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Research Article

Effect of Glutamine Stability on the Long-term Culture and Line Establishment of Chicken Primordial Germ Cells

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Abstract

Primordial germ cells (PGCs) are precursors of mature gametes, which transmit genetic information to the next generation. Due to the importance of PGCs in many fields, including developmental biology, genome editing, transgenesis, and conservation of avian genetic resources, various research aspects have focused on the cultivation of PGCs. Despite considerable progress in the establishment of specified culture media for the expansion of PGCs, a well-defined PGC culture medium has not yet been developed. This might be due to the complexity of the nutritional requirements of PGCs in the culture. Besides the nutritional needs, including vitamins, amino acids, salts, carbohydrates, and growth factors, a particular source of energy must be provided to sustain growth and viability. Glutamine is a major energy source for cultured cells, commonly added in cell culture media at higher concentrations than other amino acids. However, glutamine is very labile and rapidly degrades in solutions such as culture media. This generates ammonia as a by-product, which is toxic to the cultured cells and can affect cell viability and protein glycosylation. Therefore, the stability of glutamine in culture conditions is another concern for the long-term culture of PGCs. Here, we study the effect of glutamine stability on PGC culture using glutamine and GlutaMax (a commercial stabilized dipeptide form of glutamine). We found that the addition of GlutaMax in the medium promotes PGC proliferation. This effect might be exerted by minimizing production of toxic ammonia that results in maximizing cell performance and media stability.

***Keywords*:** Primordial Germ Cells, Long-term Culture, Glutamine Supplementation, Culture Media, Transposon, Germ Cell-specific Promoter

Introduction

Primordial germ cells (PGCs) are the embryonic precursors of germline cells that produce male and female gametes in adults. Due to the importance of chicken PGCs (cPGCs) in many fields, including developmental biology, genome editing, transgenesis, and conservation of avian genetic resources, a cost-effective and efficient *in vitro* cultivation of PGCs is necessary. In the early development of the chicken embryo, cPGCs arise from the epiblast and migrate to the germinal ridges through blood circulation during HH stages 13–17 (Hamburger and Hamilton 1992; Naito, Harumi, and Kuwana 2015). The cPGCs can be isolated from the bloodstream or the gonads in low numbers. They can be enriched by appropriate *in vitro* culture conditions (Macdonald et al. 2010; Whyte et al. 2015). Despite progress in cPGC culture methods, problems such as low proliferation rates and the low germline specification rates exist in the long-term *in vitro* culture and establishment of cPGCs (Kong et al. 2018; van de Lavoir et al. 2006).

The basic composition of culture medium has been identified by Eagle in the 1950s. Culture media provide the mixtures of substances and nutrients, including vitamins, amino acids, salts, carbohydrates, and growth factors that support the growth and propagation of cells. Reduction of any one of these factors affects cell viability and proliferation (Eagle 1959). Amino acids are the raw material for the cells to synthesize protein and thus are necessary ingredients of cell culture media. Nine essential amino acids cannot be synthesized by cells and must be included in the culture media. Glutamine is considered a conditionally essential amino acid required 10- to 100-fold greater than any other amino acid in the culture medium. Glutamine

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functions at low concentrations to support the energy required for the growth and proliferation of cells, but rapidly dividing cells such as cancer cells require much larger concentrations to survive (Chen and Cui 2015).

Glutamine (L-glutamine) is very labile in solution (including culture media) and degrade in a temperature-dependent manner during the culture period. The degradation of glutamine generates toxic by-products such as ammonia which negatively affect cell survival. Dipeptide derivatives of glutamine such as GlutaMax (a commercial stabilized dipeptide form of glutamine) are degradation-resistant and more stable during long-term culture. This stability can be important for the sensitive and challenging cultivation of cPGCs. In this study, we assayed the long-term culture and compared the establishment of chicken PGC lines using two different culture media containing glutamine (Gmin medium) and GlutaMax (Gmax medium).

Materials and Methods

Isolation, Establishment, and Maintenance of Chicken Primordial Germ Cell

The fresh fertile chicken eggs were used for the establishment of primordial germ cell lines. Blood was collected from each embryo between HH stages 13 to 16 (2.5 days old). Using a glass microcapillary attached to a pipettor, about 2µl blood was taken from the dorsal aorta under the stereomicroscope and was immediately transferred into a well of the 48-well plates containing media with glutamine (Gmin) or GlutaMax (Gmax). Gmin-containing and Gmax-containing media were prepared for feeder-free culture of cPGCs. The base medium was avian KO-DMEM basal medium (a custom modification of knockout-DMEM produced by Gibco®, USA; 250 mOsm/kg, 12.0 mM glucose, without calcium chloride), supplemented with B-27, NEAA, 0.1 mM β-mercaptoethanol, nucleosides, 1.2 mM pyruvate, 0.2% ovalbumin (Sigma-Aldrich, USA), 0.2% sodium heparin (Sigma-Aldrich, USA), 10 mg/ml ovotransferrin (Sigma-Aldrich, USA), 25 ng/ml human BMP4 (ABM, US), and 4 ng/ml human FGF2 (Sigma-Aldrich, USA). For the Gmin-containing medium, glutamine (Gibco®, USA) was added at the concentration of 2.0 mM. For the Gmax-containing medium, GlutaMax (Gibco®, USA) was added at the final concentration of 2.0 mM. All components were purchased from Gibco company unless specifically indicated.

Evaluation of Proliferation Rate

To evaluate the proliferation rate of long-term cultured cPGCs, 0.5 x 10^5 cells were seeded in 300 ul of the Gmin and Gmax media in triplicates (in 48-well culture plates). On the day 2, 4, 6, 8, and 10, the cells were pipetted gently, and 15µl of suspended cells were mixed with 15µl of trypan blue dye (0.4%). The cells were counted using a hemocytometer slide. The average of counted live cells obtained from three repeats each day was used to generate proliferation plots for each medium.

Sex Determination

Sex-determination was performed on cPGCs cultured in the Gmin and Gmax media. To this end, after the extraction of genomic DNA using genomic DNA purification kit (New England Biolabs, Ipswich, MA, USA) from cPGCs, and previously described specific primers (Fridolfsson and Ellegren 1999) (Table 1), the W-specific and Z-specific bands were amplified using Hot Start Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Electrophoresis was done on 2% agarose gel at 90V for 1h.

Table 1. Primers used in this study

GenBank accession number	Primer Sequence (5' to 3')	Product (bp)	Application
DAZL (NC_052533)	**DAZL-F:** GGCgctagcacagtcaagagtttgtcacagcatcc **DAZL-R:** GGCgctgaggtgtcagcgttagaatagcagataccg	1833	Promoter assay
W Chr. (NC_052571.1) **Z Chr.** (NC_052572.1)	**Sexing_F:** gttactgattcgtctacgaga **Sexing_R:** attgaaatgatccagtgcttg	Z Chr.: 594 W Chr.: 447	Sex determination

Underlined sequences indicate restriction sites: *NheI* in primer DAZL-F and *BbvcI* in primer DAZL-R.

Plasmid Construction, Transfection, and Promoter Assay

The functionality of germ-line-specific promoters was assessed to ensure the germ-line competency of the cells during the long-term culture of cPGCs. An expression vector containing a PGC-specific promoter (DAZL) driving tdTomato was constructed. To this end, the DAZL promoter was amplified from the chicken genome using DAZL-F, and DAZL-R primers containing *NheI* and *BbvCI* cut sites at the 5' end of each primer, respectively (Table 1). The amplified 1833-bp DAZL promoter was cloned into a plasmid at the upstream of tdTomato reporter gene containing *NheI* and *BbvCI* cut sites between two internal terminal repeats (ITRs) of the *piggyBac* transposon. The plasmid backbone was cut with *NheI- BbvCI* for 3 hrs at 37 °C, followed by dephosphorylation with fast alkaline phosphatase for 10 min at 37 °C, and heat-inactivation for 30 min at 70 °C. The amplified 1833-bp DAZL promoter was cut with the same enzymes for 3 hrs at 37 °C. A 1:3 vector to insert molar ratio was used to ligate the amplified 1833-bp DAZL promoter into the plasmid backbone by T4 ligase. 5µl of the ligation mix was transformed into chemically competent DH5alpha bacteria followed by overnight incubation at 37 °C. Colony PCR was performed on transformants by vector-specific, and insert-specific primers, and positive clones were liquid-cultured overnight for plasmid extraction using the Plasmid Isolation Kit (New England Biolabs, Ipswich, MA, USA). Plasmid subcloning was verified using restriction enzyme digestion.

To transfect cPGCs, 0.5×10^6 cells were suspended in 10µl of R buffer containing 10µg DAZL-tdTomato transposon and 10µg transposase plasmids. Using the Neon tip attached to the Neon pipette, the suspended cells were placed in a pipette station filled with buffer E. Electroporation was performed at 1000 volt, 100 ms, and 1 pulse. Immediately after, the cPGCs were transferred into a well with 300µl medium. Transfected cPGCs were evaluated for tdTomamto expression after 24h.

Results

Morphological Characteristics of freshly isolated circulating cPGCs

Initial identification of cPGCs is based on morphological features. Circulating cPGCs are distinguished by their large round-shape nuclei and glycogen granules in the cytoplasm (Meyer 1964). We examined the morphological features of freshly isolated cPGCs with an inverted microscope. A few cPGCs with previously described features were found in blood-derived samples. As the culture progressed, the proliferating cPGCs became much more visible and prominent due to the disappearance of the red blood cells, and doublets indicative of dividing cPGCs appeared in the cultured blood samples (Figure 1 A & B).

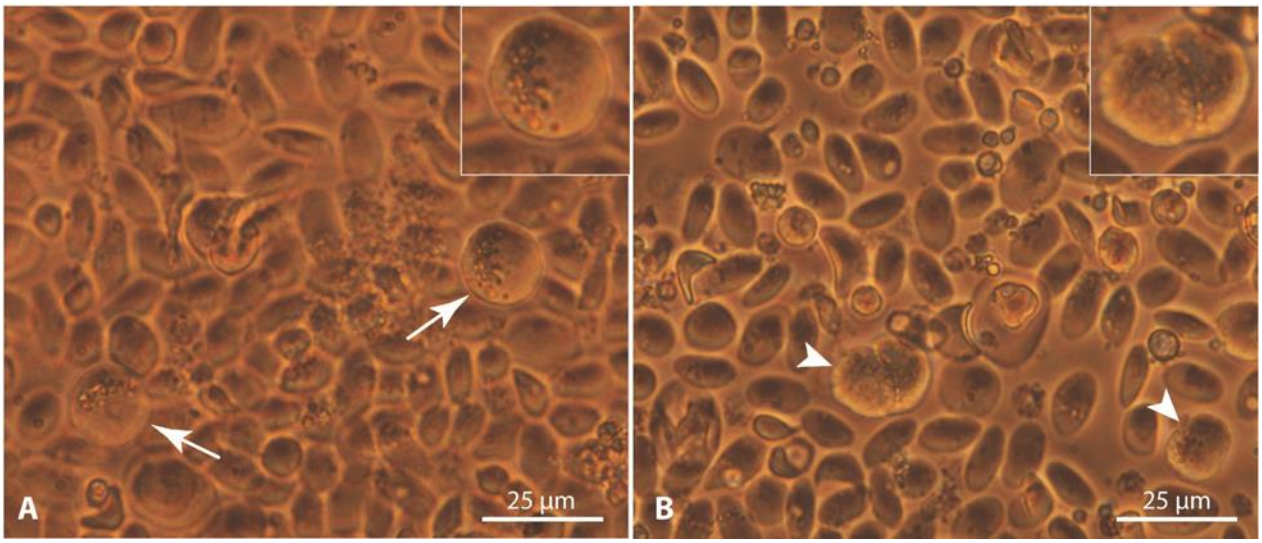

Figure 1. Morphology of circulating cPGCs in the freshly-isolated sample of embryonic blood. Circulating cPGCs, isolated from the embryonic blood (stage 13-H.H), which can easily be distinguished from red blood cells by their large size, large nuclei, and accumulated granules in the cytoplasm (arrows, inset). (B) Arrowheads (and inset) pinpoint two dividing cPGCs (doublets) in the freshly isolated embryonic blood. Scale bar = 25 µm. Magnification = ×400

Derivation of cPGCs in The Gmin and Gmax Media

To study the effects of glutamine stability on the long-term culture and establishment of chicken PGC lines, we cultured circulating-cPGCs in two different media with different energy sources. Circulating-cPGCs were grown in L-glutamine-containing (Gmin) and GlutaMAX-containing (Gmax) media.

Blood samples collected from 20 chicken embryos at stages 13–16 were individually transferred to each well of 48-well culture plates containing Gmin or Gmax media. From day 2 onward, half of the Gmin and Gmax media in each well was exchanged with the fresh medium every other day. After ~ 2 weeks of culture, from a total of 20 cPGC lines cultured in each medium, 13 and 16 lines of cPGCs were successfully derived in the Gmin and Gmax media, respectively. Apoptotic cells were more frequently observed in the Gmin-cultured wells, whereas the glycogen granules were more abundant in the derived lines cultured in the Gmax medium (Figure 2).

Evaluation of Proliferation and Viability of cPGCs Cultured in The Gmin and Gmax Media

To evaluate the cell proliferation status of derived cPGCs in the Gmin and Gmax media, a total of 0.5 x 10^5 cells were seeded in each well in 48-well culture plates in triplicates. The cPGCs were counted on culture days 2, 4, 6, 8, and 10. The results showed that on day 4 onward, cells in the Gmax medium proliferate with higher efficiency than cells in the Gmin medium (Figure 3).

Long-term Culture of cPGCs in The Gmin and Gmax Media.

To evaluate the long-term self-renewal capacity of cPGCs during *in vitro* culture, first, we determined the sex of the Gmin and Gmax cultured cPGC lines, and then we cultured one male and one female in each medium to ensure that the long-term culture of the cPGC is not sex-dependent (Figure 4).

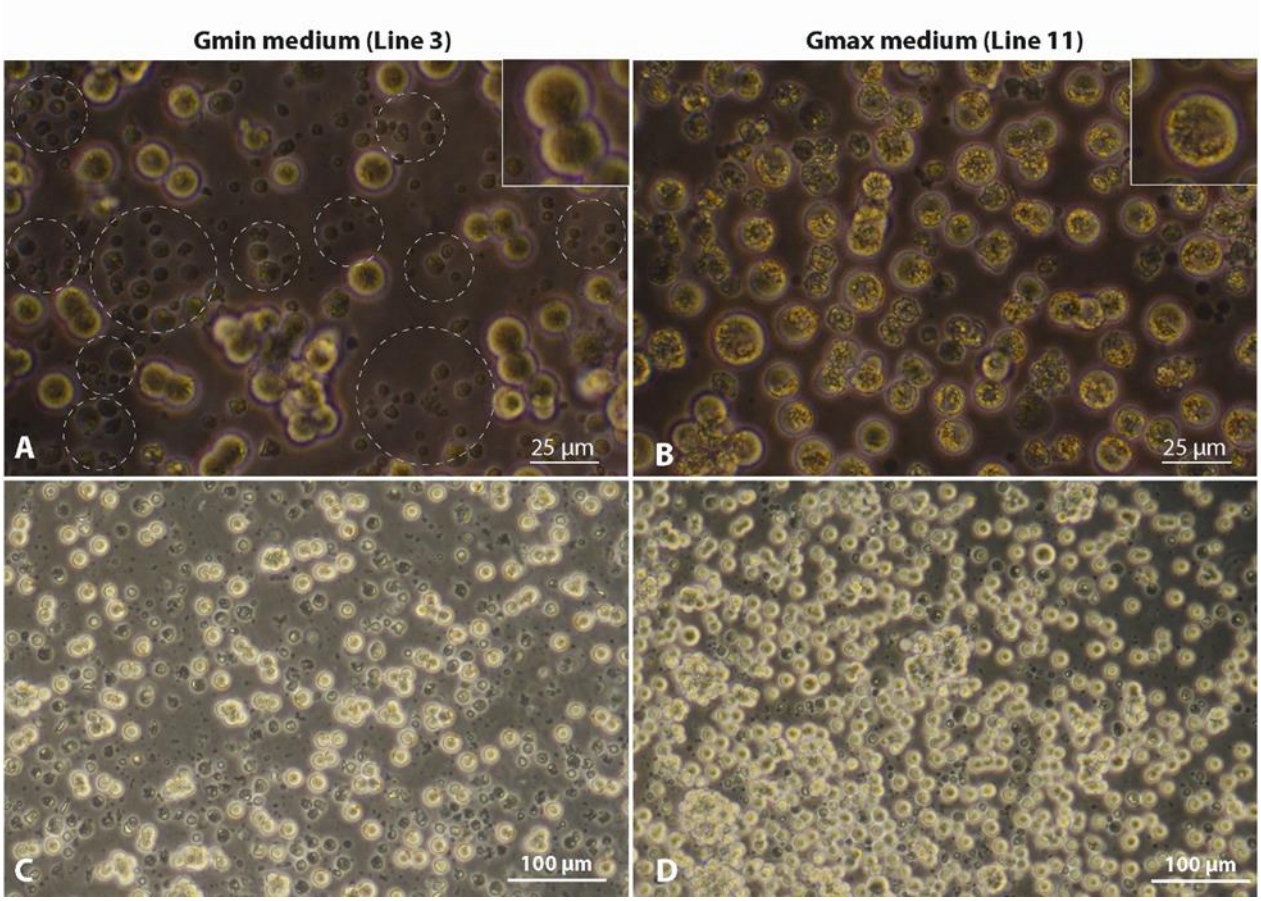

Figure 2. Derivation of cultured cPGCs in the Gmin and Gmax media. Two lines of derived cPGCs after 2 weeks of culture in the Gmin (A & C), and Gmax (B & D) media. The dashed enclosed areas in panel A show apoptotic cPGCs. The granular pattern of cytoplasm is much more prominent in panel B. The insets in panels A and B show a digitally magnified doublet and a PGC with granular cytoplasm, respectively. Scale bar = 25 µm. Magnification = ×200 (A & B). Scale bar = 100 µm. Magnification = ×100 (C & D).

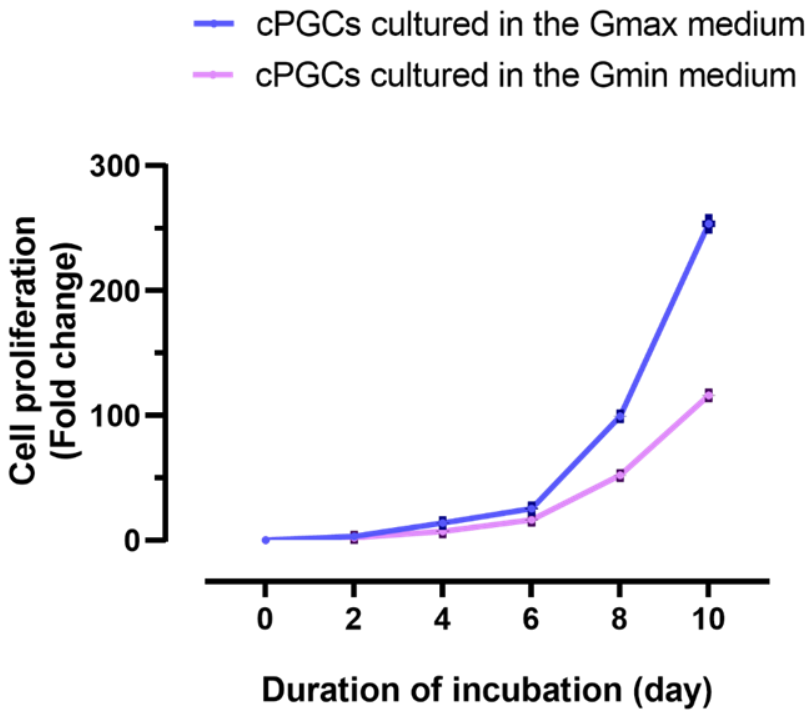

Figure 3. Growth curve of cultured cPGCs in the Gmin and Gmax media. The cPGCs cultured in the Gmin and GMax media were counted at various time points (days 2, 4, 6, 8, and 10). To calculate fold-change, the number of cells at each time point has been divided by the number of cells seeded at day 0. The results for each data point are based on 3 independent experiments. Error bars are standard deviations based on three biological replicates.

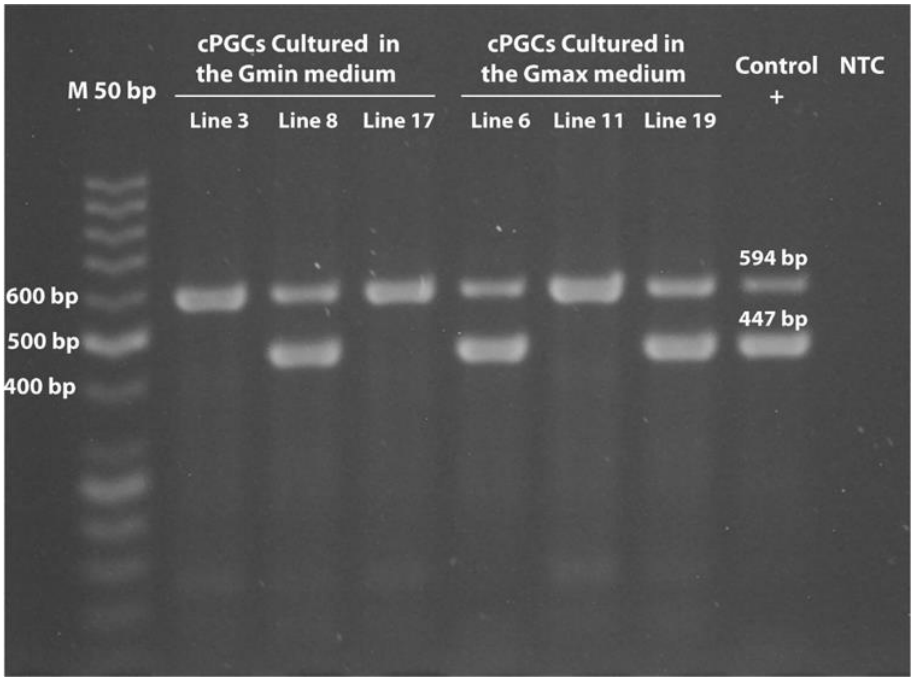

Figure 4. Sex determination of cPGCs cultured in the Gmin and Gmax media. The amplified fragments with the sexing primer set from a female's W and a male's Z chromosome were 447 bp and 594 bp, respectively. In each lane, one PCR band indicates male genotype, and two PCR bands a female genotype of the PGC line.

Selected derived-cPGCs were cultured in the Gmin and Gmax media for more than 3 months. In a long-term culture of 100 days, cultured cPGCs in the Gmin medium underwent around 21 passages, whereas cultured cPGCs in the Gmax medium underwent around 33 passages. Although the rate of proliferation in cPGCs cultured in the Gmin medium was slow with an extremely significant difference, no apparent difference was observed in the morphological characteristics of the cell lines (Figure 5 A & B).

Germ Cell-specific Promoter Assay

To examine whether our long-term cultured cPGCs in the Gmin and Gmax media are amenable to genetic modification and whether genetically manipulated cPGCs retain their germline features in the long-term culture, a promoter assay was carried out. To this end, we constructed an expression vector with a PGC-specific (DAZL) promoter driving tdTomato expression (Figure 6 A). The reporter cassette allowed the visualization of tdTomato in long-term cPGCs. These cells were successfully transfected and retained their germline features. Our results demonstrated that the long-term culture of cPGCs cultured in both the Gmin and Gmax media does not affect the transfectability of these cells (Figure 6 B).

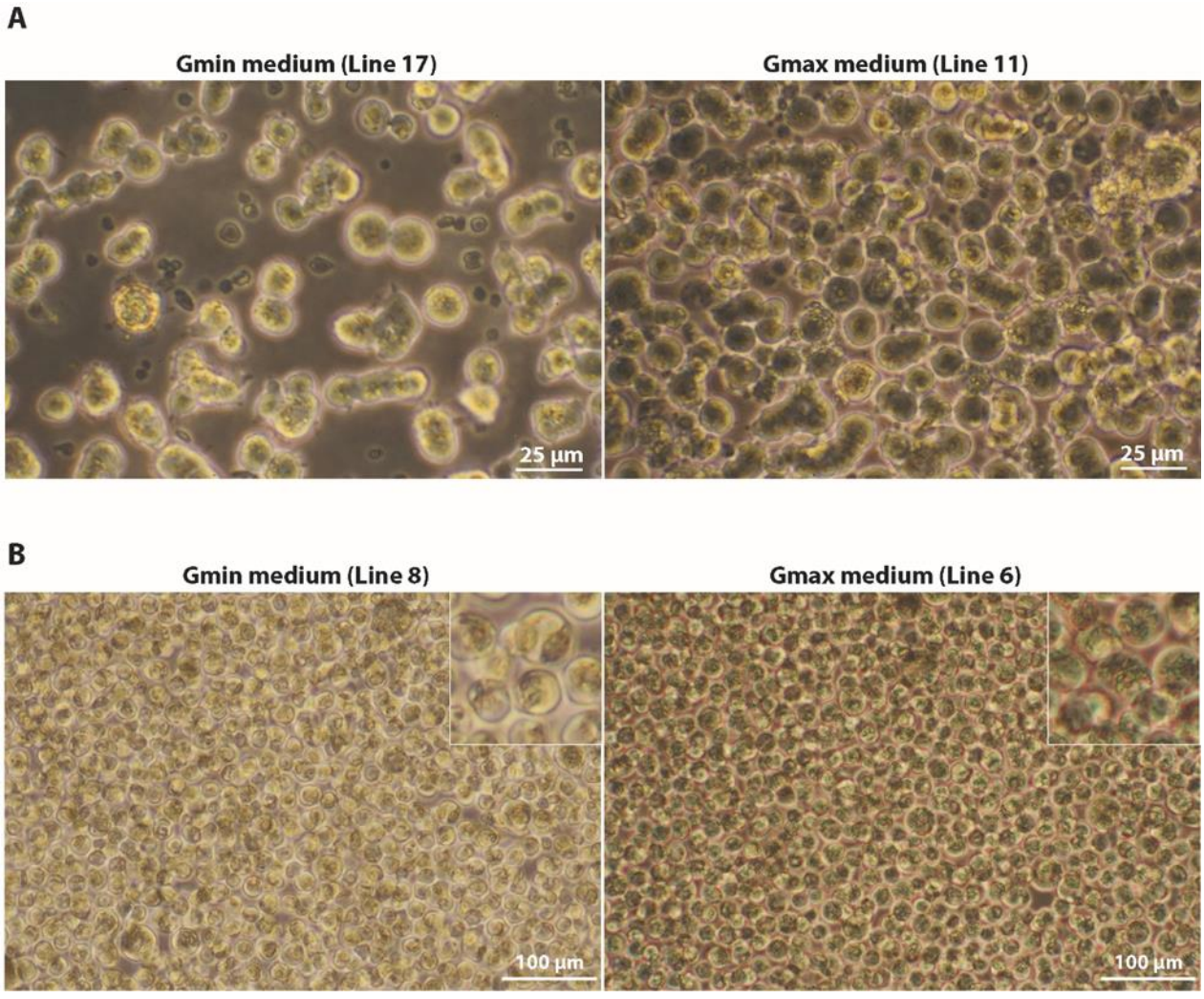

Figure 5. Morphology of the long-term cultured cPGCs in the Gmin and Gmax media. (A) Two lines of male PGCs, cultured for more than one month in the Gmin and the Gmax media. Scale bar = 25 µm. Magnification = ×200. (B) Two lines of female cPGCs, cultured for more than 3 months in the Gmin and the Gmax media. The insets in the left and right panels of B show digitally magnified long-term cultured cPGCs. Scale bar = 100 µm. Magnification = ×100

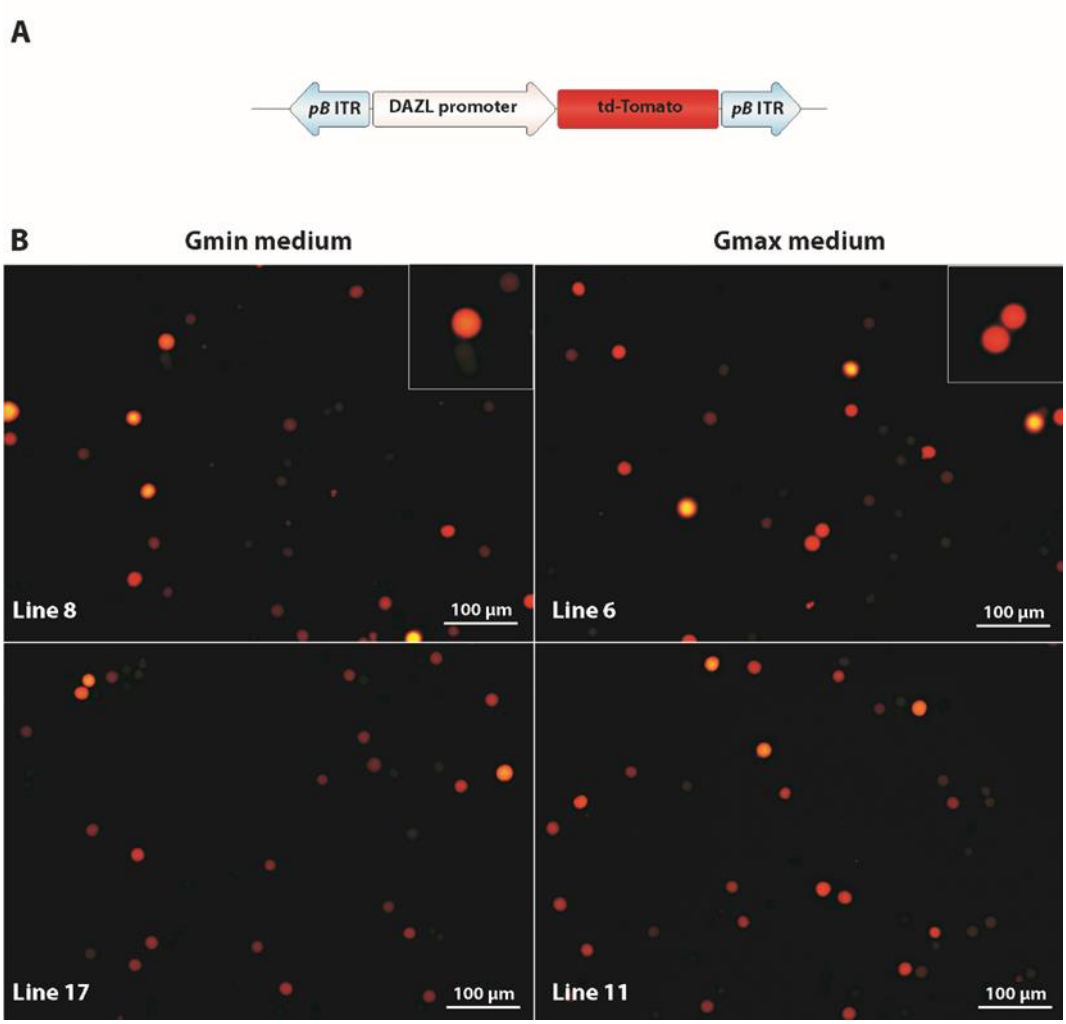

Figure 6. Promoter activity assay in cPGCs after long-term culture in the Gmin and Gmax media. (A) Schematics of the *piggyBac* transposon vector used in this study. The DAZL promoter drives the expression of tdTomato. The *piggyBac* transposon vector contains two ITRs of 206 bp. (B) cPGCs after long-term culture in the Gmin and Gmax media were transfected with the transposon construct and imaged by fluorescence microscopy. The insets in the top-left and top-right panels show digitally magnified cPGCs expressing tdTomato. Scale bar = 100 µm. Magnification = ×100

Discussion

Considering the importance of glutamine metabolism for cell survival and growth, we have evaluated the effect of glutamine stability on the long-term culture and establishment of chicken PGC lines. Despite considerable progress in the field of cPGC transgenesis, a thoroughly defined culture medium for the culture of chicken PGCs has not yet been developed. It has been reported that extrinsic factors including the feeder layer, growth factors, and the micro-environment in accordance with the intrinsic state of the cPGCs can affect the *in vitro* culture of cPGCs (Chen et al. 2018; Kong et al. 2018; Whyte et al. 2015). The source of energy is another factor that is essential to sustain cell survival and growth. The oxidation of glutamine and the metabolism of carbohydrates are two main energy sources for *in vitro* cell culture. It has been demonstrated that in the presence of both glutamine and glucose, 30-50% energy requirement is provided by glutamine. The contribution of glutamine oxidation to energy is determined by the presence of glucose. When glucose is decreased or is replaced by other carbohydrates, glutamine becomes the only source of energy in the *in vitro* cell culture (Zielke, Zielke, and Ozand 1984). Furthermore, glutamine plays an important role in cell signaling, amino acid

production, nucleotide synthesis, and redox state (Chen and Cui 2015; Gwangwa, Joubert, and Visagie 2019).

In this study, we successfully obtained cPGCs from the circulating embryonic blood. After the isolation, we cultured cPGCs using two selective media, one containing glutamine and the other containing Glutamax. As the culture progressed, along with the destruction of red blood cells, doublets became visible in both culture media. This observation confirmed the appropriate culture conditions for cPGCs and ongoing derivation of cPGCs, and also non-appropriate culture conditions for blood cells (Figure 2 B). After ~ 2 weeks of culture, we reached high cPGCs derivation efficiencies of 65% and 80% in the Gmin and Gmax media, respectively. Even though the derivation was observed in both media, our results showed higher derivation efficiency in the presence of GlutaMax. Glutamine is very labile in the culture media and spontaneously degrades over time. The rate of decomposition of glutamine is temperature-dependent, as only 2%–3% of the glutamine is available in the medium at 37 °C (Arii, Kai, and Kokuba 1999; Heeneman, Deutz, and Buurman 1993; Jagušić et al. 2016; Tritsch and Moore 1962). It has been demonstrated that restriction of glutamine metabolism through glutamine deprivation can induce autophagy and, or apoptosis both *in vivo* and *in vitro* (Chen and Cui 2015). The presence of apoptotic cells among cPGCs cultured in the Gmin medium during the derivation stage might be partly due to the depletion of the glutamine source. It has been reported that glutamine has anti-apoptotic activity independent from its application as an energy source, suggesting that glutamine plays an essential role in signaling pathways relating to cell survival (Fuchs and Bode 2006; Gwangwa, Joubert, and Visagie 2019; Harnett et al. 2013). Furthermore, the chemical degradation of glutamine generates pyroglutamate and ammonia as by-products which can negatively affect cell viability, protein glycosylation, and protein production (Ozturk and Palsson 1990).

Primary cells such as cPGCs should be gradually adapted to *in vitro* culture; otherwise, cells may encounter growth defects (ATCC Primary Cell Culture Guide, 2012). Thus, from the initial culture to the derivation stage, the medium should be refreshed in half every two days. Aggregation of ammonia in culture is toxic to the cells, especially for cells sensitive to ammonia toxicity (Bort, Stern, and Borth 2010). A diminished energy source and aggregation of ammonia resulting from the degradation of glutamine in the Gmin medium may have had a detrimental effect on cPGCs, a condition that was not observed in the Gmax medium. This could explain the presence of more apoptotic cells in cultures containing the Gmin medium (Figure 2). The viability and proliferation of cPGCs cultured in the presence of GlutaMax were more optimal (Figures 3 and 5), which can be explained by the higher stability of the Glutamx.

Although both media could successfully support the long-term culture and transfectability of cPGCs, and transfected cells retained their germline features according to the promoter assay (Figure 6), the GlutaMax medium provided better culture conditions, leading to shorter doubling time and more efficient derivation of cPGC lines. In the long-term culture of cPGCs, we didn't observe any apoptotic cells and morphological alterations in cPGC cultured both in the Gmin and Gmax media. This observation can be explained by the complete exchange of the media every two days and proper feeding. However, the proliferation rate of cPGCs cultured in the Gmax medium was higher than that in the Gmin medium. Although both Gmin and Gmax media can support the growth performance of cPGCs *in vitro*, it is reasonable to use the Gmax media for proliferation, expansion, and long-term culture of cPGCs.

In conclusion, isolation, cultivation, derivation, and establishment of chicken PGC lines are time-consuming and complicated. Therefore, using a stabilized form of glutamine such as GlutaMax could be a more reasonable and cost-effective option for the long-term culture of genetically manipulatable chicken PGCs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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