1 ng/ml, 2.5 ng/ml, and 5 ng/ml) over multiple time points (T1 to T13), with cell counts recorded at each interval. The color coding remains consistent across the graphs: dark blue for the control (untreated) group, orange for 1 ng/ml, green for 2.5 ng/ml, and light blue for 5 ng/ml, which allows for easy comparison of the results across different conditions (Figure 17), (Table 4).

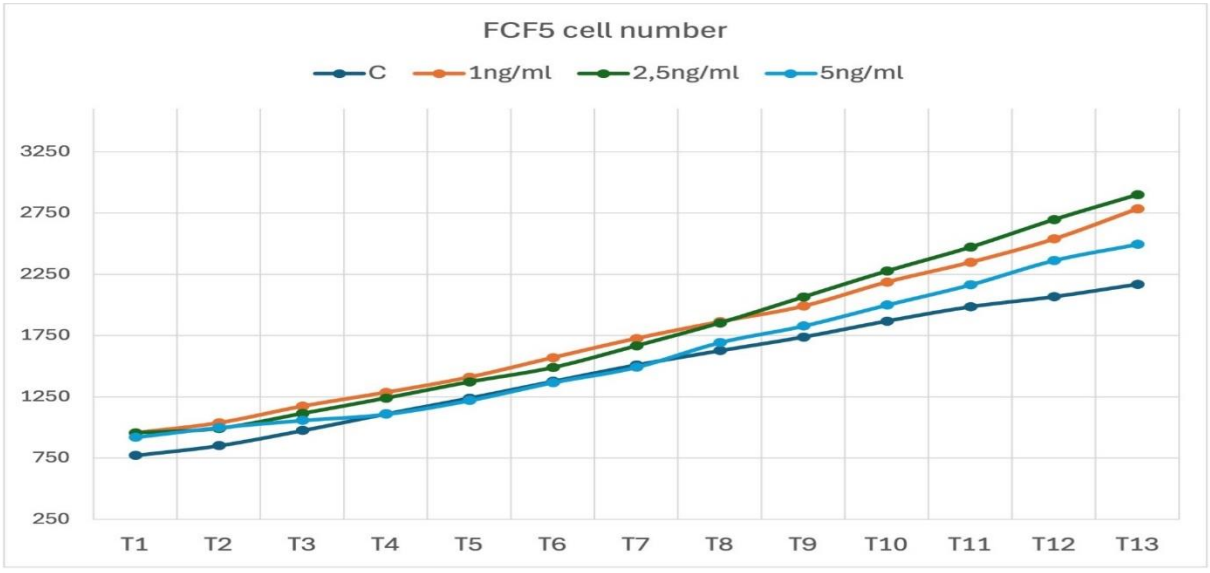

Figure 17. Proliferation of FCF5 (Female) Cell Line Under Different Toxin Concentrations

Table 4. T-test Table: Comparison of the Control and Experimental Groups (p values)

FCF5- cell number		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
C	1ng/ml	0,323	0,330	0,312	0,390	0,440	0,397	0,348	0,339	0,310	0,232	0,211	0,104	0,051
C	2,5ng/ml	0,280	0,453	0,459	0,516	0,552	0,614	0,514	0,366	0,228	0,157	0,132	0,071	0,047
C	5ng/ml	0,478	0,483	0,700	0,990	0,931	0,966	0,940	0,798	0,743	0,652	**0,566**	**0,374**	**0,355**

In the FCF5 cell line, a clear trend emerges, indicating that lower concentrations of the toxin may enhance cell proliferation relative to the control. By the final time point, T13, cells treated with 1 ng/ml and 2.5 ng/ml have average cell numbers of approximately 2,245 and 2,535, respectively, compared to the control's 1,850 cells. This increase suggests that low-dose toxin exposure might stimulate cellular pathways that promote cell division in the female cell line. Furthermore, while the 5 ng/ml concentration continues to exhibit an elevated proliferation rate, reaching around 2,720 cells by T13, this higher dose does not result in the same proportionate increase seen at 1 ng/ml and 2.5 ng/ml. This plateau in cell number at the highest concentration could imply that the toxin has a stimulatory effect up to a certain threshold, beyond which the enhancement diminishes, possibly due to a mild inhibitory impact on cellular processes. (Table 5), (Figure 17).

In contrast, the FCM5 (male) cell line shows a consistent proliferation rate across all toxin concentrations, with only minimal deviations from the control. By T13, the control group in FCM5 reaches around 1,670 cells, while the groups treated with 1 ng/ml, 2.5 ng/ml, and 5 ng/ml reach 1,750, 1,700, and 1,680 cells, respectively. These numbers demonstrate that the variations among treated and untreated groups in the male cell line are minor, and no significant dose-dependent increase is observed. This consistency across treatments suggests that the male cell line may be less responsive to the toxin's proliferative effects, or that these concentrations do not significantly impact its growth dynamics. The clustering of data points for each treatment concentration implies that, at least within this experimental range, the toxin does not influence the proliferation of the male cell line in a statistically significant way (Figure 18), (Table 6).

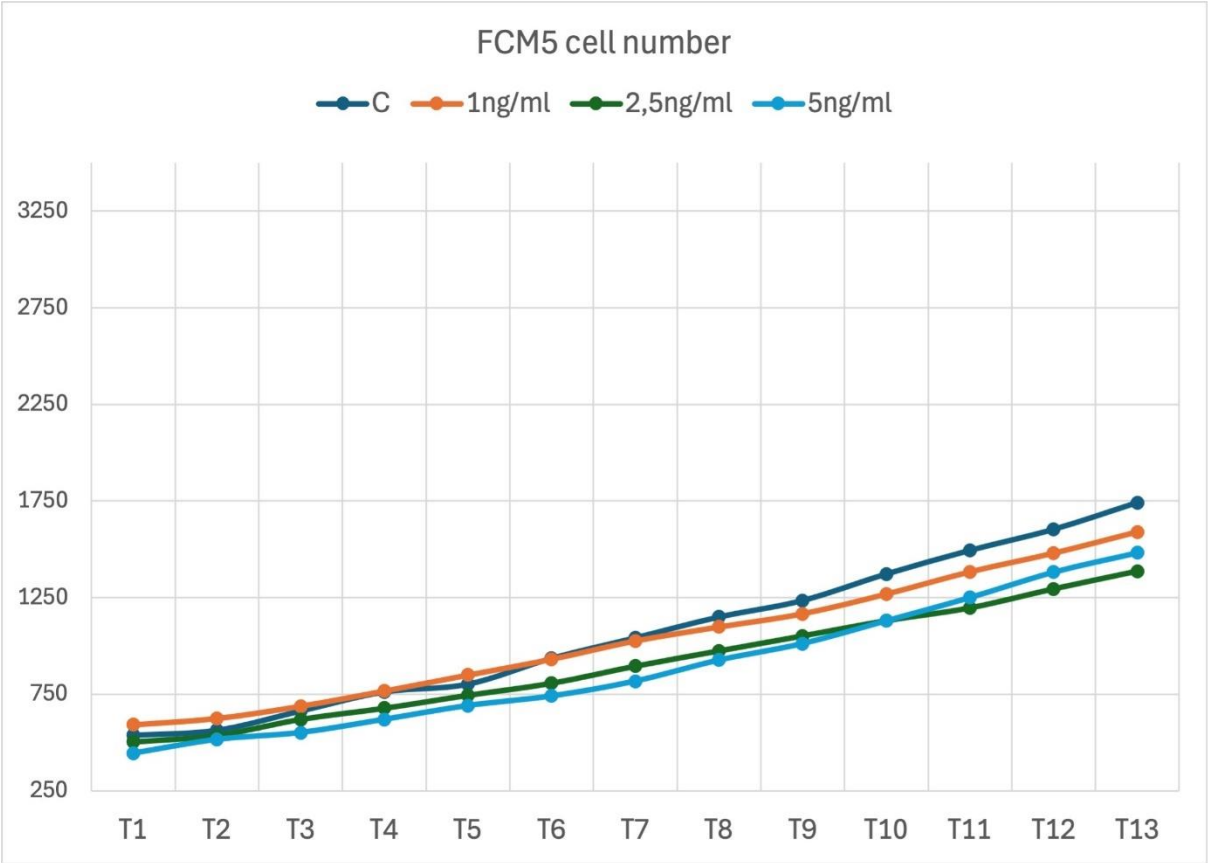

Figure 18. Proliferation of FCM5 (Male) Cell Line Under Different Toxin Concentrations

These results align with prior findings, reinforcing the notion that lower concentrations of the toxin selectively enhance proliferation in the FCF5 (female) cell line, whereas the FCM5 (male) cell line remains largely unaffected.

Table 5. T-test Table: Comparison of the Control and Experimental Groups (p values).

FCM5-cell number		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
C	**1ng/ml**	0,613	0,556	0,821	0,965	0,694	0,972	0,905	0,713	0,674	0,563	0,599	0,587	0,550
C	**2,5ng/ml**	0,770	0,807	0,707	0,493	0,647	0,334	0,290	0,242	0,281	0,206	0,182	0,202	0,185
C	**5ng/ml**	0,362	0,616	0,300	0,218	0,344	0,136	0,108	**0,140**	**0,211**	**0,224**	**0,27853**	**0,36288**	**0,32373**

4.7. Impact of Treatment Concentration on G2 Phase Cell Cycle Progression in FCF5 Cells

The graph shows the progression of green cells (representing the G2 phase in FCF5 cells) over time under different treatment conditions: control (C), 1 ng/ml, 2.5 ng/ml, and 5 ng/ml concentrations. Notably, the 5 ng/ml treatment (indicated by the blue line) significantly affects the green cell count, particularly towards the later time points (T12 and T13).

As the experiment progresses, we observe a steady decline in green cells across all treatment conditions. However, the decline is most pronounced with the 5 ng/ml treatment, where the cell count significantly drops below other conditions by T12 and T13. This suggests that at a 5 ng/ml concentration, the treatment profoundly disrupts the cell cycle, likely slowing or halting progression through the G2 phase. The p-values marked in blue (for T12 and T13) confirm the statistical significance of this difference, indicating that this concentration affects the G2 phase more substantially than lower concentrations (Figure 19).

Additionally, compared to the 1 ng/ml and 2.5 ng/ml treatments, which display a relatively gradual decline, the 5 ng/ml treatment causes a sharp drop, especially from T7 onwards. This suggests a threshold effect where higher concentrations might lead to increased toxicity or stress, potentially stalling cells in earlier phases and preventing them from progressing through the G2 phase, (Table 7).

In summary, these results highlight a dose-dependent impact of the treatment on cell cycle dynamics, with the 5 ng/ml concentration notably inhibiting green cell progression by the end of the experiment. This significant reduction in green cell count underlines the sensitivity of the cell cycle to higher concentrations, supporting the hypothesis that elevated treatment levels disrupt cellular proliferation and could have cytostatic or cytotoxic effects.

The graph shows the impact of varying toxin concentrations (1 ng/ml, 2.5 ng/ml, and 5 ng/ml) on the male PGC line, specifically examining the G2 phase of the cell cycle, represented by green fluorescence. The y-axis reflects the percentage of cells in the G2-green phase over multiple time points (T1 to T13), allowing us to observe how cell populations in this phase fluctuate in response to increasing toxin levels.

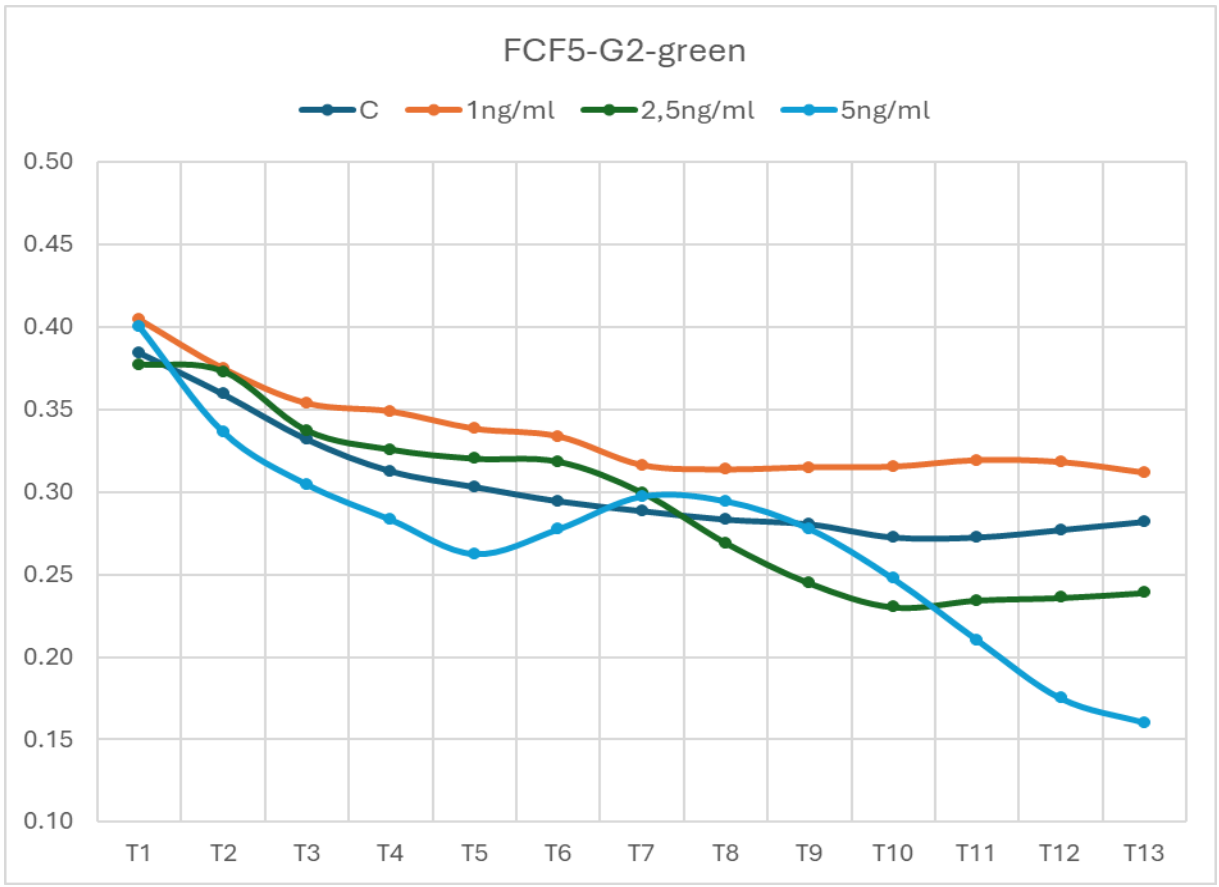

Figure 19. Effect of Treatment Concentration on G2 Phase Cell Count in FCF5 Cells Over Time

At the lowest toxin concentration of 1 ng/ml, the data indicate that the green cell population remains relatively like the control group (C). This suggests that, at low concentrations, the toxin does not substantially disrupt the G2 phase population, pointing to minimal or no cytotoxic impact. Although there are slight fluctuations, the values remain close to control levels, indicating that cells might be able to tolerate or repair minor disruptions at this concentration.

Table 6. T-test Table: Comparison of the Control and Experimental Groups (p values)

FCF5- Green - G2%		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
C	**1ng/ml**	0,282	0,489	0,296	0,061	0,098	0,113	0,246	0,279	0,218	0,087	0,072	0,132	0,301
C	**2,5ng/ml**	0,739	0,548	0,811	0,498	0,290	0,276	0,606	0,605	0,169	0,070	0,132	0,143	0,188
C	**5ng/ml**	0,337	0,343	0,264	0,171	0,058	0,513	0,765	0,721	0,928	0,296	**0,017**	**0,001**	**0,001**

Moving to a moderate toxin concentration of 2.5 ng/ml, we observe a reduction in the green cell percentage compared to both the control and the 1 ng/ml group. However, this decrease is not statistically significant, suggesting that the cell cycle begins to be affected at this concentration, though not to a degree that disrupts the overall population. The decline hints at early signs of cellular stress or toxicity, likely impacting the G2 phase, but the cells retain some resilience.

At the highest toxin concentration, 5 ng/ml, the effect on the G2 phase cells becomes significantly pronounced, with a marked and sustained decrease in the green cell population from time point T5 onward. This reflects a substantial cytotoxic effect, where the toxin likely interferes with cell cycle progression in the G2 phase. The sharp reduction in cell numbers at this concentration indicates that cells are unable to maintain normal G2 phase progression, possibly due to severe DNA damage or cellular stress that inhibits the cell cycle. From time point T9 onwards, the difference between the control and the 5 ng/ml group becomes statistically significant, underscoring the time-dependent nature of the toxin's effects; prolonged exposure exacerbates toxicity, possibly leading to cell cycle arrest or apoptosis within the G2 phase.

The data provide strong evidence that high toxin concentrations (5 ng/ml) severely disrupt the cell cycle in male PGC lines by reducing the population of cells in the G2 phase. The effect is both dose- and time-dependent, with lower concentrations showing negligible impact, while higher levels cause notable interference as exposure continues. This finding suggests that controlling toxin levels is essential to prevent disruption of cell cycle dynamics, which is critical for cellular health and proliferation, especially in a research context where maintaining healthy cell lines is essential.

4.8. Cell Cycle Disruptions in FUCCI-Labeled Cells Under Toxin Treatment: A Sensitive In Vitro Toxicity Assessment

In examining the cell cycle phase marked by red fluorescence in FCF5 and FCM5 cells, we observed significant alterations in the cell cycle profile under toxin treatment compared to the control. This change is not merely a shift in the percentage of cells within the red (G1) phase, but a broader, more profound restructuring of the cell cycle dynamics (Figures 20, 21).

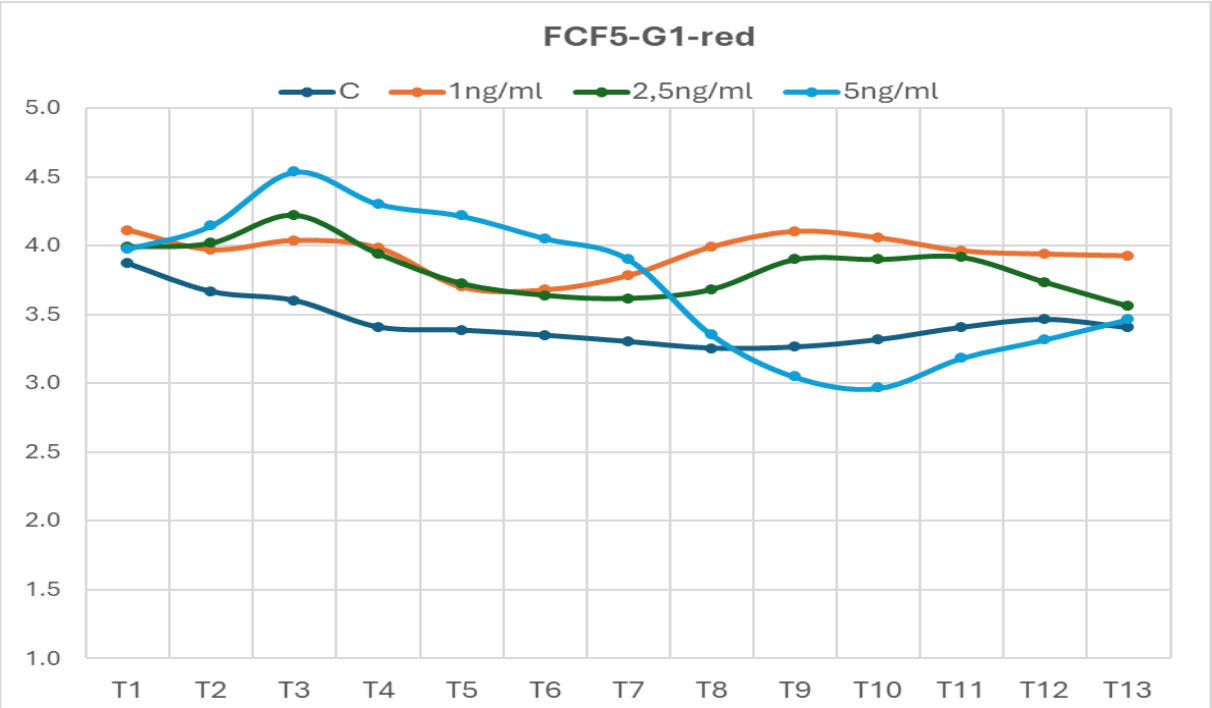

Figure 20. Effect of Toxin Treatment on G1 Phase Dynamics in FCF5 Cells

In early time points (around T1 to T3), cells exposed to the toxin at various concentrations (1 ng/mL, 2.5 ng/mL, and 5 ng/mL) demonstrated an increased percentage of red-labeled cells relative to the control, indicating an initial accumulation in the G1 phase. This suggests that toxin exposure initially causes a delay or arrest in the G1 phase. However, as the treatment continues, particularly noticeable at T10, the 5 ng/mL concentration group shows a substantial reduction in red cells compared to the control, reflecting a marked inhibition in cell proliferation or a shift in cell cycle dynamics, likely leading to fewer cells progressing through the cycle, (Table 7,8).

Table 7. T-test Table: Comparison of the Control and Experimental Groups (p values)

FCF5- red - G1%		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
C	**1ng/ml**	0,245	**0,036**	**0,007**	**0,025**	0,196	0,229	0,057	**0,007**	**0,001**	**0,003**	**0,037**	0,056	0,074
C	**2,5ng/ml**	0,560	**0,007**	**0,002**	**0,035**	0,179	0,303	0,174	0,096	**0,005**	**0,030**	0,066	0,295	0,556
C	**5ng/ml**	0,692	**0,015**	**0,001**	**0,007**	**0,009**	**0,036**	**0,040**	0,690	0,345	0,148	**0,439**	**0,630**	**0,830**

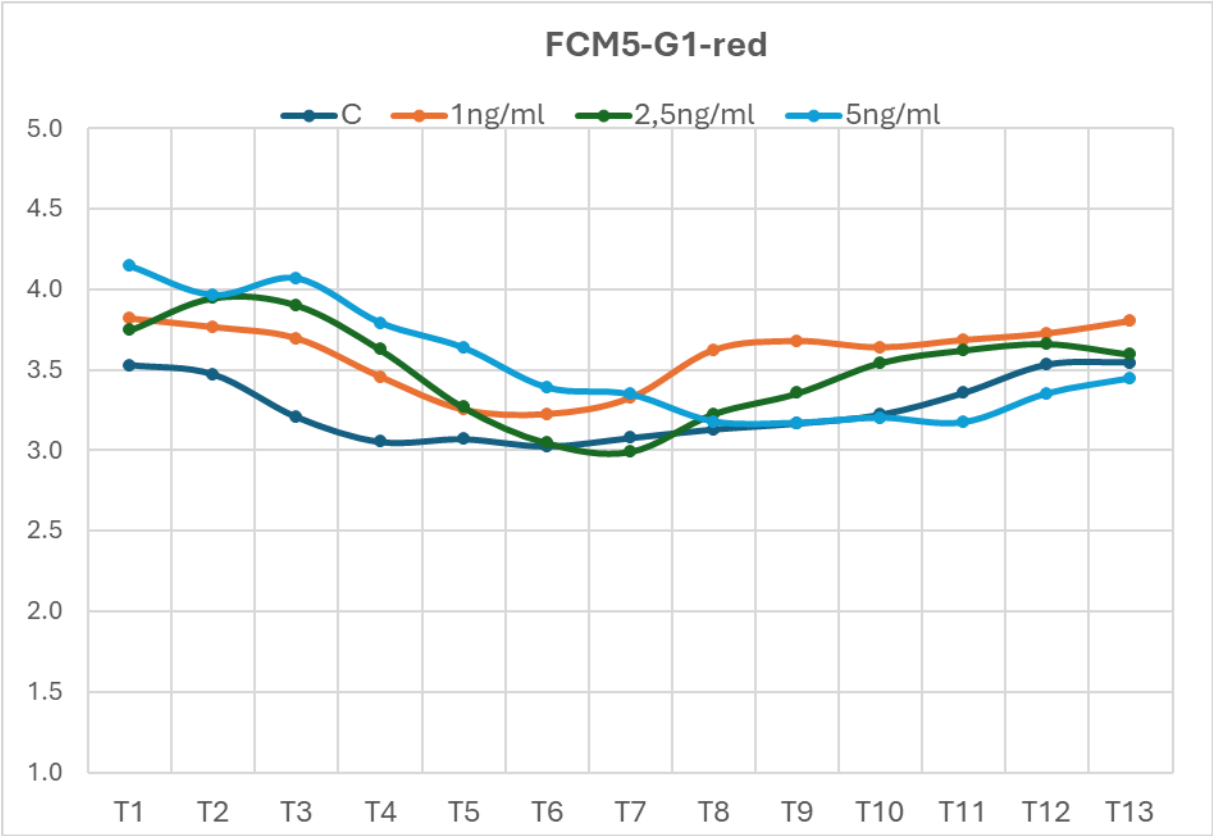

Figure 21. Impact of Toxin Treatment on G1 Phase Dynamics in FCM5 Cells

These findings underscore that toxin treatment does not merely influence the number of red-labeled cells but actively reshapes the cell cycle trajectory. Specifically, treated cells exhibit a distinct temporal pattern in G1 phase occupancy compared to the untreated controls, indicating that the toxin's effects extend beyond cell number and involve deeper disruptions to cell cycle regulation.

Table 8. T-test Table: Comparison of the Control and Experimental Groups (p values)

FCM5- red-G1%		T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13
C	**1ng/ml**	0,302	0,222	0,055	0,194	0,550	0,490	0,398	0,106	0,077	0,201	0,264	0,438	0,272
C	**2,5ng/ml**	0,419	0,032	0,016	0,064	0,437	0,940	0,756	0,732	0,480	0,294	0,361	0,637	0,846
C	**5ng/ml**	0,102	0,132	0,005	0,014	0,059	0,202	0,329	**0,843**	**0,988**	**0,943**	**0,44033**	**0,36527**	**0,61944**

When considering the implications for in vitro toxicity testing, the FUCCI system proves to be exceptionally sensitive in detecting toxin-induced changes. The differential red and green fluorescence signals provide an immediate indication of shifts in cell cycle phases, making this system potentially valuable for early-stage screening of toxic effects. The FUCCI-based fluorescence markers thus offer an advanced sensitivity that surpasses traditional cell number metrics, especially in long-term culture conditions where subtle cell cycle disruptions can be observed more clearly.

5. Conclusion

This work highlights the importance of primordial germ cells (PGCs) in genetic conservation, transgenesis, and cellular analysis, particularly in avian species such as chickens. This study, which successfully established and characterized male (FCM5-ZZ) and female (FCF5-ZW) transgenic PGC lines, indicates that PGC technology is an excellent tool for maintaining avian genetic diversity, particularly in uncommon and native chicken breeds. PGCs use cryopreservation and gene banking to safeguard genetic material in the long term without depending on continuous breeding, which is critical for species with falling populations. In this work, the transgenic PGC lines were demonstrated to be stable, viable, and capable of integrating into host germlines, suggesting their ability to successfully assist conservation programs.

The experimental results provide important insights into the cellular responses of PGC lines under various environmental circumstances, particularly when exposed to T-2 toxin. The study used FUCCI technology to assess cell cycle stages and response patterns in male and female PGCs. The findings reveal a significant sex-specific difference: while female PGCs (FCF5) showed a biphasic response, with increased proliferation at low toxin concentrations and inhibition at higher levels, male PGCs (FCM5) showed resilience and stability across the same toxin doses, with minimal change in proliferation rates. This proposes a sex-specific adaptation mechanism in female cells, allowing for brief proliferation under low stress conditions before toxic consequences take over. The findings suggest that sex chromosomes may alter cellular stress responses, with female cells potentially suffering hormesis—a process in which low amounts of a stressor drive growth.

Further investigation of the FUCCI-labeled cells after T-2 toxin administration revealed that female PGCs suffered considerable alterations in cell cycle distribution, particularly in the G1 and G2 phases, with high toxin levels causing cell cycle arrest and increased cell mortality in the late stages. Male PGCs, on the other hand, showed a consistent cell cycle distribution across doses, indicating innate resistance to the toxin's effects, possibly due to chromosomal or metabolic variations. This differential reaction highlights the importance of sex-specific considerations in toxicological research, since male and female cells may respond differently to environmental stresses and contaminants.

This finding has broader practical ramifications than only genetic conservation. The capacity to cryopreserve and subsequently regenerate species from PGC lines, along with an understanding

of sex-specific cellular responses to toxins, lays the groundwork for future advancements in environmental toxicology, agricultural biotechnology, and animal husbandry. PGC technology helps to conserve biodiversity by maintaining the distinctive genetic profiles of endangered breeds, while insights into cellular stress responses may lead to better breeding and agricultural techniques.

Finally, this paper gives a complete overview of how PGCs might be used for conservation and research, contributing to advances in genetics, toxicity, and biotechnology. Future study might investigate the processes behind the reported sex-specific responses to toxins, as well as improve PGC usage in bird and agricultural conservation initiatives.

6. Summary

The thesis explores how different culture conditions impact male (FCM5-ZZ) and female (FCF5-ZW) Primordial Germ Cell (PGC) lines that express the FUCCI reporter system. This system enables the immediate tracking of cell cycle stages, aiding in distinguishing superior PGC cultures by their ability to integrate in vitro, crucial for studying PGC developmental biology and potential harm from contaminants, specifically mycotoxins.

The goal of the thesis is to evaluate cell cycle dynamics, doubling times, and cell cycle alterations when exposed to the T-2 mycotoxin, utilizing instruments such as the Arthur cell counter and Pico cell image analyzer. The research seeks to forecast the lasting effects of mycotoxin exposure on PGCs through the creation of a cell cycle model. This has wider consequences in farming, raising livestock, and public health, providing valuable information on environmental safety concerning mycotoxin contamination in poultry and food items.

A review of the literature emphasizes important steps in the development of chicken embryos, specifically PGC migration, differentiation, and the significance of PGCs in maintaining genetic variation. The thesis explains the important functions of PGCs in preserving rare bird breeds through cryopreservation and regeneration methods in avian gene banking. It delves deeper into progress in transgenesis and genome editing by utilizing PGCs to modify genes in poultry, contributing to the creation of disease-resistant breeds and bioreactors for crucial proteins.

The techniques section describes the ethical compliance, PGC isolation, culture, and electroporation processes. The FUCCI transgene enables the monitoring of cell cycle stages in cultivated PGCs. Furthermore, the introduction of genetically engineered PGCs into chicken embryos investigates their integration and migration in the gonadal milieu. Sophisticated imaging technologies, such as the Pico machine and Arthur cell analyzer, are used to monitor cell responses in real-time under controlled conditions, especially when examining toxicological consequences.

In conclusion, this work advances our understanding of PGC biology and mycotoxin impacts, with potential applications in food safety, preservation, and agricultural technologies.

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DECLARATION

__ **GOUNI Moustafa** _____ (name) (student Neptun code: ___ **YALCOI**_____) as a consultant, I declare that I have reviewed the **final thesis**/thesis/dissertation/portfolio[1] and that I have informed the student of the requirements, legal and ethical rules for the correct handling of literary sources.

I recommend / do not recommend[2] the **final thesis** / dissertation / portfolio to be defended in the final examination.

The thesis contains a state or official secret: yes **no***[3]

Date: ____**2024**_____ year ____**November**_____ month __**04**_____ day

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