A Thesis

For The Degree of Master of Applied Biotechnology

Characterization and cardiac differentiation of chicken Spermatogonial stem cells

Nguyen Thanh Luan

DEPARTMENT OF APPLIED BIOTECHNOLOGY

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

2012. 2

Characterization and cardiac differentiation of chicken spermatogonial stem cells

Nguyen Thanh Luan

(Supervised by Professor Dong-Kee Jeong)

A Thesis submitted in partial fulfillment of the requirement for the degree of Master of Applied Biotechnology

2012. 2

This thesis has been examined and approved

DEPARTMENT OF APPLIED BIOTECHNOLOGY

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

LIST OF FIGURES

LIST OF TABLES

요약

여러가지 실험적 결과들은 다능성의 성체 생식줄기세포가 배아줄기세포처런 유사한 줄기세포의 특성을 보여주고 이를 통하여 안정적인 줄기세포로서의 특성을 보여주고 있음을 나타내었다. 본 연구의 결과는 심장질환을 치료할 수있는 세포 치료제로서의 가능성을 보여주고 있다. 본 연구에서, 우리는 성공적으로 정원줄기세포를 정소로부터 분리할 수 있었으며, 부가적인 실험을 통하여 다능성을 확인하였다. 이 실험을 증명하기 위하여 PAS 염색, AP 염색 그리고 줄기세포 특이 항체(Oct4, SSEA1, SSEA3, SSEA4, STRA 1-60, and STRA 1-81)들을 이용하여 줄기세포를 검증하였다. 본 연구결과에서는 배아줄기세포관련된 마커에서도 동일하게 강한 발현을 내었으며, 삼배엽성 분화 실험에서도 부분적으로 분화가 완성됨을 확인하였다. 본 줄기세포를 이용하여 분화시킨 심근세포에서 특이적으로 발현하는 sarcomeric alpha actinin, specific for alpha-cardiac actinin, conexin-43 등의 심장박동과 관련된 유전자가 높은 발현을 보였으며, troponin T 와 같은 근육의 수축과 팽창을 조절하는 단백질의 발현을 관찰 할 수 있었다. 추가적으로, RT-PCR 을 통하여 심근관련 전사인자 유래 유전자들의 발현이 분화가 진행되면서 증가함을 관찰하였다. 본 연구 결과들은 아마도 닭의 정원줄기세포가 심근세포로 분화하면서 배아줄기세포와 유사한 기능을 가지고, 이를 통하여 심근세포 치료에 관련한 기초자료를 제공할 것으로 예상되며, 향후 치료를 위한 적용이 가능할 것으로 사료된다.

주요어: 정원줄기세포, 심장분화, 성세포, 다능성 세포, 배아줄기세포

INTRODUCTION

Several scientific groups have reported that embryonic stem cells (ESCs) could differentiate into functional cardiomyocytes, characterized by their developmentally controlled expression of cardiac specific genes, proteins, and ion channels [3, 10]. ESCs or ESC-derived cardiomyocytes have been examined for treatment of heart failure in animal models and demonstrated beneficial effects in humans [11, 18, 26]. However, recent studies have shown that undifferentiated ESCs formed teratomas in normal and infarcted hearts of nude or immunocompetent syngeneic mice. Even allogenic ESCs caused teratomas, but these were immunologically rejected after several weeks [29].

Spermatogonial stem cells (SSCs), a unique population of germline stem cells in adult testis, have the capability to self-renew and produce daughter cells destined to differentiate into spermatozoa throughout the life of male animals [33] (Fig.1). Previous studies have found that SSCs of mice and/or their progenitors can revert spontaneously (without the addition of exogenous genes) back to pluripotent embryonic stem-like cells [5, 7, 9, 22]. The next important step is to identify with certainty the cell type in the testis that is capable of reprogramming to become embryonic stem-like cells. Secondly, identification of the regulatory molecules and signaling mechanisms that allow for this reprogramming must be investigated for potential application of SSCs in regenerative medicine [37]. In addition, SSCs are reported to have several potentials including the production of functional cells derived from human SSCs for use in cell-based therapies. SSCs were transplanted into normal mouse hearts and were able to proliferate and differentiate without tumor formation up to one

month after cell transplantation. Together, this information indicates that SSCs may provide a new source of distinct types of cardiomyocytes for basic research and potential therapeutic application [17, 19, 20].

Recently, there have been many studies using SSCs from mice, but not many experiments have used chicken SSCs, which are believed to have great potential as a germ cell [11, 14]. Chickens are considered an important animal model for developmental studies, but little is understood about the *in vitro* differentiation potential of multipotent adult germline stem cells (maGSCs) from chicken testis and the conditions needed for inducible differentiation. We performed several experiments to identify putative pluripotent cells in testicular cells and to culture and characterize those identified.

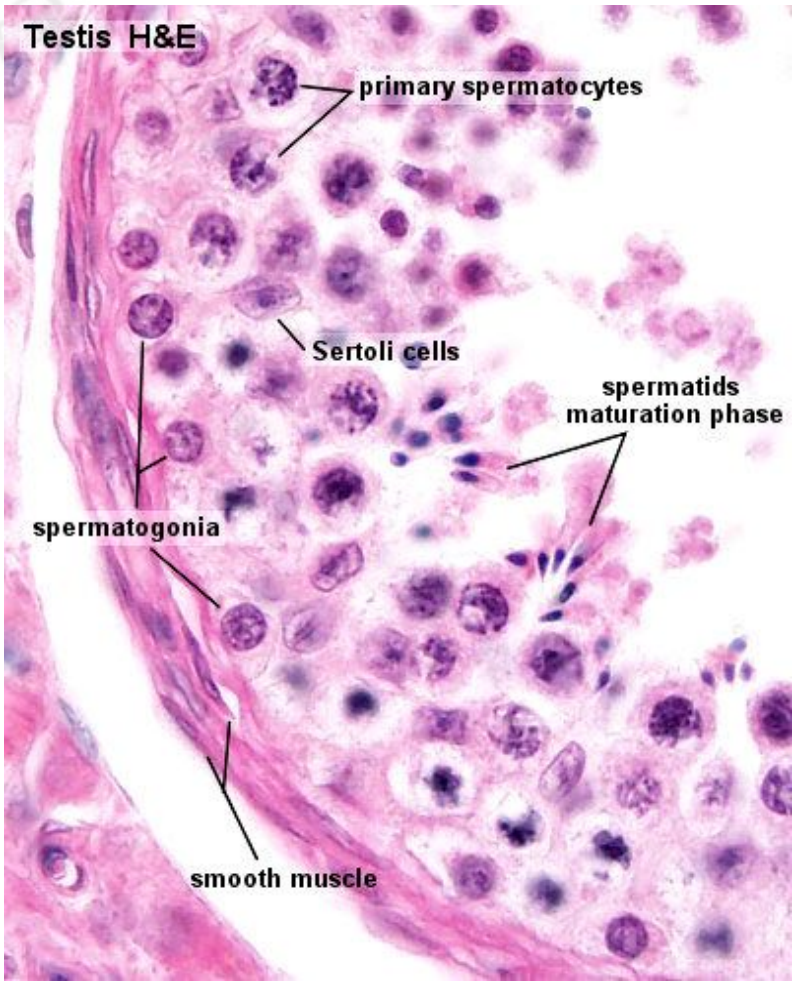

Fig.1. Schematic diagram showing the full progress of sperm forming from spermatogonia located at basement membrane of seminiferous tubules. (Seminiferous tubule cross-section and supporting cells php.med.unsw.edu.au)

In the present study, we isolated chicken SSCs from testicular cells and subsequently analyzed them to identify pluripotent cells. The cytochemical reagents investigated included periodic acid-Schiff (PAS), alkaline phosphatase (AP) stain, antibodies to stage-specific embryonic antigens (Oct4, SSEA1, SSEA3, SSEA4, STRA 1-60, and STRA 1-81)[12, 13, 15, 21, 25, 31, 32, 34]. The results showed that chicken maGSCs were stained selectively by PAS and by antibodies to Oct4, SSEA1, SSEA3, SSEA4, STRA 1-60 and STRA 1-81. The maGSC highly expressed marker characteristics related to ESCs (Fig.2) indicated that they were able to spontaneously differentiate into derivatives of the three embryonic germ (EG) layers *in vitro* [5]. Therefore, they could be used as a stem cell source for differentiating maGSCs into cardiomyocytes which intuitively manifest their functional properties. Our results contribute to information related to the ability of maGSCs to differentiate into different cells such as contraction cardiomyocytes similar to ESCs.

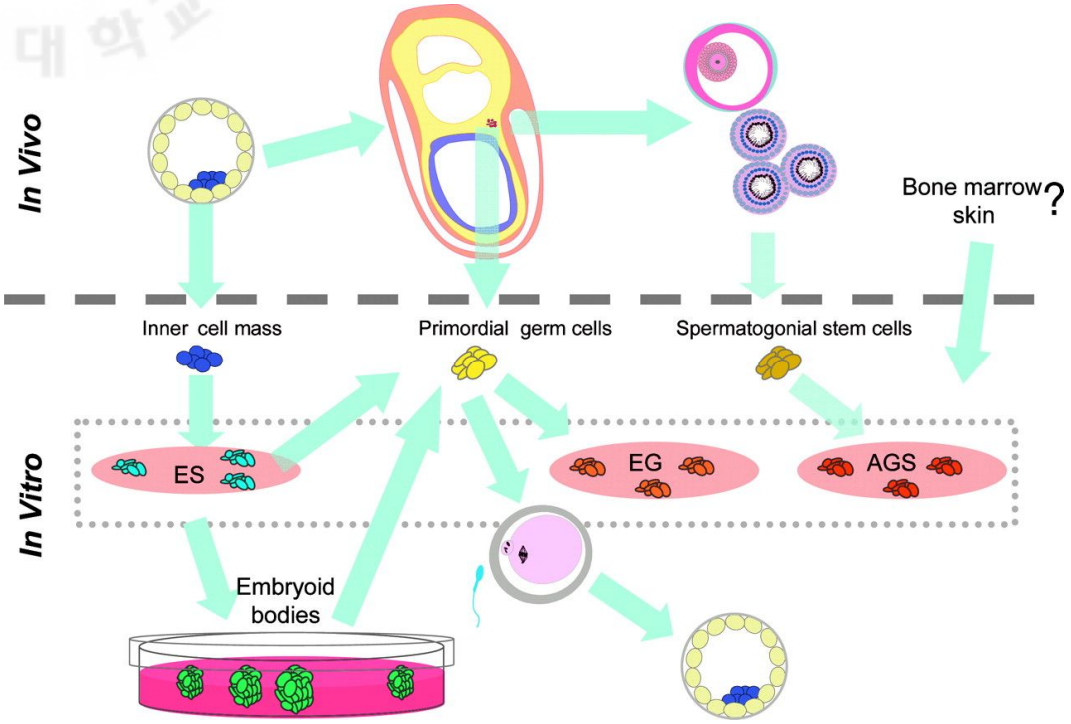

Fig.2. Schematic diagram showing the relationship between in vivo development of germ cells and the in vitro pluripotent stem cell lines that have been reported to be derived from mouse and human cells (ESC and EG) and their developmental potential. The relationship between ESC, embryonic germ cell (EG) and Adult germ line stem cell (AGS) cells still remains unclear although they express similar markers of pluripotency. (Behrouz Aflatoonian and Harry Moore, Germ cells from mouse and human embryonic stem cells. Reproduction, 2006)

Several cardiac differentiation reports were examined in relation to mouse and human ESC using different induction chemicals for each species, such as 5-aza-2-deoxycytidine, ascorbic acid, 5-azacytidine, BMP2, etc. [35]. However, differentiation into cardiac cells from chicken maGSC is quite different due to their distinct characteristics, thus addition of these substances to chicken cells is not really enough to generate cells capable of contraction. In previous studies, some chemicals including phenylephrine, isoprenaline, 3-isobutyl-1-methylxanthine (IBMX), and clenbuterol [6] were used to increase beating heart cells via specific mechanisms. In our study, we observed that IBMX plays an important role in the process of inducing stem cell differentiation into adipose cells and increases the contraction ability of cardiac cells. As such, it can be used as a supportive stimulation reagent to differentiate cardiac like cells from chicken maGSC because it regulates calcium ion channels through biological processes via a cAMP-dependent pathway.

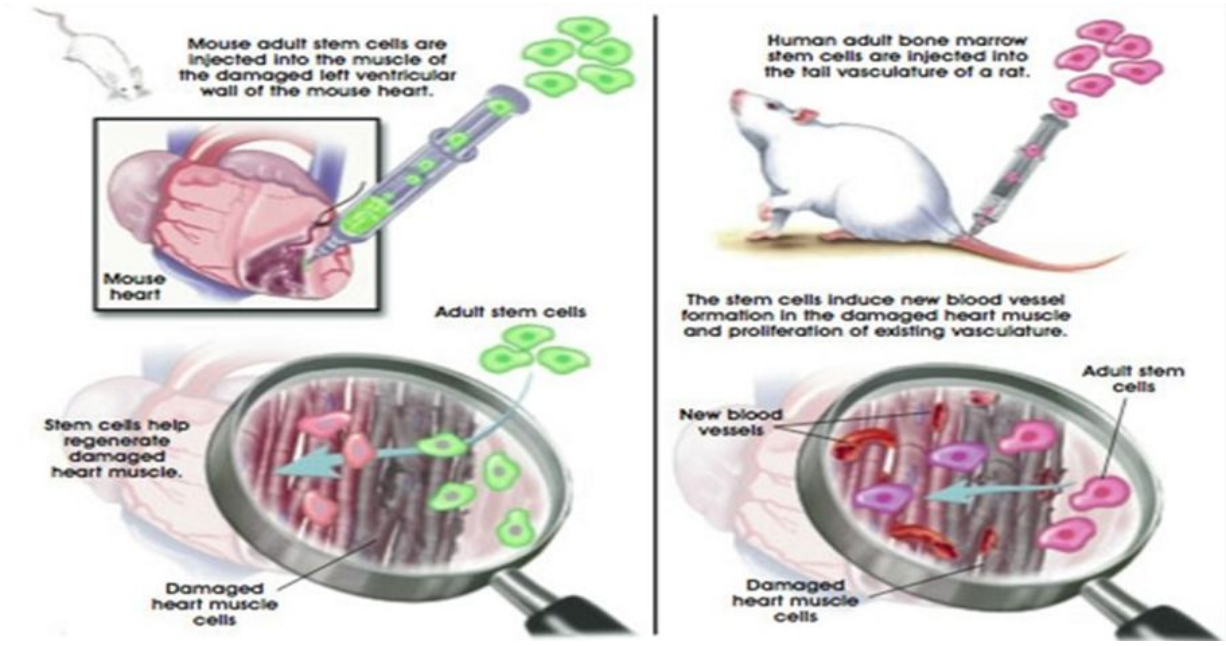

Fig.3. Schematic diagram showing the stem cell therapy was used in cardiac failure. (Terese Winslow, 2001)

The present study indicated that maGSC-derived cardiomyocytes expressed sarcomeric alpha actinin, which is known to be specific for alpha-cardiac actinin. Conexin-43 was expressed as the major protein of gap junctions in cardiac clusters, and gap junctions are thought to play a crucial role in the synchronized contraction of the heart and in embryonic development. In addition, cardiac troponin T, which is the tropomyosin binding subunit of the troponin complex which regulates muscle contraction in response to alterations in intracellular calcium ion concentration [17, 19, 20] was also expressed in this experiment. Furthermore, reverse transcription polymerase chain reaction (RT-PCR) results indicated that genes related to cardiac transcription factors were expressed after differentiation. These results suggest that maGSCs provide a new source of a distinct type of cardiomyocyte for basic research and may have potential in regenerative medicine therapies (Fig.3).

I. MATERIALS AND METHODS

SSC plays a very important role in the regeneration studies of sperm but not actually present a number of studies for use SSC as a source of stem cell potential. Therefore in this experiment I want to explore the ability to form three germ layers from chicken SSC, important characteristics of embryonic stem cells which are capable of use for therapeutic purposes.

II.1. Isolation of spermatogonial stem cell from young chick testes

Animal protocols were approved by the Jeju National University Animal Care Committee. Spermatogonial cells were isolated from 3-day-old White Leghorn chickens. Decapsulated testes were suspended in phosphate buffered saline (PBS). Teste tissues were digested using collagenase IV and trypsin ethylenediaminetetraacetic acid (EDTA) to obtain single cells. Approximately 1 x 10^6 dissociated cells were placed in a culture flask with modified Dulbecco's Modified Eagle's Medium (DMEM; Hyclone) containing 10% fetal bovine serum (FBS; Hyclone), 1% nonessential amino acids (Gibco Invitrogen), 50 μmol/L β-mercaptoethanol (Sigma), 10^3 units/mL leukemia inhibitory factor (LIF), and 1% penicillin. The seeded cells were then cultured in an incubator at 37^0C and 5% CO^2 in a humidified atmosphere.

From the mixed cell population that was initially seeded, the testicular cells formed a monolayer within three days of culture, whereas the maGSCs formed colonies by 10-15 days of culture. On day 15, the colony-forming maGSCs were passaged, and the cultures were subsequently passaged three times at intervals of five days.

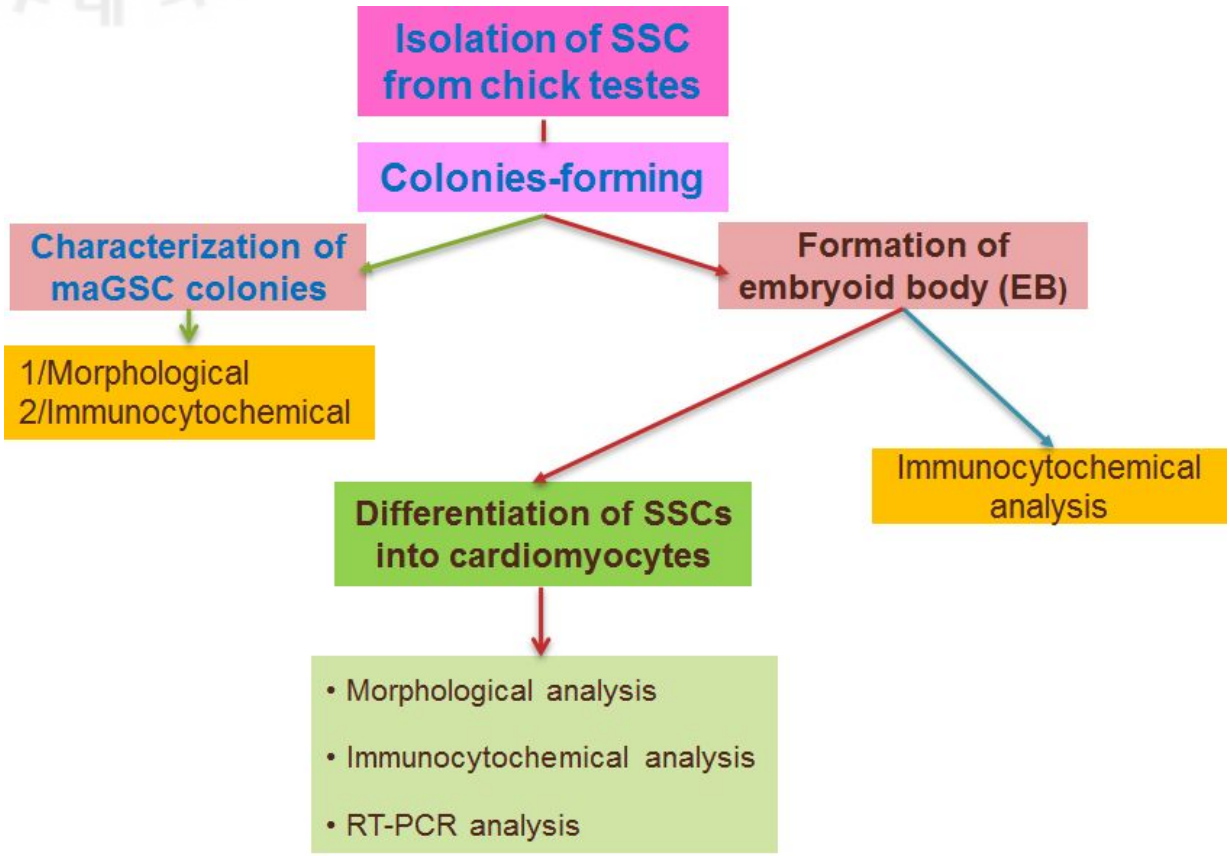

Figure 4: Study design of producing cardiomyocyte-like cell derived from chicken SSCs

II.2. Characterization of chicken maGSC colonies

Characterization of chicken maGSC colonies were accomplished using PAS, AP stain, and antibodies to stage-specific embryonic antigens (OCT-4, anti-SSEA1, anti-SSEA3, anti-SSEA4, STRA1-60, and STRA1-81). Briefly, colony-forming cells were fixed in 3-4% paraformaldehyde for 10 min. Samples were then washed twice (5-10 min each) with 1X rinse buffer and blocking solution was applied for 30 min at room temperature. Cells were then incubated with diluted primary antibodies (SSEA-1, SSEA-3, SSEA-4, STRA1-60, STRA1-81, and Oct-4 used in the range of 1:10 – 1:50) for 1 hr at room temperature. Following incubation, cells were washed three times (5-10 min each) with 1X rinse buffer and incubated with diluted secondary antibodies (fluorescein isothiocyanate [FITC]-conjugated antibody or C5-conjugated antibody) for 30 min at room temperature, followed by three more washes (5-10 min each) with 1X rinse buffer. Colonies were counterstained with 4, 6-diamino-2-phenylindole (DAPI; Sigma) and visualized using a fluorescence microscope (Olympus IX70, Japan).

II.3. AP and PAS Staining

Colony-forming of maGSCs, collected after three passages (15 or 20 days) in culture, were fixed with 3-4% buffered paraformaldehyde for 10 min and rinsed with PBS three times. The colonies were then immersed in periodic acid solution (Sigma) for 5 min and subsequently treated in Schiff solution (Sigma) for 10 min. For AP staining, fixed colonies were immersed in filtered AP staining solution (2 mg naphtol AS-MX phosphate, 200 lL N,N-dimethylformamide, 9.8 mL of 0.1M Tris [pH 8.2], and 10 mg Fast Red TR salt) for 30 min

and then rinsed three times with PBS. All procedures were performed at room temperature, and stained cells were observed under an inverted microscope (Olympus IX70, Japan).

II.4. Formation of embryoid body (EB) derived from colony-forming cells

For EB formation, maGSC colonies were resuspended and maintained in a suspended condition. Briefly, EB colonies (15-20 days in culture) were first dissociated by gentle scraping and then harvested by centrifugation. Subsequently, EB colonies were suspension cultured in ultra-low-attachment bacterial Petri dishes supplemented with 20% FBS, L-glutamine, non-essential amino acid (NEAA), and β-mercaptoethanol without LIF. The medium and morphological changes of EB cells were monitored every 12 hr. EB candidates were collected for immunocytochemical analysis after 5-7 days in the suspended state. Immunocytochemical staining was used to identify spontaneous differentiation into three EG layers *in vitro* using the primary antibodies for mesoderm (anti-desmin, anti-alpha smooth muscle actin; Abcam), endoderm (Anti-AFP; Santa Cruz Biotechnology, Inc.), and ectoderm (S100 alpha 2; Abcam). Briefly, EB candidates were seeded on 96-well plates 2-4 hours before fixing in 3-4% paraformaldehyde for 10 min. The next steps followed the procedure for fluorescent staining detailed previously until EBs were counterstained with DAPI and observed under a fluorescence microscope.

II.5. Differentiation of chicken maGSCs into cardiomyocytes

For differentiation of chicken maGSCs into cardiomyocytes, EBs were formed in ultra-low-attachment bacterial Petri dishes for a period of seven days. Subsequently, the EBs were

transferred onto gelatin (0.1%) coated 24-wells culture plates, plated at a density of 1-2 EBs in EB cell culture medium for 48 hr to initiate attachment. The attached cells were then cultured in differentiated conditioned DMEM (Hyclone) supplemented with 20% FBS, 1% NEAA, β-mercaptoethanol 50μmol/L (Sigma), 10 μmol L^{-1} 5-aza-2'-deoxycytidine (Sigma), and 10^{-5}M IBMX (Sigma) for seven days. Half of the medium was replaced daily with fresh medium. Rhythmic contraction was considered to be a functional marker of successful cardiomyocyte differentiation and verified using microscopy at various times during the differentiation process.

II.6. Analysis characterization of cardiac differentiation

Characterization of chicken maGSC-derived cardiac differentiation was analyzed using immunocytochemical staining with cardiac antigens. Isolated cardiomyocytes from differentiated EBs were first incubated with cardiac specific markers including antibody against sarcomeric α-actinin (mouse monoclonal [EA-53] antibody, Abcam), cardiac troponin T (mouse monoclonal [0.N.590]), and connexin-43 (rabbit polyclonal antibody, Abcam). Afterwards, specimens were incubated with the fluorescent antibodies: FITC-conjugated mouse IgG2b (Bioscience) for sarcomeric α-actinin; Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen) for connexin-43; and PE-Cy5-conjugated mouse IgG1 (Bioscience) for cardiac troponin T. Specimens were counter stained with DAPI and analyzed using a fluorescence microscope.

Briefly, cells derived from differentiation were fixed in 3-4% paraformaldehyde for 15 min at room temperature. The samples were washed twice with ice cold PBS and incubated for 10 min with PBS containing 0.25% Triton X-100. Cells were then washed in PBS three times for five min and subsequently incubated with 1% BSA in PBST for 30 min to block unspecific antibody binding, after which, cells were incubated in diluted antibody in 1% BSA in PBST in a humidified chamber for 1 hr at room temperature. Prior to incubating cells with the secondary antibody in 1% BSA for 1 hr at room temperature in the dark, the solution was decanted and the cells washed three times in PBS, 5 min each wash. The secondary antibody solution was then decanted and the cells washed three times with PBS for 5 min each in the dark. Cells were counter stained with DAPI and visualized using a fluorescence microscope.

Table 1: Primer sequence used for RT-PCR

Gene [a)]	Primer sequences	Product size	Annealing temp (°C)	Accession No.
Nkx2.5	CCCGCGTACCCTAACTACAA GCGAAATGACCGACTGTTTT	364	51.1	NC_006100.2
ANF	CTGATTGAAGCCCTGGAGTC GGGAGGATCAGGTTCTGTGA	323	50.1	NC_006108.2
GATA4	AGGGAGAGCCCGTCTGTAAT AGACTGGCTGATGGCTGACT	386	51	NC_006090.2
α MHC	CTTCAACCACCACATGTTCG GTTGTTGGACTTGCCCAGAT	223	50.2	NC_006105.2
GAPDH	GACGTGCAGCAGGAACACTA TCTCCATGGTGGTGAAGACA	343	50.4	NC_006088.2

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

II.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Differentiating EB areas at various stages were collected for total RNA isolation. Total RNA was extracted from dissected differentiation clusters using an extraction Aurum™ Total RNA Mini Kit (732-6820; Bio-Rad) according to the manufacturer's instructions. First-strand reactions were performed using 2 μg of total RNA with oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen). PCR was carried out in a 0.2-mL tube containing 1 μL cDNA and 1 μL Taq polymerase in a total volume of 25 μL. Primer sequences and PCR conditions are listed in Table 1(Supporting information). PCR was performed using the following program: 94°C/3 min (1 cycle); 94°C/20 s, 51°C/30 s, 72°C/30 s (39 cycles); 72°C/10 min (1 cycle), and the PCR product was analyzed on 1% agarose gel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Sequences of oligonucleotide primers used for RT-PCR analyses are available from the authors upon request.

II. RESULTS

III.1. Characterization of isolating and colony forming cells

After seeding for 18-20 hr, testicular cells were plated and formed a monolayer within three days. These cells subsequently formed colonies by 10-15 days of primary culture. The maGSC colonies formed in the primary culture were maintained and observed several times for morphological differentiation until the third passage.

Two types of colonies were observed in this study. The first type of cell colony was only present at low numbers and in few cultures. In these colonies individual cells were difficult to recognize, and these colonies grew as compact structures that were morphologically comparable to colonies derived from primordial germ cells (PGCs) (Fig. 5A and B). The second colony type morphology consisted of round proliferating cells on top of a monolayer of cells (Fig. 5C and D). This type of colony was abundant and resembled that of previously described SSC lines [2, 8, 23, 27].

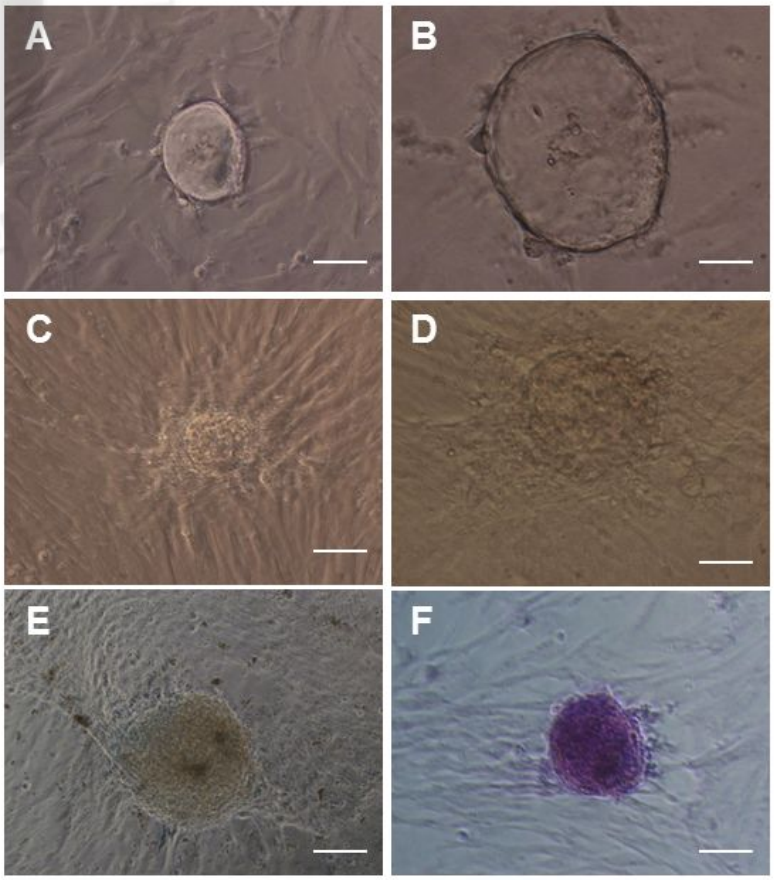

Fig.5. Morphology of chicken multipotent adult germline stem cell (maGSC) colonies. Colony-forming maGSCs from the primary culture were passaged three times. Colonies grew as compact structures (A and B). Colony type consisted of round proliferating cells on top of a monolayer of cells (C and D). Observations were made on day 15 of the third passage (A, C; 10X) (B, D; 40X). The maGSCs were characterized by periodic acid-Schiff (PAS) and alkaline phosphatase (AP) staining. The colonies were maintained with greatly observed morphological differentiation and strong reactions to PAS stain through the third passage *in vitro* (F). However, most cells of these colonies did not have AP activity, which suggests that cells of these colonies are equivalent to cell cultures derived from chicken embryonic germ (EG) cells (E). Magnifications at 20X.

Most of these colonies did not have AP activity, which suggests that cells of these colonies are equivalent to cell cultures derived from chicken EG cells. Previous studies showed different reactivates between mouse ESCs and chicken EG cells (positive for the mouse and negative for the chicken) [9, 10]. However, regardless of the cell type or source, colony-forming cells strongly reacted with PAS stain (Fig. 5E and F). Moreover, undifferentiated markers were markedly expressed in these colonies as shown in Figure 2 including anti-Oct-4 (Fig. 6A), anti-SSEA-1 (Fig. 6B), anti-SSEA-4 (Fig. 6C), anti-TRA-1-60 (Fig. 6D), anti-TRA-1-81 (Fig. 6E), and anti-SSEA-3 (Fig. 6F). Hence, these colonies could be used to spontaneously differentiate into three EG layers *in vitro* as they have pluripotent characteristics resembling embryonic properties.

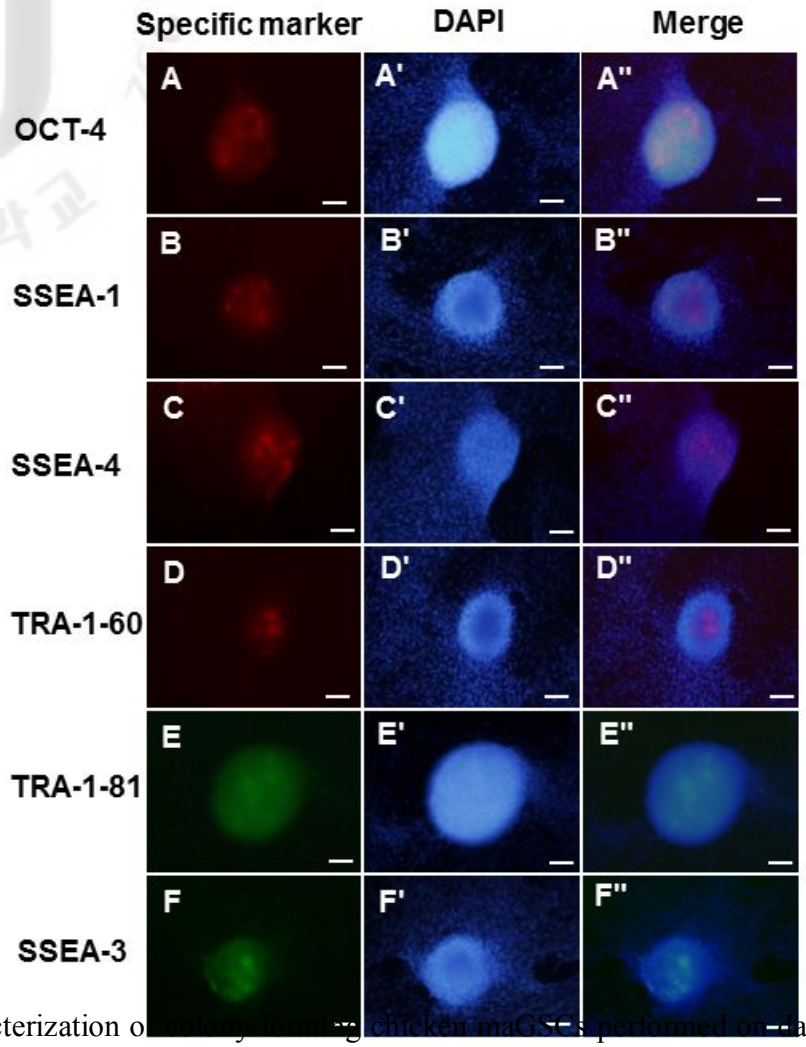

Fig.6. Characterization of colony-forming chicken maGSCs performed on day 20 (at the end of the third passage) by immunostaining. The following primary monoclonal antibodies were used to detect surface-antigen expression: anti-Oct-4 (A), anti-SSEA-1 (B), anti-SSEA-4 (C), anti-TRA-1-60 (D), anti-TRA-1-81 (E), anti-SSEA-3 (F),and fluorescein isothiocyanate (FITC)-labeled secondary antibodies (E, F) or Cy5-labeled secondary antibodies (A, B, C, D); 4',6-diamidino-2-phenylindole (DAPI) staining (A', B', C', D', E', F'); Oct-4 with DAPI overlay (A''); anti-SSEA-1 with DAPI overlay (B''); anti-SSEA-4 with DAPI overlay (C''); anti-TRA-1-60 with DAPI overlay (D''); anti-TRA-1-81 with DAPI overlay (E''); anti-TRA-1-81 with DAPI overlay (F''). Magnifications at 20X.

III.2. Assessment of EB formation derived from colony-forming cells spontaneously differentiated i*n vitro*

The undifferentiated state of chicken maGSC colonies, characterized by specific embryonic antigens, spontaneously differentiated into three EG layers *in vitro*. The embryoid bodies formed from colony-forming cells for 5–7 days in LIF-free media (Fig. 7 A, A' and A'') were strongly positive for the specific markers for three germinal lineages. Namely, differentiation of mesodermal lineages (e.g., cardiac, skeletal muscle, and vascular cells) was confirmed by expression of the early mesoderm marker α-smooth muscle actin (alpha-smooth muscle isoform of actin) as well as lineage-specific genes and proteins (Fig. 7B) and desmin for localization of intermediate filaments of the desmin group in all types of muscle cells and localization at the periphery of z-discs (Fig. 7C). Both expressed α-smooth muscle actin and desmin suggesting that the mesoderm, which was present inside the EB-derived maGSCs has great potential for cardiac differentiation.

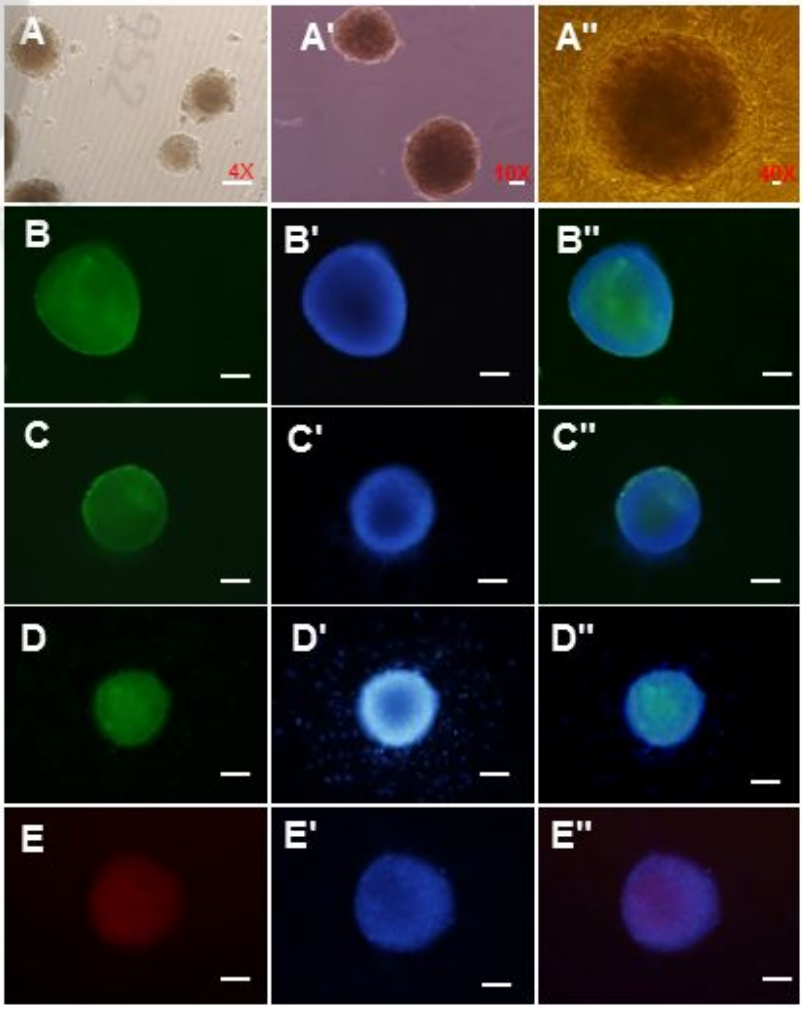

Fig.7. Embryoid body (EB) formation was characterized. The morphology of EB formation (A), (A'), and (A'') was observed at 4X, 10X, and 40X, respectively. Characterization of EB was performed on day five (2-4 hours after seeding) by immunostaining with primary monoclonal antibodies: alpha smooth muscle actin antibody (B) and desmin antibody (C) for identifying the mesoderm layer; S100 alpha 2 antibody (D) for ectoderm and anti-AFP (E) for endoderm and FITC-labeled secondary antibodies (B, C, D) or Cy5-labeled secondary antibodies (E); DAPI staining (B', C', D', E'); alpha smooth muscle actin with DAPI overlay (B''); desmin with DAPI overlay (C''); S100 alpha 2 with DAPI overlay (D''); AFP with DAPI overlay (E''). Magnifications at 20X.

To determine differentiation of endodermal lineages, one of the germ layers formed during animal embryogenesis was identified by a positive reaction with α-fetoprotein (AFP) as an expression maker in fetal liver (Fig. 7E), whereas the ectoderm which emerges first and forms from the outermost germ layers was expressed with S100 alpha 2 (Fig. 7D). S-100 protein is found in glial cells, melanocytes, chondrocytes, Schwann's cells, neurons, interdigitating reticulum, and Langerhans cells.

III.3. Results of induced cardiomyocyte differentiation of chicken maGSC

Most experimental studies have focused on SSCs for the ability to differentiate into sperm, but to date only a single report of the author Guan (2007) about the likelihood of success in the use of mouse SSC to differentiate into cardiac cells. Thus, the success of this research plays an important role in confirming the potential of this cell line SSC and opens new hope for the treatment of people with many experiments with human SSC.

The differentiating medium in this study contained DMEM supplemented with the differentiation reagent 5-aza-2-deoxycytidine (5-aza-dC), which is known to enhance cardiomyocyte differentiation in mesenchymal and ESCs, was assessed at different times during the differentiation process. 5-aza-dC has been shown to induce differentiation of MSCs presumably via demethylation of DNA. Some previous studies indicated that treatment with 5-aza-2'-deoxycytidine induced the formation of beating cells within EBs [1, 34].

Table 2: The changed of morphology and contraction state after culturing in differentiation medium

No	Supplements	Changed morphology	Contraction of cell
1	5-aza-dC 1 μmol L^{-1}	Yes	Not yet
2	5-aza-dC 1 μmol $L^{-}1$ + IBMX 10^{-5}M	Yes	Yes
3	IMBX 10^{-5}M	No	No

5-aza-dC, 5-aza deoxy cistidine (Sigma); IMBX, 3-isobutyl-1-methylxanthine (Sigma)

In an induced environment, EB differentiation was initially observed with expression of cell contraction in the area of EBs after seven days of culture. This suggests that chicken maGSCs could be induced to differentiate into cardiomyocyte-like cells (Fig. 4). In the experimental batches, EB differentiation was induced with 5-aza-2'-deoxycytidine, and expressed weak contractions (Table 2). However, after the addition of IMBX at a concentration of 10^{-5} Ma, marked contraction of cells in the area and expansion of EBs was clearly observed (Supporting information video). This result illustrates that the fact that the IBMX reagent significantly increased the expression of differentiation of EB cells generated from chicken maGSCs. Contraction activity can be observed longer than three days as we observed the occurrence of contraction at days 7-9 of the differentiation process. Moreover, the amount of EB differentiation, with contraction-like cells also increased compared to those in medium treated with 5-aza-2'-deoxycytidine alone.

In this experiment, we carried out observations on the culture wells with environmental components and different periods. Initial test results show that it is hard to beat the ability to observe the mechanical beating of it though when analyzed by RT-PCR, they showed positive for the particular marker. This suggests that the process of differentiation has occurred and the ability to recognize mechanical pulse is completely possible. So after multiple follow-up, we were lucky to record the moment of occurrence of the most resilient of specialized cells with environmental components are added to both substances: IBMX and 5-aza-2'-deoxycytidine. It concluded that chicken SSC is a great model for studying differentiation into cardiac cells.

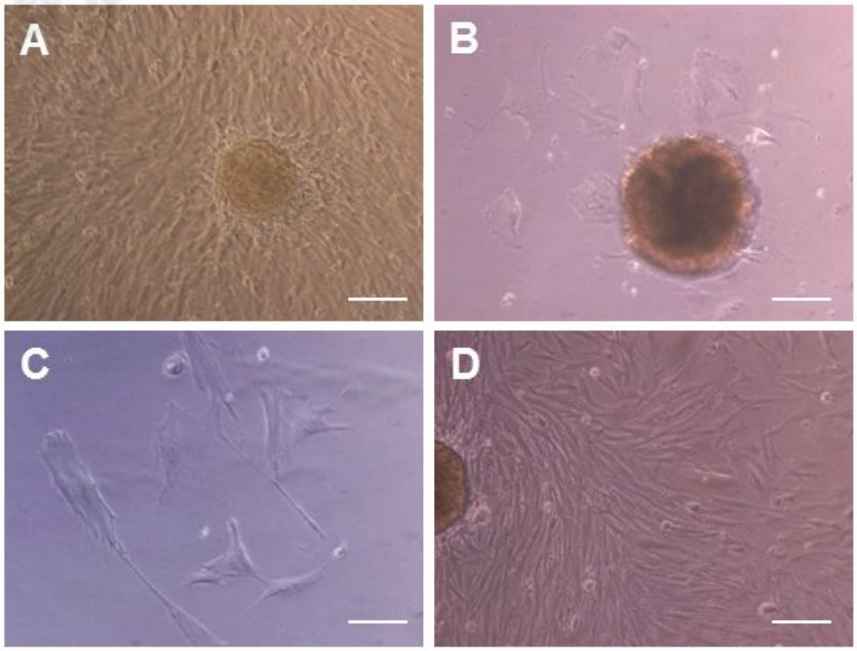

Fig.8. Morphological changes pre- and post-differentiation. Colony forming prior to differentiating (A). Differentiated EB with morphological changes around the cell (B) observed in differentiated medium. Differences around the cell between EB induced in medium supplement with differentiating agents (B, C) and without differentiating agents (D). Magnifications at 20X.

III.4 Cardiac-related gene expression in chicken maGSC-derived cardiomyocytes

Morphological changes following differentiating were observed in this study with differences in EB cells induced in medium supplemented with differentiating agents (Fig. 8B and C) with regard to colony formation before and after differentiation (Fig. 8A) and with and without differentiating agents (Fig. 8D).

The cardiomyocytes derived from the maGSCs in this study were characterized by detection of cardiac-related genes and cell markers. These markers were exclusively detected within the contracted clusters, and not in the non-contracted cell outgrowths. Sarcomeric α-actinin is a microfilament protein that is necessary for the attachment of actin filaments to the Z-lines in striated muscle cells. It is typically used as a myocardial cell marker (Fig. 9A). Cardiac-specific troponin T (Fig. 9B) is a subunit of the troponin complex which regulates striated muscle contraction, and is used as a specific biochemical marker of myocardial injury [19] and cardiac-derived cells. In addition, expression of the gap-junction protein connexin-43 at cell-to-cell contacts in cardiac clusters (Fig. 9C) indicates cell-to-cell communication.

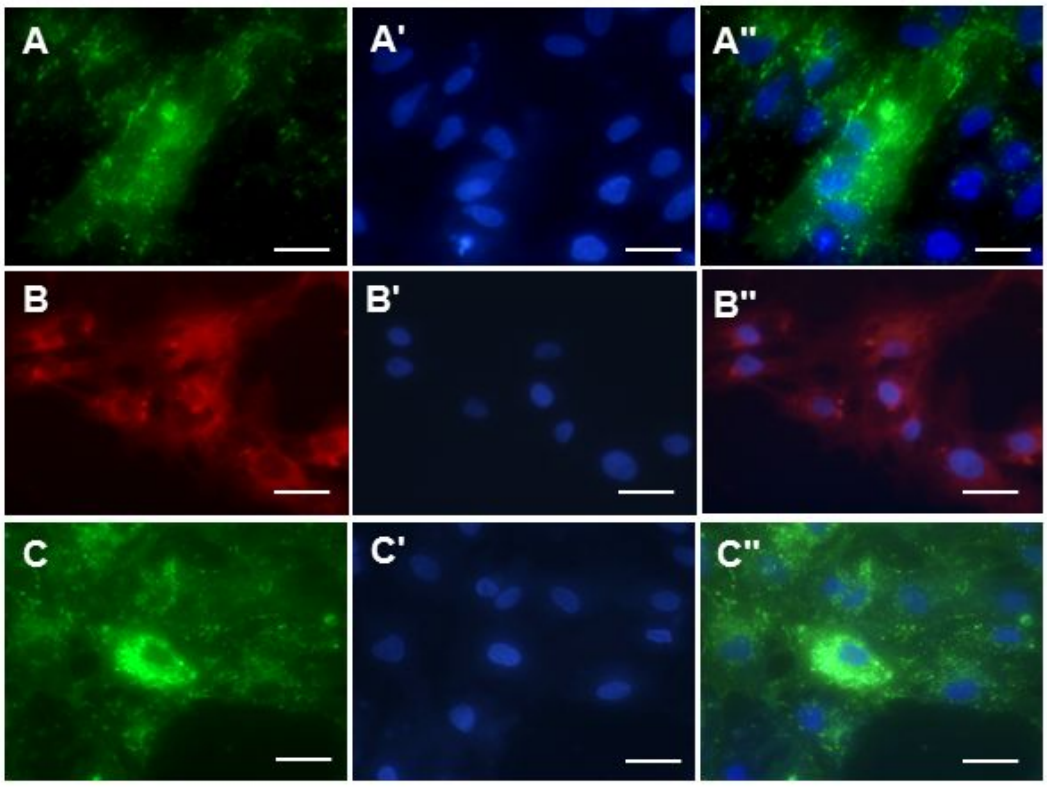

Fig.9. Identify of cardiac antigen expression. Analysis performed on day 10 after differentiation using immunocytochemicals with cardiac markers: sarcomeric α-actinin antibody (A), cardiac troponin T antibody (B), connexin-43/GJA1 antibody (C), with FITC Alexa Fluor® 488-labeled secondary antibodies (A, C) or PE-Cy5-labeled secondary antibodies (B); DAPI staining (A', B', C'), sarcomeric alpha actin with DAPI overlay (A''), troponin T with DAPI overlay (B''), connexin-43/GJA1with DAPI overlay (C''). Magnifications at 20X.

According to RT-PCR analysis, several cardiac transcription factors were also expressed in isolated contraction areas after three and seven days in plated cultures with the addition of 10 μmol L^{-1} 5-aza-2'-deoxycytidine and 10^{-5}M IBMX, or without these compounds, but with 20% serum in the medium (Fig. 10). These cells expressed GATA-4, which is a highly conserved transcription factor that is a key factor in regulating embryonic morphogenesis and cellular differentiation. Tissue-restricted expression of GATA-4 transcription factor and Nkx2-5 homeodomain protein are two early markers of precardiac cells, suggesting that the differentiated cardiomyocytes derived from maGSCs were not fully mature. Alpha myosin heavy chain (α-MHC) and atrial natriuretic factor (ANF, also known as Nppa) are gene sequences coding myocardial structural proteins.

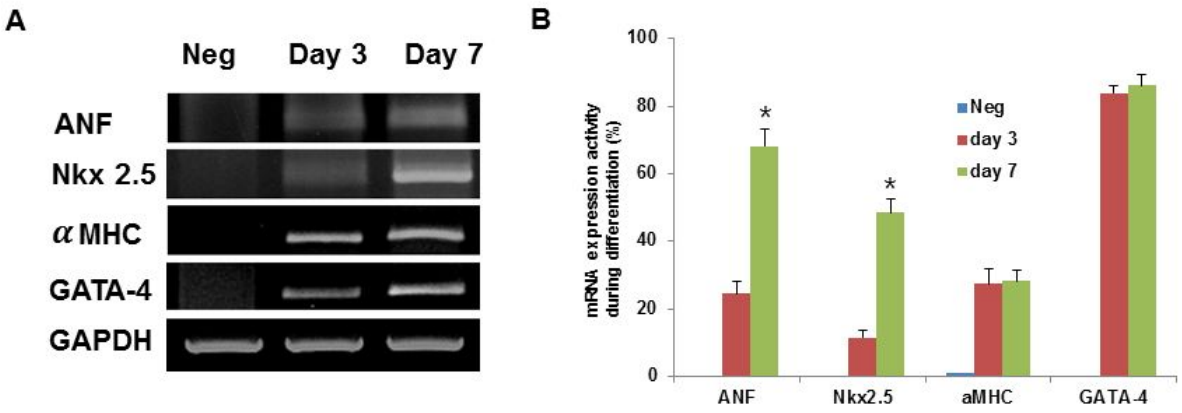

Fig.10. Cardiac-related gene expressed after differentiation *in vitro*. (A) RT-PCR analysis showing the expression of gene related cardiac differentiation. (B) Comparison of the expressed genes on days 0, 3, and 7 of differentiation. Data represent means±SEM of experiment. P values are indicated in the graph ($^{*}p<0.05$).

III. DISCUSSION

Multipotent adult germline stem cells (maGSCs) have not only served as an experimental model for early development of various cell lineages but are also known to provide an unlimited supply of various cell types for drug screening and cell therapy in regenerative medicine [12]. They can proliferate virtually indefinitely in culture when kept in an undifferentiated state, being characterized by surface marker antigens such as stage-specific embryonic antigens SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and expression of transcription factor OCT-4 [15, 35]. These characteristics have proved to be useful for assessing the culture condition of SSCs.

Our results show that maGSCs in culture are similar to ESCs. When maGSCs were cultivated as colonies in a suspended condition using an ultra-low-attachment bacterial Petri dish, as described for mouse ESCs, they spontaneously differentiated into three EG layers *in vitro* with efficiency similar to that seen with ESCs [20]. They could differentiated into cardiomyocytes under the stimulation of 5-aza-2'-deoxycytidine and IBMX, which are two important components which support the regulating effect on differentiation of the maGSCs into cardiac-like cells *in vitro* via EB differentiation. IBMX has been shown to be a potent inhibitor of adenosine 3',5'-cyclic monophosphate (cAMP PDE), significantly more effective than theophylline. IBMX inhibits the cyclic nucleotide PDE with subsequent inhibition of cyclic nucleotide hydrolysis, resulting in accumulation of cyclic AMP and guanosine 3',5'-cyclic monophosphate. In a study of cyclic AMP and insulin release by islets of Langerhans, IBMX caused a marked increase in the intracellular concentration of cyclic AMP in the

presence of glucose. Moreover, the increase in cAMP level as a result of phosphodiesterase inhibition by IBMX activates PKA leading to decreased proliferation and increased differentiation.

Furthermore, cAMP is known as a second messenger used for intracellular signal transduction, such as transferring the effects of hormones like glucagon and adrenaline, which cannot pass through the cell membrane. It is involved in the activation of protein kinases and regulates the effects of adrenaline and glucagon. It also regulates the passage of intracellular calcium through ion channels. In addition, cAMP-associated kinases function in several biochemical processes. The effects of protein kinase are cAMP-dependent and based on the type of cell. However, there are some minor PKA-independent functions of cAMP, e.g., activation of calcium channels, and providing a minor pathway by which growth hormone-releasing hormone causes a release of growth hormone. Protein kinase A can also phosphorylate specific proteins that bind to promoter regions of DNA, causing increased expression of specific genes.

In this study, the identity of the cardiomyocytes was confirmed by ultrastructural examination and by expression of cardiac-related cell markers and genes. The staining results also revealed the cardiomyocyte-like ultrastructure of the derived cells. The myofibrillar structure developed from an irregular myofilament distribution to a more mature sarcomeric organization during the course of differentiation. These results are consistent with those of previous studies, which also showed the developmental patterns of cardiomyocytes *in vitro* [31].

The ability of maGSCs to differentiate into cardiomyocytes similar to the differentiation of ESCs indicates that they may open a new movement in research to replace ESCs, which are more ethically controversy. Besides the ability to differentiate into cardiac-like cells, the maGSCs also show a great potential to differentiate into other cell types in the body (e.g., neurons, osteoblast, and cartilage) because of the characteristic formation of all three germ layers similar to ESCs. Most importantly, if the present study could be performed on human tissue, this would open new options for human cardiac regeneration without the ethical problem associated with ESCs. SSCs and maGSCs could be obtained from testicular biopsies without the use of human embryonic tissue. Moreover, the availability of immunocompatible tissue for autotransplantation would circumvent immunological problems associated with ESC-based therapy [20, 22].

In conclusion, we suggest that regeneration strategies using maGSCs may be based on techniques that have been developed previously for ESCs. These maGSC-derived cardiomyocytes can now be tested for their ability to restore the function of damaged hearts in animal models. A critical challenge will be to produce functional cardiomyocytes derived from human SSCs and to use them in cell-based therapies for heart disease.

ABSTRACT

Cumulative experiments have illustrated that multipotent adult germline stem cells (maGSCs) have been successfully established and show phenotypic characteristics similar to embryonic stem cells (ESCs). As such, this would be an ideal cell source for stem cell–based regenerative therapy which is a promising approach for the treatment of heart failure. In the present study, we successfully isolated chicken spermatogonial stem cells (SSCs) from testicular cells and performed subsequent analysis to identify pluripotent cells by investigation with cytochemical reagents including periodic acid-Schiff (PAS), alkaline phosphatase (AP), and antibodies to stage-specific embryonic antigens (Oct4, SSEA1, SSEA3, SSEA4, STRA 1-60, and STRA 1-81). Our results show that maGSCs highly express these markers related to ESCs and could spontaneously differentiate into three embryonic germ (EG) layers *in vitro*. The maGSC-derived cardiomyocytes expressed some cardiac-specific markers such as sarcomeric alpha actinin, specific for alpha-cardiac actinin; conexin-43, the major protein of gap junctions which are thought to play a crucial role in the synchronized contraction of the heart and in embryonic development; and cardiac troponin T, the tropomyosin binding subunit of the troponin complex which regulates muscle contraction in response to alterations in intracellular calcium ion concentration. Furthermore, reverse transcription polymerase chain reaction (RT-PCR) results indicated these genes related to cardiac transcription factors were expressed following differentiation. Our results contribute information related to the ability of maGSCs to differentiate into cells such as contraction cardiomyocytes similar to ESCs providing a new source of distinct cardiomyocyte types for basic research and potential therapeutic application.

Keywords: Spermatogonial stem cells, cardiac differentiation, germ cell.

Abbreviations: SSCs, spermatogonial stem cells; E*SC*, Embryonic stem cell; maGSC, multipotent adult germ line stem cell.

REFERENCE

1. A. van Dijk, HWM, Niessen B, ZandiehDoulabi, FC et al. (2008). Differentiation of human adipose-derived stem cells towards cardiomyocytes is facilitated by laminin. Cell Tissue Res 334:457–467.
2. Aponte PM, Soda T, Teerds KJ et al. (2008). Propagation of bovine spermatogonial stem cells in vitro. Reproduction 136:543–557.
3. Boheler KR, Czyz J, Tweedie D et al. (2002). Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ Res 91:189–201.
4. Brinster RL. (2002). Germline stem cell transplantation and transgenesis. Science 296:2174–2176.
5. Brulet P, Babinet C, Kemler R et al. (1980). Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation. ProcNatlAcadSci U S A 77:4113–4117.
6. Xu C, Police S,Rao N et al. (2002). Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells. Circulation Research 91:501–508.
7. Conrad S, Renninger M, Hennenlotter J et al. (2008). Generation of pluripotent stem cells from adult human testis. Nature 456:344–349.
8. Ewart WK, Ben C, Bernard AJR. (2009). The effects of growth factors on in vitro-cultured porcine testicular cells. Reproduction 138:721–731.
9. Golestaneh N, Gallicano GI, Kokkinaki M et al. (2009). Pluripotent stem cells derived from adult human testes. Stem Cells Dev 18:1115–1126.

10. Guan K, Furst DO, Wobus AM. (1999). Modulation of sarcomere organization during embryonic stem cell-derived cardiomyocyte differentiation. Eur J Cell Biol 78:813–823.
11. Guan K, Rohwedel J, Wobus AM. (1999). Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro. Cytotechnology 30: 211–226.
12. Thomson JA, Itskovitz-Elder J, Shapro SS et al. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282:1145.
13. Jung JG, Kim DK, Park TS et al. (2005). Development of Novel Markers for the Characterization of Chicken Primordial Germ Cells. Stem Cells 23:689–698.
14. Jung JG, Lee YM, Park TS et al. (2007). Identification, Culture, and Characterization of Germline Stem Cell-Like Cells in Chicken Testes. Biology of reproduction 76:173–182.
15. Draper JS, Pigott C, Thomson JA et al. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J. Anat 200:249–258.
16. Kanatsu-Shinohara M, Ogonuki N, Inoue K et al. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69: 612–616.
17. Kanatsu-Shinohara M, Inoue K, Lee JY et al. (2004). Generation of pluripotent stem cells from neonatal mouse testis. Cell 119:1001–1012.
18. Guan K, Rohwedel J. (1999). Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro. Cytotechnology 30: 211–226.

19. Guan K, Nayernia K, Maier LS et al. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440:1199-1203.
20. Guan K, Wagner S, Unsöld B et al. (2007). Generation of Functional Cardiomyocytes from Adult Mouse Spermatogonial Stem Cells. Circ Res 100:1615-1625.
21. Johkura K, Li C, Asanuma K et al. (2004). Cytochemical and ultrastructural characterization of growing colonies of human embryonic stem cells. J. Anat 205:247–255
22. Kossack N, Meneses J, Shefi S et al. (2009). Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells 27:138–149.
23. Kubota H, Avarbock MR, Brinster RL. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. PNAS 101:16489–16494.
24. Maeda S, Ohsako S, Kurohmaru M et al. (1994). Analysis for the stage specific antigen of the primordial germ cells in the chick embryo. J Vet Med Sci 56:315–320.
25. Menard C, Hagege AA, Agbulut O et al. (2005). Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. Lancet 366:1005–1012.
26. Kanatsu-Shinohara M, Inoue K, Lee J et al. (2004). Generation of pluripotent stem cells from neonatal mouse testis. Cell 119:1001–1012.
27. Nagano M, Ryu BY, Brinster CJ et al. (2003). Maintenance of mouse male germ line stem cells in vitro. Biol. Reprod 68:2207–-2214.

28. Nussbaum J, Minami E, Laflamme MA et al. CE. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J.
29. O'Brien PJ, Smith DEC, Knechtel TJ et al. (2006). Cardiac troponin I is a sensitive, specific biomarker of cardiac injury in laboratory animals. Lab Anim 40:153–171.
30. Snir M, Kehat I, Gepstein A, et al. (2003). Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. Am J Physiol Heart Circ Physiol 285:2355–2363.
31. Solter D, Knowles BB. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). ProcNatlAcadSci U S A 75:5565–5569.
32. Spradling A, Drummond-Barbosa D, Kai T. (2001). Stem cells find their niche. Nature 414:98–104.
33. Wang YX, Chen GA, Song TR et al. (2010). Enhancement of cardiomyocyte differentiation from human embryonic stem cells. Sci China Life Sci 53:581–589.
34. Kim YY, Ku SY, Jang J et al. (2008). Use of long-term cultured embryoid bodies may enhance cardiomyocyte differentiation by BMP2. Yonsei Med J 49(5):819 – 827.
35. He Z, Kokkinaki M, Jiang J et al. (2010). Isolation, characterization, and culture of human spermatogonia. Biology of reproduction 82:363–372.

ACKNOWLEDGEMENT

I am deeply indebted to my supervisor, Professor Dong-Kee Jeong who stimulated suggestions and encouragement helped me in all the time of research in Korea. I had been getting much knowledge from his ordinary life to his great scientific because of his wide knowledge, his enthusiasm, his inspiration and logical way of thinking. During my thesis experiment and writing period, he always provided encouragement, sound advice, great teaching, good supervisor, and lots of good ideas.

I am deeply grateful to my reviewers, Professor Tae-Young Kang, Professor Wang-Shik Lee left great comments to correct and improve my thesis.

I am indebted to other Professors at the Department of Animal Biotechnology, Prof. Moon-Chul Kim, Prof Young-Hoon Yang, Prof. Se-Pill Park and Prof. Min-Soo Kang who taught me during the Master's course. They continually and convincingly conveyed a spirit of adventure in regard to research and scholarship, and an excitement in regard to teaching. Without their guidance and persistent help this dissertation would not have been possible.

I would like to send my sincere gratitude to my former Professor Phan Kim Ngoc and my senior Pham Van Phuc, Laboratory of Animal Physiology and Biotechnology, University of Science, Vietnam National University for encouraging and teaching me some modern techniques.

I would like to express my sincere gratitude to all lab members and everyone at department of biotechnology who gave me the professional environment of study and cultural life during master's course at Jeju National University.

I would like to thank the seniors Uk-Deuk Kang; Yong-Jun Kang and my friends Han Ki Eun, Kim Tae Soo, Quynh Anh, and Luong Do at Department of Animal Biotechnology for helping and providing great environment in which I have studied and stayed in Jeju.

Especially, I would like to give my special thanks to my family, my lover, Mss. Hai Ha, as well as her family as well as whose patient love enabled me to complete this work.