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ISOLATION, CHARACTERIZATION AND DIFFERENTIATION POTENTIAL OF CHICKEN SPERMATOGONIAL STEM CELL DERIVED EMBRYOID BODIES*

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Abstract

Human, murine and monkey spermatogonial stem cells (SSCs) have the capability to undergo self-renewal and differentiation into different body cell types *in vitro*, which are expected to serve as a powerful tool and resource for the developmental biology and regenerative medicine. We have successfully isolated and characterized the chicken SSCs from 3-day-old chicken testicular cells. The pluripotency was using Periodic Acid-Schiff (PAS) staining or alkaline phosphatase staining, and antibodies to stage-specific embryonic antigens. In suspension culture conditions SSCs formed embryoid bodies (EBs) like embryonic stem (ES) cells. Subsequently EB differentiated into osteoblasts, adipocytes and most importantly into cardiomyocytes under induced differentiation conditions. The differentiation potential of EBs into cardiomyocyte-like cells was confirmed by using antibodies against sarcomeric α-actinin, cardiac troponin T and connexin 43. Cardiomyocytes-like cells were also confirmed by RT-PCR analysis for several cardiac cell genes like GATA-4, Nkx2-5, α-MHC, and ANF. We have successfully established an *in vitro* differentiation system for chicken SSCs into different body cells such as osteoblasts, adipocytes and cardiomyocytes. The most significant finding of this study is the differentiation potential of chicken SSCs into cardiomyocytes. Our findings may have implication in developmental biology and regenerative medicine by using chicken as the most potential animal model.

Key words: chicken cardiomyocytes, differentiation, embryoid bodies, spermatogonial stem cells.

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Spermatogonial stem cells (SSCs) are a unique population of germline stem cells in adult testis cells having the capability to undergo self-renewal and produce daughter cells destined for differentiation into spermatozoa throughout the life cycle of male animals (Aponte et al., 2008). In previous studies, SSCs from neonatal and adult mice were found to be capable of developing into multipotent cells under specific *in vitro* conditions (Kanatsu-Shinohara et al., 2004; Guan et al., 2006; de Rooij and Mixrak, 2008). In addition, SSCs of mice were found to be capable of reverting spontaneously (without the addition of exogenous genes) to pluripotent embryonic stem (ES) like cells (Guan et al., 2006). Other groups have identified the formation of ES-like cells after exposure of human testicular cells to ES conditions *in vitro* (Ge et al., 2007; Conrad et al., 2008; Kossack et al., 2009). Few studies have also confirmed the pluripotency in avian/chicken gonadal primordial germ cells or SSCs (Petitte et al., 2004; Intarapat and Stern, 2013; Zuo et al., 2015).

Reports on the generation of SSCs, encouraged further investigations into application of gene targeting to create models for the study of human diseases (Han, 2009; Momeni-Moghaddam et al., 2014; Zuo et al., 2015). In addition, the *in vitro* system based on differentiation of ES cells into 3 germ layers, serves as a powerful tool and resource for the investigation of mammalian development, cell function, tissue repair, and drug discovery (Cao et al., 2011). The embryoid body (EB) is a three-dimensional structure, which creates appropriate conditions for the developing embryo and generates the three germ layers (endoderm, mesoderm, and ectoderm). Osteoblasts, adipocytes, and cardiomyocytes are derived from the mesoderm. The previous studies involving EB generation have demonstrated the capacity of ES cells (mouse, human, or monkey) to generate osteoblasts, adipocytes, and cardiomyocytes within 3 to 4 weeks in the presence of differentiated factors (ascorbic acid, β-glycerophosphate, 1α,25-OH vitamin D3, 5-aza-2'-deoxycytidine, IBMX, and dexamethasone) (Coelho and Fernandes, 2000; Buttery et al., 2001; George et al., 2006; de Rooij and Mizrak, 2008; Jia et al., 2008). But no experiments have been conducted on chicken SSCs, which are believed to have great potential as germ cells. Mammalian SSC are able to differentiate *in vitro* into different cell types and are valuable sources for regenerative medicine and gene transfer studies (Li et al., 2010).

The chicken has had a long association with man, probably dating back more than 8,000 years when humans ceased to be hunter-gatherers (Burt et al., 2007). Chickens particularly chick embryo are known as an important animal model for developmental studies (Jung et al., 2005, 2007). The key advantages of the chicken embryo for studies such as these include low cost, easy handling, the ability to time incubations and stage embryos accurately for experimental standardization, high accessibility for a variety of surgical implantation procedures, and its lack of immune response has made it a popular model for xenotransplantation (Mitsiadis et al., 2003; Glover et al., 2010). However, use of chick embryos in human clinical and stem cell research has some disadvantages (Glover et al., 2010). The disadvantages are that chicken embryos are very small and thus poor models for human organs in terms of size, and they have a number of physiological and anatomical peculiarities not found in humans, although the basic organization of many organs is similar to that of human organs; in particular, the organization of the brain stem and spinal cord is

very similar to that in mammals, and the fact that chickens use bipedal locomotion has suggested a greater similarity to human locomotion than for quadrupedal rodents (Jacobson and Hollyday, 1982).

The research on chicken SSCs is still in the infancy stage. We conducted several experiments to isolate and identify putative pluripotent cells in testicular cells and to differentiate the identified cells. The aims of this study were to establish an *in vitro* differentiation system of SSCs and to investigate whether SSCs are capable of forming terminal-differentiated osteoblasts, adipocytes, and cardiomyocytes.

Material and methods

Isolation of spermatogonial stem cells from chick testes

Spermatogonial cells were isolated from 3-day-old five White Leghorn chickens testes (testis weight ranged from 1 to 2 mg) tissue after surgical removal. The experiment was approved by the animal ethics committee of the Faculty of Biotechnology, Jeju National University, Jeju, South Korea and birds were slaughtered according to the standard protocols of Jeju National University. The decapsulated testes were resuspended in phosphate-buffered saline (PBS). Testes tissues were digested using collagenase IV and trypsin EDTA to isolate single cells. Approximately 1×10^6 dissociated cells were cultured in the culture flask with standard media (Jung et al., 2005) after slight modifications, modified Dulbecco's modified eagle medium (DMEM; Hyclone, USA) containing 10% fetal bovine serum (FBS; Hyclone, USA), 1% non-essential amino acids (Gibco, Invitrogen, USA), 50 µmol/L β-mercaptoethanol (Sigma, USA), 10^3 units/mL leukemia inhibitory factor (LIF), and 1% penicillin at 37°C and 5% CO_2 in a humidified atmosphere. The testicular cells formed a monolayer after 3 days of culture, whereas the spermatogonia containing SSCs started to form colonies after 10 to 15 days of culture. On day 15, the colony-forming cells were passaged, and the cultures were subsequently passaged thrice at 5-day intervals.

Characterization of chicken SSCS colonies

The chicken SSC colonies were characterized using periodic acid-Schiff (PAS) staining, alkaline phosphatase (AP) according to the manufacturer's instructions and antibodies to stage-specific embryonic antigens. Colony-forming cells were fixed in 4% paraformaldehyde for 10 min. The sample was then washed twice (5 to 10 min for each wash) with 1× rinse buffer. Blocking solution (1% bovine serum albumin) was applied for 30 min at room temperature, followed by incubation with primary antibodies (SSEA1, SSEA3, SSEA4, STRA 1-60, STRA 1-81, and Oct4) with recommended dilutions (1:10–1:50) for 1 h at room temperature. After incubation, the solution was washed thrice (5 to 10 min for each wash) with 1× rinse buffer. After that stained with fluorescent secondary antibodies (FITC-conjugated antibody or C5-conjugated antibody) for 30 min at room temperature and washed thrice (5 to 10 min for each wash) with 1× rinse buffer. Colonies were then stained with nuclear dye 4′,6-diamino-2-phenylindole (DAPI; Sigma, USA) and were examined using

a fluorescence microscope (Olympus IX70, Japan). Different genes expression level was quantified using ImageJ software as relative expression in per cent.

Formation of EB derived from colony-forming cells

For EB formation, colonies were re-suspended and maintained under suspension conditions (Ge et al., 2007). Briefly, EB colonies were first dissociated by gentle scraping, and then harvested by centrifugation. Subsequently, EB colonies were sub-cultured in suspension culture in an ultra-low-attachment bacterial petri dish supplemented with 20% FBS, L-glutamine, non-essential amino acid (NEAA), and β-mercaptoethanol (Sigma, USA) without LIF. The cultured dishes were regularly examined at 12 h interval to monitor morphological changes in EB cells. EB candidates were subjected to immunocytochemical analysis after 5 to 7 days in the suspension state, by using primary antibodies for mesoderm (anti-desmin, 1:200 dilution; anti-α smooth muscle actin, 1:150 dilution, Abcam, UK), endoderm (anti-AFP, Santa Cruz Biotechnology Inc), and ectoderm (S100 alpha 2, Abcam, UK). Different genes expression level was quantified using ImageJ software as relative expression in per cent.

***In vitro* differentiation potential of EBS into osteoblast like cells**

To induce directed formation of osteoblasts, EBs were incubated in ultra-low-attachment bacterial petri dishes for 7 days. Ge et al. (2007) protocol was followed for osteogenic differentiation after own optimization. Subsequently, the EBs were plated at a density of 2–3 EBs/well in an EB cell culture medium for 24 h in 24-well dishes until they became attached. The attached cells were then cultured in osteoblast-conditioned DMEM/F12 medium supplemented with 10% FBS, 5.5×10^{-2} µmol/mL β-mercaptoethanol, 1×10^{-4} µmol/mL dexamethasone, 10 µmol/mL β-sodium glycerophosphate (Sigma, USA), and 0.05 mg/mL ascorbic acid for 20 days. Half of the medium was replaced daily with fresh medium. For the control, all the procedures were followed same as test group, except inclusion of osteogenic differentiating agents. When the osteoblast-like cells were formed, they were stained with alizarin red and examined under microscope. Briefly, the osteocyte-like cells were first rinsed with PBS and fixed by incubating them in ice-cold 70% ethanol for 1 h at room temperature. The cells were then washed twice (5–10 min for each) with water and sufficient alizarin red solution was added to cover the wells (500 µL per well in a 24-well plate). After incubating for 30 min, the alizarin red solution was removed, and the cells were washed 4 times with 1 mL of water. The stained cells were then examined using a microscope.

***In vitro* differentiation potential of EBS into adipocyte-like cells**

After the EBs were retained for 7 days in suspension culture, they were plated at a density of 2 to 3 EBs/well in EB cell culture medium for 24 h in 24-well dishes to induce attachment of cells prior to induction of directed adipocytes. The attached cells were then cultured in adipocyte-conditioned DMEM/F12 medium supplemented with 10% FBS, 5.5×10^{-2} µmol/mL β-ME, 1×10^{-3} µmol/mL dexamethasone, 0.01 mg/mL insulin (Sigma, USA), and 0.5 µmol/mL IBMX (Sigma, USA). After

3 days, the induction medium was replaced with DMEM supplemented with 10% FBS, 5.5×10^{-2} μmol/mL β-ME, 1×10^{-3} μmol/mL dexamethasone, and 0.01 mg/mL insulin and cultured for 15 days. Subsequently, half of the medium was replaced daily with fresh medium. For the control, all the procedures were the same as that of directed differentiation without the inclusion of the agents mentioned above. When the adipocyte-like cells were formed, they were stained with oil red O. The adipocyte-like cells were first fixed by incubating them in 10% formalin solution for 10 min at room temperature. Then, the formalin was removed, and 60% isopropanol was added to each well. The cells were then left undisturbed for 5 min and isopropanol was removed. After that, oil red O stain was added, and the cells were incubated for 5 min at room temperature. Subsequently, the plate was rinsed with tap water until the rinse became clear. The cells were then incubated with hematoxylin for 1 min at room temperature. The cells were rinsed again with tap water until the rinse became clear. The cells were then observed under a microscope.

Directed differentiation of cardiomyocyte-like cell from embroid bodies

To initiate differentiation into cardiomyocyte-like cells, after 5 to 7 days in the suspension culture, the EBs were transferred onto gelatin (0.1%) coated 24-well culture plates (Life Technologies, Korea) at a density of 1–2 EBs/well in EB cell culture medium for 24 h until the cells became attached (Fukuda, 2001). Subsequently, the attached cells were cultured for 7 days under cardiac cell differentiation medium consisting of DMEM/F12 (Hyclone, USA) supplemented with 20% FBS, 1% NEAA, 50 μmol/L β-mercaptoethanol (Sigma, USA), 10 μmol/L 5-aza-2'-deoxycytidine (Sigma, USA). Half of the medium was replaced with fresh medium after every 2 days. After the incubation, cardiomyocyte-like cells were characterized by immunostaining using cardiac specific markers including antibody against cardiac troponin T (1:150 dilution, mouse monoclonal [0.N.590], Abcam, UK), sarcomeric α-actinin (1:250 dilution, mouse monoclonal [EA-53] antibody, Abcam, UK) and connexin-43 (5 μg/ml dilution, rabbit polyclonal antibody, Abcam, UK). After that, specimens were incubated with the fluorescent secondary antibodies: FITC-conjugated mouse IgG2b (1:300 dilution, Abcam, UK) for sarcomeric α-actinin; Alexa Fluor® 488 goat anti-rabbit IgG (1:1000 dilution, Abcam, UK) for connexin-43; and PE-Cy5-conjugated mouse IgG1 (1:250 dilution, BD Biosciences, Korea) for cardiac troponin T. Specimens were counterstained with DAPI and analyzed using a fluorescence microscope.

Table 1. Primer sequence for RT-PCR

Gene[a)]	Forward primer	Reverse primer
Nkx2.5	CCCGCGTACCCTAACTACAA	GCGAAATGACCGACTGTTTT
ANF	CTGATTGAAGCCCTGGAGTC	GGGAGGATCAGGTTCTGTGA
GATA4	AGGGAGAGCCCGTCTGTAAT	AGACTGGCTGATGGCTGACT
α MHC	CTTCAACCACCACATGTTCG	GTTGTTGGACTTGCCCAGAT
GAPDH	GACGTGCAGCAGGAACACTA	TCTCCATGGTGGTGAAGACA

[a)]Nkx2.5, NK2 transcription factor-related locus 5; ANF, atrial natriuretic factor; GATA4, transcription factor GATA-4; α-MHC, α-myosin heavy chain.

For RT-PCR analysis, total RNA of cardiomyocyte-like cells was extracted by using the Aurum Total RNA Mini Kit 732-6820 (Bio-Rad, Canada), as per manufacturer's instructions. Primer sequences are listed in Table 1. PCR was performed using the conditions: 94°C for 3 min; 94°C for 20 s, 51°C for 30 s, 72°C for 30 s (39 cycles); 72°C for 10 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Different genes expression level was quantified using imageJ software as relative expression in per cent.

Results

Characterization of isolated and colony forming cells

After 18 to 20 h of seeding, testicular cells were plated and formed a monolayer within 3 days. These cells subsequently formed colonies after 10 to 15 days of primary culture. The SSC colonies have a morphology consisting of round proliferating cells over cell monolayer (Figure 1). Most of these colonies did not have alkaline phosphatase activity. However, regardless of the cell type or source, colony-forming cells react strongly with PAS stain (Figure 2 A). Moreover, markers of the undifferentiated cells were markedly expressed in these colonies including anti-Oct4 (Figure 2 B), anti-SSEA1 (Figure 2 C), anti-SSEA4 (Figure 2 D), anti-SSEA3 (Figure 2 E) and anti-STRA 1-81 (Figure 2 F). Figure showed the rational expression of ES markers (Figure 2 G). It appears that the cells of these colonies could spontaneously differentiate into three embryonic germ layers *in vitro* because they have pluripotent properties resembling embryonic characteristics.

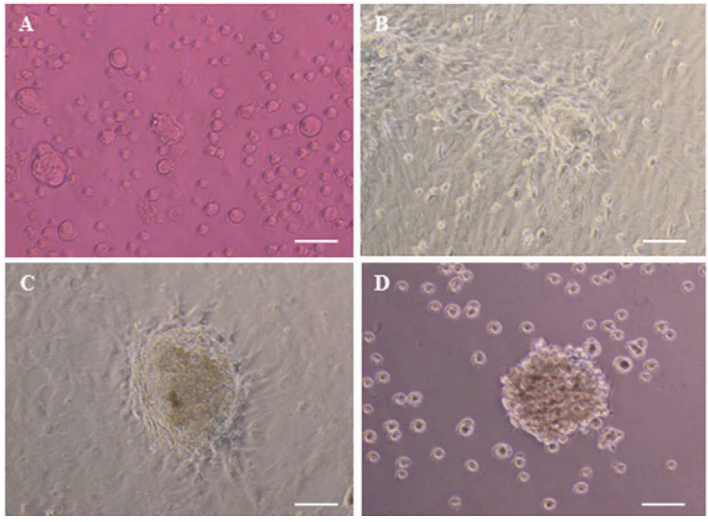

Figure 1. Morphology of chicken SSCs. Cell population obtained from the testis of a chick (A). Initiation of cell aggregations after day 10 (B). Proliferation of cell aggregations and formation of colony after day 15 (C). EB formation from suspended spermatogonial stem cell colonies after day 35 (D)

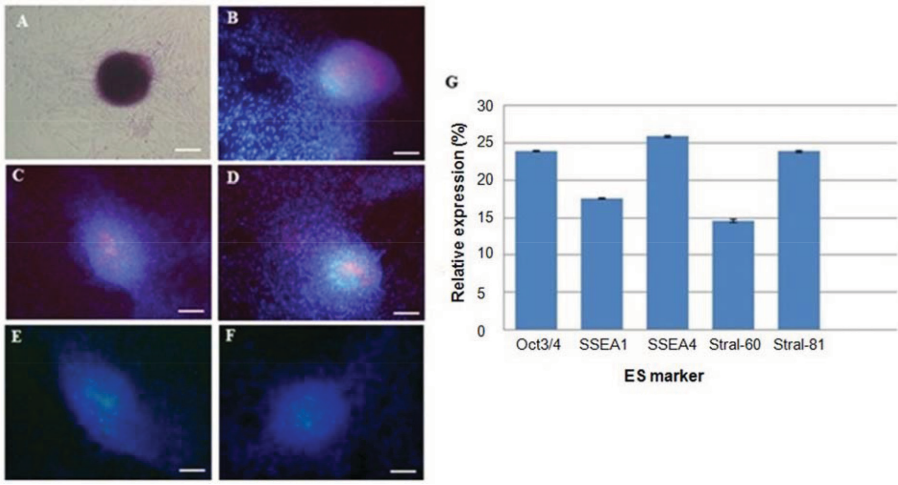

Figure 2. Characterization of chicken SSC colonies and EB formation. Characterization of colony-forming chicken SSCs was performed on day 20 by detecting surface-antigen expression. The colonies strongly reacted with PAS stain (passage 3) *in vitro* (A), anti-Oct4 (B), anti-SSEA1 (C), anti-SSEA4 (D), anti-SSEA3 (E) and anti-STRA 1-81 (F). Fluorescein isothiocyanate (FITC)-labeled secondary antibodies (B, C, D) or Cy5-labeled secondary antibodies (E, F) merged with 4',6-diamidino-2-phenylindole (DAPI). Figure (G) shows the expression ratio of ES markers by ImageJ analysis

Assessment of formation of EB derived from SSC colonies *in vitro*

The undifferentiated state of chicken SSC colonies was used to induce spontaneous differentiation into three embryonic germ layers *in vitro*. In LIF-free media, an EB, which was formed successfully over the course of 5 to 7 days (Figure 3 A), reacted strongly with the specific markers of 3 germinal lineages. Differentiation of mesodermal lineages was confirmed by the expression of the early mesoderm marker desmin and a protein involved in the localization of intermediate filaments of the desmin group in all types of muscle cells. This protein is seen localized at the periphery of z-discs (Figure 3 B). Expression of desmin suggests that the mesoderm, which is present inside the EB-derived SSCs, has significant potential for differentiation into different mesodermal cell types. Differentiation of the ectoderm that emerges first and is formed from the outermost layer of the germ layers expresses S100 alpha 2 (Figure 3C). Endodermal lineages were identified by a positive reaction with α-fetoprotein (AFP), which is a marker for development of the fetal liver (Figure 3 D). The rational expression of three embryonic germ layers related markers are shown in Figure 3 E.

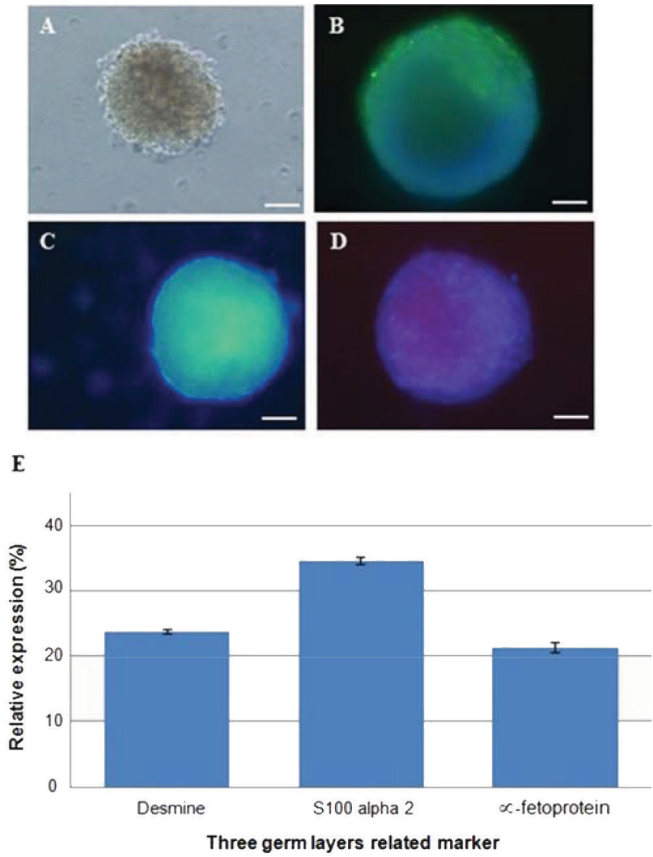

Figure 3. Characterization of chicken EB formation. EB morphology (A) characterized on day 5 with primary monoclonal antibodies: anti-desmin (B) for identifying the mesoderm layer, anti-S100 alpha 2 (C) for the ectoderm layer, and anti-AFP (D) for the endoderm. Fluorescein isothiocyanate (FITC)-labeled secondary antibodies (B, C) or Cy5-labeled secondary antibodies (D) merged with 4',6-diamidino-2-phenylindole (DAPI). Figure (E) shows the expression ratio of 3 embryonic germ layers related markers by ImageJ analysis

***In vitro* differentiation potential of EBS into osteoblast-like cells**

After culture for 2 days in the induction media for osteoblast differentiation, the cells derived from EB were easy to obtain with different morphologies compared to the original SSC morphology (Figure 4 A). We found that from third day after osteogenic stimulus, the outer differentiated cells began to expand and showed different morphology than original SSCs. After 21 days onward, these cells morphologically started to become as fibroblast-like cells. On day 15, the osteoblast-like cells became more dispersed and gained the appearance of crowded granular mineralized nodules and gradually lost their fibroblast-like morphology. The alizarin red dye strongly reacted with osteoblast-like cells derived from the EB (Figure 4 C) at day 20 relative to the negative control (Figure 4 E).

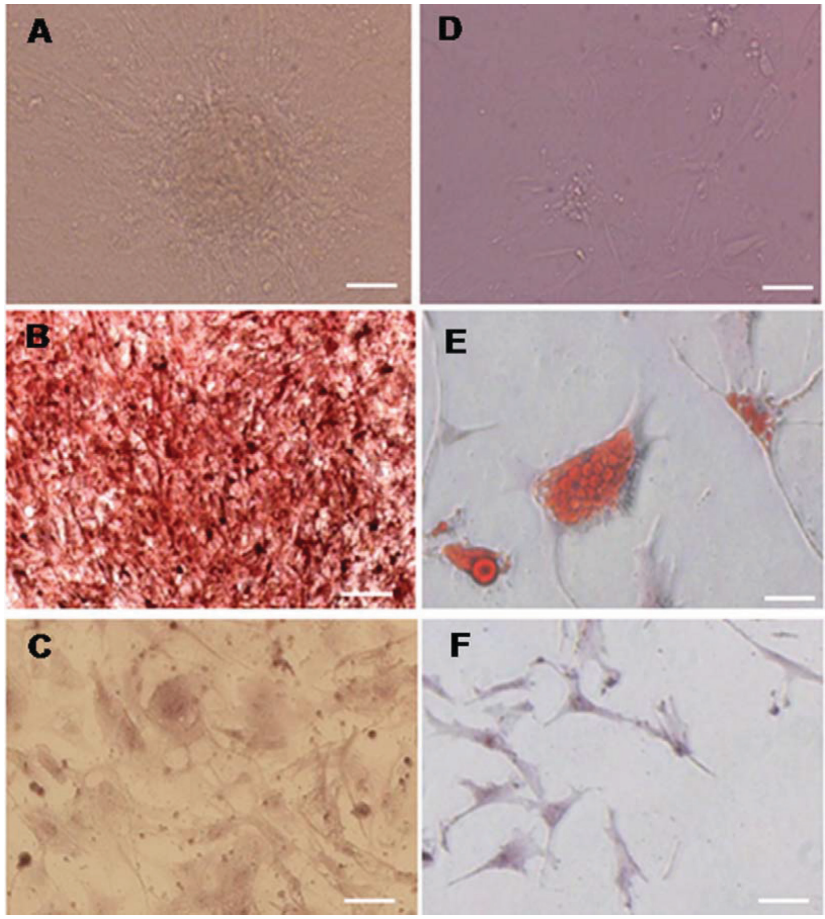

Figure 4. Identification of chicken SSC-derived osteoblasts and adipocytes. The morphology of differentiated EBs in osteoblast conditioned medium (A); Alizarin red showing strong positive staining of differentiated cells (B); Negative control group for osteoblasts (C); Morphology of the differentiated EB in adipocyte conditioned medium (D); Oil red O staining of adipogenic differentiated cells (E) and the negative control group for adipocytes (F)

***In vitro* differentiation potential of EBS into adipocyte-like cells**

On the third day of the induction process, the cells derived from EB were observed to initially grow with different morphologies and contain small droplets near the nucleus (Figure 4 B). In addition to the changes in expression that occurs upon differentiation, on day 10, the EBs derived from differentiated cells also underwent a marked change in morphology with accumulation of larger lipids, and began to resemble adipocyte-like cells (Figure 4 D). After a significant growth period under adipocyte differentiation conditions, we observed that the spontaneous differentiation of EBs into cells with typical adipocyte morphology produces a higher accumulation of lipids in the cells on day 18. These cell aggregates formed a typical toroidal structure that stained red after oil red O staining compared to the control group (Figure 4 F).

***In vitro* differentiation potential of EBS into cardiomyocyte-like cells**

In the differentiation media, the differentiated cells significantly changed and attained different shapes (Figure 5 A), relative to the shapes of the cells of the control group that began to expand, and took up the appearance of fibroblast-like cells (Figure 5 B). Differentiation activity continued for 10 days, and the cardiomyocyte-like cells were observed to have cardiac cell-related characteristics as confirmed by the detection of cardiac cell-related genes and cell markers. These markers were exclusively detected within the differentiated clusters and not in the undifferentiated cell outgrowths. Cardiomyocyte-like cells markedly expressed the sarcomeric α-actinin (Figure 5 C, a myocardial cell marker) and cardiac-specific troponin T (Figure 5 D). In addition, cardiomyocyte-like cells also expressed the gap-junction protein connexin-43 marker (Figure 5 E), which is involved in cell-to-cell communication. According to RT-PCR analysis, several cardiac cell genes related to the transcription factors such as GATA-4, Nkx2-5, α-MHC, and ANF were also expressed in the differentiation group (Figure 5 F). These are the most important findings in the present study and it is first time reported that chicken SSCs have bounteous potential to differentiate into beating cardiomyocytes.

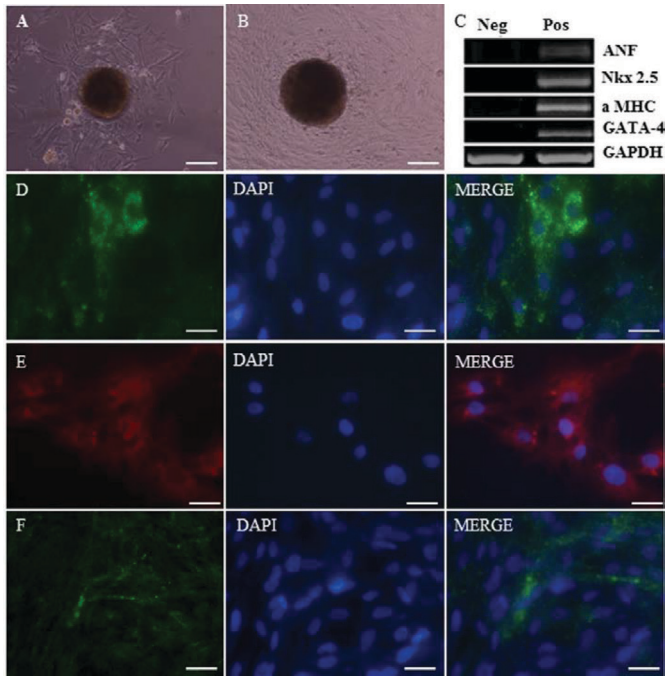

Figure 5. The change in morphology after differentiation compared to the negative control. The differences in morphology between the induced EBs in a medium supplemented with differentiation agents (A) and without differentiation agents (B). Identification of cardiac antigen on day 10 by immunocytochemical agents and cardiac markers: sarcomeric α-actinin antibody (D), cardiac troponin T antibody (E), connexin 43/GJA1 antibody (F) and fluorescein isothiocyanate (FITC), Alexa Fluor® 488-labeled secondary antibodies (D, F), or PE-Cy5-labeled secondary antibodies (E) merged with 4',6-diamidino-2-phenylindole (DAPI) dye. RT-PCR comparing the expression of genes related to cardiac differentiation (positive/negative) (C)

Discussion

Spermatogonial stem cells from human and mice testes have been isolated successfully and have confirmed potential to differentiation into different body cells (Coelho and Fernandes, 2000; Buttery et al., 2001). In the present study, we isolated chicken SSCs from testicular cells at day three because our previous experiments showed that more SSCs could be isolated easily after this period and subsequently analyzed them to identify pluripotent cells. The cytochemical reagents used included PAS stain, alkaline phosphatase stain, and antibodies to stage-specific embryonic antigens (Oct4, SSEA1, SSEA3, SSEA4, STRA 1-60, and STRA 1-81) (Brulet et al., 1980; Guan et al., 1999; Maeda et al., 1994; Draper et al., 2002). Therefore, this system can be used as a source for stem cells capable of differentiating into various cell types of the mesoderm.

In the present study most of colonies SSCs did not have alkaline phosphatase activity. Cells of these colonies are similar to the cell cultures derived from chicken EB cells that have different reactivity with mouse ES cells (positive for the mouse cells and negative for the chicken cells) (Ge et al., 2007; Golestaneh et al., 2009).

The induction of differentiation of SSCs *in vitro* occurs through an intermediate step in the formation of EB-like ESCs, which are complex three-dimensional cell aggregates (Guan et al., 1999). In our experiments, morphologically EBs were similar to the previous report (Jokura et al., 2004). The formation and early differentiation of EBs occur in two phases, which begin after 2 to 4 days in suspension culture when the endoderm forms on the surface of the EBs and gives rise to simple EB structures. Subsequently, at about day 4, EB cysts develop with the formation of a central cavity and differentiation of a columnar epithelium with basal laminin. In our study, differentiation of the ectoderm that emerges first and is formed from the outermost layer of the germ layers expressed S100 alpha. S-100 alpha protein is found in glial cells, melanocytes, chondrocytes, Schwann's cells, neurons, interdigitating reticulum, and Langerhans' cells. Continuous culture can produce additional differentiated cell types, including insulin-producing cells, neuronal cells, and hematopoietic cells in human (Golestaneh et al., 2009; Jia et al., 2008). But in the present study we found that chicken SSCs can also produce different types of body cells including cardiomyocytes, osteoblasts and adipocytes. Specialized cells derived through differentiation procedures may be useful in future stem cell therapies. This is the first report that chicken SSCs can differentiate into important body cells including cardiomyocytes, osteoblasts and adipocytes. We believe that these findings may be breakthrough in the field of regenerative medicine. Because, unlike other domestic animals, they have high prolificacy (a single bird can have thousands of offspring), mature quickly, have bounteous quantity of sperm production and are easy to handle and manage. As the use of embryos has some ethical issues, hence use of adult chicken SSCs may be good source of pluripotent stem cells for the repair of body tissues.

SSCs may provide alternative sources of pluripotent stem cells since SSCs are highly similar to ES cells (Kanatsu-Shinohara et al., 2004). From the present study, we are confident or hope that chicken SSCs may be good alternative resource of pluripotent stem cells for the application in human regenerative medicine (repair

of body tissues) after *in vivo* studies. For efficient derivation of adipocytes, dexamethasone, insulin, and IBMX were included in the induction medium; differentiation could be achieved at an early stage. This indicates that both dexamethasone and IBMX are important induction factors for the differentiation of EB-derived chicken SSCs into adipocytes. In addition, the proper concentration of β-mercaptoethanol in the induction media was found to be paramount for efficient differentiation into adipocytes.

Dexamethasone, β-glycerol phosphate sodium, and ascorbic acid are the most common inducers for osteoblast differentiation from EB-derived chicken SSCs and mammalian PGCs. Dexamethasone promotes their proliferation, and promotes expression of osteocalcin and type-I collagen. β-glycerol phosphate can promote calcium deposition, and thus, promote osteocalcin calcification, while ascorbic acid is involved in the synthesis of collagen to osteocalcin and can promote the formation of calcified nodules. By including these compounds in the induction media, we observed differentiation of EB-derived chicken SSCs into osteoblasts. From these findings, we can strongly recommend that chicken SSCs may provide an alternative source of stem cells in the orthopedic medicine and limb regeneration.

The differentiation reagent 5-aza-2-deoxycytidine (5-aza-dC) is used to enhance cardiomyocyte differentiation in mesenchymal stem cells from ES cell. This compound has been shown to induce differentiation of mesenchymal stem cells, presumably via demethylation of DNA. A previous study has indicated that a treatment combination, which includes 5-aza-dC, induces the formation of beating cells within EBs (Fukuda, 2001). Therefore, in the present study, induction of differentiation of cardiomyocytes by 5-aza-dC indicates that these compounds support the regulatory effect on the differentiation of the SSC into cardiac-like cells *in vitro* via EB differentiation.

In our study, differentiated cardiomyocyte-like cells markedly expressed the cardiac specific markers such as sarcomeric α-actinin, myocardial cell marker and cardiac-specific troponin T, a subunit of the troponin complex regulating striated muscle contraction that is used as a specific biochemical marker of myocardial injury. The most important finding of the present work is the differentiation ability of chicken SSCs into cardiomyocytes, which may be a new avenue for regenerative medicine especially for human cardiology research. For more than two decades turkey poults have been recommended as a more cost-effective model than rodents for investigating therapeutic drug-induced cardiomyopathy in tumor patients. The chicken may be used as the most potential animal model for stem cell research and to understand developmental biology. Finally, the chicken model has several advantages over mammalian systems for *in vitro* and *in vivo* studies, as it is cost effective (cheap), readily available, bred and managed conveniently, and easily manipulated. Moreover, the most prized feature of the chicken is that the developing embryo can be conveniently studied (vivisected and otherwise manipulated) outside the mother.

In this study, in addition to the ability to differentiate into different cell types in the mesoderm layer, SSCs have been demonstrated to possess great potential for differentiating into other cell types in the body, such as hepatocytes, pancreatic cells, cartilage, and hormone-secreting cells (Duplomb et al., 2007). Chick embryo,

including its extraembryonic membranes, has long been used as a developmental model (Wolpert, 2004) because of its accessibility for surgical manipulations and abundance of data elucidating cellular signaling and interactions during embryonic development. Our findings may provide a new idea for the use of chicken SSCs for cardiology research after more detailed experiments, including *in vivo*. We believe that our findings may prove to be an excellent model to adopt in order to tackle some classical issues in regenerative medicine including limb regeneration, orthopedic medicine and developmental biology.

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