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COMPARISON OF THE DEVELOPMENTAL POTENCIAL OF MALE (ZZ) AND FEMALE (ZW) PRIMORDIAL GERM CELL LINES

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2. *Introduction and aims*

The protection of endangered species, which are being pushed out of production, is being addressed through gene preservation by various state and civil organizations. Several gene preservation methods exist, within which *in vivo* and *in vitro* options are distinguished. The type of cell or tissue used for gene preservation depends on the species to be preserved. For mammals, sperm cryopreservation technology is the most widely used method (Ugur et al., 2019; Yánez-Ortiz et al., 2022). However, in the case of bird species, due to their unique reproductive biological characteristics, this technology only provides a partial solution. Promising results in this area have been demonstrated with primordial germ cells (PGCs) (Petitte, 2006) or the cryopreservation of gonadal tissues (Hu et al., 2022; Liptoi et al., 2013; Liptoi et al., 2020; Tiambo et al., 2021), although these methods still require development for most of the species.

Primordial germ cells are unipotent cells, which are the precursors of gametes (Kimura and Nakano, 2011). Their use in research is extremely diverse, as they play a significant role not only in gene preservation (Lázár et al., 2021) but also in veterinary health experiments related to reproductive biology (Trefil et al., 2017), the production of gonadal chimeric animals (Tajima et al., 1998), and in avian transgenesis (Divya et al., 2021). In domestic chickens, the isolation and culture of PG cells is well-established, thanks to the description of effective methods and culture media. These *in vitro* cultures provide an excellent foundation for the above-mentioned research, provided that the cell cultures consist of a sufficient number of healthy cells. Several methods are available to assess the condition of the cultures, but many of them can be harmful to the cells, meaning that conclusions about the overall state of the culture can only be drawn based on measurements performed on a small number of cells.

The FUCCI (Fluorescent Ubiquitination-based Cell Cycle Indicator) transgenic complex provides a tool for monitoring an entire cell culture. This tool is based on the phenomenon of proteins that are periodically expressed during the

cell cycle (Blow and Dutta, 2005). Since specific proteins are produced in the cell at certain stages of the cell cycle, by linking reporter genes labeled with different colors of fluorescence to these proteins, the cell can undergo a cyclic color change during the stages of cell division. This allows the creation of cell cultures that, with appropriate excitation, enable the assessment of the current health status and suitability of the cells for experimental use, considering the entire culture.

Although, recently researchers managed to adept the FUCCI transgene to several species, there hasn't been any occasion for creating FUCCI positive chicken cells. This experiment aimed to achieve this.

Aims:

During the experiment, the aims were as follows:

- establishment, maintenance, and characterization of chicken PGC lines,
- creating the FUCCI plasmid using copetent cells,
- transfection of the FUCCI complex into chicken PGCs,
- creation and characterization of stable transgenic FUCCI cell lines,
- creation and characterization of stable FUCCI cell lines from single cell origin,
- proving the presence of the FUCCI transgene in cells,
- examination of mycotoxin effects on FUCCI cells.

3. Materials and methods

Introduction of the White Hungarian chicken breed

Hungarian chicken breeds are generally characterized by medium body size (2.0-2.3 kg for hens, 2.5-3.0 kg on average for roosters), well-developed, rounded breast muscles, high-set wings, and a deep abdomen. Their legs are of medium length and yellow. Their heads are small, with a rounded skull, and their beaks are short but strong at the base. The comb is medium-sized, serrated, and backward-reaching, upright, though often tilted in hens. The wattle is rounded, and the earlobe is oval and bright red. Since the breed is dual-purpose, it produces high-quality, finely-textured, flavorful meat, but its egg production is also noteworthy (140-150 eggs per year). We chose this breed for our experiment because the in vitro procedures for this breed are already well-established and have been carefully documented by my colleague, Bence Lázár (Lázár et al., 2021).

Ethics approval

Animals were kept and maintained according to general animal welfare prescriptions of the Hungarian Animal Protection Law (1998; XXVIII). All experimental methods described herein were approved by the Institutional Ethics Review Board of the Institute for Farm Animal Gene Conservation (No. 7/2011). The experiments were done in the Applied Embryology and Stem Cell Biology Group's laboratory on the Institute of Genetics and Biotechnology of the Hungarian University of Agriculture and Life Sciences, with the approval and animal experimental and transgenic lisenses of the institute.

Maintenance of chicken experimental stocks

The eggs used to establish the primordial germ cell cultures came from the NBGC Institute of Farm Animal Gene Conservation gene bank stock. The animals were housed in outdoor runs with barns, at a stocking density of 5-6 individuals per square meter and a sex ratio of 7 hens to 1 rooster. Laying took place in two-

tiered nests, with an intensity of 5 hens per nest. The animals were fed laying hen feed *ad libitum*. To improve egg quality, the feed was supplemented with lime grit. The eggs were collected twice a day and then placed in a refrigerated egg storage. The incubation took place in the Institute of Genetics and Biotechnology laboratory, using a Midi F500S incubator (PL Machine Ltd., Tárnok, Hungary), at a temperature of 37.8°C, with 70% humidity and 45° rotation every hour.

Isolation of PGCs

The blood samples were taken from Hamburger & Hamilton stage 15-17 embryos (approximately 51-56 hours). After removing the eggs from the incubator, I disinfected them while maintaining their position in the incubator, then cracked them into a petri dish, ensuring that the yolk membrane remained intact and that the embryo was positioned on top of the yolk. The blood collection itself was performed under a microscope using a sterile glass micropipette attached to a mouth pipette, with a diameter of approximately 30-40 µm. The penetration occurred in the dorsal aorta of the embryo, from where I drew approximately 1.5-2.0 µl of blood. Simultaneously, I also isolated tissue samples from each embryo for later sex determination, which I stored at -20°C until further analysis. All blood collection data (serial number, date, developmental stage, deformities) were documented.

Establishment and maintenance of the PGC lines

I transfered the blood containing the primordial germ cells into a PGC culture medium. Using this solution, stable primordial germ cell cultures can be obtained within 2-3 weeks after isolation. The medium was replaced three times a week (Monday, Wednesday, Friday) on the cultures. The cell cultures were maintained in a CO2 incubator (Sanyo MCO-19AIC (UV) Incubator, Sanyo, Japan) at 38°C, with high humidity and 5% CO_2 concentration.

Sex determination

For sex determination, I thawed the frozen embryonic tissue samples and isolated DNA from them using the High Pure PCR Template Preparation Kit (Roche Diagnostics, 11796828001, USA). For sex determination, I used the CHD1 primer pair (Griffiths et al., 1996; Lee et al., 2010). The PCR was performed using the ProFlex PCR System (Applied Biosystems, USA). The obtained products were analyzed by gel electrophoresis.

RNA expression measurements

I collected cell samples from the stable cell cultures. After thawing, I performed RNA isolation according to the protocol of the RNAquarious™-Micro Total RNA Isolation Kit (Thermo Fisher Scientific, AM1931, USA) by adding 125 µl of pure ethanol. I mixed 15 µl of the sample with 15 µl of MasterMix, which I prepared according to the instructions of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814, USA).

After PCR, the obtained cDNA samples were analyzed using quantitative real-time PCR. The reagents used for this contained nuclease-free water, Power SYBR Green Master Mix (Applied Biosystems, 4368575, USA), forward and reverse primers, and cDNA sample per reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Integrated DNA Technologies, USA) primer was used as a control, while the chicken VASA homolog (CVH) (Integrated DNA Technologies, USA) and the *DAZL* gene primer (Deleted in azoospermia-like gene) (Integrated DNA Technologies, USA) were used as stem cell-specific markers. The qPCR was performed using the Mastercycler® Realplex Real-Time PCR System (Eppendorf, Germany). ROX was used as a reference dye.

Immunohistochemical staining

The fixation was performed with 4% PFA (Fluka, 30525-89-4, Switzerland). Afterward, I applied a blocking solution (PBS, 0.1% BSA, 0.1% TritonX (Fluka, 93426, Switzerland), 2.5% donkey serum) to the samples. For the primary

antibodies, I used stem cell-specific anti-SSEA-1 (Millipore, MC480, Germany) and germ cell-specific anti-CVH (Dr. Bertrand Pain, Stem Cell and Brain Research Institute (SBRI), Lyon, France). The anti-SSEA-1 was paired with the Anti-Mouse-IgM-rD549® (Jackson ImmunoResearch, 715-505-140, USA) red secondary antibody, while the anti-CVH was paired with the Alexa Fluor® 488 Anti-Rabbit-IgG (H+L) (Life Technologies/Molecular Probes, A-21207, USA) green secondary antibody. I used deep red TO-PRO™-3 iodide (642-661) (Invitrogen, T3605, USA) nuclear stain for all samples (Bink et al., 2001), which was digitally modified and displayed as blue in the images. The samples were then covered with ProLong Diamond Antifade Mountant with DAPI (Invitrogen, P36962, USA) and a coverslip. The evaluation of the prepared samples was performed using a Leica TCS SP8 confocal microscope (Leica, Germany).

Freezing and thawing of cell lines

I performed the check for the ideal cell count (approximately 80,000 cells/300 µl medium) and purity using the NanoEntek Arthur fluorescent cell counter (NanoEntek, South Korea), based on the cell size and shape. For cryopreservation, I used the FAM2 cryopreservation medium developed by Kong et al. (Kong et al., 2018). The samples were stored in a -150°C freezer.

During thawing, I placed the samples in a 38°C water bath for 90 seconds, then quickly added 900 µl of a 3:1 DMEM+water mixture to dilute the DMSO and mitigate its harmful effects. I centrifuged the cells, discarded the supernatant, and after another wash, I resuspended the cells in culture medium and put them onto a culture plate. After the cell lines were established, I changed the medium daily for two days to ensure optimal conditions for the cells' acclimatization.

Plasmid isolation

The FUCCI transgene, the piggyBac transposon, and the hyPBase transposase were provided by Dr. András Nagy (Lunenfeld-Tanenbaum Research Institute, Toronto, Canada). The plasmids were transformed into competent

Escherichia coli cells with the help of Dr. Orsolya Hoffmann (Hungarian University of Agriculture and Life Sciences, Institute of Genetics and Biotechnology, Model Animal Genetics Group), and these bacteria were cultured on agar plates to obtain colonies. I collected individual colonies and transferred them into separate liquid media. When the cultures reached the desired cell density through liquid culture, I isolated the plasmid DNA using the EndoFree® Plasmid Maxi Kit (Qiagen, 169026122, Germany). The samples were then diluted either in OptiMEM medium (Gibco, 11058-021, USA) or PBS to the desired 1200 ng/µl concentration, in order to compare the potential effects of the two solutions on the success of electroporation.

Electroporation

Based on the characterization of the cell lines, I selected the female cell line coded '1111' and the male cell line coded '1116' for the transgenesis, as these were pre-characterized, stable cultures. I collected the two lines separately, then set aside 20 µl from each for further cultivation as an absolute control, and I created a concentration of 1 million cells/100 µl from the rest. From this cell suspension, I aliquoted three 100 µl samples: one for plasmids diluted in OptiMEM, one for plasmids diluted in PBS, and one as an electroporation control. Additionally, I kept 30 µl of cell suspension in OptiMEM for 15 minutes to observe the effects of the solution on the cells.

For electroporation of 100 µl of cell suspension, 10 µg of plasmid DNA was required, which I dissolved in 8 µl of solution. The ratio of FUCCI plasmid to hyPBase enzyme in the reaction was 1:1, so 4 µl of each solution contained 5 µg of plasmid, resulting in a final plasmid concentration of 1.2 µg/µl. For transfection, I used the Neon Transfection System (Invitrogen, MPK5000, USA) electroporator and its accessories. The settings for the device were as follows: 1300 V voltage, 10 ms duration, and 4 pulses.

Establishment and maintenance of transgenic cell lines

After electroporation, I placed the transfected cell solution into pre-prepared culture medium in a 12-well tissue culture plate, with two separate wells for each. The following day, I examined the cell lines using a Leica DFC 7000T stereo microscope (Leica, Germany) to check their fluorescence and, thereby, the success of the electroporation.

I checked the established fluorescent transgenic lines every other day under the stereo microscope. Additionally, over the following month, I regularly checked the cell count and the green/red cell ratio using the Arthur fluorescent cell counter, and also took photographs with the Leica confocal microscope. For these latter analyses, I used TO-PRO-3™ nuclear stain. Based on these observations, I was able to reliably monitor the development of the cell lines. Ten days after electroporation, the cultures reached the appropriate cell count, allowing me to cryopreserve two samples per line, ensuring their long-term preservation.

Establishment of single-cell-based cultures

To establish pure transgenic cultures, I initiated single-cell-based clonal cultures. I selected the fluorescent cells from drops using a mouth pipette with an attached glass microcapillary. The selected cells were then placed into the wells of a 96-well tissue culture plate, each containing 70 µl of medium.

I labeled the single-cell cultures established from the female 1111 line as FCF (FUCCI female) and those from the male 1116 line as FCM (FUCCI male). I attempted to establish 20 cultures from each sex within a 10-day period (FCF1-9: 23.11.2022; FCF10-20 + FCM1-5: 24.11.2022; FCM6-8: 30.11.2022; FCM9-20: 02.12.2022).

Three weeks after the initial establishments, 6 clone lines remained, consisting of three male (FCM5-7-8) and three female (FCF3-4-5) cell cultures. Of these, the FCF3-4 lines, although initially displaying fluorescent signals, had lost the fluorescence by this point. I cryopreserved the six lines in separate tubes

for later use (1 tube per line). Since the cell quality and fluorescence intensity were most prominent in the FCF5 and FCM5 lines, I selected these two clonal cultures for further experiments.

Examination of integrational potential: injecting FUCCI cells into recipient embryos

Using cell counting, I established the ideal concentration of 5000 cells/µl for injection. The suspensions were placed in 1.5 ml tubes in the incubator at 38°C until the start of the experiment (but for no longer than a few hours).

For the injection, I incubated White Hungarian chicken eggs. The eggs were disinfected in the laboratory, and then, using tweezers, I carefully made a window on the top of the eggshell, as well as at the air sac. I removed the membrane at the window and prevented the embryo from drying out by adding a few drops of PBS. Then, using a glass microcapillary attached to a mouth pipette, I drew up 2 µl of the warmed cell suspension and injected the cells into the bloodstream of the embryo by guiding the capillary into the embryo's heart tube. Afterward, I sealed the egg with UV sterilized parafilm, and by rotating the embryo to the opposite side of the window, I returned it to the incubator.

On the 7th day of incubation, I removed the injected embryos from the eggs and isolated the gonads. The isolated gonads were examined and photographed immediately under a stereomicroscope to confirm the presence of the fluorescent signal.

Chromosomal analysis

For chromosome analysis, I added 20 µl of KaryoMAX™ Colcemid solution (10 µg/mL, Gibco, USA) to the cell suspension, then after a two-hour incubation, I collected the cultures into 1.5 ml Eppendorf tubes. The cells were centrifuged for 7 minutes at 300 g, then suspended in 0.56% KCl solution (hypotonic treatment), and after 10 minutes of incubation at room temperature, I fixed them in a 3:1 methanol–acetic acid mixture in three steps. I dropped the cell suspension

onto a wet microscope slide, air-dried it at room temperature, and then stained it for 7 minutes with 5% Giemsa stain in phosphate buffer (Alfi et al., 1973; Anand et al., 2018). After washing the slides with distilled water, I air-dried them at room temperature and examined them under a microscope (Zeiss Axioskop2 Plus) at 1200x magnification.

DNA sequencing to prove the integration of the transgene

Genomic DNA was isolated from cell pellets with the Roche High Pure PCR Template Preparation Kit and sequenced on an Illumina NovaSeq (Illumina, San Diego, CA) instrument, paired end, with a read length of 150 nt.

Sequencing quality was controlled using FastQC, before alignment (Andrews, 2010). Reads were then aligned to the whole genome (bGalGal1.mat.broiler.GRCg7b, Ensembl release 110)(Martin et al., 2023), complemented with the fasta sequence of the FUCCI transgene system. The PB_CAG_FUCCI sequence was appended to the top level genomic fasta as an extra contig. The complemented genome was then indexed and the reads were aligned using bwa-mem2 (Vasimuddin et al., 2019). The alignment parameters were set to default.

First, aligned reads, that were at least partially aligned to the FUCCI sequence, were selected, after which the whole set of aligned reads was filtered based on the selected read IDs using Samtools 1.16 (Danecek et al., 2021). Reads that were chimerically aligned to the FUCCI sequence and a genomic scaffold or read pairs of which one read was aligned to the FUCCI sequence and the other to a genomic scaffold were used to determine the exact location of the transgene integrations, using coverage and clipping information. In the cases we judged as true positive hits, multiple reads were overlapping the integration site. In these cases, coverage data and read alignment positions were extracted from the alignment files to identify the exact coordinates.

Affected genes were determined by intersecting the integration coordinates with the relevant annotation file (Ensembl release 110). Position of the integration

sites and relevant reads were visually controlled using IGV as well (Robinson et al., 2011)

Using mycotoxin treatment to alter the cell cycle

In the experiment, I investigated the effects of 5 ng/ml T-2 and 20 ng/ml zearalenone concentrations, as well as their combined effect. The mycotoxins were provided by Dr. Zsuzsanna Szőke (Hungarian University of Agriculture and Life Sciences, Institute of Genetics and Biotechnology, Reproductive Biology and Toxicology Group) for the experiment.

I dissolved the mycotoxins in PGC culture medium. Throughout the experiment, I adhered to the mandatory safety and hazardous waste disposal regulations for handling mycotoxins. I transfered cells from the FCF5 and FCM5 lines into a 12-well plate at a density of 20,000 cells per well, and treated the PGCs with mycotoxin-containing medium for three days without changing the medium. Before and after the experiment, I examined the fluorescence of the cells under a confocal microscope. For each sample, I took 5 images with a 10x objective lens, which were analyzed using the KALSZÁM program (a Java-based program developed by Ákos Kalcevszki for analyzing confocal microscope images) to determine the fluorescence ratio in the cultures.

Creating timelapse videos to prove the color change in the cells

To create the timelapse videos, I seeded the FCF5 and FCM5 cells into a 96-well plate at a concentration of 9,000 cells per well. The measurements were performed using an ImageXpress Pico Cell Imaging System (Molecular Devices, USA), an automated microscope, with the assistance of Dr. Ferenc Uher and Dr. Zsolt Matula (South Pest Central Hospital, National Institute of Hematology and Infectious Diseases), as well as Dr. Zoltán Hegyi (Bio-Science Ltd.). The measurement lasted for 29 hours, with photos taken every 10 minutes. The obtained data were then analyzed using the Fiji image editing and analysis program (Schindelin et al., 2012) and timelapse videos were created.

Statistical analysis

For the qPCR results, we calculated the target gene expression to internal control gene expression ratio for each sample. We used the GenEx 7.0 software (Multid Analyses AB, Sweden) with the $2^{-\Delta\Delta Ct}$ formula, where ΔCt = Ct of the target gene — Ct of the internal control, and $\Delta\Delta Ct$ = ΔCt of the test sample — ΔCt of the control sample. For statistical analysis between groups, we used the t-test, also with the GenEx 7.0 software. The presented data are shown as the mean ± standard deviation, and a result with a p-value below 0.05 was considered statistically significant. The significance levels were as follows: $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***.

4. Results and their discussion

Establishment statistics

The cell lines involved in the experiment were established and cryopreserved in 2019 by my colleague Bence Lázár and form part of our institute's gene bank collection. A total of 42 blood samples were taken from the White Hungarian breed, of which 20 successful cell line establishments were achieved, corresponding to a 47.6% efficiency rate. A total of 120 samples were cryopreserved from these establishments, which are stored in both our gene bank at -150°C and the NBGC Institute of Farm Animal Gene Conservation gene bank (Lázár, 2020). With the exception of the establishment, the characterization of the cell lines is the result of my own work.

Sex determination

Based on sex determination via PCR, it can be stated that out of the 20 established cell lines, 14 were male, and 6 were female. This corresponds to a 70% male and 30% female cell line ratio. For the experiment, we selected the female cell line marked 1111 and the male cell line marked 1116, as they exhibited the most promising proliferation capacity.

RNA expression measurements

Based on qPCR, I determined that both of the cell lines I selected expressed the germ cell-specific markers, making them suitable not only for gene banking but also for use in further experiments.

Immunohistochemical experiment

Based on the photos taken with the confocal microscope, both cell lines expressed both markers, further confirming that I had started the experiment with properly conditioned PG cells. I did not repeat the immunostaining step on the transgenic

cell lines, as the FUCCI green/red fluorescence would have interfered with the signal from the secondary antibodies available to us.

Transgene expression results after the electroporation

On the first day following electroporation, the presence of FUCCI transgene fluorescence was successfully detectable, both under the stereomicroscope and using the Arthur fluorescent cell counter. The data supported the presence of transgenic cells in the cultures. According to the measurements from the Arthur cell counter, the proportion of fluorescent cells in the male cell line was 26%, while in the female cell line, it was 32%. However, since FUCCI cells do not show fluorescence in the M phase, the actual transgenic proportion was likely higher than these values.

Transgene expression results in the clone lines

Through continuous monitoring of the culture quality, I was able to determine the point at which the lines were suitable for initiating single-cell-based cultures. This time point was identified based on the distribution of fluorescence present in the culture, using images from the Arthur fluorescent cell counter and photos from the confocal microscope. The data clearly showed that the fluorescent cells were present in higher proportions in the cloned cultures than in the starting transgenic lines. This increase was 52% in the male line and 8% in the female line. The smaller increase observed in the female line is likely due, in part, to the lower proliferation rate observed in the line and, in part, to the aggregation formation typically seen in female PGC cultures.

Characterization of the clone cell lines

I characterized the FCF5 and FCM5 single-cell lines as described above, as well as with additional details. Based on the results, both cell lines preserved their characteristic sex- and stem cell gene expression properties despite long-term in vitro cultivation. For both lines, I obtained the expected sex outcome (FCF5 was

indeed female-originating, FCM5 was indeed male-originating, with no technical errors occurring during the extended culture period), and despite the transgenesis, they stably expressed the CVH and DAZL markers. The gene expression results indicated that electroporation did not have any significant effect on the function of these two genes. Repetition of the immunohistochemical analysis was not possible due to fluorescent interference. No chromosomal abnormalities were found in either clone cell line. According to the analyses, both cell cultures contained 8 pairs of macrochromosomes and 30 pairs of microchromosomes, with FCF5 carrying the ZW sex chromosome pair and FCM5 carrying the ZZ sex chromosome pair. Therefore, the diploid chromosomal number of the cell lines is 2n = 78.

Effectivity of integration into recipient embryos

I injected FUCCI transgene-containing PGC cells into recipient embryos to investigate their integration ability. In the male group, a total of 10 injections were performed, of which 5 embryos survived to day 6.5. Of these, 3 contained fluorescent cells integrated into the gonads. In the female group, I conducted more experiments; out of 31 injections, I was able to isolate gonads from 20 embryos, of which 3 contained fluorescent cells. However, it is important to note that in several cases, I observed fluorescent cells in the blood vessels outside the gonads, meaning that the injection was successful, but integration had not yet occurred. This could easily be attributed to the relatively early gonad isolation, meaning that not all cells had migrated to the gonads yet. Although attempts were made to fix the chimeric gonads for later sectioning and confocal analysis, these efforts were unsuccessful, likely due to the rapid degradation of the cell cycle-specific proteins used by FUCCI.

DNA sequencing results

To confirm the integration of the transgene, we performed sequencing on two single-cell-derived cell lines that I created, in order to determine the number of

integrations and their location within the genome. We identified plasmid integration in 2 genomic loci of the FCM5 PGC line, at the genomic coordinates of NC052533.1: 50 890 131 (chr2) and NC_052543.1: 13 339 941 (chr12), which are located in the genes GLI3 and CADPS, respectively (Fig. 3E-F.). Both integrations occurred in an intronic sequence. In the FCF5 PGC line, we detected one integration site at the coordinates (chr27) NC052558.1:3028482. It is located in an intergenic region, the closest genes being (Ensembl ID, no name) LOC121107680 and NGFR (annotation version: Ensembl release 110). In all three cases, the insertion is positioned after a TTAA sequence.

In the case of the FCM5 PGC line, at the integration sites on chromosome 12 and in the FCF5 PGC line, the whole FUCCI transgene was integrated (vector coordinates: PB_CAG_FUCCI:2338-8864). It is likely the case in the other integration in the FCM5 PGC line as well, although it cannot be stated with certainty, due to a few overlapping reads at the integration site. Overall, aligned reads are within the vector coordinates PB_CAG_FUCCI:2335-8864. Interestingly, we observed consistent positive alignment across the region at the (chr14) NC_052545.1:4183720-4185523 coordinates in all – transgenic and non-transgenic - samples. This site is within the ACTB gene, and shows a similar number of reads aligned in all samples. However, I believe this can be explained by the fact that the beta-actin promoter we used is also present in the genome of the Hungarian White chicken, and this signal was detected during the measurement.

The effects of mycotoxin treatment and the lack of medium change on the cell cycle

Based on the obtained data, it appeared that the T2 toxin significantly increased the proportion of red cells in the G1 phase over three days in both cultures, while the proportion of green cells in the G2 phase significantly decreased in both cultures, and the proportion of yellow cells in the S phase significantly decreased in the male cell line.

In the second phase, the cultures were treated with 20 ng/ml zearalenone toxin, and the measurement procedure was the same as described above. The results were also very similar to those observed after the T2 treatment. In the male cell line, all three phases showed significant differences, while in the female cell line, significant effects were observed in the G1 and G2 phases.

Finally, we were curious about the combined effect of the two mycotoxins to check for possible synergistic or antagonistic interactions between them. To this end, we used a medium containing 5 ng/ml T2 and 20 ng/ml zearalenone simultaneously on the cells. The result again followed the pattern previously described: the increase in the proportion of red cells and the decrease in the proportion of green cells were significant in both cell lines, while the decrease in the proportion of yellow cells was significant only in the male cell line.

However, since no medium change was performed during the mycotoxin treatment, I had the opportunity to investigate how a three-day period without fresh medium affects the proliferation of the cells. To this end, I repeated the experiment without the mycotoxin treatment. The results obtained were strikingly similar to those obtained after the mycotoxin treatment. Although the cell count significantly increased between the 24th and 72nd hours in both cell lines, the proportion of red cells increased in both cases, while the proportion of green and yellow cells decreased. The increase in red cell proportion and the decrease in green cell proportion were significant in the female cell line, while the decrease in yellow cell proportion was significant in the male cell line. However, the trends themselves were observed in both cell lines.

Based on these results, it can be concluded that although the absence of medium change during the mycotoxin treatment could have influenced the outcome, the data dispersion was much smaller in the mycotoxin treatment group, which indicates more drastic effects caused by the presence of the mycotoxins. To confirm this, I plan to repeat the mycotoxin treatment experiment with regular medium changes to eliminate the potential influence of the lack of fresh medium on the results.

Proving the transgene expression changes in the cells

I examined the cyclic changes in the fluorescence of the cells using an automated microscope. The obtained image series was analyzed with the Fiji program and a timelapse video was created for better visualization. Based on this, it is clearly observable that after cell division, the cells fluoresce red, then transition to yellow after a short period, followed by a change to green, and finally lose their color before the next cell division (*Figure 1*).

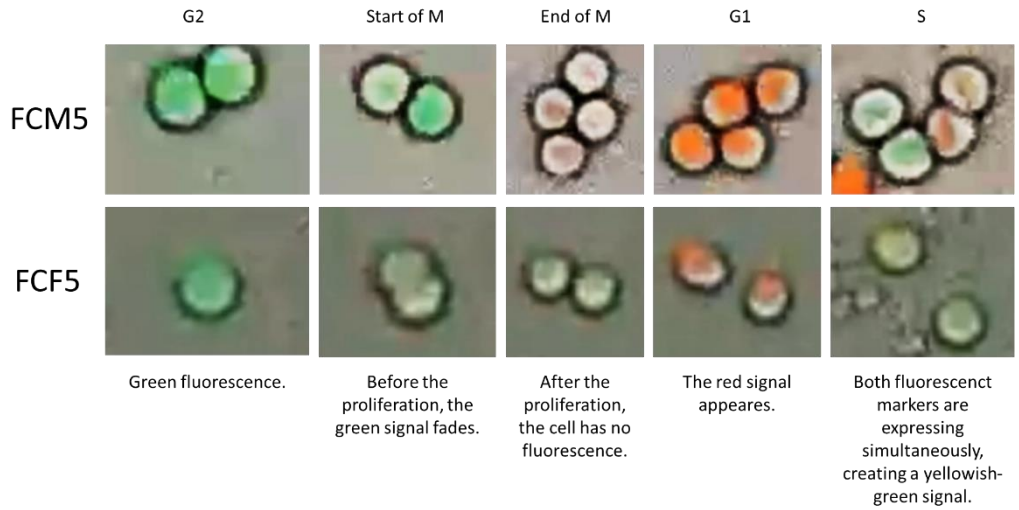

Figure 1: The color changes of FUCCI primordial germ cells in different stages of the cell cycle. *The distinct individual expression of the two reporters (red or green) and their combined expression (red and green → yellow/greenish-yellow) can be clearly observed in the different stages of the cell cycle.*

5. Conclusions and recommendations

Different domesticated bird species are of significant importance for humanity, both from an economic and nutritional standpoint, and thus also in experimental contexts. Economically, they are the world's most important source of animal protein, making poultry meat the most widely produced meat product, thanks to their ease of management, good feed conversion rates, and the fact that their consumption is permitted across all major cultures and religions. Besides meat, egg and feather production also represent significant industries.

From an experimental perspective, birds serve as excellent models for reproductive biology and embryology (Bednarczyk et al., 2021), and due to their high protein content, their eggs are promising candidates for use as bioreactors for the production of various proteins (Sheridan, 2016). Therefore, the design and successful execution of experiments involving birds are of great importance. The research I have conducted may contribute to this effort.

Avian primordial germ cells (PGCs) are increasingly gaining importance in various fields of research. Their role has already been demonstrated in animal health (Trefil et al., 2017), in gene preservation (Chaipipat et al., 2022; Lázár et al., 2021), and in transgenesis (Petitte and Mozdziak, 2014). For chickens, long-term culture of PGCs is already well-established (Whyte et al., 2015), and there are ongoing developments for the use of PGCs from other bird species (Chen et al., 2019b; Nakamura, 2015). Based on these advancements, the use of avian primordial germ cells is expected to play an increasingly significant role in the near future.

In the literature review, I aimed to outline the challenges involved in adapting a transgenic technology to a new species, as well as how past research has contributed to the development of a well-established, fully functional tool. Additionally, these studies highlight the potential of FUCCI transgenic lines. From my results, it can be concluded that adapting this technique to economically important animal species could provide valuable insights into their developmental

biology, responses to external factors, and reproductive biology. The domestic chicken species, which we selected, is an excellent model for studying embryological development, while the established primordial germ cell lines can offer valuable data on the factors influencing the formation of gonads and germ cells.

In my experiments, I observed that in the presence of mycotoxins, the cell cultures exhibited behavior indicative of cell cycle synchronization. Specifically, there were samples where 100% of the cells showed red fluorescence, indicating that they remained in the G1/G0 phase. This finding is consistent with previous observations suggesting that stress induced by low temperatures can also trigger cell cycle synchronization (Enninga et al., 1984). Various methods have been developed for cell cycle synchronization, including physical techniques (such as centrifugal separation, flow cytometry, or dielectrophoresis based on cell size or density) and chemical methods (such as mitotic selection, DNA synthesis inhibition, or serum withdrawal) (Banfalvi, 2016). The phenomenon we observed strongly supports the idea that FUCCI cell lines are well-suited for investigating the consequences of different environmental stressors.

The stable FUCCI-positive transgenic chicken PGC lines I successfully established have great potential for use in various research fields. At the end of my experiments, I briefly explored the effects of different mycotoxins using FUCCI-PGC lines, primarily to demonstrate the functionality of the transgene. This topic is of considerable importance, as mycotoxins not only pose harmful effects on livestock but can also accumulate in animal-derived products, thereby endangering human health through consumption. Therefore, there is a significant need for research the effects of mycotoxins, from both an economic and health perspective. In the experiment presented in this thesis, I investigated the effects of only two mycotoxins; however, I recommend including several others (such as deoxynivalenol, aflatoxin, fumonisin B1) in future studies of this nature. It is also advisable to investigate the combined effects of various mycotoxins.

Primordial germ cells provide an excellent model for studying embryonic development, specifically the development of the different sexes and their gametes in avian species. As these cells migrate through various parts of the body during development, they are continuously exposed to different environmental factors. Among these factors, the negative effects generated by mycotoxins can also occur. Certain toxins can have particularly detrimental effects on embryonic development, and researching these effects can offer not only veterinary and production-related benefits but also reveal important trends for human medicine.

In addition to the toxic effects, research on the cellular-level impacts of temperature increases due to climate change is highly relevant. This is especially important for avian species, as their offspring develop outside the mother's body, making them more exposed to environmental temperature changes. Our group has previously studied the heat treatment of chicks and its effects on embryo development and RNA expression (Tóth et al., 2021). FUCCI cell lines offer a new opportunity to study the developmental and gene expression responses of primordial germ cells to heat stress. Furthermore, by incorporating additional Hungarian breeds, we could more accurately assess their temperature tolerance, thus determining their genetic value in the fight against global warming.

This experiment has already been initiated through the work of my colleague, Arnold Tóth, and his B.Sc. student, Júlia Bognár. In their study, they are working with samples treated at temperatures of 40, 41, 42, and 43 °C, alongside a control group held at 38°C. Even in the early stages of the experiment, they have already demonstrated that the FUCCI model I developed is excellent for studying the effects of heat stress.

The cell lines I have created may also be useful for developing techniques commonly used in laboratories. During embryonic development, PGCs do not proliferate uniformly, so certain developmental stages may be more optimal for performing specific operations (e.g., chimeric gonad isolation) than others. The FUCCI cell lines provide a useful tool for this. It may also be worthwhile to develop a protocol for synchronizing the cell cycle of an entire culture, as cells at

specific developmental stages may integrate into the gonads more efficiently and establish colonies more reliably than other cells. Additionally, by creating synchronized cell lines, we could greatly improve the efficiency of both gonadal chimera production and PGC-based transgenesis.

However, in addition to PGCs, it could also be valuable to investigate other cell types using the FUCCI system. To achieve this, the production of transgenic gonadal chimeras would be necessary, followed by their crossbreeding to create transgenic animals. These individuals, as they would carry the transgene in every cell, would open new avenues for studying the effects of external factors on the cell cycle of various cell types. This approach could significantly enhance our understanding of how different environmental and physiological conditions influence cellular processes across various tissues, providing insights into broader biological mechanisms.

The researches presented above clearly demonstrate that the FUCCI system can be adapted to new species. In the case of avian primordial germ cells (PGCs), the primary challenge lies in the lack of universal culture media. Currently, the only species for which a stable culture medium is available for PGC maintenance is the domestic chicken. However, this does not mean that efforts to incorporate other species have not been made. Culture media have already been developed for species such as the domestic duck (Anas platyrhynchos domestica) and quail (Chen et al., 2019b; Tae et al., 2008), and there have also been attempts in our laboratory to include the domestic goose and the helmeted guinea fowl (Numida meleagris). Additionally, experiments related to the bronze turkey are planned. If these medium development efforts succeed in enabling the long-term in vitro maintenance of PGCs from these species, it will open up new possibilities for transgenic studies in economically important poultry species.

6. New scientific achievements

My new scientific achievements are the followings:

1. I successfully optimized and applied the piggyBac transposon system for efficient delivery of the FUCCI reporter vector into chicken primordial germ cells
2. I was the first to establish both male (ZZ genotype) and female (ZW genotype) chicken PGC lines, each stably containing the FUCCI vector integrated into their genomes.
3. I demonstrated that the FUCCI-transfected PGC lines (FCF5 for ZW and FCM5 for ZZ) could integrate into the gonads of recipient embryos, confirming their potential for transgenesis.
4. Through whole-genome sequencing, I confirmed that the FUCCI vector integrated into the FCF5 cell line as a single copy and into the FCM5 cell line in two copies, confirming stable integration in both cases.
5. Using timelapse video analysis, I validated that the expression of the fluorescent proteins in the FCF5 and FCM5 cell lines fluctuates according to the cell cycle phases, providing visual confirmation of FUCCI's functionality.
6. I demonstrated that under conditions where culture medium changes were not performed, as well as under T-2 and zearalenone treatment, the percentage of cells in the G1 phase increased significantly in both the FCF5 and FCM5 lines, confirming that these cells respond to environmental changes and stressors.

7. Important scientific publications

Journal articles with an impact factor related to the topic of the thesis:

- Bence Lázár, Nikolett Tokodyné Szabadi, Mahek Anand, Roland Tóth, **András Ecker**, Martin Urbán, Maria Teresa Salinas Aponte, Ganna Stepanova, Zoltán Hegyi, László Homolya, Eszter Patakiné Várkonyi, Bertrand Pain, Elen Gócza: Effect of miR-302b MicroRNA Inhibition on Chicken Primordial Germ Cell Proliferation and Apoptosis Rate. 2022, ***GENES***, 13(1), 82, **IF: 3,5**, doi: 10.3390/genes13010082. **Q2**
- **András Ecker**, Bence Lázár, Roland Imre Tóth, Martin Urbán, Nikolett Tokodyné Szabadi, Maria Teresa Salinas Aponte, Mohd Adnan, Eszter Várkonyi, Elen Gócza: The Effects of Freezing Media on the Characteristics of Male and Female Chicken Primordial Germ Cell Lines. 2023, ***LIFE***, 13, 867. **IF: 3,2**, doi: 10.3390/ life13040867. **Q1**
- Pouneh Maraghechi, Maria Teresa Salinas Aponte, **András Ecker**, Bence Lázár, Roland Tóth, Nikolett Tokodyné Szabadi, Elen Gócza: Pluripotency-Associated microRNAs in Early Vertebrate Embryos and Stem Cells. 2023, ***GENES***, 14(7), 1434. **IF: 3,5**, doi: 10.3390/genes14071434. **Q2**
- Tokodyné Szabadi Nikolett, Tóth Roland, Lázár Bence, **Ecker András**, Urbán Martin, Várkonyi Eszter, Liptói Krisztina, Gócza Elen: Hőkondicionálás hatására létrejövő molekuláris változások vizsgálata a házityúkok agyszövetében. 2024, ***MAGYAR ÁLLATORVOSOK LAPJA***, 146. **IF: 0,1**, doi: 10.56385/magyallorv.2024.02.67-75. **Q4**
- **András Ecker**, Bence Lázár, Roland I. Tóth, Martin Urbán, Orsolya I. Hoffmann, Zsófia Fekete, Endre Barta, Ferenc Uher, Zsolt Matula, Eszter Várkonyi, Elen Gócza: Creating a novel method for chicken

primordial germ cell health monitoring using the fluorescent ubiquitination-based cell cycle indicator reporter system. 2024, ***POULTRY SCIENCE***, 103:104144, **IF: 3,8**, doi: 10.1016/j.psj.2024.104144. **D1**

Journal articles without an impact factor related to the topic of the thesis:

- **Ecker András**, Lázár Bence, Tóth Roland, Várkonyi Eszter, Gócza Elen: Embrionális gonádból származó sejtszuszpenziók beépülésének vizsgálata magyar parlagi gyöngytyúkban. 2022, ***ANIMAL WELFARE, ETHOLOGY AND HOUSING SYSTEMS***, 19(1). doi: 10.17205/SZIE.AWETH.2023.1.018
- Lázár Bence, Tokodyné Szabadi Nikolett, Tóth Roland, **Ecker András**, Urbán Martin, Várkonyi Eszter, Liptói Krisztina, Gócza Elen: A napos kori hőkezelés transzgenerációs hatásainak vizsgálata házityúkban. 2023, ***ÁLLATTENYÉSZTÉS ÉS TAKARMÁNYOZÁS***, 72(3). 220-230.

Educational articles related to the topic of the thesis:

- **Ecker András**, Lázár Bence, Tóth Roland, Patakiné Várkonyi Eszter, Gócza Elen: A génmegőrzés jelentősége a baromfitenyésztésben. 2022, Magyar Mezőgazdaság, 6. szám, 2022.02.09.
- **Ecker András**, Lázár Bence, Várkonyi Eszter, Gócza Elen: Mi volt előbb: a tyúk vagy a CRISPR/Cas9? 2024, Baromfiágazat, 24. évfolyam, 2024/3.

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