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ENHANCEMENT OF CHICKEN PRIMORDIAL GERM CELL *IN VITRO* MAINTENANCE USING AN AUTOMATED CELL IMAGE ANALYSER

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Primordial germ cells (PGCs) were isolated from blood samples of chicken embryos. We established four PGC lines: two males (FS-ZZ-101, GFP-ZZ-4ZP) and two females (FS-ZW-111, GFP-ZW-5ZP). We could not detect a significant difference in the marker expression profile, but there was a remarkable difference between the proliferation rates of these PGC lines. We monitored the number of PGCs throughout a three-day period using a high-content screening cell imaging and analysing system (HCS). We compared three different initial cell concentrations in the wells: ~1000 cells (1×), ~4000 (4×) and ~8000 (8×). For the GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111 PGC lines the lowest doubling time was observed at 4× concentration, while for GFP-ZZ-4ZP we found the lowest doubling time at 1× concentration. At 8× initial concentration, the growth rate was high during the first two days for all cell lines, but this was followed by the appearance of cell aggregates decreasing the cell growth rate. We could conclude that the difference in proliferation rate could mainly be attributed to genotypic variation in the established PGC lines, but external factors such as cell concentration and quality of the culture medium also affect the growth rate of PGCs.

Key words: Chicken, primordial germ cells, cell culture, proliferation rate, doubling time, high-content screening

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Avian primordial germ cells (PGCs) are upcoming pioneers in the field of stem cell and developmental biology. The chicken, due to its relatively short reproductive cycle and easy access of embryos, is an important animal model among vertebrates for *in vitro* studies (Nakamura et al., 2013). PGCs are the precursors of functional gametes and they tend to migrate from the hypoblast via the circulatory system to the genital ridge – the place of the final development, where based on the sex of the embryo they will develop into male or female gametes (Kagami, 2016). It is possible to collect avian blastodermal cells from the blastodisc at stage X (Sztán et al., 2017) or PGCs from the blood between stages 13–17 (Raucci et al., 2015) and culture the isolated cells *in vitro*. In order to fully reserve the developmental potential of PGCs, an optimal cell culture medium should be developed. A well-defined culture medium plays an important role in expanding the population of PGCs (Van Der Sanden et al., 2010) and promotes healthy cell proliferation (Kagami, 2016; Nakamura, 2016). Using the culture protocol developed by McGrew et al. (2004), it became possible to culture both female and male chicken PGCs *in vitro* for a long time (Whyte et al., 2015).

The components of the culture medium, cell concentration in the culture plate along with physical factors like pH, oxygen, and the level of carbon dioxide play a major role in regulating the proliferation, growth and quality of PGCs (Whyte et al., 2015). The culture medium can effectively mimic the *in vivo* microenvironment during *in vitro* expansion; also, the components tend to interact with each other and with the metabolites or factors secreted by the cells.

Cell competition is present under both *in vitro* and *in vivo* conditions. This system acts as a filtering method of removing stem cells having acquired deleterious mutations following the onset of proliferation. Under *in vitro* conditions, a similar mechanism has been observed. When male and female PGCs were co-cultured, it was observed that male PGCs grow faster and female PGCs tend to disappear from the culture after an extended period of culturing (Whyte et al., 2015; Nakamura, 2016). This observation was consistent with the findings of Sancho et al. (2013) who observed competition between the different genotypes when cells were cultured in a heterogeneous population.

Bertocchini and Chuva de Sousa Lopes (2016) reported that PGCs tend to acquire mutations via migration. This may turn them ectopic or tumorigenic in nature (De Melo Bernardo et al., 2012; Bertocchini and Chuva de Sousa Lopes, 2016), which affects the proliferation rate.

The main aim of our investigation was to explore the optimal culture conditions along with environmental and physical factors that promote the growth of PGCs maintained *in vitro*, facilitating the future of PGC-based bio-banking.

Materials and methods

Experimental animals and animal welfare

The animals were maintained according to the rules set up by the Hungarian Animal Protection Law (Act No. XXVII of 1998). The permission to perform experiments on animals at the Research Centre for Farm Animal Gene Conservation (Gödöllő, Hungary) was granted by the National Food Chain Safety Office, Animal Health and Animal Welfare Directorate, Budapest (no.: 1/1512/49/15/2/2012). The Partridge Colour Chicken breed used in our study is being kept at the Research Centre for Farm Animal Gene Conservation (Gödöllő, Hungary) *in vivo*. The White Leghorn chicken breed expressing the GFP gene was identical to the one established by McGrew and colleagues as described before (McGrew et al., 2004).

Establishment of the PGC lines

The eggs were collected and then incubated for the establishment of the Partridge Colour PGC lines (PGC lines FS-ZZ-101 and FS-ZW-111) and the GFP-expressing White Leghorn lines (GFP lines GFP-ZZ-4ZP and GFP-ZW-5ZP). The blood was isolated (approx. 1 µl) from the HH stage 14–17 embryos and added to the selective PGC culture medium developed by McGrew and colleagues (Whyte et al., 2015), to remove the blood cells completely and to support the growth and division of PGCs. A one-third proportion of the medium was changed every day. A PGC line was considered successfully established if the cell number has reached 1×10^5 by the end of the third week (Whyte et al., 2015).

During our study, two male PGC lines (FS-ZZ-101, GFP-ZZ-4ZP) and two female PGC lines (FS-ZW-111, GFP-ZW-5ZP) were compared (Fig. 1).

Detailed analysis of the established FS-ZZ-101 and FS-ZW-111 was described by Lázár et al. (2017) and that of the GFP-ZZ-4ZP and GFP-ZW-5ZP lines by Tóth et al. (2017). The stem cell and germ cell specific marker and miRNA expression profiles of the PGC lines used have been described recently by our group (Lázár et al., 2018).

Culture medium for primordial germ cells

The PGC culture medium was prepared according to the medium protocol formulated by McGrew and his colleagues (Whyte et al., 2015). The basis of the PGC culture medium is a special AVIAN DMEM containing B27, ovalbumin and heparin. The medium contains the growth factors h-Activin A (25 ng/µl) and h-FGF2 (10 ng/µl). The stock medium was filtered using a 0.22-µl syringe filter. The filtered medium was kept at 4 °C.

DNA isolation and sex determination

The DNA extraction procedure was performed using the High Pure PCR Template Preparation Kit (Roche, Cat. No. 11796828001), according to the manu-

facturer's instructions. The samples were stored at –20 °C. The sex of the donor embryos and the established PGC lines were determined using the *CHD1* primer set (FW: 5'-TATCGTCAGTTTCCTTTTCAGGT-3'; RV: 5'-CCTTTTATTGAT CCATCAAGCCT-3') as described previously by Lee et al. (2010). The extracted DNA was diluted to 25 ng/µl concentration for PCR reaction and gel electrophoresis. MyTaq Red Mix was used for the reaction (Bioline, Cat. No. BIO-25043). The PCR products were then separated by electrophoresis, using 1% agarose gel stained with ethidium bromide, at 100 V for 1.5–2.0 h. The DNA bands were then visualised and photographed under UV illumination.

Chromosome analysis

The metaphase chromosomes were prepared by methanol-acetic acid fixation and air-drying technique (Alfi et al., 1973). To the intensively proliferating PGC culture one drop of vinblastine solution (10 µg/ml, Sigma) was added. After 2 h, the medium containing the PG cells was harvested from the plate, collected in an Eppendorf tube, and then the cell suspension was centrifuged. The supernatant was removed, and the cells were suspended in ice-cold 0.56% KCl solution. Hypotonic treatment took 10 min at room temperature and was followed by 3 changes of methanol/acetic acid (3:1) fixative. The cell suspension was spread on slides, dried at room temperature, and stained with 5% fresh Giemsa (in phosphate buffer, pH 7.0) for 7–8 min. Five slides of each cell line were prepared and at least 50 metaphase spreads per line were examined.

Cell counting

The cell counting of the PGCs before preparing the examined 96-well plates was performed using the Arthur Novel Fluorescence Cell Counter (NanoEnTek, Pleasanton, USA). This cell counter is a 3-channel desktop image analyser which allows users to perform assays for cells in suspension, including GFP and RFP expression, apoptosis, cell viability, cell cycle, and cell counting.

Doubling time measurement and time lapse video analysis

High-content screening and analysis were performed by a Molecular Devices ImageXpress Micro XLS Imaging System with a built-in incubator, which equipment also allows acquisition of time lapse videos (Kecse-Nagy et al., 2016; Hegedüs et al., 2017). Twelve fields of view were monitored of each well of a 96-well culturing plate for 64 h. The cell number was determined every 4 h. Doubling times were calculated from 12 repeats, two biological parallels of the four PGC lines (FS-ZZ-101, FS-ZW-111, GFP-ZZ-4ZP, GFP-ZW-5ZP) at 1×, 4× and 8× concentration.

Doubling time calculation

The doubling time is the time required for a culture to double in number. We calculated the doubling time using the following formula. Gr (growth rate) = ln[N(t)/N(0)]/t, where N(t) is the number of cells at time t, N(0) is the number of cells at time t = 0 (t expressed in days). Therefore, Doubling Time = ln(2)/growth rate (Gr). The doubling time is inversely proportional to the proliferation rate.

Statistical analysis

All data were analysed by R Studio (version 1.0.136), R (version R-3.2.2.) and GenEx (version 6.0). R software was used to build a multiple linear regression model in which 'doubling time' was the response variable, while 'concentration' and 'cell line' were predictor variables. Both predictor variables showed a significant effect on doubling time; therefore, multiple comparisons of means (Tukey contrasts) were performed to further analyse the differences between groups. $P < 0.05$ was considered significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

Characterisation and sex determination of PGC lines

As a first step, we characterised the established PGC lines. We performed chromosome analysis (Fig. 1A1, A2) and sex PCR (Fig. 1B) to detect the sex of the PGC lines. According to our results, two male PGC lines (FS-ZZ-101, GFP-ZZ-4ZP) and two female PGC lines (FS-ZW-111, GFP-ZW-5ZP) were identified. There was no difference in the marker expression profile between the stem cell-specific (SSEA-1) and germ cell-specific (CVH, DAZL) markers in the PGC lines examined. Figure 1C shows the results of the SSEA-1, CVH and DAZL immunostaining of FS-ZZ-101 and FS-ZW-111 PGC lines (SSEA-1: Fig. 1/C1, C2, C3, C4; CVH: Fig. 1/C1, C3; DAZL: Fig. 1/C2, C4).

Tracking the cell number growth using time lapse video image analyser in chicken PGCs cultured in vitro

The total number of PGCs was calculated by measuring the cell number in 12 small squares in each well. Two biological parallels and three different initial concentrations [~1000 cells (1×), ~4000 (4×) and ~8000 (8×)] were examined and compared in all four cell lines (GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111; Fig. 2/A, B, C, D).

The cell number increased to ~3000 cells (1×), ~17,000 (4×) and ~26,000 (8×) in the case of the GFP-ZZ-4ZP (Fig. 2A); ~2000 cells (1×), ~10,000 (4×) and ~10,000 (8×) for the GFP-ZW-5ZP (Fig. 2B); ~3000 cells (1×), ~12,000 (4×) and ~20,000 (8×) in the case of the FS-ZZ-101 (Fig. 2C), and ~3000 cells (1×),

~12,000 (4×) and ~17,000 (8×) for the FS-ZW-111 (Fig. 2D). We observed the highest growth rate at a high (8×) initial cell concentration; however, despite the increased cell number, we detected high standard deviation (SD) between the two parallel samples after 32 h. The high value of SD corresponds to the random processes going on under *in vitro* cell culture conditions. The cell number of the GFP-ZW-5ZP PGC line increased from 0 to 32 h, but subsequently there was a regression in the cell numbers from 32 to 64 h [32 h: ~11,000 (8×); 64 h: ~10,000 (8×); Fig. 2B]. After 64 h of cultivation, we obtained the highest cell number of ~26,000 (8×) in GFP-ZZ-4ZP line (Fig. 2A). The lowest cell number after 64 h of cultivation at 8× initial cell concentration was observed for the GFP-ZW-5ZP PGC cell line [64 h: ~10,000 (8×); Fig. 2B]. The decrease in cell number can be caused by the formation of clumps in the *in vitro* culture but other on-going cellular processes such as apoptosis or cellular toxicity could also be involved.

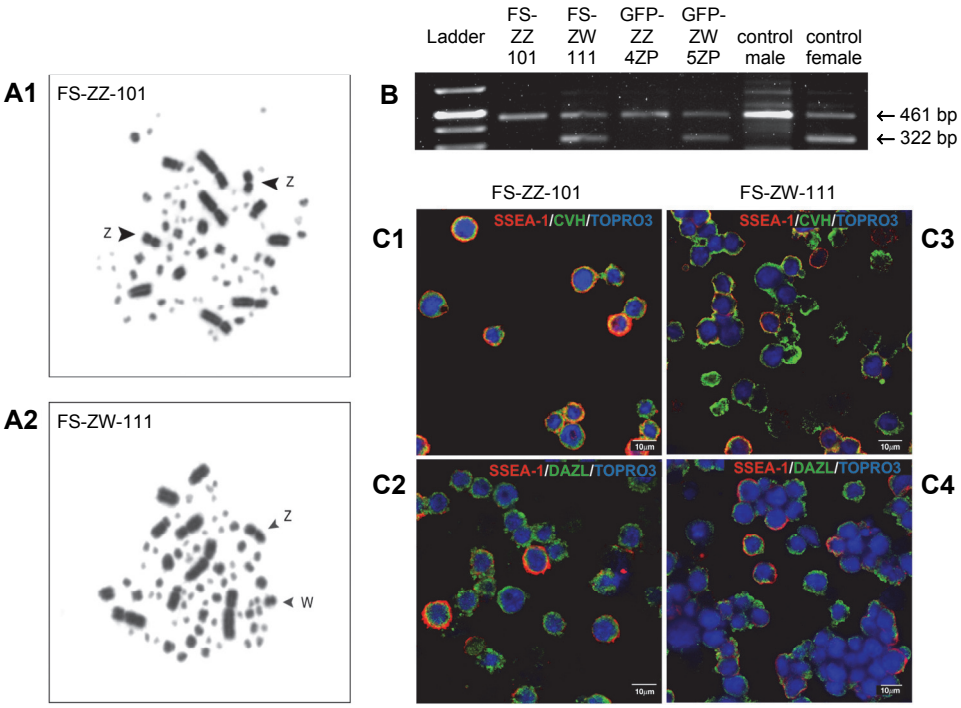

Fig. 1. SSEA-1, CVH and DAZL expression in FS-ZZ-101 and FS-ZW-111 PGC lines was performed (SSEA-1: *Fig. 1/C1, C2, C3, C4*; CVH: *Fig. 1/C1, C3*; DAZL: *Fig. 1/C2, C4*). We performed chromosome analysis *(A)* and sex PCR to detect the sex *(B)* of PGC lines. A: Metaphase spread of FS-ZZ-101 *(A1)* and FS-ZW-111 cell lines *(A2).* B: Sex determination of PGC lines using CHD1 primers. Sex determination was performed by using the CHD1 FW and RV primer sets. The size of the PCR products was 322 bp and 461 bp, respectively. In case of the female cell lines, two bands were detected. C: SSEA-1 (red), CVH (green) and nuclear staining (blue) of FS-ZZ-101 *(C1)* and FS-ZW-111 cell lines *(C3).* SSEA-1 (red), DAZL (green), nuclear staining (blue) in FS-ZZ-101 *(C2)* and FS-ZW-111 cell lines *(C4).* Scale bars: 10 µm

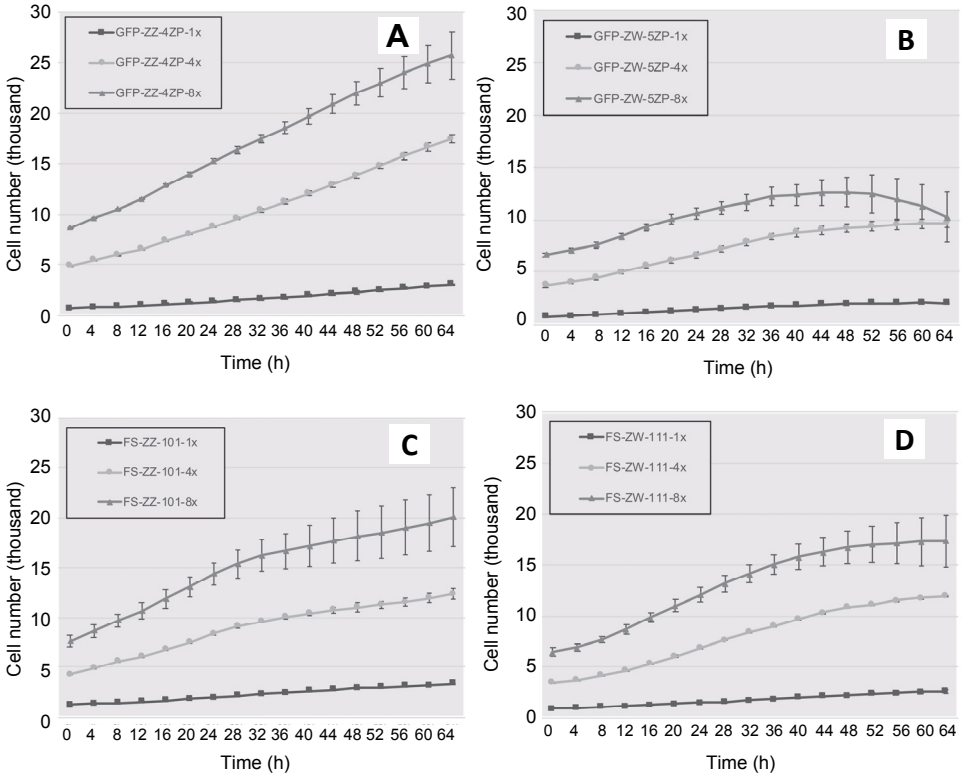

Fig. 2. High-content screening using Molecular Devices ImageXpress Micro XLS Imaging System with a built-in incubator was performed at the Molecular Cell Biology Research Group. The cell number was measured at 4-h intervals, at 17 different measurement points, for 64 h. For the calculation of doubling time, we used data collected from 12 repeats, two biological parallels of the four PGC lines (FS-ZZ-101, FS-ZW-111, GFP-ZZ-4ZP, GFP-ZW-5ZP). The four cell lines (GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111) were compared by examining three different initial concentrations [~1000 cells (1×), ~4000 (4×) and ~8000 (8×)] (Fig. 2/A, B, C, D)

We followed the cell proliferation by capturing time lapse pictures. The initial concentration was 4×, and the image analyser took pictures at 4-h intervals. In the case of GFP-ZZ-4ZP PGC line the formation of small aggregates was observed after 48 h (Fig. 3A). In the case of the GFP-ZW-5ZP PGC line, we recognised small aggregates from the beginning of culture. After 48 h of culture, these small aggregates formed big clumps (Fig. 3B).

Determination of the doubling time in chicken PGC lines cultured in vitro

The doubling time (the time required for a culture to double in number) of PGCs was calculated by measuring the cell numbers in 12 small squares in each

well. Four cell lines (GFP-ZZ-4ZP, GFP-ZW-5ZP, FS-ZZ-101, FS-ZW-111) were compared using two biological parallels and three different initial concentrations [~1000 cells (1×), ~4000 (4×) and ~8000 (8×); Fig. 4/A, B, C, D].

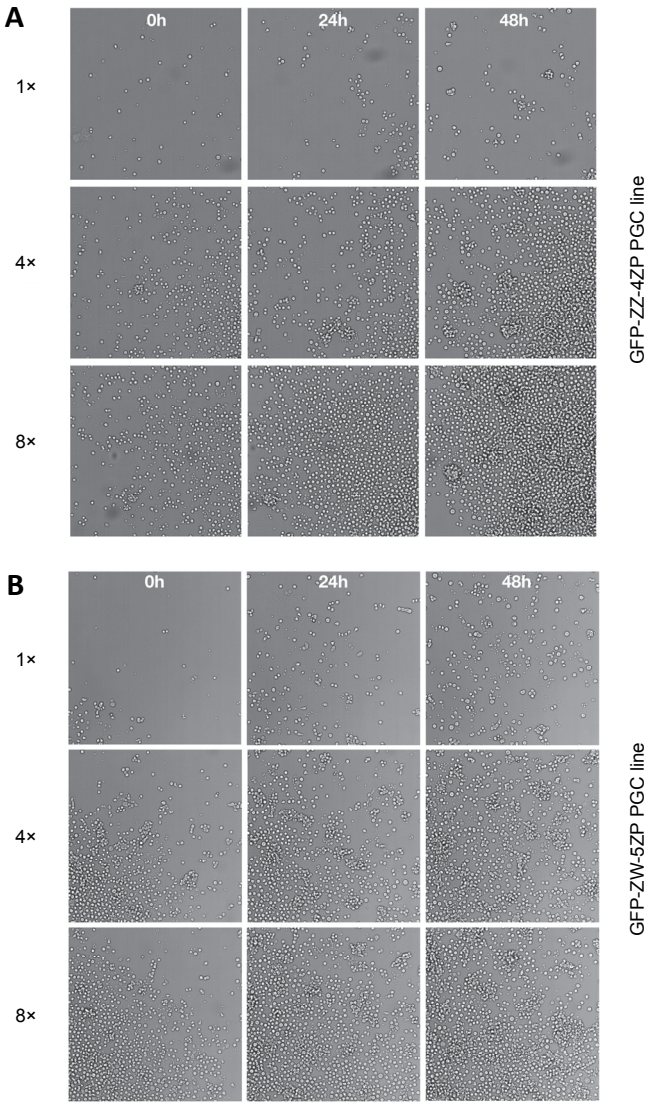

Fig. 3. A: Time lapse captures of the GFP-ZZ-4ZP PGC line. The initial concentration was 4×, the image analyser took pictures at 4-h intervals. After 48 h of culture it was possible to observe the formation of small aggregates. B: Time lapse captures of the GFP-ZW-5ZP PGC line. The initial concentration was 4×, and the image analyser took pictures at 4-h intervals. At the beginning of the culture it was possible to recognise small aggregates. After 48 h of culture, these small aggregates formed big clumps

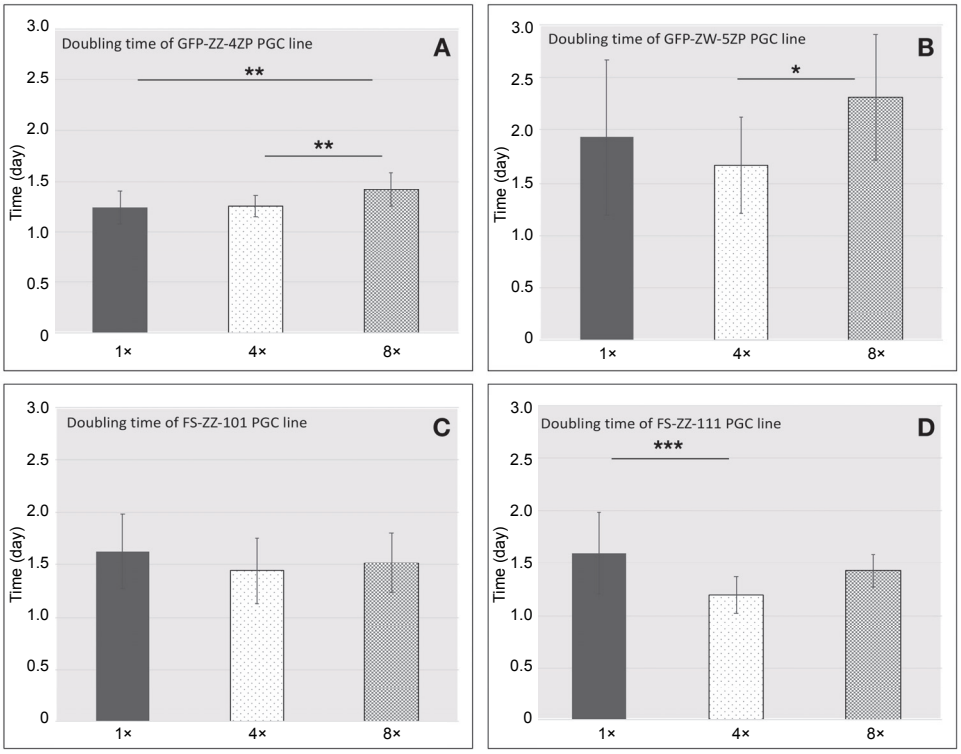

Fig. 4. Summary of the doubling times of PGC lines at different concentrations. The FS-ZW-111 PGC line showed the lowest doubling time at 4× concentration (*Fig. 4D*). The highest doubling times were calculated in the case of the GFP-ZW-5ZP PGC line *(Fig. 4B).* The doubling times of the GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111 lines were the lowest at the 4× initial concentration *(Fig. 4B, C and D)*, while the GFP-ZZ-4ZP line showed the highest proliferation rate at 1× concentration. $P < 0.05$ was considered significant ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$)

According to the multiple linear regression model, in which 'doubling time' was the response variable while 'concentration' and 'cell line' were predictor variables, we found a significant difference between the PGC lines except the FS-ZW-111–GFP-ZZ-4ZP and the FS-ZW-111–FS-ZZ-101 pairs. In cell concentration, a significant difference was found between 4×–8× and 4×–1× but not between 1×–8×. Comparing the doubling times of cell lines at different concentrations, the FS-ZW-111 PGC line showed the lowest doubling time (the maximum proliferation rate) with a value of 1.19 (4×) (Fig. 4D). The highest doubling time was calculated for the GFP-ZW-5ZP PGC line [with 2.31 (8×), 1.94 (1×) and 1.67 (4×); Fig. 4B]. It was followed by the FS-ZZ-101 PGC line with a value of 1.63 (1×) (Fig. 4C) and the FS-ZW-111 PGC line with a value of 1.59 (1×) (Fig. 4D). Interestingly, the doubling time of the GFP-ZW-5ZP, FS-ZZ-101 and FS-

ZW-111 lines was the lowest at 4× initial concentration [GFP-ZW-5ZP: 1.67 (4×); FS-ZZ-101: 1.45 (4×); FS-ZW-111: 1.19 (4×); Fig. 4B, C, D].

From the above results, it can be concluded that the doubling time is genotype dependent, but it is also affected by the initial cell concentration because the cell metabolism modifies the *in vitro* culture conditions.

Discussion

In our laboratory, male and female PGCs were cultured in a defined medium (Whyte et al., 2015).

As in chickens the females are heterogametic and the males are homogametic, some of the genes show sex-dependent expression and, hence, it is speculated that this phenomenon affects the proliferation rate differently in male and female PGCs (Ronen and Benvenisty, 2014). According to a study (Van Der Sanden et al., 2010), many intrinsic and extrinsic factors affect cell proliferation. Cell concentration is an important extrinsic factor. It was observed that at an optimal cell number the cells tend to show higher proliferation rate. In our study, the optimal concentration was cell line dependent. The best initial concentration for the cell lines GFP-ZW-5ZP, FS-ZZ-101 and FS-ZW-111 was ~4000 cells/well (in a 96-well plate), while in the case of the GFP-ZZ-4ZP it was ~1000 cells/well. The GFP-ZZ-4ZP cell line showed the lowest doubling rate at an initial concentration of ~1000 cells/well.

Cells are in a state of dynamic interaction with one another while growing. Also, there may be some competition between the cells. In a few cells, new mutations could arise which may cause a higher proliferation rate (Bertocchini and Chuva de Sousa Lopes, 2016), or cell may acquire a mutation that decreases the proliferation rate. There is interaction between cells via cell to cell adhesion or chemical attraction (Renner et al., 1993; Agnew et al., 2014). PGCs secrete chemical molecules (glycoproteins, cell surface proteins), which attract PGCs towards each other, resulting in clump or aggregate formation. At high concentration, there is exacerbated proliferation followed by clump formation. These aggregates eventually increase cellular toxicity in the culture (Yao and Asayama, 2017). These results were consistent with our findings. Both male and female PGCs form aggregates, but in the case of female cell lines this was observable at lower cell concentrations. For the GFP-ZW-5ZP PGC line we could detect increased cell clump formation from 32 h of culturing. The clump formation was thus decreasing the proliferation rate.

The primary mechanisms of molecular signalling pathways controlling the proliferation rate or growth pattern of PGCs are not yet fully elucidated. Despite the establishment of an optimum PGC culture medium there is clump/aggregation formation noticed during *in vitro* culturing of PGCs. Hence, understanding these

mechanisms and identifying the main molecules or genes responsible for controlling the growth would open doors for future work and applications related to the culturing of PGCs (Nakamura, 2016). The maintenance of PG cells under optimal culture conditions can improve the efficiency of cryopreservation, genome modification or drug design studies. Our study demonstrated that, using an automated cell image analyser, the optimal PGC culture condition can be identified in a short period of time.

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