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Evaluating potential of H'mong chicken bone marrow-derived mesenchymal stem cells at different ages for primordial germ cells' feeder layer

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Abstract

Primordial germ cells (PGCs) have potential applications in genetic conservation, vaccination, tissue repair therapies, and genetic research. Chicken bone marrow-derived mesenchymal stem cells (cbMSCs) is a good candidate for co-culture with PGCs. However, there is no consensus on the optimal age of donors. In this study, we aimed to compare specific parameters of H'Mong cbMSCs obtained from day 14th and 19th embryos, and day 3rd newborns. Isolated cbMSCs showed characteristics of MSCs. Cells had fibroblast-like morphology, plastic-adherent, expressed specific markers of MSCs and multilineage differentiation potential. The growth rate of cells from day 19th embryos was higher than from other ages. Moreover, cells expressed markers of pluripotency such as *Nanog*, *PouV*, *Sox2*, *CVH*, *DAZL*, and *KIT*, known for their role in maintaining stem cell self-renewal and pluripotency. As feeder cells, cbMSCs from three different ages promoted proliferation of H'Mong PGCs during co-culture. These results suggested that cbMSCs from different ages can be used for co-culture H'Mong PGCs which were further used for genetic preservation of H'Mong chicken or gene editing research.

Introduction

H'Mong chicken, the native breed raised by H'mong minority tribes, is a unique breed of chicken known for its distinctive black meat, skin, and bones. H'Mong chicken is rare breed which find in the mountainous region of Vietnam, particularly in the northern provinces such as Lao Cai, Ha Giang, and Cao Bang.[1] Recently, H'Mong chicken population has been declining rapidly due to the expansion of industrial poultry farming and the introduction of other chicken breeds that are more productive but less resilient to the local climate and diseases.[2] This has led to concerns about the loss of genetic diversity and cultural heritage associated with the breed. There have been initiatives to establish conservation programs and protected areas for the H'Mong chicken in Vietnam.[3] However, these projects still focus on implementing *in vivo* conditions, while currently, diseases are always at risk of wiping out the whole farm. Therefore, cryopreservation of cells under *in vitro* conditions is a direction that needs to be studied.

Chicken stem cell research has gained significant attention in recent years due to the potential applications of chicken stem cells in various fields. Stem cells are undifferentiated cells that have the ability to differentiate into different cell types and have tremendous potential in regenerative medicine, tissue engineering, and drug discovery.[4] The advantage of using chicken stem cells is that they are easy to obtain and culture, and their use is ethically acceptable. They can be isolated from various tissues, including embryonic tissues,[5] bone marrow,[6] adipose tissue,[7] and umbilical cord tissue,[8] and can be cultured *in vitro* for long periods without losing their stemness properties.[9] Chicken stem cells have been shown to have the ability to differentiate into various cell types, including muscle, bone, cartilage, and nerve cells, making them a promising source of cells for tissue engineering and regenerative medicine.[10] Moreover, chicken stem cells have been used to produce viral vectors for vaccine delivery, and chicken mesenchymal stem cells (MSCs) have been shown to have immunomodulatory properties that can enhance the immune response to vaccines.[4]

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The age of donor can have a significant impact on the potential of MSCs.[11] Reportedly, the age of donor can affect the proliferation, differentiation, and immunomodulatory properties of MSCs.[12] The MSCs can be used as feeder layer of primordial germ cells (PGCs).[13] The collection of tissues during specific developmental stages can be a convenient approach. However, the timing of tissue sampling can significantly impact the activity and proliferation potential of MSCs. If tissues are sampled too early, they may not have formed yet,[14] while sampling too late may result in a significant decrease in MSCs viability.[15] Thus, conducting research on MSCs during optimal developmental stages can reduce costs and facilitate further studies and applications of MSCs.This research aims to evaluate potential of H'mong chicken bone marrow-derived MSCs (cbMSCs) at different ages for PGCs' feeder layer.

Materials and Methods

Experimental animals and chicken embryos. Specific pathogen-free fertilized eggs of H'Mong chicken were supported by the Center for Conservation of Genetic Resources of Animal Breeds (Animal Husbandry Institute, Hanoi, Vietnam). Chicken embryos were incubated in a rotary egg incubator (CNE, Truongsa, Hochiminh, Vietnam) at 38.00 ˚C and 55.00% humidity, with rocking at an angle of 90° every 1 hr for the following test. All experiments were performed in accordance with the Animal Care guidelinesas stated by University of Science (Vietnam national university Ho Chi Minh, Decided no. 2487/QĐ-KHTN). On the 14th and 19th day of incubation, chicken embryos were sacrificed by decapitation. Three-day old chickens were sacrificed using carbon dioxide inhalation.

Isolation and culture of cbMSCs. Femurs and tibia bones were obtained from H'mong chicken embryos on the day 14th (group 1), and 19th (group 2), as well as from newborns (day 3 post hatching, group 3). The cbMSCs were isolated as previously described.[16] After removing muscles and connective tissues around fumurs and tibia, the epiphyses of these bones were removed. Bone marrow inside these bones were collected by flushing with Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, USA). Subsequently, the bone marrow cells were passed through a 70.00 µm nylon mesh filter (Falcon, North Carolina, USA), and filtered contents were centrifuged at 1,000 rpm for 10 min. Afterwards, the supernatant was discarded and the cell pellet was suspended with DMEM supplemented with 10.00 % fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, USA). These cells were then plated at a density of 1.00 × 10^5 cells per cm^2 in 6-well culture dishes and cultured in DMEM/F12 supplemented with 10.00% FBS, 2.00 mm L-glutamine (Gibco), and 1.00% antibiotic solution (Gibco). The cultures were maintained at 37.00 ˚C in a humidified incubator containing 5.00% CO_2. After 24 hr of culture, media was changed to remove the non-adherent cells. Once the primary cells reached 90.00% confluence, the cells were detached using 0.25.00% trypsin (Gibco) supplemented with 0.02% ethylenediamine tetraacetic acid (EDTA; Gibco) and were then utilized for subsequent experiments.

Growth kinetics of cbMSCs. Cells at passage (P)2 - P5 were plated within 24-well plates with a density of 1.00 × 10^4 cells per well. The cell number was counted by a viability detection method using a Trypan Blue (Sigma-Aldrich) exclusion test. Population doubling time (PDT) was calculated using the formula:

$$PDT = T\ ln2/ln\ (Xei/Xbi)$$

where, *T* means incubation time in hours, *Xbi* is the number of cells at the beginning of the incubation, and *Xei* corresponds to the number of cells at the end of incubation.[17] Growth curves were plotted for P3. Experiment was repeated three times.

Differentiation potential of cbMSCs. Adipogenic differentiation capacity of cbMSCs was evaluated as previously described.[18] Initially, cells were seeded in 6-well plates at a density of 1.00 × 10^5 cells per well with expansion medium until they reached 70.00 - 80.00% confluence. Adipogenic medium comprised DMEM supplemented with 10.00% FBS, 1.00 µM dexamethasone (Sigma-Aldrich), 10.00 µg mL^{-1} insulin, and 0.20 mM indomethacin (Sigma-Aldrich). The medium was changed every 3 - 4 days. After 21 days of induction, cells were stained with Oil Red O (Bio Basic Inc., Markham, Canada) for determining lipid droplets or harvested for gene expression using qRT-PCR with two key adipogenic genes (*peroxisome proliferator-activated receptor gamma* [*PPARγ*], and *apetala 2* [*aP2*]). For Oil Red O staining, cells were washed with phosphate buffered saline (PBS), fixed with 10.00 % formalin for 20 minat room temperature, and then incubated with 0.30 % Oil Red O (Sigma-Aldrich) in 60.00 % isopropanol for 20 min. After washing cells with PBS, cell images were captured with a Nikon Eclipse Ti with DS-Fi2 camera (Nikon, Tokyo, Japan). The osteogenic differentiation potential was assayed as previously described,[6] cbMSCs were plated in 6-well plates at a density of 3.00 × 10^5 cells per well with expansion medium until they reached 90.00 - 100% confluence. Osteogenic medium was consisted of DMEM supplemented with 100 nM dexamethasone, 10.00 mM β-glycerophosphate (Sigma-Aldrich), 0.05 mM l-ascorbic acid-2-phosphate (Sigma-Aldrich), and 10.00% FBS for 21 days. The medium was replaced every 3 - 4 days. Calcium deposition after 21 days was evaluated by Alizarin Red staining technique. After fixing with 10.00% formalin, cells were washed with distilled water, stained with Alizarin red (Bio Basic Inc., Ontario, Canada) for 20 min. Afterwards, the cells underwent three additional washes with distilled water before being photographed.

Quantitative RT-PCR analysis. Total RNA was isolated using the TRIzol RNA isolation reagent (Ambion, Life Technologies, Carlsbad, USA). The RNA concentration of each sample was measured using NanoDrop™ Lite Spectrophotometer (ND-LITE-PR, Thermo Fisher Scientific, Waltham, USA). Subsequently, the extracted RNA (1.00 µg) was subjected to synthesize first-strand cDNA using GostriptTM Reverse Transcription system (A5001; Promega, Madison, USA). Then qPCR reactions were carried out with cDNA, GoTaq® qPCR Master Mix (A6001; Promega), and primers showed in Table 1.[19,20] *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as a housekeeping gene. Quantitative expression analysis was conducted based on the $2^{-\Delta\Delta CT}$ method.[21]

Feeder cell preparation. Chicken MSCs within passages 3 - 5 were placed into 6-well plates. Once cells reached 80.00% confluence, cells underwent a 2-hr treatment with 10.00 µg mL^{-1} Mitomycin C (Sigma-Aldrich) in culture medium. After washing five times with PBS, the mitotically inactivated cells were used as feeder cells in the PGCs culture medium (DMEM supplemented with 7.50% FBS, 2.50% chicken serum (Sigma-Aldrich), 2.00 mM GlutaMAX-I Supplement (Invitrogen, Waltham, USA), 1.00% nucleosides (100 X, Millipore, Billerica, USA), 1.00% nonessential amino acids (100 X, Invitrogen), 0.10% β-mercaptoethanol (1,000 X, Sigma-Aldrich), 5.00 ng mL^{-1} human SCF (Peprotech, Cranbury, USA), 10.00 ng mL^{-1} human basic fibroblast growth factor (Sigma-Aldrich), and 1.00% Antibiotic-Antimycotic (100 X, Invitrogen).

Primordial germ cells isolation and proliferation assays. Embryos at stage 28 - 29 HH (Hamburger Hamilton Stages; incubated for 6 days) were rinsed three times with PBS. Afterwards, gonadal ridges were isolated by a medial incision in the abdomen using fine-tipped tweezers under a stereomicroscope. The gonadal tissues were disaggregated into individual cells using 0.25% trypsin with 0.02% EDTA. Then cells were washed with PBS and centrifuged at 1,000 rpm for 5 min. The primary gonadal cells (comprising both PGCs and somatic cells) were resuspended in PGCs culture medium and seeded in 35.00 mm dish. After 24 hr, somatic cells adhered to the dish surface entirely and the suspended PGCs were collected and co-cultured with or without the mitotically inactivated feeder cells (MSCs-feeder). Following a 3-day culture period, PGCs were transferred to 96-well plates to assess cell proliferation efficiency using the PrestoBlue™ Cell Viability Reagent (Invitrogen). Subsequently, the plates with the treated cells were analyzed for absorbance values at optical density 450 using Multiskan™ Sky microplate spectrophotometer (Thermo Fisher Scientific).

Statistical analysis. The data were analyzed through ANOVA followed by Tukey's Post Hoc test for making pairwise comparisons between individual means using SPSS Software (version 20.0; IBM Corp., Armonk, USA). A significance threshold of $p < 0.05$ was applied to establish statistical significance.

Results

Isolation, culture, and morphology of H'Mong cbMSCs. Collected femurs and tibia bones of three groups are shown in Figure 1. In group 1, the femur and shin bone of H'Mong chicken embryos on the 14th day had a completely calcified structure and formed tubular bones similar to the bone structure in group 2 (embryo on the 19th day) and in group 3 (day 3rd postpartum group). However, black pigmented tissue has only formed at the two ends of the bone, but not the entire bone yet (Fig. 1A). On the 19th day, pigmentation gradually increased throughout the entire bone, but the color was still pale (Fig. 1B). In group 3, the bone has a completely dark black bone structure (Fig. 1C). Morphology of isolated H'Mong cbMSCs are shown in figure 2. After 24 hr, the primary cbMSCs adhered to the plates and had fibroblast-like morphology (Fig. 2A). Cells grew well and exhibited spindle-shaped and plastic-adherent after subculture (Figs. 2B and 2C). Cells at passage 3 reached > 90.00 % confluence on the 3rd day (Fig. 2C).

Table 1. Quantitative polymerase chain reaction primer sequences

Genes	Primer's sequence		Product size (bp)
	Forward (5' - 3')	Reverse (5' - 3')	
GAPDH	CACAGCCACACAGAAGACGG	CCATCAAGTCCACAACACGG	443
CD44	GGTTTTATAGTGGGGCATATTGTTATCCC	TTAACCGCGATGCACACGGC	700
CD90	GGTCTACATGTGCGAGCTGA	AAAGCTAAGGGGTGGGAGAA	471
CD105	ACGGATGACACCATGGAAAT	ATGAGGAAGGCTCCAAAGGT	704
PouV	AAATGTGTGAAGCCCAGTCC	TTGTGGAAAGGTGGCATGTA	401
Sox2	TCCGGCGGTAATAATAGCAG	TTGCTGATCTCCGAGTTGTG	312
Nanog	CAGCAGACCTCTCCTTGACC	CCAGATACGCAGCTTGATGA	371
CVH	GGGAAGATCAGTTTGGTGGA	GACAAAGAAAGGCTGCAAGG	388
DAZL	CGTCAACAACCTGCCAAGGA	TTCTTTGCTCCCCAGGAACC	540
KIT	GTGGGCAAGAAGTGGAAGCC	GCAAACCAAGCATCTCATCCC	239
PPAR-γ	TACATAAAGTCCTTCCCGCTGACC	TCCAGTGCGTTGAACTTCACAGC	401
Ap	GAGTTTGATGAGACCACAGCAGA	ATAACAGTCTCTTTGCCATCCCA	312
Osteopontin (SPP1)	TGCCAGGAAGCTCATTGAGGATG	GCGTCTACATTTACAAACACACGTC	371

Fig. 1. The morphology of chicken femur and tibia bones at different stages. **A)** Chicken bones after incubation for 14 days, **B)** Chicken bones after incubation for 19 days, and **C)** Chicken bones after hatching for 3 days.

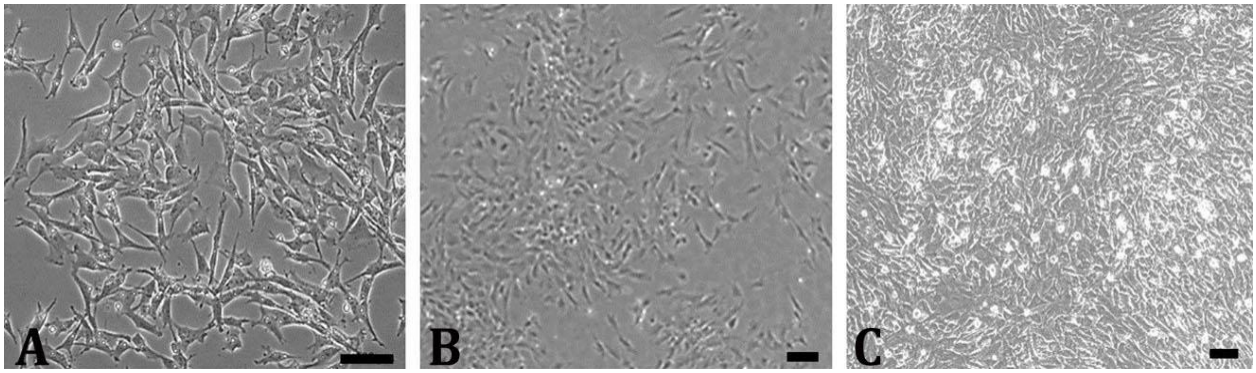

Fig. 2. Cell morphology at chicken bone marrow-derived mesenchymal stem cells (cbMSCs). **A)** cbMSCs on day 2 of primary culture, **B)** cbMSCs on day 4, and **C)** cbMSCs on day 3 at passage 3. Scale bars = 100 µm.

Growth kinetics of cbMSCs. The PDT of H'Mong cbMSCs in three groups at P2 - P5 was shown in Table 2. The PDT of group 1 was ranged from 79.48 ± 5.02 to 96.04 ± 4.16 hr while PDT of group 3 were varied from 82.23 ± 4.86 to 93.61 ± 4.26 hr. However, the highest value of PDT of group 2 were 79.02 ± 5.05 hr at P2. The PDT of group 2 at P3 - P5 was slightly changed from 74.59 ± 5.35 to 75.13 ± 5.31 hr. These results showed that cbMSCs collected from chicken embryo on the 19th day had higher self-renew capacity than other collection times in this study. The proliferation profile of cbMSCs at P3 in three groups was shown in Figure 3. Cells in group 1 had similar proliferation potential to cells in group 3. Group 2 showed higher growth rate than other groups. Cells in group 1 and 3 initially had a lag phase of 1 - 2 days, a log phase for 3 - 6 days, and reached a stationary phase in 7 - 8 days. However, cells in group 2 had a lag phase in the first day, a log phase of 2 - 6 days, and reached a stationary phase after 7 days. These results indicated that cbMSCs in three groups were capable of self-renewal and cells in group 2 had the highest proliferation property.

Gene expression of cbMSCs. The qPCR results of cbMSCs with MSCs phenotypic markers was shown in Figure 4A. Results showed that cbMSCs in all groups expressed MSCs surface markers *CD44*, *CD90*, and *CD105*. Expression of these markers was significant higher in group 2 than in groups 1 and 3 ($p < 0.05$). Expression of *Nanog*, *Pouv*, *Sox2*, *CVH*, *DAZL*, *KIT* was shown in Figure 4B.

Table 2. Population doubling time of chicken bone marrow-derived mesenchymal stem cells at different ages. Data are presented as mean ± SD.

Groups	Population doubling time (hr)			
	Passage 2	Passage 3	Passage 4	Passage 5
1	96.04 ± 4.16	79.48 ± 5.02	84.64 ± 4.72	79.74 ± 5.01
2	79.02 ± 5.05	74.59 ± 5.35	75.1 3± 5.31	74.86 ± 5.33
3	90.89 ± 4.39	93.61 ± 4.26	82.23 ± 4.86	91.51 ± 4.36

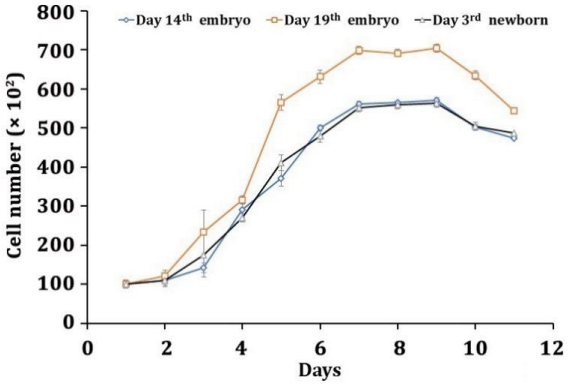

Fig. 3. Growth curves of chicken bone marrow-derived mesenchymal stem cells at passage 3 in three groups. Values are expressed the mean ± SEM (n = 3 in each group)

Gene expression of these markers was detected in all groups. However, expression of these genes in group 2 was significantly higher than other groups ($p < 0.05$). These results indicated that cbMSCs in all groups showed characteristics of MSCs. Group 2 showed higher expression of MSCs marker than other groups.

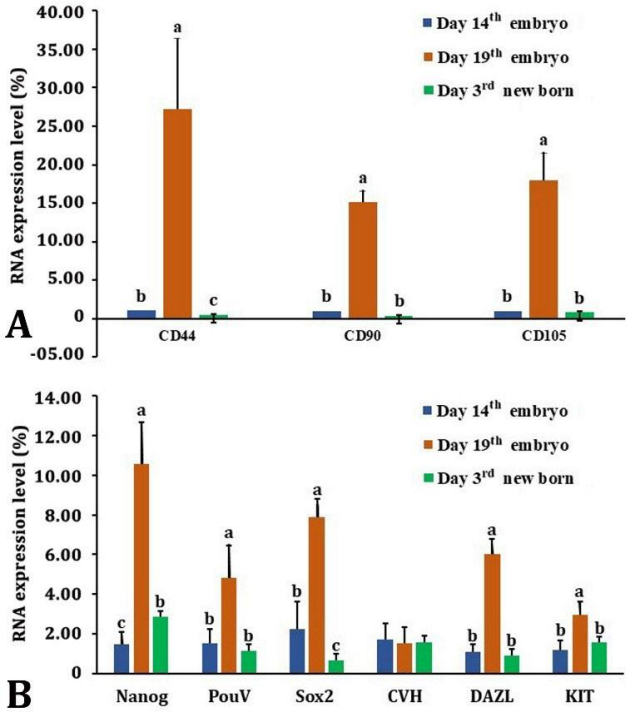

Fig. 4. Qualitative marker expression of chicken bone marrow-derived mesenchymal stem cellswere detected by quantitative polymerase chain reaction at passage 3 in three groups. With the marker specific for **A)** mesenchymal stem cell, and **B)** embryonic stem cell.
[abc] Different letters indicate significant differences at $p < 0.05$.

Differentiation potential of cbMSCs. The results of osteogenic differentiation of the cbMSCs in three groups were shown in Figure 5. After 21 days of osteogenic induction, the calcium nodules in three induced groups were stained positively with Alizarin Red in these groups (Fig. 5) while there was negatively stained in control group. Furthermore, mRNA expression level of osteopontin gene was significantly increased in an age-dependent manner (Fig. 6A; $p < 0.05$). These results showed that cbMSCs in three groups had osteogenic potential. Besides, the results of differentiation into adipocytes are illustrated in Figure 5. After Oi Red O staining, the results showed that the induced groups had a clear positive expression (Fig. 5E to 5H) while the control group had no differentiated cells. The mRNA expression level of *PPARγ*, and *aP2* genes of treated cells in group 2 were significantly higher when compared to groups 1 and 3 (Figs. 6B and 6D; $p < 0.05$). These results indicated that cbMSCs in three experimental groups had adipogenic capacity.

Primordial germ cells isolation and proliferation assays. Gonad buds were isolated as small crescents, uniformly white in color (Fig. 7A). After trypsin-treatment, gonad cells were separated (Fig. 7B). The PGCs were spherical, relatively uniform in size, and had large nuclei and cytoplasm. These cells showed positive expression with anti-SSEA1antibody (Fig. 7C). The results showed that the isolated PGCs had morphological characteristics and indicators of chicken PGCs. The proliferation results of PGCs on MSCs feeder layers are shown in Figures 7D to 7G. The absorbance values of PGCs were significant higher in three co-cultured groups than control group (without feeder layer; Fig. 7H). The results showed that feeder cells could promote the proliferation of PGCs *in vitro* culture.

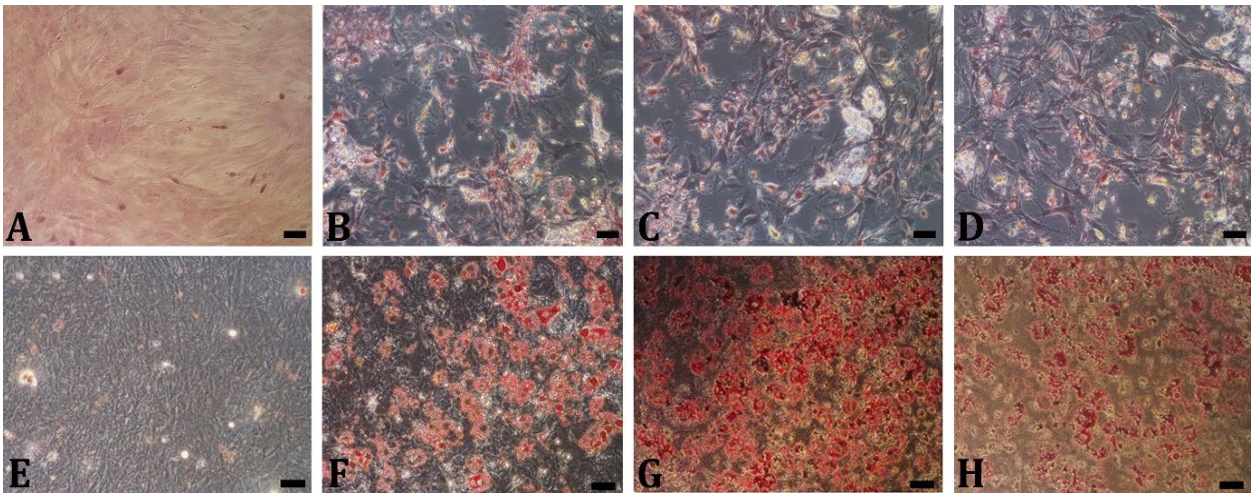

Fig. 5. Osteogenic differentiation potential of chicken bone marrow-derived mesenchymal stem cells. With, **A)** control cells, **B)** day 14th embryo, **C)** day 19th embryo, **D)** day 3rd newborn, (Alizarin Red staining; bars = 100 µm). Adipogenic differentiation potential of chicken bone marrow-derived mesenchymal stem cells. stain with **E)** control cells, **F)** day 14th embryo, **G)** day 19th embryo, and **H)** day 3rd newborn (Oil Red O staining; bars = 100 µm).

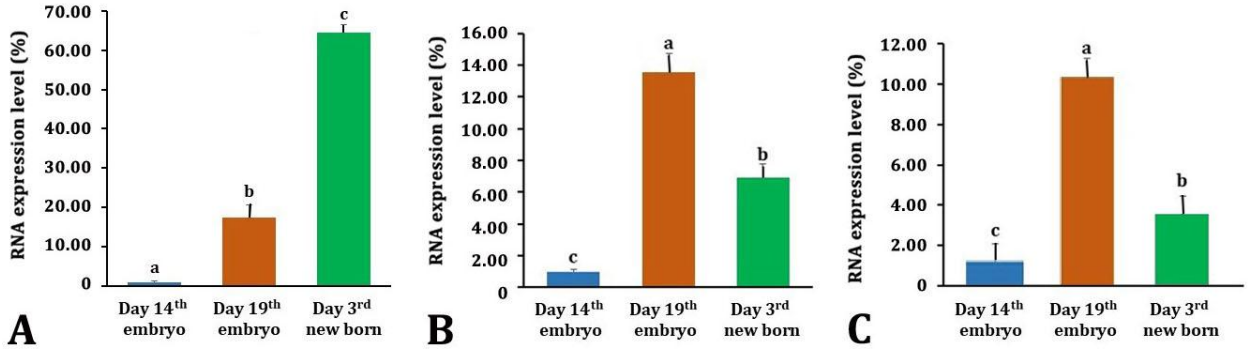

Fig. 6. Level of gene expression of **A)** osteopontin, **B)** *Peroxisome proliferator-activated receptor gamma* (*PPARγ*) gene expression, and **C)** *Apetala 2 (aP2).*

[abc] Different letters indicate significant differences at the $p < 0.05$ level.

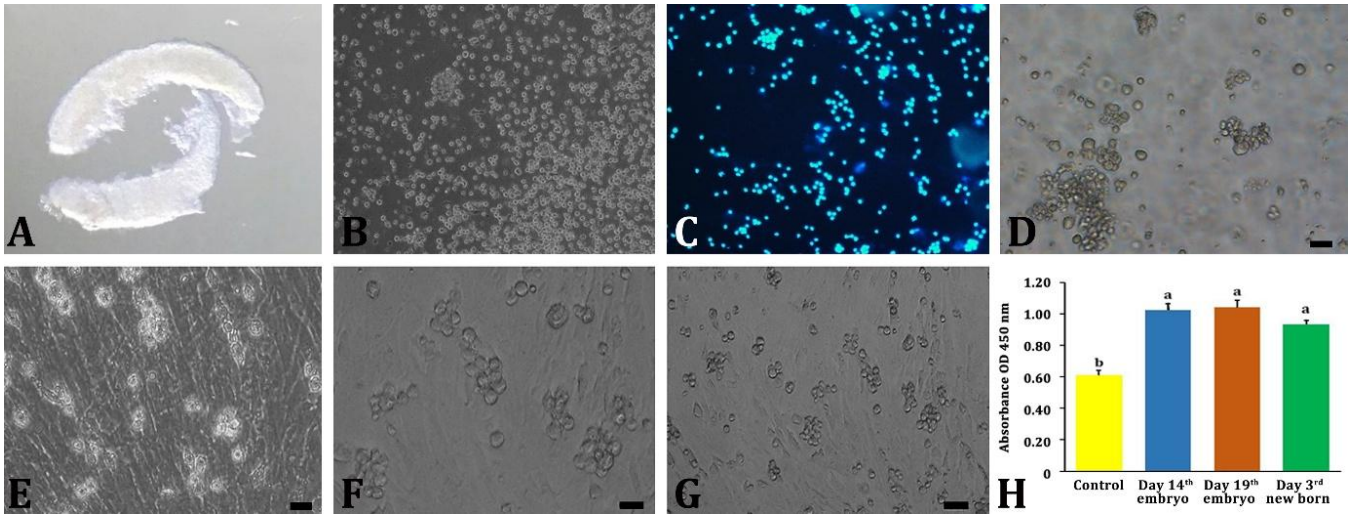

Fig. 7. Results of primordial germ cells (PGCs) isolation. **A)** Gonad germ morphology at day 6, **B)** PGCs after enzymatic digestion, **C)** PGCs stained with SSEA1. Proliferation of chicken primordial germ cells (PGCs) cultured with and without mesenchymal stem cells (MSCs) feeders at different ages. **D)** Morphology of PGCs cultured without feeder cells, **E)** with MSCs feeder cells isolated from embryo on the 14th day, **F)** on the 19th day, and **G)** 3-day-old chicks. **H)** The absorbance values of optical density 450 of PGCs co-cultured with or without MSCs-feeder cells. Values are shown as mean ± standard deviation; n = 5. *$p < 0.05$.

Discussion

H'mong cbMSCs were successfully isolated from femurs and tibia bones at different ages on the day 14th (group 1), and 19th (group 2) of embryos and day 3 post hatching (group 3). The cells were plastic-adherent, spindle-shape and able to self-renewal. Besides, cbMSCs showed specific markers of MSCs (*CD44*, *CD90*, and *CD105*) and had ability to differentiate into osteocytes, adipocytes. These results indicated that cbMSCs from H'Mong chicken at different ages had characterizations of chicken MSCs.[6,22,23] Moreover, cbMSCs also expressed pluripotent stem cell markers (*Nanog*, *Pouv*, *Sox2*, *CVH*, *DAZL*, and *KIT*). Expression of Pouv (a chicken homologue of mammalian Oct4)[24] was reported in chicken MSCs derived from lung,[22] bone marrow.[16] *Nanog* and *Sox2* are involved in the maintenance of stemness in undifferentiated embryonic stem cells.[25,26] The *Pouv*, *Nanog*, *Sox2* were expressed in multipotent Turkey tendon-derived stem cells.[19]

The PDT of cbMSCs from day 19th embryos were 79.02, 74.59, 75.13, and 74.86 hr for P2, P3, P4, and P5, respectively, which was similar to the PDT of cbMSCs from compact bones.[6] However, PDT of cbMSC from day 14th embryos and day 3rd newborn was higher than the PDT of cbMSC from day 19th embryos. These results indicated that the growth potential of MSCs was varied with different ages. The growth curve of the cbMSCs in this study was similar to growth curve of cbMSCs in previous studies.[6,16]

The cbMSCs of 3 different ages showed multilineage differentiation potential. After 21 days of treatment, cells expressed *osteopotin* (secreted phosphoprotein-1, *SPP1*) gene which played a role in bone metabolism and homeostasis[27] and induced cells produced mineralization of extracellular matrix that positively stained with Alizarin Red. Osteopotin and bone morphogenetic protein were used as the osteogenic specific genes that determined osteogenic differentiation of stem cells.[6,16,23] Otherwise, BSP, collagen type 1 alpha 2, and BGLAP were also used for

confirmation of osteogenic potential of MSCs.[6,19] Several staining methods could determine osteogenic differentiation.[28-30] After staining, Alizarin Red reacts with calcium cations to form Alizarin Red-calcium complex, an orange-red chelate thus confirming the deposits of Ca in cells.[31] In this study, calcium deposits produced by differentiated osteoblasts derived cbMSCs were positively stained with Alizarin Red that was similar to previous studies.[6,16,19,23] Both evaluating gene expression and Alizarin Red staining confirmed osteogenic potential of cbMSCs. Adipogenic differentiation of cbMSCs in this study was evaluated by both gene expressions by qPCR and Oil Red O staining method. The *PPARγ* and *aP2* are both adipocyte-specific genes. While *PPARγ* is a primary regulator in adipocyte differentiation and maintenace,[32,33] The *aP2* is exhibited in the late stage of the adipocyte differentiation and identified as an important link between lipid metabolism and cellular functions in adipocytes.[34] After treatment with adipogenic medium, both *PPARγ* and, *aP2* genes were expressed in induced cells in 3 groups that were in accordance with previous studies.[6,16,19,23] Other genes (c/EBPα, c/EBPβ, and FAS) were also used to detect adipogenic induction.[6,23] Oil Red O staining is general method to determine lipid droplet formation after adipogenic induction. Similar to previous studies, lipid droplets, positively stained with Alizarin Red, were induced in 3 groups in this study after adipogenic differentiation.[6,16,19,23] These results indicated that cbMSCs from different ages had adipogenic potential.

The PGCs, precursor cells of spermatozoa and ova, are promising genetic resources for avian studies, including modified animals.[33,34] Generals, PGCs were co-cultured with several cell types such mouse embryonic fibroblasts, buffalo rat liver cells, and cell line established from Sandos inbred mouse (SIM) embryonic fibroblasts which support self-renewal and proliferation of chicken PGCs for *in vitro* cultures.[35,36] However, the feeders from cross species animal had the risk of releasing animal materials and unknown pathogens. Co-cultured PGCs with chicken MSCs as feeder could maintain characteristics of PGCs and limit the disadvantage of feeders from cross species animal.[23] In this study, H'Mong cbMSCs from different ages were used for PGCs derived from same species. The cbMSCs-feeders from three different ages promoted proliferation of PGCs. These results suggested that MSCs-feeder from both embryos and newborn chickens is a good candidate for co-culture H'Mong PGCs which were further used for genetic preservation of H'Mong chicken or gene editing research.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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