A Dissertation
For the Degree of Doctor of Philosophy

Studies on the Establishment of Optimal Culture Conditions of Chicken Sox9 Positive Cells and the Exploration of Putative Bioactive Substances

닭 Sox9 발현 세포의 최적화 배양 환경 구축 및 생리 활성 효과 탐색에 관한 연구

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SUMMARY

Bone marrow cells are considered as the prominent cell source for clinical application to injuries and degenerative diseases. For cell-based therapies, the optimal cell source must be readily accessible with easily isolated and large-scale expanded cells maintenance is important for clinical application. Optimizing manipulation protocol of chicken bone marrow cells have not been reported because of their heterogeneous phenotype and mixed cell population. In this study, I conducted a study on the cell characteristics and the culture environment for a mass culture environment using chicken Sox9 positive cells. And the possibility of applying the treatment confirmed through consideration of the application in terms of regenerative medicine using the cell secretions.

First, I observed the histology of the long bone and cellular properties of bone marrow cells retrieved from 4-day-old neonatal chicks. Long bones, such as the femur, have been a major source of bone marrow cells in which endochondral ossification is responsible for bone formation. Histology, cell culture outcomes, specific marker expression, and differentiation

potential were evaluated the academic and industrial feasibility of chick bone marrow cells as a new biomaterial. The results of this study clearly demonstrated that bone marrow tissue of neonatal chicks was premature at least up to 4 days after hatching. This tissue contains a variety of cells involved in endochondral ossification as well as mature bone marrow cells. The majority of these cells became cuboidal until the end of primary culture, and more than 80% of cells were Sox9-positive. The results of the osteogenic and chondrogenic differentiation ability suggest the feasibility of Sox9 for regenerating bone marrow tissues.

Next, I investigated the necessity culture regime for enhancing cell maintenance, expanding the Sox9-rich neonatal bone marrow cells and differentiation through a suspended culture. Neonatal bone marrow cells retrieved from 4-day-old white leghorn (WL) were subcultured in high glucose DMEM, low glucose DMEM or DMEM/F12. Use of DMEM/F12 as a basic medium for chicken bone marrow cell culture was found suitable for the culture environment from the results of this study. In retrieved cells from 4-day-old chicken, osteogenic and

chondrogenic related gene expression were expressed regardless types of culture media. But, changing of basic medium to DMEM/F12 mainly serves as the activator of cell proliferation. Also, this beneficial function directly enhances osteogenic or chondrogenic differentiations potential of sox9 positive cells using DMEM/F12 media although mixed population of the cells involving endochondral ossification. In conclusion, the 4-day-old chicken bone marrow cells culture in vitro amplifies Sox9- and Col II-positive cells, and optimized culture environment of DMEM/F12 was useful for customizing culture regime for cell maintenance and differentiation.

Third study is the analysis on effectiveness of basic fibroblast growth factor (bFGF) and stem cell culture medium like primordial germ cell (PGC) culture media for expanding germness and pluripotent related expression. First, In DMEM/F12 culture media with different concentrations of bFGF (0ng/ml, 1ng/ml, 5ng/ml, 10ng/ml) are added to the optimized sox9 positive cell culture medium. Basic fibroblast growth factor (bFGF) is a member of the FGF family and is a critical component of stem cell culture field. However, the role and optimized

concentration of bFGF on promoting the chicken derived sox9 positive cells has not been reported. To monitor the influence of bFGF on retrieved cells in vitro, proliferative capacity, cell morphology, gene expression, immunophenotype and differentiation potential and functional tests like cell migration and invasion assay were evaluated as experimental parameters. Second, the PCG culture medium is similar to the medium condition of embryonic stem cells, which has been amplification of the germness and pluripotent gene expression of primary cells in 4-day-old whole bone marrow. In conclusion, the results of this study suggested that addition of 5 ng/ml bFGF in vitro on sox9 positive cells was effective for proliferation, differentiation, migration and invasion ability. Also using PGC media for culture of isolated cells shows different cell characteristics. In cell specific marker expression, stromal cell marker (CD44) expressed on PGC culture media and in differentiation assay shows adipogenic differentiation ability on PGC medium cultured cell population.

Finally, I applied putative bioactive substances substance to human adipose derived mesenchymal stromal cells (hADSC)

that is the origin of cell secretion and explored the effect of regenerative medicine such as bone differentiation potential. Bone marrow cells from four-day-old white leghorn chicks differentiated into osteogenic lineages. After 3weeks differentiation time, media changes serum-free fresh culture media. These Collected media were concentrated until total protein concentration reached to 50, 100 or 200 ug/ml.

Using these concentration soup, osteogenesis was induced either by routine protocol or by the protocol using at different concentrations of total protein. From the results of this experimentation suggests that certain substrates secreted by chick bone marrow cells stimulate osteogenic differentiation of hADSCs.

Keywords : Bone marrow-derived cells, Chicken, Cell characteristic, Putative bioactive substance of cell secretion, Culture environment

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LIST OF ABBREVIATIONS

ADSC : adipose derived stromal cell

ARS : alizarin red s

BM : bone marrow

BMC : bone marrow derived-adherent cell

BMP : bone morphogenetic protein

BSA : bovine serum albumin

CFU-F: colony forming unit-fibroblast

CMBC : chicken bone marrow derived cell

CO_2 : carbon dioxide

CREB : cyclic AMP-responsive element binding protein

DMEM : dulbecco's modified Eagle's medium

DMSO : dimethyl sulfoxide

DPBS : dubecco' s phosphate buffer saline

ECM : extra cellular matrix

FACS : fluorescence activated cell sorter

FBS : fetal bovine serum

FGF-2 : fibroblast growth factor-2

GAG : glycosaminoclycan

GMP : good manufacturing practices

GP130 : glycoprotein 130

H_2O : water

HA : Hyaluronic Acid/Hyaluronan

HPSGs : heparin sulfate proteoglycans

IF : immunofluorescence

LIF : leukemia inhibitory factor

MACS : magnetic-activated cell sorting

MHC : major histocompatibility complex

MSC : mesenchymal stem cell

OA : osteoarthritis

ORO : oil red o

PBS : phosphate buffer saline

PCR : polymerase chain reaction

RBC : red blood cell

RT-PCR : Reverse transcriptase polymerase chain reaction

SAS : statistical analysis system

SMA : smooth muscle actin

SOP : standard operating procedures

TGF-β : transforming growth factor beta

CHAPTER 1.

GENERAL INTRODUCTION

The chicken is a popular experimental model animal and research is underway in various fields (Davey and Tickle, 2007; Kain et al., 2014). As an experimental animal, chickens have the advantage of being easy to access and easy to manipulation (Cloney and Franz-Odendaal, 2015). However, in experiments with chickens, the lack of unusual markers like chicken specific antibodies can be seen as a disadvantage. Research on chickens has been conducted since the early 2000s. The study used over 11 weeks old chickens and the isolated cells were typical morphology of the stromal cells (Wu et al., 2010b; Bai et al., 2012). But my previous study showed that the properties of cells from 4-day-old chicken differed from the commonly known mesenchymal stem cells properties.

The avian species like chickens, quails and pheasants have unique characteristics based on their evolutionary position between mammals and other species. Development stage is the most significant difference from mammals performing organogenic reproduction in the body, and relevant or additional differences are found in the process of embryonic production. In bone development stage, two different processes involve the

formation of the bones such as endochondral and intramembraneous ossifications play a pivotal role in inducing bone morphogenesis then differentiated into osteoblasts and chondrocytes (Shapiro, 2008; Zhang et al., 2011a). Endochondral ossification is responsible for the formation of the long bones such as the femurs and tibia. Cartilage tissue is formed prior to ossification, and subsequent ossification acquires the activities of rudimentary long bone formation, extension of its length and natural healing against various damages. Intramembranous ossification induces bone formation without cartilage development. In both process, bone formation is completed before birth, while the extension of bone length continues until puberty. In mammals, endochondral ossification was completed until the birth and primitive cells for ossification usually disappeared in neonatal offsprings. The long bones are the source of bone marrow cells, of which endochondral ossification following hypertrophic proliferation and calcium deposition occurs.

Bone marrow-derived cells have an enormous value for the research on mesenchymal stems cell and cell transformation

(Pal and Das, 2017). In recent studies, mesenchymal-derived multipotent cells have become a valuable resource for cell-to-tissue regeneration and experimental modelling for differentiation and reprogramming (Akiyama et al., 2012; Goldberg et al., 2017). Bone marrow cells are comprised of various undifferentiated progenitors and precursors, which could provide enormous information on cell differentiation using an ex vivo system (Tevlin et al., 2016). It can differentiate into multi lineages (Woodbury et al., 2002) such as bone, fat, cartilage, muscle, neuron and liver cells under differentiate culture condition in vitro (Pittenger et al., 1999; Campagnoli et al., 2001). Differentiation is controlled by specific genetic regulation pathway which can be induce into a specific lineage. Also induction chemicals, growth factors are accelerate appropriate differentiation, proliferation (Indrawattana et al., 2004; Kim et al., 2005; Li et al., 2007). And bone marrow cells are considered as the basic material for clinical application to injuries and degenerative diseases (Chanda et al., 2010; Chu, 2015; Wang et al., 2016). For clinical implementation of bone marrow cells, their stable maintenance is important for optimizing manipulation protocol of bone marrow cells and the large-scale expanded

culture system has been necessary to high reproducibility of bone marrow cells. Also bone marrow-derived cells give rise to a hierarchy of cell population within the bone. In bone, it can be divided into several development stages on osteogenic and chondrogenic progenitor cells like osteochondro progenitor cell, preosteoblast, osteoblast, osteocytes, chondrocyte, proliferate chondrocyte and hypertrophic chondrocyte (Hofstetter et al., 1991; Aubin, 2001). Osteochondral progenitor cells are located in the inner layer of the perichondrium, the inner layer of the periosteum, and in the endosteum. From these locations, they can be a potential source of new osteoblasts or chondroblasts. Also it can be differentiated into osteoblastic, chondroblast lineages (Friedenstein et al., 1966; Orlic et al., 2001; Toma et al., 2002; Morikawa et al., 2009). Osteochondro progenitor cells are very important for making a bone and cartilage. They can be found inner of the long bone and can be differentiated into osteoblast and chondrocyte. Isolation of a more homogeneous population of progenitors may be necessary for more efficacious study and adaptations on regenerative medicine. For using cells in a clinical application, cells can be easily isolation, accessible and abundant. As the fully differentiated bone and cartilage develops from

mesodermal lineages like mesenchymal stem cells that differentiate into osteochondro progenitors and finally differentiated into mature bone and chondrocytes.

Especially, osteo/chondro progenitor cells are one of the kind of cells in bone marrow, which are also known as sox9 positive cells. In osteo/chondro progenitor cells, Sox9 is a transcriptional factor that responsible for expression of Runx2 (Leung et al., 2011; Chen et al., 2014; Loebel et al., 2015), Collagen related various type, aggrecan and the other multiple ECM related markers for differentiation into ostegenic and chondrogenic differentiations (Day et al., 2005; Nooeaid et al., 2012). In osteogenic differentiation, differentiation medium consisting of culture media supplemented with dexamethasone, ß-glycerophosphate, ascorbic-2-phosphate was conducted to induce differentiation into osteoblast (Eslaminejad et al., 2013). And it can be demonstrated by calcium accumulation using Alizarin Red S (ARS) (Gregory et al., 2004). In chonrogenic differentiation, differentiation medium consisting of culture media supplemented with Insulin-transferrin-selenium x, bovine serum album, linoleic, ascorbic acid-2-phosphate, TGF-

β1 (Joyce et al., 1990; Roark and Greer, 1994; Day et al., 2005; Kolambkar et al., 2007). And it can be demonstrated by formation of proteoglycan using alcian blue staining solution (Akiyama et al., 2002; Day et al., 2005).

Over the past few years, many study groups have studied using conditioned media to evaluate efficiency as a generative biomarker. Released protein from conditioned media by cultured cells is a rich source of materials for biomarker as an application of novel treatment (Xue et al., 2008). Useful proteins released from cells culture medium can be used as novel bio-active substances to apply for the disease or regenerative treatment effects. This technique provides a useful, easy and effective method for discovering new biomarkers. The point here is that protein analysis is enhanced due to finding identification of biomarker candidate in which grown serum-free culture conditions (Righetti et al., 2003). Protein found in the medium controlled by the cell lines will come from cells through many different channels. The development of mass spectroscopy (MS), unlabeled techniques, LC systems, and biometrics approaches had a profound impact on protein dynamics (Wong et al., 2009).

These advances, combined with analysis of conditioned media, will undoubtedly facilitate identification of candidate biomarkers in cell culture environments.

The standard procedure for the osteogenic differentiation of mesenchymal stem cells is addition with dexamethasone (Dex), ascorbic acid (Asc) and β−glycerophosphate (β−Gly). Osteogenic differentiation protocols using these three chemicals are frequently used for many experimental studies, including tissue engineering or detection of differentiation capabilities of particular cell types. These complex chemical mechanisms involved in osteogenic differentiation of stem cell cultures under the influence of Dex, Asc and β−Gly. Dex, Asc and β−Gly orchestrate several regulatory mechanisms during the differentiation process and does not start with an initial differentiation that if any insufficient. In some reports, Dex prevents apoptosis of BMSCs in confluent cultures (Song et al., 2009) and promotes mesenchymal stem cell proliferation (Wang et al., 2012). In osteogenic differentiation, Dexamethasone acts as a key molecule to differentiate into osteogenic lineage. FHL2 signaling is a critical signal in the osteogenic differentiation of

BMSCs and that Dexamethasone induces osteogenesis through the upregulation of FHL2 expression (Hamidouche et al., 2008). Then FHL2 induces the upregulation of the key transcription factor Runx2. Furthermore, Dexamethasone induces upregulation of TAZ, which binds to Runx2 (Hong et al., 2009). Additionally, Dexamethasone further induces osteogenic differentiation is the modulation of Runx2 phosphorylation through MKP-1 (Langenbach and Handschel, 2013). Ascorbic acid facilitates osteogenic differentiation by increasing the secretion of Collagen type I, as the binding increases integrin $\alpha 2\beta 1$ to Col1 (Kishimoto et al., 2013). This signal pathway increased phosphorylation of ERK1/2 in the MAPK signaling pathway and then PERK1/2 moved into the nucleus, where it binds to Runx2 and increase the osteogenic related lower signals then make expression of osteogenic proteins (Chaudhary and Avioli, 2000). β-Glycerophosphate increases osteogenic differentiation by the phosphorylation of ERK1/2 (Langenbach and Handschel, 2013).

The objective of this study is to harvest a large amount of reliable sox9 positive cells as a new biomaterial in chicken

bone marrow. In chapters 3–6, the different culture conditions and insertion of growth factors (bFGF) were optimized for promoting and scale–up of cell proliferation and different cellular activities of isolated cells to tissue culture plate. By using these optimized culture media, the effects of on the cell expansion and their utility potential value of chicken bone marrow–derived sox9 positive cells were examined. Finally, in chapter 7, a study was conducted on the use of regenerative medicine using cellular secretions factors. The application of cellular secretions was considered to overcome the shortcomings of using cells directly like genome effect from different animals. I provide a clue about the possibility as a regenerative medicine using sox9 positive cell secretion bioactive substance to enhance bone regeneration. Given these observations, I provide establishment of a condition about the optimized culture environment of chicken sox9 positive cells for the feasibility of large–scale expansion and suggests about the possibility as a regenerative medicine. And I emphasize a necessity of more advanced research studies about identifying the soluble factors from cell secretion and how it could be a newly alternative medicines. And it brings advances in therapy of several chronic and degenerative diseases.

CHAPTER 2.

LITERATURE REVIEW

1. Chicken as an experimental animal

In recent years, chicken has been used as experimental animal models in pharmaceutical and medical studies and also in the pharmaceutical immunotherapy field due to its advantages such as short reproduction time, simple management, and high productivity in the egg (Farzaneh et al., 2017; Sudjarwo et al., 2017). Especially, Chicken embryos are a significant useful historical research model in development sciences. However, in experiments with chickens, the lack of unusual markers like chicken specific antibodies can be seen as a disadvantage. There are many advantages to using eggs in developmental biology research. As an experimental animal, chicken egg have the advantage of being easy to access and easy to manipulation (Cloney and Franz-Odendaal, 2015). In the case of cell line derived from eggs and chicken embryos, it is used in a variety of therapeutic areas, including the production of vaccines. The line of cells derived by birds is an alternative to the production of viruses. Because using eggs are simple, flexible system that can be controlled with the ability to produce relatively easy and rapid vaccines. Recently, research is being done on the production of

transgenic animals using chickens, and pharmaceutical industries are developing in the field of medical research and research using transgenic animals.

2. Difference in developmental stages between aves and mammals

The avian species like chickens, quails and pheasants have unique characteristics based on their evolutionary position between mammals and other species. Development stage is the most significant difference from mammals performing organogenic reproduction in the body, and relevant or additional differences are found in the process of embryonic production. Unlike mammals, physiological polyspermy is usually induced at the time of fertilization, and numerous sperm are visible regardless of the formation of embryonic nuclei. Consequently, asymmetric cleavage yielding pre-blastodermal cells is observed throughout early embryogenesis and three stages of egg shell formation are placed before laying. According to the developmental stage, eggs from 1 to 3,000 cells form the soft

yellow eggshell of stage 1. The second stage of the egg, which consists of 3,000 to 30,000 cells, has a flexible egg shell that is light yellow, and the third stage, which consists of 30,000 to 60,000 cells, is white eggshell. These differences with mammals can lead to unique cell fate decisions in various systems that can be prominent in the tissues and organs.

3. Bone formation

Two different processes involve the formation of the bones such as endochondral and intramembraneous ossifications play a pivotal role in inducing bone morphogenesis then differentiated into osteoblasts and chondrocytes (Shapiro, 2008; Zhang et al., 2011a). Endochondral ossification is responsible for the formation of the long bones such as the femurs and tibia. Cartilage tissue is formed prior to ossification, and subsequent ossification acquires the activities of rudimentary long bone formation, extension of its length and natural healing against various damages. Intramembranous ossification induces bone formation without cartilage development. In both process, bone formation is completed before birth, while the extension of bone

length continues until puberty. In mammals, endochondral ossification was completed until the birth and primitive cells for ossification usually disappeared in neonatal offsprings. The long bones are the source of bone marrow cells, of which endochondral ossification following hypertrophic proliferation and calcium deposition occurs.

4. Stem cells

First stem cells using study conducted more than 140 years ago, and since 1868 stem cells have appeared in scientific literature in the works of famous German biologist Ernst Hatchell (Haeckel, 1879). From this start, many scientists tried to understand how these cells became different organs and tissues in the body (Becker et al., 1963; Ramalho-Santos and Willenbring, 2007). The culture of mouse embryonic stem cells was first reported by Evans and Kaufman in 1981.

In fertilized embryo at the blastocyst stage (BL), embryonic stem cells exist in the inner cell mass and are an attractive therapeutic cell type due to their pluripotency

(Thomson et al., 1998). These embryonic stem cells (ES) have the unlimited self-renewal ability and differentiated into multi lineage adult type cells. Stem cells provide an infinite supply of cells that development part or all of the body's tissue, and this type of stem cell is called a pluripotent stem cell. They have the ability to sustain themselves throughout life. (Barry and Murphy, 2004). Current study using stem cells is based on the facilitate the differentiation of stem cells into the required lineage and introduction of a form to replace or improve the functioning for the treatment disease (Odorico et al., 2001; Daadi and Steinberg, 2009; Pal, 2009). For example, in vitro studies using ES cells can sustain indefinitely without differentiation, but can be divided into wanted cell types when providing appropriate environment or chemicals. (Thomson et al., 1998; Amit et al., 2000; Daadi and Steinberg, 2009; Lu et al., 2009; Pal, 2009).

5. Bone marrow cells

Bone marrow tissue consists of a variety of cells and been employed for stem cell engineering and clinical therapy (Haynesworth et al., 1992; Krebsbach et al., 1999; Bai et al.,

2012; Bai et al., 2013). Bone marrow cells of different types have been established to date (Bianco et al., 2001; Jiang et al., 2002; Schwartz et al., 2002) and in the chicken, somatic chimeras have been derived from the transplantation of bone marrow cells into recipient developing eggs (Heo et al., 2011; Heo et al., 2012). An attempt to produce germline chimera and induce transgenesis has progressed rapidly in this species (Han et al., 2002; Han, 2009; Han et al., 2015). Bone marrow is spongy tissue and consists multi different types of population like single nucleus cells that include monocytes, lymphocytes, stem related cells and progenitor cells (Alvarez-Viejo et al., 2013). Bone marrow derived single nuclear cells are very valuable for tissue engineering, such as genetic and regenerative disease studies. (Henrich et al., 2015b, a). Bone marrow is the soft and flexible tissue that found in the interior of hollow spaces of bones. Bone marrow is consists of red and yellow marrow that depending on the predominance of hematopoietic or fatty tissue(Malkiewicz and Dziedzic, 2012). Red marrow is also known as hematopoietic cells are produces red blood cells, white blood cells and platelets. Hematopoietic cells mature and migrate into sinusoids to enter the circulation when they are formed(Lang et al., 1992).

According to ages, red bone marrow is found on the flat bones such as the pelvic girdle and the sternum in adult stage. But in children, it is found in the medullary cavity of the long bones, such as the femur (Moore and Dawson, 1990; Gurevitch et al., 2009). Yellow marrow is also known as stromal cells are produces fat, cartilage and bone (Zakaria and Shafrir, 1967; Gurevitch et al., 2007). Almost all of bone marrow is yellow bone marrow by the time a getting old age. Its main function is to store adipocytes that can serve as a source for energy (Tavassoli et al., 1977; Ortiz-Nieto et al., 2010). Bone marrow constitute multi cell types that include fibroblasts, macrophages, which contribute especially to red blood cell production, as they deliver iron for hemoglobin production, fat cells, osteoblasts, osteoclasts, endothelial cells, which form the sinusoids(Yin and Li, 2006; Mansour et al., 2012; Gordon et al., 2014). These derive from endothelial stem cells, which are also present in the bone marrow.

Mesenchyme is embryonic connective tissue that is derived from the mesoderm and that differentiates into hematopoietic and connective tissue, whereas MSCs do not differentiate into hematopoietic cells(Phinney and Prockop,

2007). Mesenchymal stem cells are multipotent stem cells that have various differentiation ability into multi-lineage(Pittenger et al., 1999) such as including osteoblasts(Heino and Hentunen, 2008), adipocytes, chondrocytes(Mackay et al., 1998), myocytes(Xie et al., 2006). Also it can be isolated using detection of cell surface marker. Mesenchymal stem cells are expressed CD44, CD73, CD90, CD105, while do not express CD11b, CD14, CD19, CD34 and CD45 surface marker(Kern et al., 2006; Jin et al., 2013).

Stem cells can be divided into two types according to their ability to differentiate. Pluripotent stem cells can generate every cell type of the organism (Verfaillie, 2009). Whereas multipotent stem cells are restricted to differentiated into more than one cell type. Mesenchymal stem cells has ability of mesodermal differentiation capacity that can generate cartilage, bone, adipocytes and fibrous connective tissue (Pittenger et al., 1999). Also mesenchymal stem cells give rise to a hierarchy of cell population within the bone. In bone, it can be divided into a several development stages including mesenchymal stem cells like osteoprogenitor cell, preosteoblast, osteoblast and

osteocytes (Hofstetter et al., 1991; Aubin, 2001).

Mesenchymal stem cells (MSCs) are type of adult stem cells and isolation and culture method is easy in in vitro culture. MSCs are have great plasticity and it can be great therapeutic tool (Camassola et al., 2012). MSCs are mainly found in bone marrow and also other specific tissue like amniotic fluide, cord blood, chorion and placenta tissue (Asgari et al., 2015; Gonzalez et al., 2015; Zhu et al., 2015). Mesenchymal-derived, multipotent cells have become a valuable resource for cell-to-tissue regeneration and experimental modelling for differentiation and reprogramming(Anbari et al., 2014). Also, these cells are crucial to the development of novel biotechnologies for stem cells. From the various characteristics of bone marrow cells are considered as the basic material for clinical application to injuries and degenerative diseases(Krebsbach et al., 1999). For clinical implementation of bone marrow cells, their stable maintenance is important for optimizing manipulation protocol of chicken bone marrow cells and the large-scale expanded culture system has been used to high reproducibility of bone marrow cells.

5.1. Chicken cell culture medium

Several culture systems have been developed for effectively maintaining chicken bone marrow cells. In most chicken bone marrow cell culture systems, Low doses of glucose have been employed (Khatri et al., 2009; Bai et al., 2013). Responding to recent demand on optimizing stem cell manipulation system, the establishment of a standard culture system and the development of simplified, optimized medium have become a part of major research objectives. Basically Dulbecco's modified Eagle's medium (DMEM) is in general use of supporting the growth of chicken stem cells, but its high osmolarity may have an adverse effect in the middle of cultivation on the cells (van de Lavoir et al., 2006). In chicken stem cell culture, Knockout Dulbecco's modified Eagle's medium (Knockout DMEM or KO/DMEM) is a basic culture medium which can support the growth ability in vitro. Although DMEM based culture medium can support the growth of various type of stem cells, the composition of KO/DMEM is suitable for all kinds of mammalian and non-mammalian stem cells and optimized culture environment of their growth rate (van de Lavoir and Mather-

Love 2006; Amit and Itskovitz-Eldor 2016). Traditionally, the presence of fetal bovine serum (FBS) in KO/DMEM has been commonly used in cell proliferation due to the presence of growth factors. The fetal bovine serum routinely used for mouse embryonic stem cell culture may not be optimized for chicken cell culture environment; because of the fetal environment from which the FBS is derived is rich in growth factors (Horiuchi et al. 2006). One of the fundamental problems with using FBS or chicken serum is that they are both very complex because they contain unknown compounds, including self-reproduction and differentiation factors. (Skottman and Hovatta 2006).

Knowing the response of isolated bone marrow cells to addition of growth factors like bFGF and LIF will directly contribute to developing a culture system. It improves the efficiency of isolated cells culture system. Leukemia inhibitory factors is generally addition on culture medium and it typically reduce spontaneous differentiation. In mouse embryonic stem cells, it was discovered that in vitro propagation required the activity of the cytokine leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988), and also it activates the Janus

kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway (Boeuf et al., 1997). Fibroblast growth factors (FGFs) regulate developmental pathways like mesoderm patterning in embryo development stage through to the organ systems (Nutt et al., 2001; Deimling and Drysdale, 2011). The major function of FGF signaling in bone, FGF2, FGF9 and FGF18 transcripts are found in differentiating osteoblasts during bone development and in the long bones (Ohbayashi et al., 2002). The FGF1 and FGF2 are expressed in mesenchymal cells prior accumulation of bone matrix at long bone development. FGFR3 are found in preosteoblasts and osteoblasts. FGF2 signaling is important for development of bone. Overexpressed FGF2 causes abnormal bone development, but its inhibition caused to malformation of bones (Iseki et al., 1997). Also FGF signaling regulate cell proliferation and osteoblast precursor cells replication into osteoblasts and controlled apoptosis of immature osteoblasts (Mansukhani et al., 2000). Activation of FGF signaling can regulate genes involved at full steps of osteogenesis.

5.2. Culture conditions of osteogenic differentiation

The standard procedure for the osteogenic differentiation of mesenchymal stem cells is addition with dexamethasone (Dex), ascorbic acid (Asc) and β−glycerophosphate (β−Gly). Osteogenic differentiation protocols using these three chemicals are frequently used for many experimental studies, including tissue engineering or detection of differentiation capabilities of particular cell types. These complex chemical mechanisms involved in osteogenic differentiation of stem cell cultures under the influence of Dex, Asc and β−Gly. Dex, Asc and β−Gly orchestrate several regulatory mechanisms during the differentiation process and does not start with an initial differentiation that if any insufficient. In some reports, Dex prevents apoptosis of BMSCs in confluent cultures (Song et al., 2009) and promotes mesenchymal stem cell proliferation (Wang et al., 2012). In osteogenic differentiation, Dexamethasone acts as a key molecule to differentiate into osteogenic lineage. FHL2 signaling is a critical signal in the osteogenic differentiation of BMSCs and that Dexamethasone induces osteogenesis through the upregulation of FHL2 expression (Hamidouche et al., 2008). Then FHL2 induces the upregulation of the key transcription factor Runx2. Furthermore, Dexamethasone induces

upregulation of TAZ, which binds to Runx2 (Hong et al., 2009). Additionally, Dexamethasone further induces osteogenic differentiation is the modulation of Runx2 phosphorylation through MKP-1 (Langenbach and Handschel, 2013). Ascorbic acid facilitates osteogenic differentiation by increasing the secretion of Collagen type I, as the binding increases integrin $\alpha 2\beta 1$ to Col1 (Kishimoto et al., 2013). This signal pathway increased phosphorylation of ERK1/2 in the MAPK signaling pathway and then PERK1/2 moved into the nucleus, where it binds to Runx2 and increase the osteogenic related lower signals then make expression of osteogenic proteins (Chaudhary and Avioli, 2000). β-Glycerophosphate increases osteogenic differentiation by the phosphorylation of ERK1/2 (Langenbach and Handschel, 2013).

5.3. Characterization of Bone marrow-derived cells

As described above, mesenchymal stem cells were mononuclear cells with different capacities that could be separated from bone marrow. However, the biological characteristics of cells either do not meet all of the permitted

criteria and lack for typical stem cell activity. The isolation of uniform cell populations will help with treatment using MSC for specific tissue repairs. Bone marrow derived stem cells are clearly defined and stated criteria. Human MSCs must express CD105, CD73 and CD90 and not CD34, CD45, HLADR, CD14 or CD11b, CD19 or CD79α and be able to differentiate into osteoblasts, adipocytes and chondrocytes under standard in vitro differentiating conditions. Much of the current data, technique and tools are insufficient to characterize bone marrow cells as stem cells. More specific marker or method to isolate a homogenous population of marrow cells are required for classified as stem cells with accuracy.

Progenitor cells have been described as cells that exist in gaps adjacent to mature and differentiated cells both in vivo and in vitro. Progenitor cells have been successfully isolated from several compartment, including fat tissue, synovium and bone marrow. Depending on the tissue source in which you are isolated, the progenitor cells has a distinct probability of differentiation and varies in frequency. There is evidence that there is uncommitted type of cells in culture derived from bone

marrow. Each cell in standby state was shown to represent a group of non-committed MSCs. After exposure to the fetal bovine serum, these same atmospheric cells generate a committed precursor to rapid growth and terminal differentiation. Within the stem cell hierarchy, several types of progenitor cells have been found to exist in bone marrow culture. Some research group have used non-staged cell replication to investigate the nature of committed programmers. The assessment of their differentiation showed that 30 % of fractions showed three germ layer differentiation potential and the other 70 % showed osteogenic and chondrogenic differentiation potential. There is a difference in our knowledge of stem cells that we need to fill in to make the most of their potential. We need to know more about the essential control of stem cells and what drives them to a particular differentiation path. The micro-environmental in which cells are commonly present affects these cellular properties. In medical field, many research conducted to adapt on application of stem cell or different type of progenitor cells. Because many specific tissues can be used to treat injuries and diseases. For example, chondroprogenitor cells can use on repairing articular lesions in articular cartilage.

6. Sox9 positive cells

Chicken bone marrow cells are comprised of population may contain various undifferentiated progenitors and precursors, which could provide enormous information on cell differentiation using an ex vivo system.(Friedenstein et al., 1987; Pittenger et al., 1999; Csaki et al., 2007; Hudson et al., 2011; Dai et al., 2014). Also bone marrow derived cells give rise to a hierarchy of cell population within the bone. In bone, it can be divided into a several development stages on osteochondro progenitor cells like mesenchymal stem cells. Osteochondro progenitor cell, preosteoblast, osteoblast, osteocytes, chondrocyte, proliferate chondrocyte and hypertrophic chondrocyte (Hofstetter et al., 1991; Aubin, 2001). Osteochondral progenitor cells have the ability to become osteoblasts or chondroblasts. Osteochondral progenitor cells are located in the inner layer of the perichondrium, the inner layer of the periosteum, and in the endosteum. From these locations, they can be a potential source of new osteoblasts or chondroblasts. Also it can be differentiated into osteoblastic, chondroblast lineages (Friedenstein et al., 1966; Orlic et al., 2001; Toma et al., 2002; Morikawa et al., 2009).

Osteochondro progenitor cells are very important for making a bone and cartilage. They can be found inner of the long bone and can be differentiated into osteoblast and chondrocyte.

A more homogeneous osteogenic and chondrogenic progenitor cells may be necessary to treat bone or cartilage more effectively. The differentiation of chemical cells into chondrocytes and their associated accumulation in the ECM are carefully controlled by the Paracrine factor.

Sox9 as a transcriptional mediator, transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signalling are responsible for initiating expression of cartilaginous ECM such as aggrecan, collagen types II and XI, fibronectin and tenascin as shown in in vitro murine micro mass cultures (Chimal-Monroy and Diaz de Leon, 1999; Hatakeyama et al., 2004). The chondrogenic differentiation of the cells in vitro through TGF-β1 is mediated by both Smad3 and Wnt-associated β-catenin (Church et al., 2002). In clinical applications, ideal cell sources are easily isolated, plentiful, and can pose minimal risk to patients. In cartilage regeneration, there are special features needed to produce a viable and durable

cartilage. These features include the dominance of collagen type II, and the binding of subcutaneous plates and surrounding cartilage. Mature articular cartilage develops from the embryonic mesodermal precursors and is differentiated into chondroprogenitogers and finally differentiated into mature adult chondrocytes or synoviocs. There is a hypothesis that the progenitors holding in these adult joint tissues provides a potential reservoir for chondroprogenitors. The application of chondroprogenitors to treat and subsequently suppress the onset of disease is the focus of current research efforts.

6.1. Characterization and Signal Pathway

Multiple type of osteochondro progenitor cells marker is used for detection. Generally various gene and surface protein markers are expressed on the osteochondro progenitor cells. Sox9 is a transcription factor and regulate chondrocyte differentiation and regulates transcription of the anti-Mullrian hormone (AMH) gene (Garside et al., 2015). Sox9 is key regulator signal for the osteochondro progenitor cells undergo osteogenic and chondrogenic differentiation. The transcription

factor Sox9 can be involved and control many signal pathways and it is also found in osteochondro progenitor cells, suggesting that they are important in early signal pathways (Pan et al., 2008). End of each signal pathways, osteoblasts make a bone and chondroblast make a cartilage. Runx2 also known as core-binding factors subunit alpha-1 (CBF-alpha-1) and Osx (a zinc finger containing transcription factor) are necessary for osteochondro progenitor cells to differentiate into the osteoblast cell lineage (Chen et al., 2009; Shahi et al., 2017). Furthermore, Runx2 regulates the expression of major bone matrix genes on differentiation (Chung, 2004; Fujita et al., 2004). Runx2 is a factor of role in hypertrophic chondrocyte maturation. It is effected by Wnt/β-catenin signaling. β-catenin signal pathways are important role in cell fate determination. WNT/β-catenin signaling-dependent Runx2 expression (Hill et al., 2005; Dong et al., 2006). In the presence of Wnt3a, an activator of the canonical WNT signaling pathway, β- catenin transport to the nucleus, where it binds TCF/LEF-1 and leads to the transcription of Runx2 (Komori, 2011; Voronkov and Krauss, 2013). Through Sox9 as a transcriptional mediator, transforming growth factor beta (TGF-β) and bone morphogenetic protein

(BMP) signaling are responsible for initiating expression of cartilaginous ECM such as aggrecan, collagen types II and XI, fibronectin and tenascin as shown in in vitro cultures (Hatakeyama et al., 2004; Chimal-Monroy and Diaz de Leon, 1999). TGF-β is representative markers for cell proliferation and differentiation during bone and cartilage development stage (Sakaki-Yumoto et al., 2013). TGF-β signals relations with Sox9 and Runx2 signalling pathways and regulates cell lineages during endochondral ossification development stage (Yang et al., 2001; Chen et al., 2012). TGF-β signal stimulates differentiation potential into chondrocytes and osteoblasts with multiple signals like FGF, Msx1, and Ctgf signalling pathways (Guo and Wang, 2009; Parada et al., 2013). Alkaline phosphatase (ALP) is the early expressed functional genes in the process of calcification and cartilage formation (Amano et al., 2009). It is expressed early development stages and decreased in later development. The regulatory signaling pathways are controlling osteogenic and chondrogenic differentiations. And ALP expression are controlled with WNT/beta-catenin, BMP, Runx2 signaling cascades. Collagen type I is most abundant extracellular matrix component and is marker of the end product like bone and

tendons (Liu et al., 1995). Fully differentiated cells reduction expression levels of collagen type I and II and an increase of collagen type X and Aggrecan (Aigner et al., 1995; Haaijman et al., 1997).

7. Extracellular matrix and cell-to-cell interaction

The ECM plays an important role in regulating the biological characteristics of the growth factors and the cytokine. Growth factors can be isolated and inhibited from binding into the receptor, or the ECM can directly affect the growth factor receptor (Hildebrand et al., 1994; Santra et al., 2002). In addition to receiving signals from the ECM, cells recite by releasing enzymes and ECM components within the ECM that produce protein for growth factors. ECM is influenced by hierarchical structures and is adapted to the functions of specific tissue or cell types. ECM components perform prominent functions only after relying on the network in which the tissues or tissues are assembled in turn, or on structural elements of insoluble, such as fiber or microstructure. These interactions represent the give-and-take relationships that define the behavior of cells

(Behonick and Werb, 2003). ECM have physical functions as well as biochemical functions; they are bound polymeric bonds that form tissue and form tissue. The ECM appears in many different forms because it is present in a wide variety of tissues (Knudsen, 2003). Since the cell itself produces the biochemical, surface interactions are likely to be associated with the cell and typically work as needed by specific tissues and networks (Dityatev et al., 2010). The ECM is an important substrate for cellular communication and is suitable for providing signal molecules to guide cells.

The components of the ECM include a fiber protein such as collagen and elastin, glycoproteins such as fibronetin and tenasin (Faissner et al., 1994; Bandtlow and Zimmermann, 2000). GAG consists of a long unit of disaccharide composition which is the disaccharide formulation that distinguishes between chondroitin sulfate (CS), heparan sulfate (HS), keratin sulfate (KS), dermatan sulfate (DS) and hyaluronan (HA). The unique composition of the disaccharides makes GAGs truly information dense biological molecules (Turnbull et al., 2001). Because of their molecular structure, GAG is suitable for combining many

different signal molecules. Hyaluronic acid (HA) is an exception because it is not a protein binding, it is not self-supplied and consists of the same breakdown enzyme unit (Toole, 2001). HA is synchronized to the plasma membrane and forms the backbone of the cartilage ECM. Most GAG are shared in key proteins. It is called a proteoglycan. According to a recent study, cells are surrounded by peri-cellular matrix that contain proteoglycan and thin collagen filaments without direct contact with the surrounding substrate (Hunziker et al., 1997). It is well established that ECM components play an important role in the differentiation of hematopoietic stem cells (Drzeniek et al., 1997). However, although little is known about the effect the ECM has on MSC differentiation, some studies show that ECM also plays an important role in MSC differentiation (Xu et al., 1998; Corsi et al., 2002; Hwang et al., 2011).

7.1. Hyaluronan

The fact that HA has many physiological features has drawn great attention over the past few decades. It was initially assumed that the HAs function was based on physical

characteristics such as joint lubrication, tissue homeostasis, and tissue adhesion. However, it is also a major regulator for cell behavior during such processes as embryogenesis, reproduction, morphogenesis, migration, proliferation and differentiation (Chen et al., 2006; Solis et al., 2012). HA has also been used clinically as a medical product and has become an important component in creating new biomaterials that are useful in tissue engineering and regenerative medicine (Allison and Grande-Allen, 2006; Serban and Prestwich, 2008; Burdick and Prestwich, 2011; Prestwich, 2011). As a pain relief treatment, HA is injected into bone or cartilage disease patient joints. The injection is not only to relieve pain and replace insufficient fluids but also such as the stimulation of chondrocyte growth, facilitate cell destruction and the production of cartilage (Goldberg and Buckwalter., 2005).

7.2. Chondroitin Sulfate

CS is a key component of the ECM and is critical to maintaining the structural integrity of the organization (Wu et al., 2010a). CS has useful biological properties used in cartilage engineering. These traits include anti-inflammatory activities,

water and nutrient absorption, wound therapy, and cellular activity that will help restore arthritis joint function (Pipitone, 1991; Ronca et al., 1998; Li et al., 2004). Effect of CS on chondrocyte culture improved produce cartilage specific matrix and sustaining of cellular activity (Sechriest et al., 2000). Some results show modulatory effect of extracellular CS on chondrocytes and a stimulatory effect of CS on proteoglycan and GAG production in cartilage ECM has also been shown (Huang, 1974; Huskisson, 2008). Importantly, CS is highly expressed during the chondrogenic progenitor cells condensation of MSCs, suggesting its importance in chondrogenesis (Barry et al., 2001; Kamiya et al., 2006). Another study showed CS increased chondrogenesis while down regulation of osteogenic related gene and cell proliferatio(Uygun et al., 2009). This positive impact of CS on bone marrow derived stem cell becomes an interesting molecule that tests its impact on directly from the marrow.

7.3. ECM and the Stem Cell Niche

The term “niche” was first described in 1978 (Schofield et al., 1978). Research into cell biology has emerged

in relation to the discovery of niche, focused on the micro-environmental support of stem cells. Stem cell niche include surrounding cell components associated signals from these supporting cells (Li and Xie, 2005). A niche requires a balanced environment that determines the fate of cells between self-renewal and differentiation. Without a balanced environment, improper differentiation occurs (Solis et al., 2012). Niches is a specific anatomical location that regulates how stem cells participate in repair, regeneration, and maintenance (Scadden, 2006). According to a recent study analyzing stem cell gaps, cell types such as enthelial cells, osteo and chondro related cells and bone marrow derived stem cells are essential to building functions. The pressure on the metabolic activity of stem cells may require special support compared to other cells in the body. Feedback control is typically given to stem cell pools because they are expandable and collapsible and can sometimes suffer large probability variations under certain assumptions (Lam et al., 2006; Morrison and Spradling, 2008; Vazin and Schaffer, 2010). The use of ECM molecules while separating them from bone marrow may provide a biomimicry environment in which can receive signals, as in vivo joints. This may lead to the

production of more stable target tissue for use in the regeneration of the degraded tissue in turn.

8. Conditioned media

Over the past few years, many study groups have studied using conditioned media to evaluate efficiency as a generative biomarker. Released protein from conditioned media by cultured cells is a rich source of materials for biomarker as an application of novel treatment (Xue et al., 2008). Useful proteins released from cells culture medium can be used as novel bio-active substances to apply for the disease or regenerative treatment effects. This technique provides a useful, easy and effective method for discovering new biomarkers. The point here is that protein analysis is enhanced due to finding identification of biomarker candidate in which grown serum-free culture conditions (Righetti et al., 2003). Protein found in the medium controlled by the cell lines will come from cells through many different channels.

The development of mass spectroscopy (MS), unlabeled

techniques, LC systems, and biometrics approaches had a profound impact on protein dynamics (Wong et al., 2009). These advances, combined with analysis of conditioned media, will undoubtedly facilitate identification of candidate biomarkers in cell culture environments.

9. Future directions, Perspective

Bone marrow-derived, multipotent cells are crucial to the development of novel biotechnologies for cell therapy. And also it become a valuable resource for cell-to-cell tissue regeneration and experimental modelling for differentiation and reprogramming(Wagers and Weissman, 2004). Bone marrow cells are considered as the basic material for clinical application to injuries and degenerative diseases(Lu et al., 1996). For clinical implementation of bone marrow cells, their stable maintenance is important for optimizing manipulation protocols and the large-scale expanded culture system has been used to high reproducibility of bone marrow cells.

9.1. Limitation of regenerative medicine using stem cell

therapy

Stem cell therapy majorly targets autologous and allogenic cell transplantation into patients by local transplantation or systemic infusion. Regarding bone-derived, cell therapy, mesenchymal stem cells may be available for various clinical applications, which has been continuously studied in regards to stem cell transplantation and autoimmunity (Daikeler and Tyndall, 2007; LoCascio et al., 2011; Alexander et al., 2013). Mesenchymal stem cells have low levels of major histocompatibility complex (MHC) class I antigens and do not express MHC class II and other stimulatory molecules (Koch et al., 2015). However, different expression pattern is detected, which is influenced by cell type and the cell characteristics and culture condition may effect on mesenchymal stem cells immunogenicity. Based on stem cells characteristic, immunosuppressive properties of transformed (differentiated) cells can be imaged as a potential risk (Flores-Figueroa et al., 2006; Merino-Gonzalez et al., 2016).

This multiple range of adaptable characteristics are

increasing uses of mesenchymal stem cells in tissue regenerative field (Caplan, 2007). But differentiation character into unwanted lineage and immunosuppressive have potential risks of use of mesenchymal stem cells. Differentiation into unwanted lineage means cell transformation of MSCs that can occur in long time cell culture. And immunosuppressive properties mean development into tumor. So for answering to the risk and question in using mesenchymal stem cells in regenerative therapeutic field, must be prepare and make according to good manufacturing practices (GMP), standard operating procedures (SOP) to obtain efficient and safe cell therapy.

9.2. Use of chicken bone marrow-derived progenitor cells in regenerative medicine

Bone marrow derived stem cells can differentiate into specific target cell type. The bone formation related cells develop into bone and cartilage related cells and then accumulate in the outer layer of the bone during intramembranous and endochondral ossification (Shapiro, 2008). In current clinical treatments, bone marrow derived mesenchymal stem cells are

used directly for cartilage regeneration. (Jin et al., 2007).

In order to develop cell transplantation technology, the retrieved target progenitor cells and optimization of culture environment and techniques are necessary. Advanced regenerative medicine currently uses in implantation system. The current trend leads to short-term recovery of bone and cartilage disease related symptoms using cells to prevent symptom (Ankrum et al., 2010).

CHAPTER 3.

General materials and methods

Experimental animals and collection of bone marrow cells.

4-day-old White leghorn(WL) being maintained at the University Animal Farm, Seoul National University were employed as laying hens and either hatched or 4-day-old chicks were euthanized for retrieving bone marrow cells by our standard protocols. All procedures of herd management and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU 140912-4).

Culture of bone marrow-derived cells

One million bone marrow cells were spread into a 100-mm tissue culture dish and subsequently cultured in high glucose DMEM (Gibco Invitogen) supplemented with 10% (v/v) FBS at 37℃, 5% CO_2 in a humidified air atmosphere. On day 3 of culture, erythrocytes and non-adherent cells were removed and the medium was changed every three days' intervals. When bone marrow-derived cells reached to 80% confluence (at the end of P0), the cultured cells were dispersed with 0.25% trypsin-

EDTA (Gibco Invitogen). After total cell number was counted by hemocytometer, the cells were replated into the dish for subculture. At the end of each passage, morphology of the cells cultured in the dishes was observed under an inverted microscope (TS 100-F, Nikon, Tokyo, Japan).

Assessing of cell proliferation during in vitro-culture of cells

Cell number was monitored at the end of each passage up to 12 passages. Both the cell number accumulated from primary passage and actual cell number proliferated at each passage were counted. Calculation of actual cell number was the subtraction of the cell number counted at the end of passage from that counted at its prior passage.

Colony forming unit fibroblast (CFU-F) assay

To quantify cell proliferation, CFU-F assay was conducted. One million cells were seeded on a 100 mm culture plate, and the medium was changed at interval of 3 days. On day 14 of culture,

some were stained with crystal violet and others were subcultured continuously and the colonies of more than 55 cells were provided for measuring cell size.

Analysis of the relative mRNA levels using real-time PCR

The bone marrow cells were transferred into RNAlater, and subsequently stored at -80℃ until qRT-PCR analysis. Total RNA from chicken bone marrow cells was extracted using RNeasyTM Mini Kit and cDNAs were synthesized from the total RNA using the M-MLV reverse transcriptase. The expression of specific chicken bone marrow cells genes was quantified by real-time PCR using iQTM SYBR® Green Supermix. The beta-actin primer was included in every treatment groups for standardization and the mRNA level of each gene was normalized to that of beta-actin. The total primer sequences are listed in Table 1.

Induction of differentiation

To confirm differentiation of chicken bone marrow cells in vitro,

ostogenic, adipogenic and chondrogenic differentiations in vitro were induced by standard protocols.

Osteogenic differentiation

Basic culture medium for osteogenesis was high glucose DMEM supplemented with 100 μM dexamethasone, 10mM ß-glycerophosphate and 50 μM ascorbate-2-phosphate. At the end of culture for 3 weeks, the cells were fixed with cold 70% (v/v) ethanol and the Alizarin Red S staining (ARS) was conducted.

Adipogenic differentiation

For adipogenic differentiation, high glucose DMEM supplemented with 0.5 mM isobutyl-methylxanthine, 200 μM indomethacin, 1 μM dexamethasone and 10 ug/ml human insulin was used. The cells were cultured for 3 weeks and medium change was conducted every three days. After being fixed with 10% (v/v) paraformaldehyde, the fixed cells were stained with an Oil Red O staining.

Chondrogenic differentiation

Chondrogenic differentiation was monitored in 15-ml polypropylene centrifuge tubes to create 3-dimensional environment. DMEM supplemented with 10 ng/ml TGF- ß 1, 1x Insulin-Transferrin-Selenium, 10^{-7} M dexamethasone, 1.25 mg/ml BSA, 50 μg/ml ascorbic acid, 5.33 μg/ml linoleic acid and 40 μg/ml L-proline was employed for the chondrogenesis. At the end of the culture for differentiation (3 weeks), the pellets were embedded in paraffin block and Alcian blue and nuclear fast-red staining was conducted.

Statistical analysis

All experiments were replicated more than three times. A generalized linear model (PROC-GLM) created using Statistical Analysis System (SAS) software version 9.4 (SAS Inst. Inc., Cary, NC) was used to analyze the data. When a significant model effect was detected, comparisons among groups were subsequently conducted the Duncan methods. A p value of less than 0.05 determined as a significant difference.

Table 1. Oligonucleotide primer sequences and thermal cycling conditions used in real-time and reverse transcriptase PCR.

Genes	GeneBank number	Primer sequence		Size (bp)	Temp (℃)
		Sense (5' >3')	Anti-sence (5' >3')		
β-actin	JN639846.1	ATGAAGCCCAGAGCAAAAGA	GGGGTGTTGAAGGTCTCAAA	223	60
Pou V	NM_001309372.1	TCAATGAGGCAGAGAACACG	GGACTGGGCTTCACACATTT	154	54
Nanog	NM_001146142.1	CAGCAGACCTCTCCTTGACC	TTCCTTGTCCCACTCTCACC	187	54
Sox2	AB092842.1	AGGCTATGGGATGATGCAAG	GTAGGTAGGCGATCCGTTCA	163	54
CD31	XM_004946203	GAAGAAAGAGATGAGCAGGCAGGAC	TGTAGCAAGGCAGATCGAGACA	204	60
CD34	XM_015299049.1	GTGCCACAACATCAAAGACG	GGAGCACATCCGTAGCAGGA	239	54
CD44	NM_204860.2	CATCGTTGCTGCCCTCCT	ACCGCTACACTCCACTCTTCAT	290	55
CD45	NM_204417.2	CACTGGGAATCGAGAGGAAA	CTGGTCTGGATGGCACTTTT	212	57
CD105	NM_001080887.1	ACGGATGACACCATGGAAAT	ATGAGGAAGGCTCCAAAGGT	217	57
Sox9	AB012236.1	GCTTTCTCGCATGAATCTCC	TTGGGGAAGGTGTTCTCTTG	174	57
Runx2	NM_204128.1	CAGACCAGCAGCACTCCATA	TTGGGCAAGTTTGGGTTTAG	154	54
CoLIa2	NM_001079714.2	CAAACCAGGCGAAAGGGGTC	AATGGACCACGGCTTCCAA	133	60
CoLIIa1	NM_204426.1	AAGATGTTGTAGGACCCCGA	CATCTGCGCCGCAAAGTTTC	212	60
CoLXa1	XM_003641007.3	GGCCAATCCACAATCCCAGA	CCCCAGGGTAGGCTTTTGAG	148	59
Aggrecan	NM_204955.2	AACCTTCAGCATCTGGAGCC	GAGGAAGCCACTTTCTCC	154	59
PPARγ	NM_6045588.1	GATCGCCCAGGTTTGTTAAA	TGCACGTGTTCCGTTACAAT	138	60

CHAPTER 4.

Characterization of Bone Marrow Cells Retrieved from Neonatal Chicks; Cellular Properties and Differentiation Potential

1. Introduction

Bone marrow tissue consists of a variety of cells and has been employed for stem cell engineering and clinical therapy (Haynesworth et al., 1992; Krebsbach et al., 1999; Bai et al., 2012; Bai et al., 2013). Bone marrow cells of different types have been established to date (Bianco et al., 2001; Jiang et al., 2002; Schwartz et al., 2002) and in the chicken, somatic chimeras have been derived from the transplantation of bone marrow cells into recipient developing eggs (Heo et al., 2011; Heo et al., 2012). An attempt to produce germline chimera and induce transgenesis has progressed rapidly in this species (Han et al., 2002; Han, 2009; Han et al., 2015).

Nevertheless, basic information on chicken bone marrow cells culture has not reported yet and a standard protocol of manipulation and cellular properties have not been established. Furthermore, the feasibility of the culture environment should be confirmed in chicken bone marrow cells for both preclinical model researches and clinical application of novel biotechnologies.

Long bones, such as the femur, have been a major source of bone marrow cells in which endochondral ossification is responsible for bone formation (Reddi, 1981; Provot and Schipani, 2005). In mammals, bone morphogenesis roughly completes until hatching and primordial cells of the bone marrow usually mature in neonatal offspring (Ortega et al., 2004; Mackie et al., 2008). It is hypothesized that bone formation in chickens differs from that in mammals considering their ex vivo development. The collection of bone marrow-derived progenitor cells could allow for the development of bone marrow-mediated biotechnologies for tissue regeneration (Salgado et al., 2004).

This study was conducted to understand both the histology and cellular properties of bone marrow cells retrieved from 4-day-old neonatal chicks. Histology, cell culture outcomes, specific marker expression, and differentiation potential were evaluated to describe the academic and industrial feasibility of chick bone marrow cells as a new biomaterial.

2. Materials and Methods

Experimental design

In experiment 1, histological examination was conducted for the femurs collected from either hatched or 4-day-old chicks. In experiment 2, the bone marrow cells retrieved from 4-day-old chicks were subcultured and cell morphology and proliferation profile were monitored. As experimental parameters, both cumulative cell number from the time of seeding and actual cell number counted at the end of each subpassage were employed. In experiment 3, the reactivity of the P5 cells to the specific markers for osteochondrogenesis (Sox9, Col I, Col II and AP) and gene expression of the P0 and P5 cells were evaluated. The expression of specific genes for pluripotency (pouV, Nanog and Sox2), endothelial cell (CD31), hematopoietic cell (CD34 and CD45), mesenchymal stem cell (CD44), endoglin (CD105) and osteo-chondrogenesis (Sox9, Col I, Col II, Col X and Aggrecan) was subsequently monitored. In experiment 4, osteogenic, adipogenic and chondrogenic differentiation of the P5 cells was induced.

Histological examination of the femur

The femurs were collected after euthanizing and subsequently rinsed with Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea) supplemented with 1% (v/v) antibiotics and 2% (v/v) fetal bovine serum (FBS; Welgene Inc., Daegu, Korea). After muscles and other connective tissues were mechanically removed, the collected bones were fixed with 4% (v/v) paraformaldehyde. Then the fixed long bone tissue was decalcified in 15% (v/v) EDTA in order to soften the tissue. Then tissues were embedded in paraffin blocks, which were consequently dissected into the specimen of 6 μm in thickness. Hematoxylin and eosin staining was conducted for tissue examination.

Immunocytochemistry

The bone marrow cells were washed with PBS, fixed with 4% (v/v) paraformaldehyde and subsequently permeabilized with 0.1% (v/v) of Triton X-100. After washing, the cells were blocked

with 10% (v/v) normal goat serum and treated with Sox9, Collagen type I, Collagen type II antibodies during overnight at 4℃ and secondary antibody for 2 hours at room temperature. After 3 times washing step, stained with DAPI staining solution to enable nuclear visualization and images captured via fluorescent microscopy using software from NIS Elements, Inc.

Alkaline phosphatase (AP) activity assay

At Passage 5 cells were fixed with 4% (v/v) paraformaldehyde and then NBT/BCIP solution (Roche Applied Science, 11681451001) treated in Buffer 3 (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) at room temperature until desired staining was obtained.

3. Result

Femur histology

A total of 110 chicks were euthanized for histological and cytological examinations. As shown in Figure 1, the bone marrow matrix consisted of a variety of cells, including osteochondrogenic cells and mature bone marrow cells such as osteoblasts, osteoclasts, chondrocytes, hypertrophic chondrocytes, hematoblasts, granular leukocytes, and erythrocytes. Numerous positive cells had spread into both the epiphyseal and diaphyseal regions, while the epiphyseal line was not distinct. There were no histological differences between hatched and 4-day-old chick femurs. Four distinct regions (zone of resting, zone of hypertrophy, zone of proliferation, and erosion zone) were visible. Proliferating chondrocytes began to transform into hypertrophic chondrocytes in the center of the bone matrix.

Characterization of bone marrow cells following in vitro culture

As shown in Figure 2, culture of bone marrow cells resulted in a cuboidal cell-dominant population. At the time of seeding, cells of different types were visible, but cuboidal cells formed a colony-like structure and were continuously established up to the end of primary culture. Subculture of this cell population enabled dispersion of the cuboidal cells, and the process of subpassage was conducted when the cells reached 80% confluency. A significant ($p<0.0001$) model effect was detected in both parameters (cumulative and actual cell number) when the number at the end of each passage was compared (Figure 3). Vigorous proliferation was detected, which peaked at P2 and P3. The cumulative cell number plateaued between P6 and P7, but decreased proliferation activity was detected between P4 and P7. Compared with P2 and P3 cells showing the best proliferation, significantly ($p<0.05$) decreased activity was detected in P8 cells.

Gene and marker expression of cultured bone marrow cells

Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that the expression of Col X and Aggrecan was prominent in P0 cells. As shown in Figure 4, strong expression

Figure 1. Morphology of the femur retrieved from immediately after hatching (C) or 4-day-old chicks (A, B). (A) Four-day-old chick. Epiphyseal line was not formed and the stromal tissue of the diaphysis and the epiphysis was mixed. Hematoxylin and eosin staining demonstrated numerous positive cells spreaded into both the epiphyseal and the diaphyseal regions. Bar=250 um. Four-day-old (B1-3) and hatched (C1-3) chicks, which observed low, middle and high maginifications. Bar=100, 50 and 25 um, respectively. Bone matrix consisted of a variety of the cells that involved in endochondral ossification. Four distinct regions, zone of resting, zone of hypertrophy, zone of proliferation and erosion zone, were visible at low magnication, which consisted of osteogenic and mature bone marrow cells such osteoblast (ob), osteoclast (oc), chondrocyte (c), hypertrophic chondrocyte (hc), hematoblast (h), granular leukocyte (gl) and erythrocyte (e).

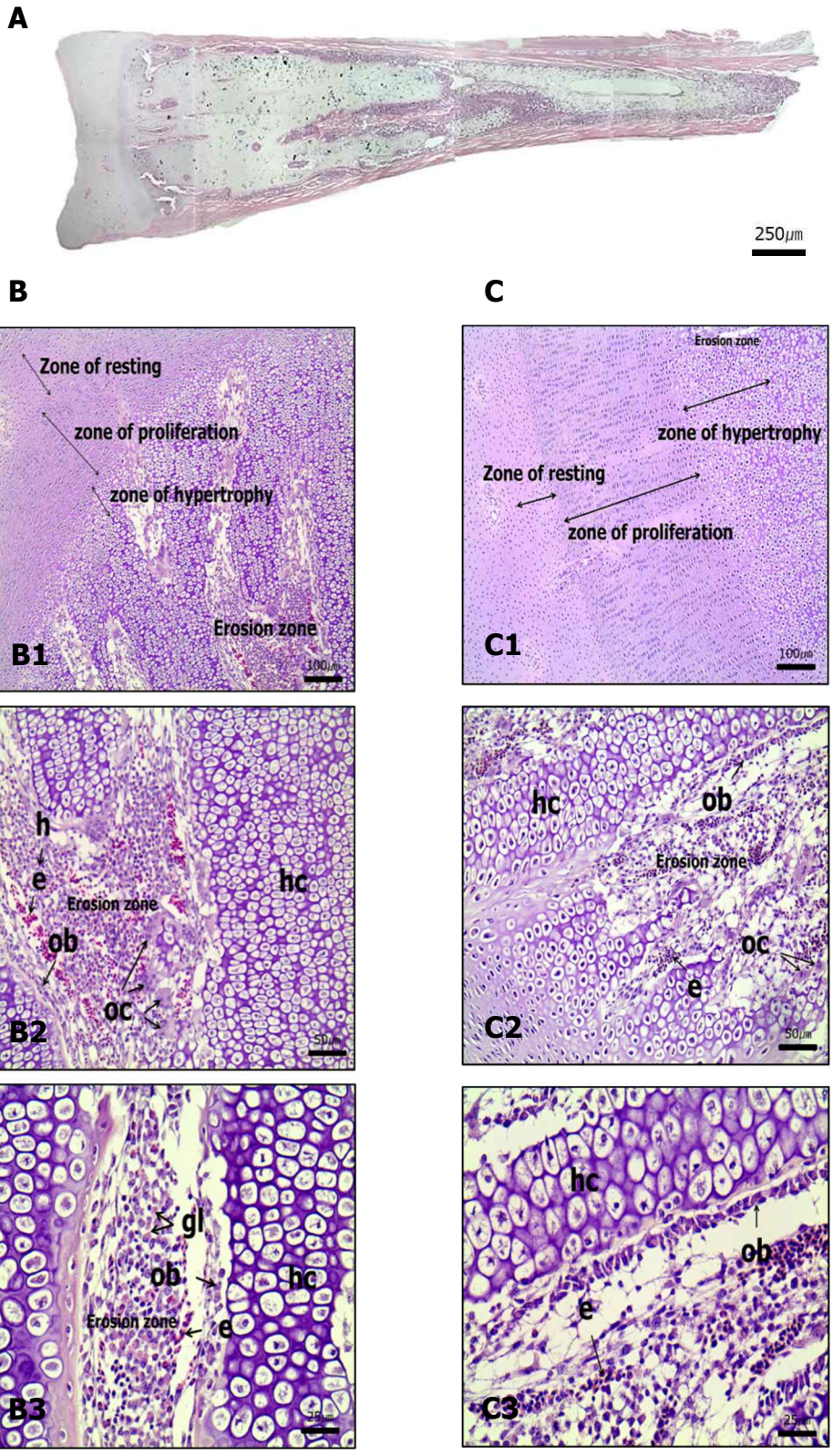

A
250μm
B
Zone of resting
zone of proliferation
zone of hypertrophy
Erosion zone
B1
100μm
h
e
Erosion zone
hc
ob
oc
B2
50μm
gl
ob
hc
Erosion zone
e
B3
25μm
C
Erosion zone
zone of hypertrophy
Zone of resting
zone of proliferation
C1
100μm
hc
ob
Erosion zone
oc
e
C2
50μm
hc
ob
e
C3
25μm

Figure 2. Morphology of the bone marrow cells retrieved from 4-day-old chicks. After being collected by enzymatic digestion, the cells were seeded onto a 100mm tissue culture dish and subsequently cultured in high glucose Dulbecco's minimal essential medium (Welgene Inc., Daegu, Korea) supplemented with 10% (v/v) fetal bovine serum (Welgene Inc., Daegu, Korea). Morphology of the cells cultured were monitored at the end of the passage 0, 5 and 10. The cultured cells transformed into amorphous morphology of either cuboidal or stromal cell-dominant. Bar=250 um

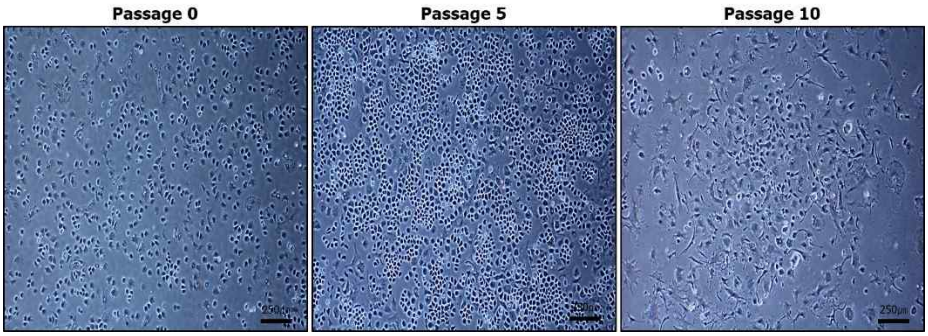
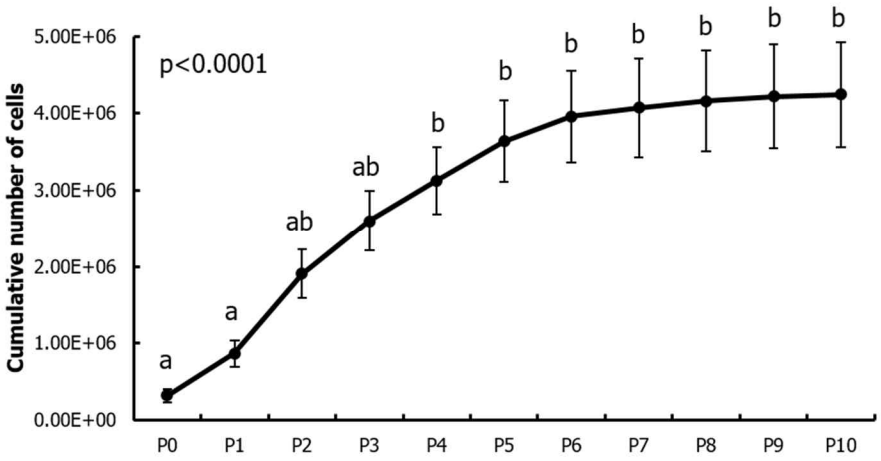


Figure 3. Proliferation of the bone marrow cells retrieved from 4-day-old chicks. Retrieved cell number at the end of each passage up to the passage 10 (P10) was counted. (A) Cumulative number of the cells cultured from P0 and (B) Actual number of the cells retrieved from each subpassage. Significant ($p<0.0001$) model effect was detected in each parameters and vigorous proliferation was gradually decreased from passage 4. Compared with P2 and P3 showing the best proliferation, significant retardation of the cell activity was detected from P8. Data were indicated as Mean±SE and different script in each passage indicated significant ($p<0.05$) difference.

A


p<0.0001
Cumulative number of cells
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
a
a
ab
ab
b
b
b
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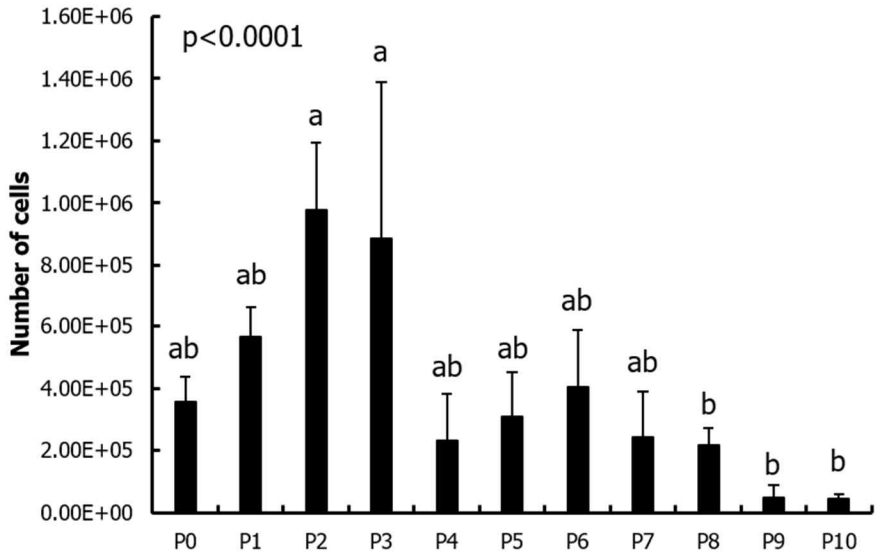
P0
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10

B


p<0.0001
Number of cells
1.60E+06
1.40E+06
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
ab
ab
a
a
ab
ab
ab
ab
b
b
b
P0
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10

of Col I, Sox9, Sox2, CD44, CD45, and CD105 was also detected. In contrast, Col II, PouV, Nanog, CD31, and CD34 were weakly expressed or not detected. In P5 cells, all genes related to osteochondrogenesis (Sox9, Aggrecan, Col I, Col II, and Col X) were expressed at P5, while the expression of Nanog and CD34 was not detected. The expression of CD31, an endothelial cell marker, was also strong. Other genes were expressed similarly compared with P0 cells.

Most P5 cells (82.2 ± 4.2% of the total population) were Sox9-positive (Figure 5). Considerable expression of Col I (67.0 ± 6.2) was detected, which was higher than that of Col II (32.2 ± 6.4). However, Col I and Col II expression was much less than the reactivity to Sox9. Counterstaining of each marker with DAPI clearly demonstrated cell-specific expression. In the co-expression of Sox9- and Col I-positive cells and of Col I and Col II was observed. As shown in Figure 6, AP activity was strongly positive in P5 cells.

Figure 4. Expression of various genes in bone marrow-derived

cells collected from 4-day-old chicks. The cells collected at the end of passage 0 (A) and 5 (B) were provided for RT-PCR. The expression of specific genes for pluripotent cell (pouV, Nanog and Sox2), endothelial cell (CD31), hematopoietic cell (CD34 and CD45), mesenchymal stem cell (CD44) and endoglin (CD105) and osteo-chondrogenesis (Sox9, Col I, Col II, Col X and Aggrecan) was monitored. (A) Prominent expression of Col X and Aggrecan was detected, and the expression of Col I, Sox9, Sox2, CD44, CD45 and CD105 was also detected. Col II, PouV, Nanog, CD31 and CD34 expression was weakly or not detected. (B) All genes related osteo-chondrogenesis were expressed at P5, while expression of Nanog and CD34 was not detected. Other genes were concomitantly expressed with the genes related to osteo-chondrogenesis. β-actin was positive control.

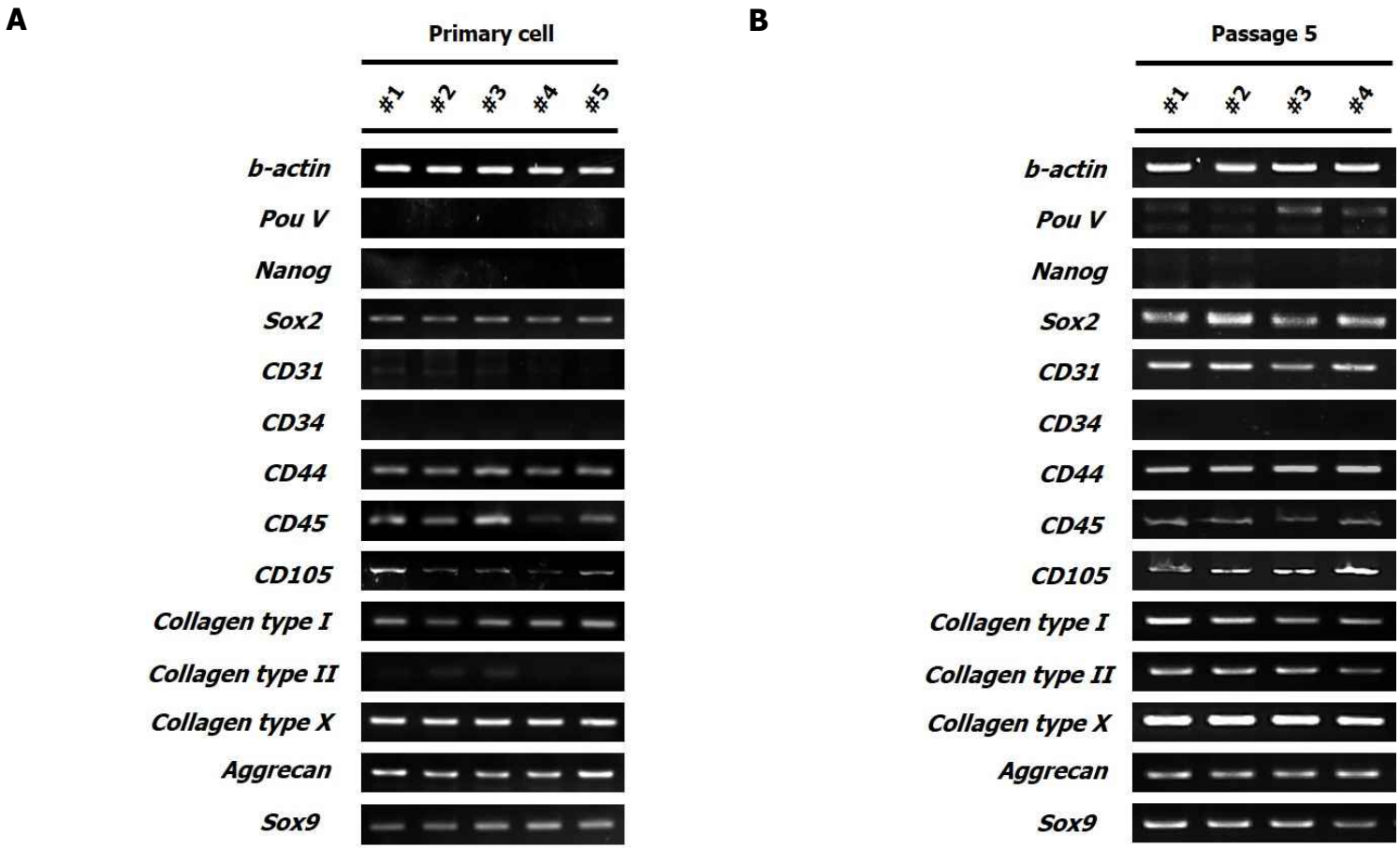

A
Primary cell
#1
#2
#3
#4
#5
b-actin
Pou V
Nanog
Sox2
CD31
CD34
CD44
CD45
CD105
Collagen type I
Collagen type II
Collagen type X
Aggrecan
Sox9
B
Passage 5
#1
#2
#3
#4
b-actin
Pou V
Nanog
Sox2
CD31
CD34
CD44
CD45
CD105
Collagen type I
Collagen type II
Collagen type X
Aggrecan
Sox9

Figure 5. Expression of specific marker proteins for osteo-chondrogenesis. Expressions of Sox9 and Sox9-related, collagen type I (Col I, fibrous cartilage marker) and collagen type II (Col II, proliferative chondrocyte marker) proteins were monitored in the bone marrow of 4-day-old chick bone marrow cells. The cells were collected from passage 5 and provided for immunocytochemistry. (A) Expression of Sox9, Col I and Col II (A1-3), and their percentage of expression to total cultured cells (A4) were evaluated. Sox9 expression were significantly ($p<0.05$) dominant than the expression of Col I and Col II, and more than 80% of the cell cultured expressed the Sox9. Data were indicated as Mean±SE. (B) Immunofluorescence counter staining of Sox9, Col I or and Col II with DAPI. P5 bone marrow cells were provided for this analysis. Prominent expression of Sox9 (red color), Col I (red color) and Col II (green color) was detected with nuclear-specific DAPI straining (blue color), which indicate, cell-specific reactivity.

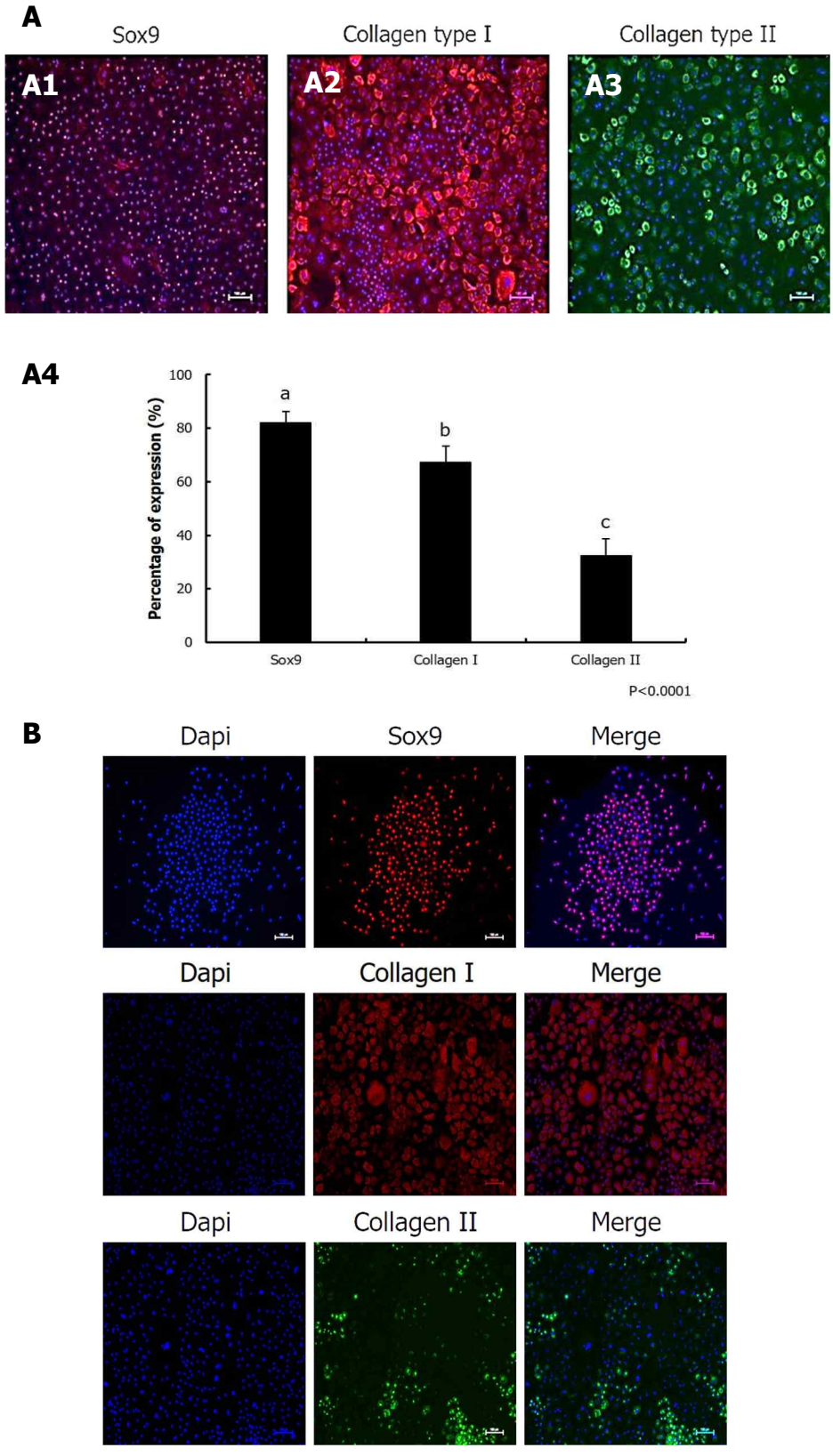
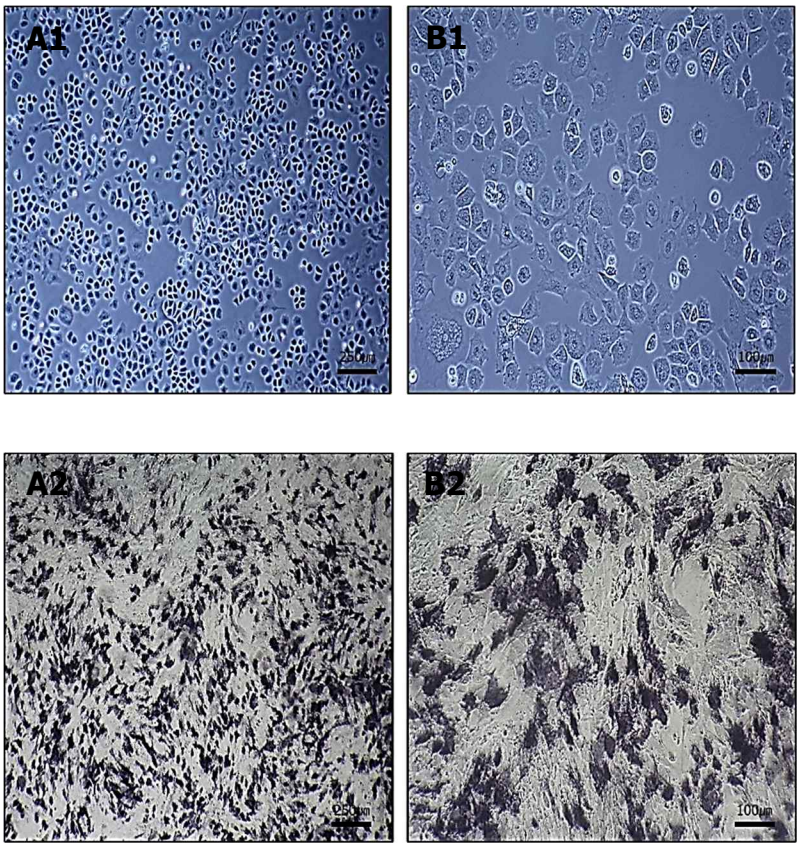

A
Sox9
Collagen type I
Collagen type II
A1
A2
A3
A4
Percentage of expression (%)
100
80
60
40
20
0
a
b
c
Sox9
Collagen I
Collagen II
P<0.0001
B
Dapi
Sox9
Merge
Dapi
Collagen I
Merge
Dapi
Collagen II
Merge

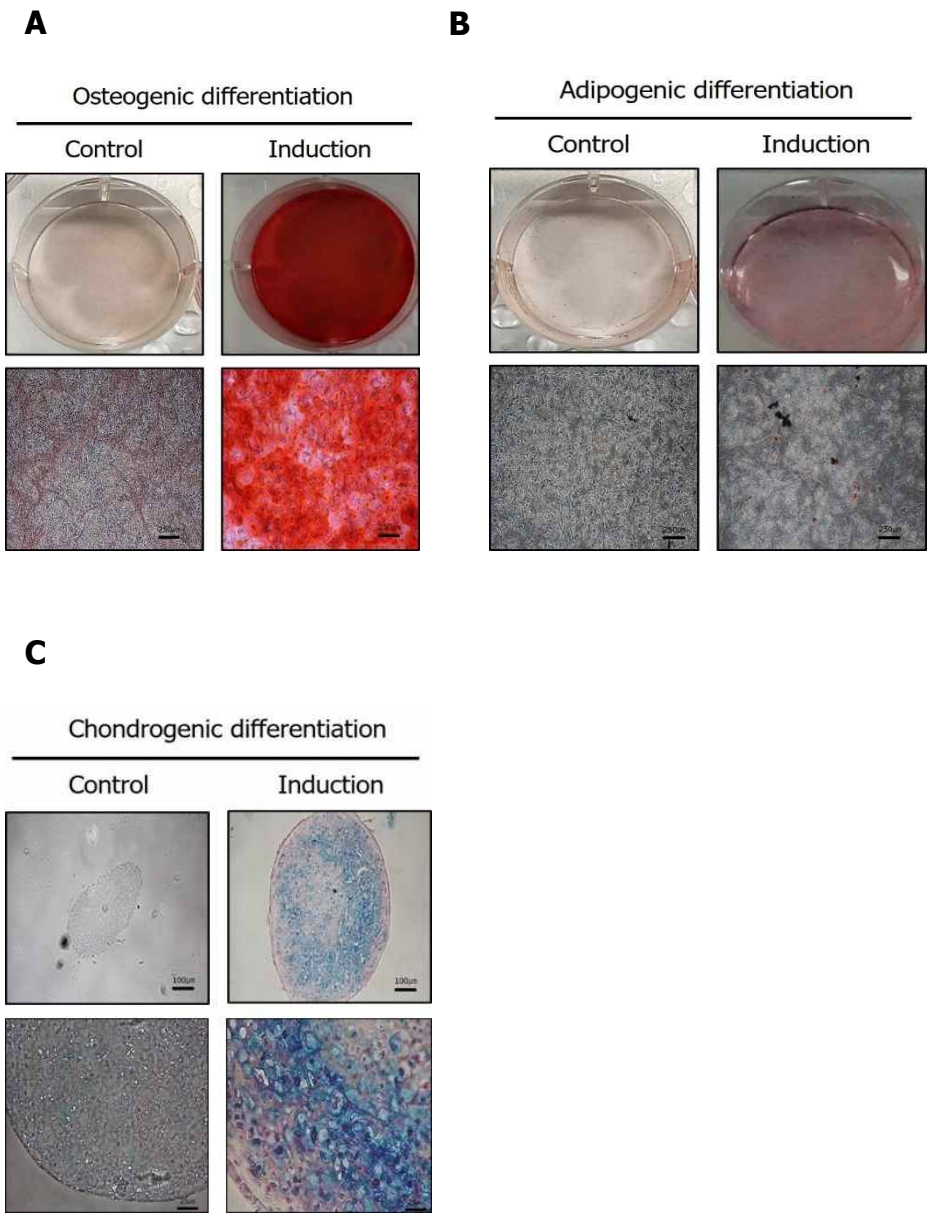
Figure 6. Staining of bone marrow cells by alkaline phosphatase (AP), a hypertophic cartilage marker. Bone marrow cells collected from 4-day-old chicks were subcultured 5 times and were subsequently provided for AP staining. Observation of morphology before (A1, B1) and after (A2, B2) staining at low (Bar=250 um) and high (Bar=100 um) magnifications. Strong reactivity of AP was detected, which indicates ossification is under progress and either osteoblasts or progenitor cells of osteo-chondrogenesis involves.



Comparison of the multi-lineage differentiation potential of chicken bone marrow cells

As shown in Figure 7, both osteogenic and chondrogenic differentiation were detected, while adipogenesis was not noticeable under this differentiation regime. After three weeks of osteogenic differentiation, P5 cells induced for osteogenesis exhibited strong Alizarin Red S staining. Alcian blue staining was also strongly detected following adipogenic differentiation. However, Oil Red O staining was negative in all replications.

Figure 7. Differentiation of 4-day-old, chick bone marrow-derived cells collected from passage 5. (A) Osteogenic differentiation. Total $3x10^5$ cells were seeded into 6-well plate and subsequently cultured in an osteogenic induction media for 21 days. Alizarin Red S staining was conducted for detecting calcium deposition and mineralization. Scale bars =250 μm. (B) Adipogenic differentiation. The same $3x10^5$ cells seeded were cultured for 21 days in an adipogenic induction media supplemented with insulin. Oil Red O staining for detecting lipid accumulation was negative in monolayer, while morphogenic transformation was noticed. Scale bars =250 μm. (C) Chondrogenic differentiation. Total $2.5x10^5$ cells were seeded into a 15ml conical tube for the pellet culture using chondrogenic induction media for 21 days. Alcian blue staining for detecting glycosaminoglycan secretion was positive and morphogenic transformation was also detected. Scale bars =100 μm.


A
Osteogenic differentiation
Control
Induction
B
Adipogenic differentiation
Control
Induction
C
Chondrogenic differentiation
Control
Induction

4. Discussion

The results of this study clearly demonstrate that bone marrow tissue of neonatal chicks was premature at least up to 4 days after hatching. This tissue contains a variety of cells involved in endochondral ossification as well as mature bone marrow cells. The majority of these cells became cuboidal until the end of primary culture, and more than 80% of cells were Sox9-positive. The results of the differentiation induction suggest the feasibility of Sox9 for regenerating bone marrow tissues.

In addition to the expression of specific markers for osteochondrogenesis, the expression of other markers, such as those associated with pluripotency, endothelial cells, hematopoietic cells, and mesenchymal cells, may indicate an amorphous structure of 4-day-old long bone (femur) tissue. Apparent differences between neonatal (hatched or 4-day-old) and adult femurs were detected, and a unique gene expression profile of neonatal bone marrow cells may be representative of cells and tissues undergoing endochondral ossification. This

tissue contains progenitor cells of osteogenesis, osteoclasts and macrophages, and blood vessel cells that trigger bone morphogenesis. The bone marrow cells of hatched or neonatal chicks, which contain a large number of Sox9-positive cells, can either directly support osteogenesis or secrete both chondrotrophic and osteotrophic factors *in vitro.*

Despite a mixed cell population, most cells collected at the end of P5 were Sox9-positive. Subculture of chick bone marrow cells may enable the vigorous proliferation of Sox9-positive cells. Although a quantitative analysis was not performed, RT-PCR results demonstrated that Sox9 expression in P0 cells was weaker than that in P5 cells. Otherwise, a mixed culture of Sox9-positive cells with other cells may increase their action when they are co-cultured at least up to five times, and the culture of premature bone marrow cells can trigger certain cells with a specific function. These results are supported by our finding that the proliferation of endothelial cell- and macrophage-specific CD31-positive cells was strongly enhanced, and that all osteogenesis- and chondrogenesis-related markers were expressed until P5.

Different culture systems may be specific to the proliferation of a specific cell type. The positive role of the culture system employed in this study in amplifying osteochondrognic cells was confirmed. The chick bone marrow cells established in this study directed their differentiation towards osteogenesis and chondrogenesis, not adipogenesis, which confirmed their specific expression for osteochondrogenesis-related genes and markers. Taken together with the results of histological, molecular, and cytological analyses, our results suggest the potential use of chick bone marrow cells as a basic biomaterial for clinical applications for injuries and degenerative diseases (Barry and Murphy, 2004; Dezawa et al., 2005; Nandoe Tewarie et al., 2006; Kassem and Abdallah, 2008).

CHAPTER 5.

CULTURE PROFILE FOR CHICKEN NEONATAL BONE MARROW CELLS AND CUSTOMIZATION FOR EXPANDING SOX9−RICH FRACTION

1. Introduction

Embryonic mesenchyme is a pivotal tissue of organogenesis and the mesenchyme-derived (mesoderm) cells retain the multipotency into committed cells (Hay, 2005), as long as have a pluripotent activity (Kitagawa and Era, 2010; Moon et al., 2011). I recently found that the bone marrow of neonatal (0- to 4-day-old) chicks is premature tissue, which derives a variety of the cells involving in endochondral ossification as well as of mature bone marrow cells. Furthermore, the neonatal bone marrow cells (nBMCs) collected from subculture mainly consist of Sox9-positive cells that prefer to differentiating into osteogenesis or chondrogenesis in vitro.

Number of questions might be answered for establishing nBMC-based biotechnologies by applying our results. First of all, it is urged to understand nBMC properties following in vitro culture for evaluating the necessity of culture system. Nevertheless, basic information on chicken bone marrow cells culture has not reported yet and a standard protocol of manipulation and cellular properties have not been established.

Furthermore, the feasibility of the culture environment should be confirmed in chicken bone marrow cells for both preclinical model researches and clinical application of novel biotechnologies.

Second, if culture is useful, the customization of the nBMC culture system for maintenance, transformation and differentiation is required. Consequently, I first examined the origin of Sox9−positive cells; from intact BMCs or by in vitro culture? Also, we evaluated whether the nBMC subculture amplify Sox9−positive cells. Second, I customized the nBMC culture system for cell maintenance and ostrochondrogenic differentiation. Three conventional media without any supplementation except for serum were evaluated. Major criteria were how many subculture can be made for nBMCs without loss of cellular function. Various culture profiles and differentiation tendency were monitored and the gene and marker expressions for osteogenesis and mesenchymal cells were monitored.

2. Materials and Methods

Experimental design and parameters

General procedures of this study consisted of 1) the collection of the femur from 4-day-old chicken, 2) the isolation and preparation of nBMCs for in vitro-culture 3) the execution of the nBMC culture using different culture environments. The outcome of nBMC culture and the expression of genes and proteins were employed as the experimental parameters for understanding the relationship between culture and nBMC properties and for optimizing the culture system of cell maintenance and differentiation, respectively. The culture outcome was represented by cell morphology and number (both cumulative and actual number) at each passage up to P12, colony-forming activity, senescence after 5 times subculture and the P5 cell differentiation (osteogenesis, chondrogenesis and adipogenesis). Subsequently, the expression of specific genes (Sox9, Runx2, Col I, Col II, Col X and Aggrecan) and proteins (Sox9, Col I and Col II) for endochondral ossification were monitored in the P0 and P5 cells by RT-PCR and immunocytochemistry, respectively.

Maintenance of nBMCs

Chicken nBMCs were culture on the monolayer and subcultured when they reached 70-80%. Three basic media, high-glucose DMEM (high), low-glucose DMEM (low) and DMEM/F-12 were employed, to which 10% (v/v) FBS and 1% (v/v) antibiotics solution were supplemented. Cell culture was conducted at 37℃, 5% CO_2 in a humidified atmosphere.

Assessment of chicken bone marrow cells senescence assay

Cell survival and senescence of nBMCs cultured were evaluated using a Senescence ß-Galactosidase Staining Kit. At the end of P5, nBMCs cultured in different media were provided for the assay and the number of senescent cells was counted under an inverted microscope.

Immunocytochemistry

nBMCs collected from P0 and P5 were washed with PBS, fixed with 4% (v/v) paraformaldehyde and subsequently permeabilized

with 0.1% (v/v) of Triton X-100. After washing, the cells were blocked with 10% (v/v) normal goat serum and treated with Sox9, Collagen type I and Collagen type II antibodies overnight at 4℃. After being treated with secondary antibody for 2 hours at room temperature, the fixed cells were stained in DAPI staining solution. Images were captured via fluorescent microscopy using software from NIS Elements, Inc.

3. Results

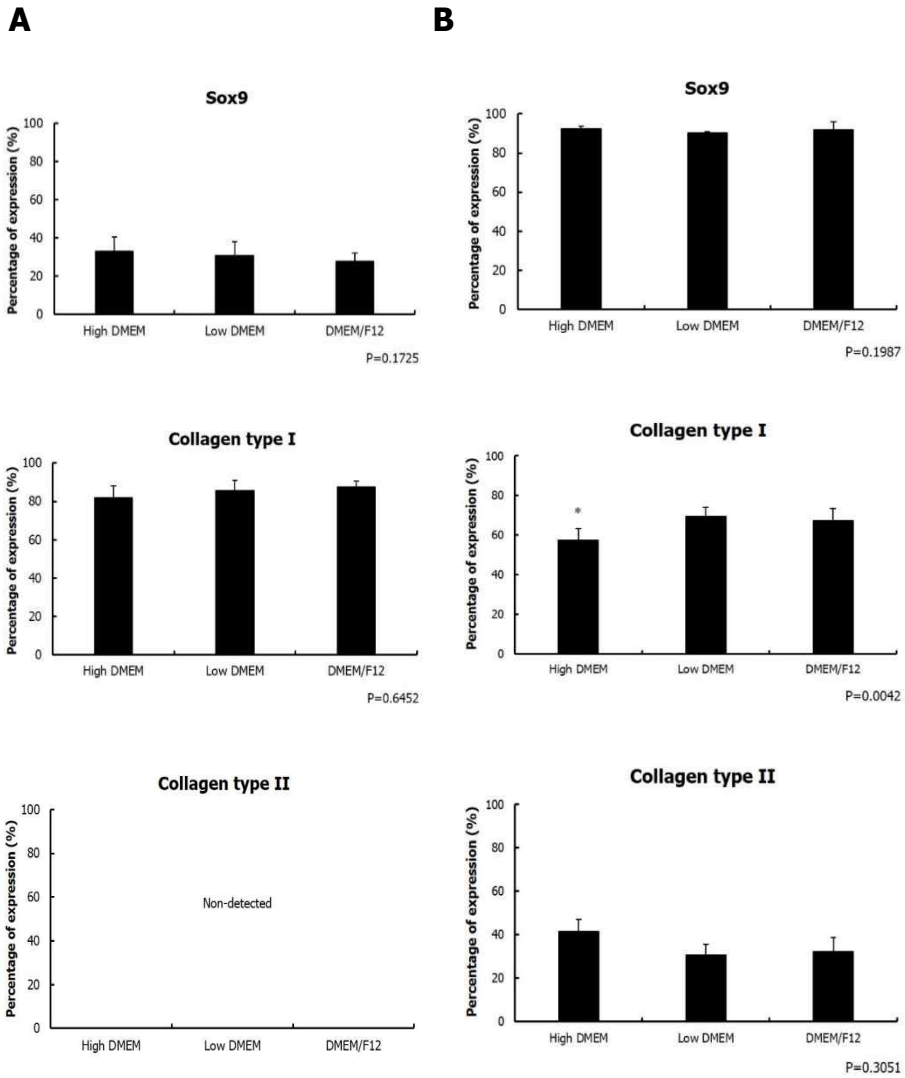
Whether the in vitro culture could expand the Sox9-rich nBMCs?

Comparing marker reactivity (Figure 8), different expression was detected between the P0 and the P5 cells. The P0 cells expressed Sox9 and Col I, not Col II. Col II became expressed with Sox9 and Col I in the P5 cells. In the P0 cells (Figure 8A), most cell were positive for Col I (85.0±1.6%) and Sox9 expression was within the range of 30.6±1.5%, regardless of media. In the P5 cells (Figure 8B), the percentage average of Sox9-positive, Col I-positive and Col II-positive cells to the total cell number was 91.7±0.5%, 64.3±3.6% and 34.4±3.4%, respectively. Significant ($p=0.0042$) effect of media was detected only in Col I expression and better expression was detected in low DMEM and DMEM/F12 than in high DMEM. Sox9 expression were dominant in the P5 cell, while Col I in the P0.

Whether the culture regime could be customized for enhancing cell maintenance and differentiation?

Figure 8. Expression of specific marker proteins for ostechondrogenesis in the bone marrow cells retrieved from 4-day-old chick femurs. Expressions of Sox9 and Sox9-related, collagen type I (Col I, fibrous cartilage marker) and collagen type II (Col II, proliferative chondrocyte marker) proteins were monitored in the bone marrow cells subcultured in different media, high glucose Dulbecco's minimal essential medium (high DMEM), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12). (A) Expression at the end of primary culture (P0) and their percentage of expression to total cultured cells. Only Sox9 and Col I were expressed, but the model effect of media on the expression was not detected in each markers. The percentage average of Sox9-positive and Col I-positive cells to the total cell number was 30.6 ±1.5% and 85.0±1.6%, respectively. (B) Expression at the end of P5. Expression of all three markers were detected. The percentage average of Sox9-positive, Col I-positive and Col II-positive cells to the total cell number was 91.7±0.5%, 64.3±3.6% and 34.4±3.4%, respectively. Only Col I expression was greatly ($p=0.0042$ of model effect) influenced by the medium, while the

others were not, and both low DMEM and DMEM/F12 yielded the largest number.



Comparing cell morphology, cuboidal cell became dominant population in the P5 cells regardless of culture media and as shown in Figure 9, no different morphology except for cell density was detected. Significant ($p=0.0207$) model effect was detected and more cells were maintained at the P5 in DMEM/F12 than in high DMEM.

Media effect become apparent when nBMC subculture were extended up to P12. As shown in Figure 10A, more ($p<0.0408$) cells were retained after the subculture with DMEM/F12 than after the subculture with high DMEM and low DMEM except for P0 ($p=0.313$). The cell number accumulated became plateau between P5 to P7, which seemed to be earlier in high DMEM compared with DMEM/F12. Significant ($p<0.0001$) effect of subculture was also detected in the actual proliferation of nBMC at the end of each passage. As shown in Fig 10B, greater proliferation was detected in P2 and P3 than in other passages, regardless of culture media. Minimized proliferation were detected from P8 in high DMEM and from P10 in low DMEM and the DMEM/F12. No more increase in cell number by nBMC subculture was detected from P11 in high DMEM and P12 in low

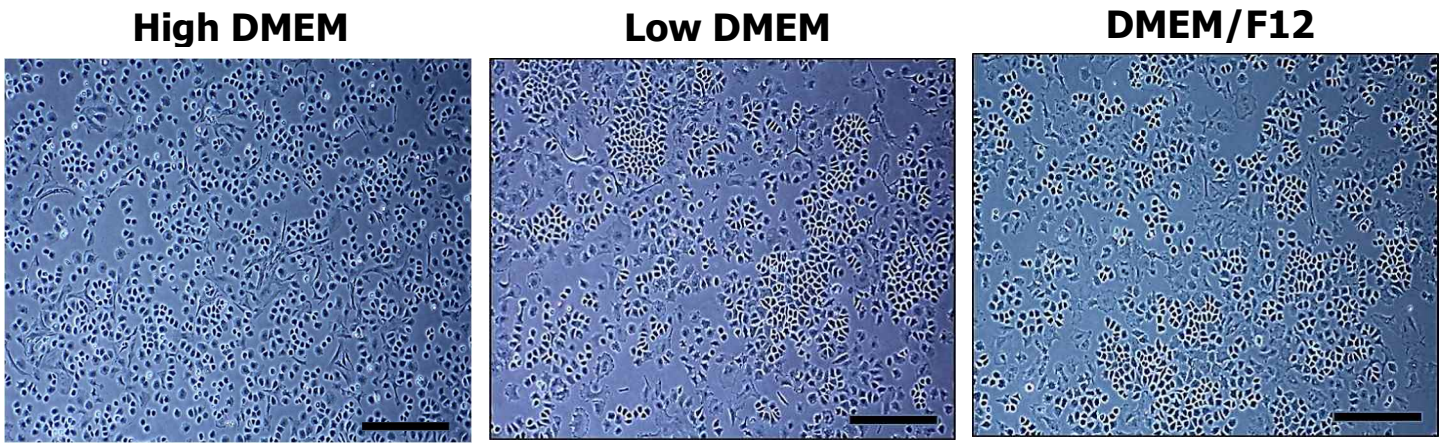
DMEM and DMEM/F12.

As shown in Figure 11, no significant difference in total cell number was detected (p=0.4789) and the number of CFU colonies was not different among the media. However, significant (p=0.0229) model effect was detected in the size of the colonies and DMEM/F12 yielded the largest colony than any others. Senescence assay showed that less senescent cells were derived from the subculture in DMEM/F12 than from the subculture in high and low DMEM (Figure 12). Significant (p=0.046) model effect was detected. When monitored gene expression in the P5 cells (Figure 13), all expressions of *Sox9*, *Runx2*, *Col I*, *Col II*, *Col X* and *Aggrecan*, the endochondral ossification-related genes tested in this study, were detected. Percentage of expression results show in the P5 cells were increased expression early osteochondrogenic related markers of Sox9 and Col I. Col II than P0 (Figure 14). As shown in Figure 15, both osteogenic and chondrogenic differentiation was detected. Significant (p=0.0058) model effect was detected in osteogenic differentiation and DMEM/F12 induced more than the others. Considering chondrogenic differentiation, larger mass of

differentiating cells was observed in DMEM/F12 than high or low DMEM

Figure 9. Morphology of the bone marrow cells retrieved from the femur of 4-day-old chicks. The bone marrow cells were cultured in different media and were subcultured at P5. High glucose Dulbecco's minimal essential medium (high DMEM), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12) was employed for the culture of bone marrow cells. (A) Regardless of culture media, the major population of the P5 cells and no different morphology except for cell density was detected. Scale bar=100μm. (B) Quantitative comparison of the numbers of the P5 cells maintained in different media. Significant model effect of media was detected and more cells were maintained at the P5 in DMEM/F12 than high DMEM and low DMEM. Different script indicated significant difference, $p<0.05$.

A


High DMEM
Low DMEM
DMEM/F12

B

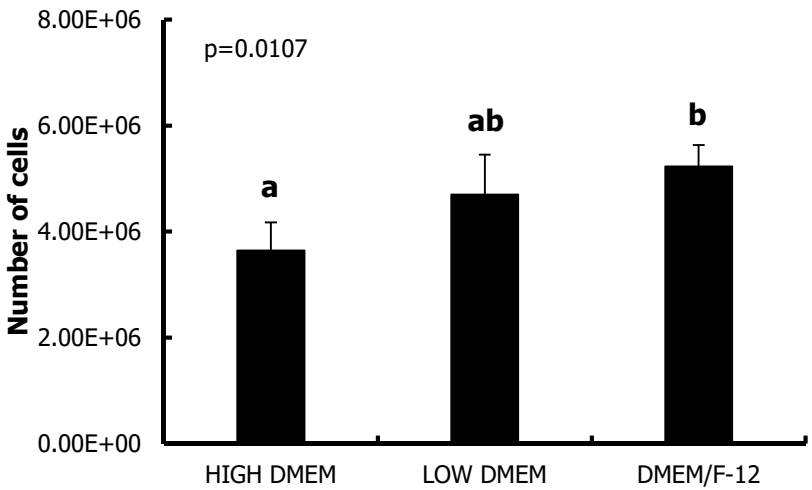

8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Number of cells
p=0.0107
a
ab
b
HIGH DMEM
LOW DMEM
DMEM/F-12

Figure 10. Proliferation of the bone marrow cells retrieved from the femur of 4-day-old chicks following subculture in vitro. The bone marrow cells were cultured in different media, high glucose Dulbecco's minimal essential medium (high DMEM), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12). Total cell number was counted at the end of each passage up to 12 and either cumulative cell number since P0 culture **(A)** or actual cell number at the end of each passage (B) was compared among the media. **(A)** Significant ($p<0.0408$) model effects of the media were detected from P1 and DMEM/F12 yielded the largest cells. Actual cell number after culture in high DMEM **(B1)**, low DMEM **(B2)** and DMEM/F12 **(B3)**. Significant ($p<0.0001$) model effect was detected in each parameter. Greater proliferation was detected in P2 and P3 than in other passages, regardless of the kinds of medium, while minimal proliferation was detected from P8 in high DMEM and from P10 in low DMEM and the DMEM/F12. Increase in cell number by subpassage was not detected from P11 in high DMEM and P12 in low DMEM and DMEM/F12.

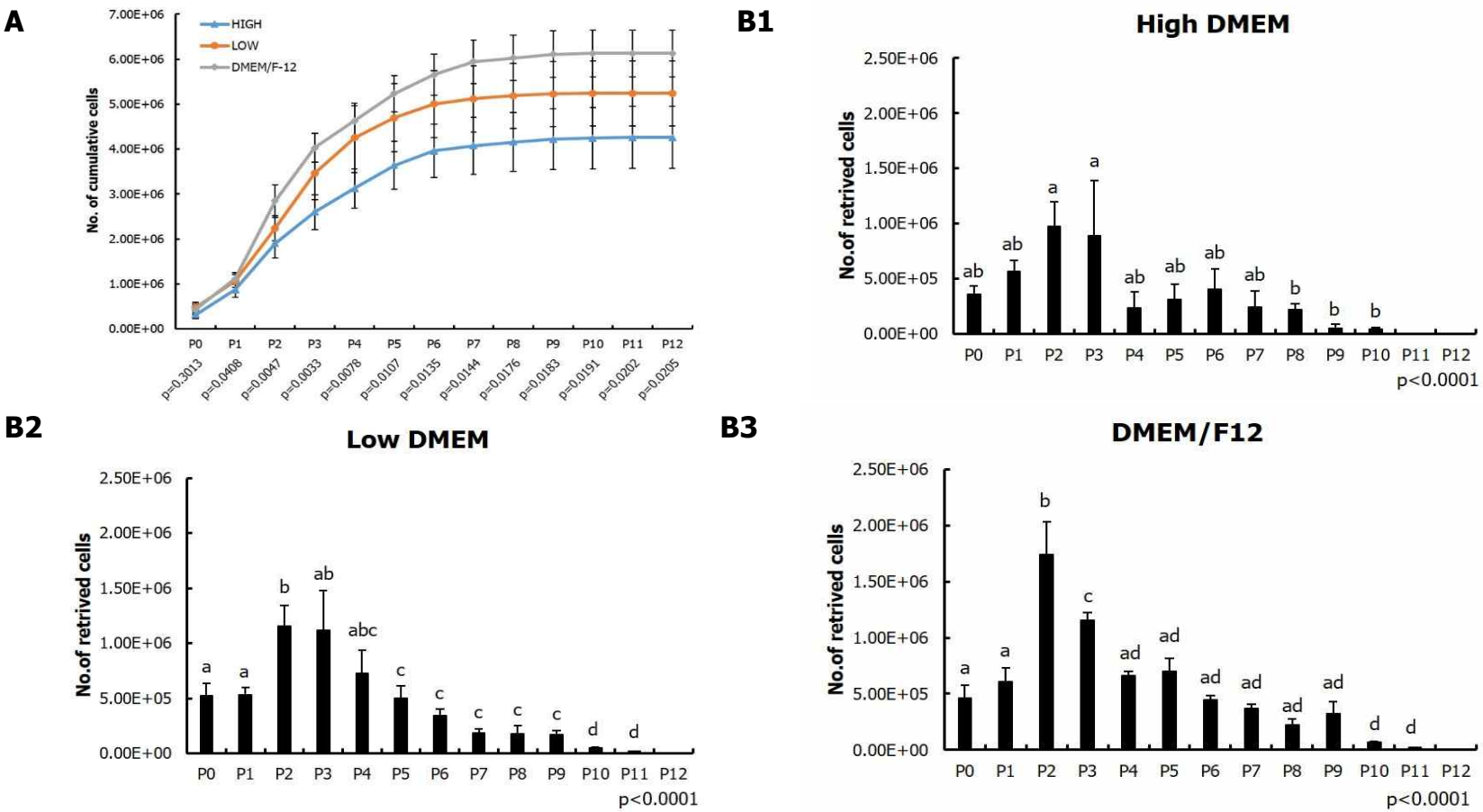
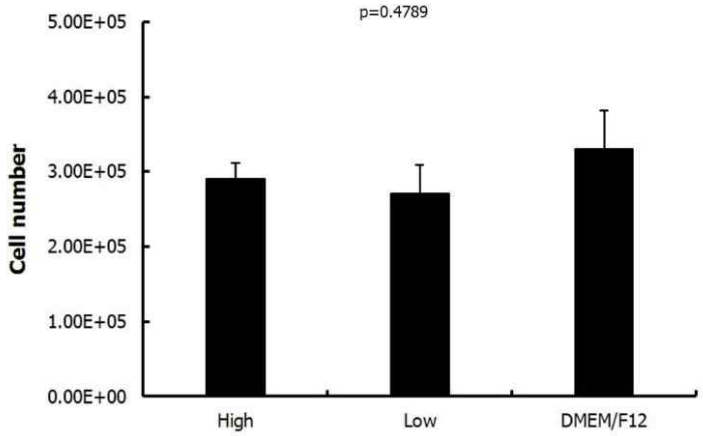
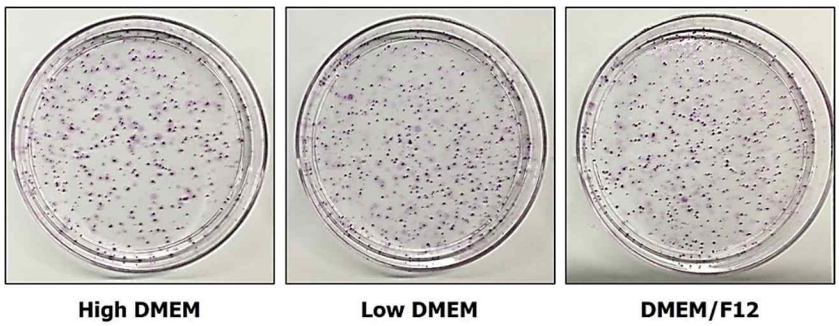

A
HIGH
LOW
DMEM/F-12
No. of cumulative cells
7.00E+06
6.00E+06
5.00E+06
4.00E+06
3.00E+06
2.00E+06
1.00E+06
0.00E+00
P0 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12
p=0.3013 p=0.0408 p=0.0047 p=0.0033 p=0.0078 p=0.0107 p=0.0135 p=0.0144 p=0.0176 p=0.0183 p=0.0191 p=0.0202 p=0.0205
B1
High DMEM
No.of retrived cells
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
ab ab a a ab ab ab ab b b b
P0 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12
p<0.0001
B2
Low DMEM
No.of retrived cells
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
a a b ab abc c c c c c d d
P0 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12
p<0.0001
B3
DMEM/F12
No.of retrived cells
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
a a b c ad ad ad ad ad ad d d
P0 P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12
p<0.0001

Figure 11. Seeding efficiency of chick bone marrow cells retrieved from 4-day-old femur. The bone marrow cells were cultured in high glucose Dulbecco's minimal essential medium (high DMEM), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12). (A) One million cells retrieved at the end of primary culture were subsequently seeded into a well of 6-well plate and the cell number was counted at the end of the 7 day. (B1 and B2) Colony-forming activity of the bone marrow cells, which was evaluated by colony forming unit (CFU) analysis. One million cells retrieved at the end of primary culture were seeded into 100-mm cell culture plate and crystal violet staining was performed to detect single cell-derived clones 14 days after culture. Positive staining was defined as a colony that contained 50 positive cells on average. Significant ($p=0.0229$) model effect of media was detected and DMEM/F12 yielded the best forming activity (B2). The number of the CFU colonies were not different significantly among the media (B1).

A


p=0.4789
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
Cell number
High
Low
DMEM/F12

B1


High DMEM
Low DMEM
DMEM/F12

B2

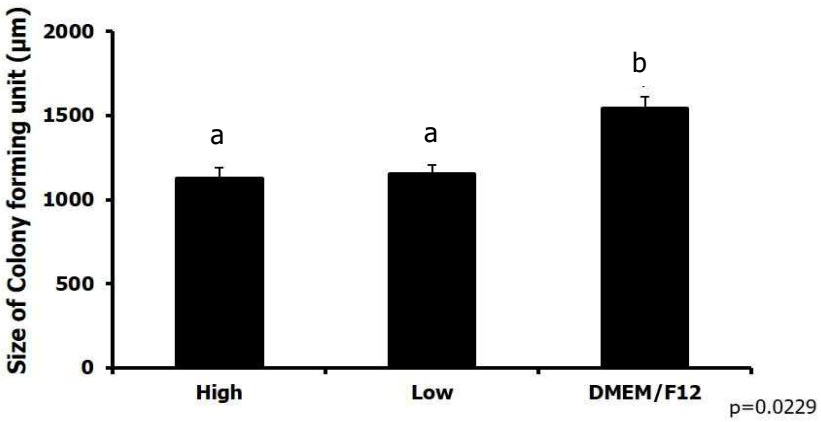
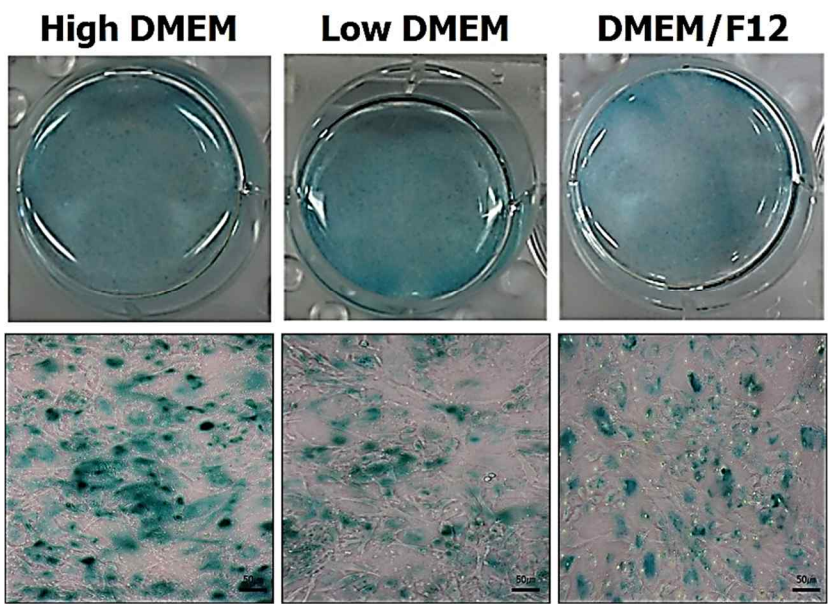

Size of Colony forming unit (μm)
2000
1500
1000
500
0
a
a
b
High
Low
DMEM/F12
p=0.0229

Figure 12. Senescence of the bone marrow cells cultured in three different media. The bone marrow cells retrieved from 4-day-old chick femur were cultured in different media, high glucose Dulbecco's minimal essential medium (high DMEM), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12). β-galactosidase (SA-β-Gal) was used for the senescence assay for the bone marrow cells cultured to passage 5 and the number of SA-β-Gal positive cells was counted. Significant model effect was detected and both low DMEM and DMEM/F12 yielded less senescent cells than high DMEM. Images showed the cells positive for SA-β-Gal or not. Data were indicated as Mean±SE. Scale bars =200μm.


High DMEM
Low DMEM
DMEM/F12

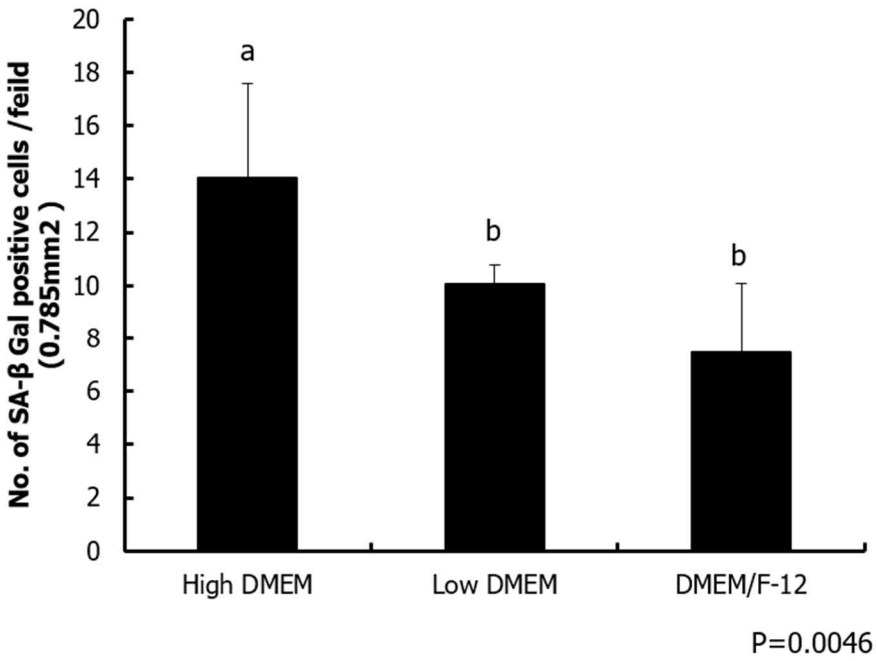
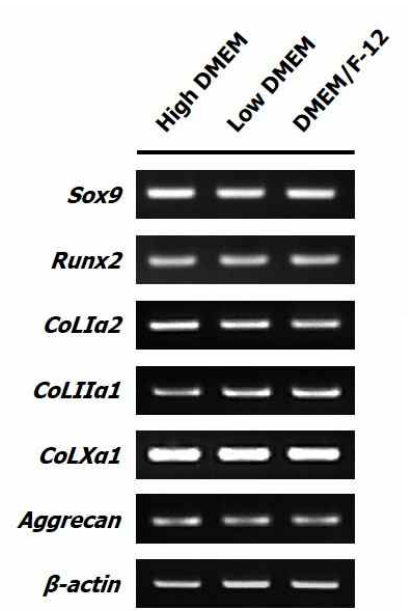
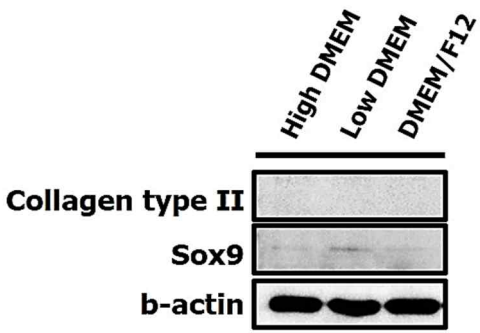

No. of SA-β Gal positive cells /feild
(0.785mm2)
20
18
16
14
12
10
8
6
4
2
0
a
b
b
High DMEM
Low DMEM
DMEM/F-12
P=0.0046

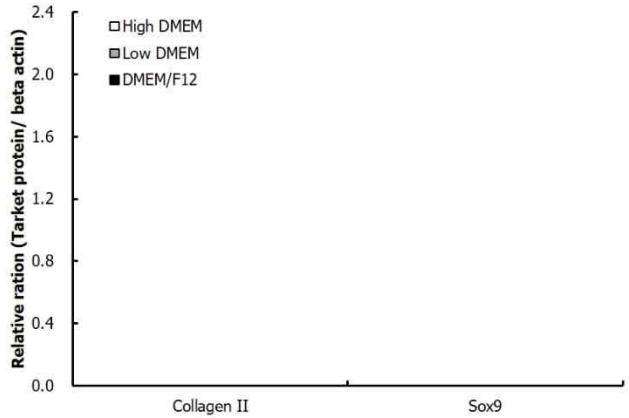
Figure 13. Assessment of gene and protein expression of the bone marrow cells cultured in different media until passage 5 (P5). The bone marrow cells retrieved from 4-day-old chick femur were cultured in different media, high glucose Dulbecco's minimal essential medium (high DMEM), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12). (A) RT-PCR analysis showing that ostengenic and chondrogenic related genes such as *Runx2, Sox9, Col I, Col II, Col X*, *Aggrecan* were expressed in the cells collected from P5. *β-actin* was positive control. Comparisons of protein expression analysis for the bone marrow cells collected at the end of primary culture (B) and the P5 cells (C). Western blot results showed Col II and Sox9 protein level increased in the P5 cells, while no or weak expression of Col II and Sox9 was detected in P0.

A (At P5)

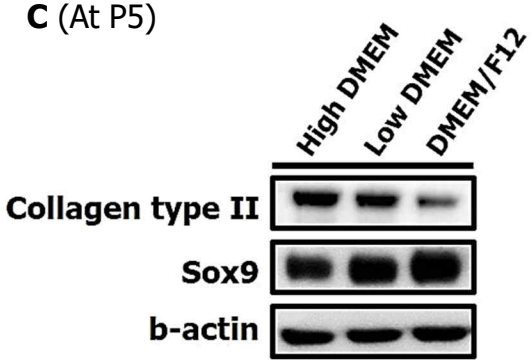


B (Primary)





C (At P5)



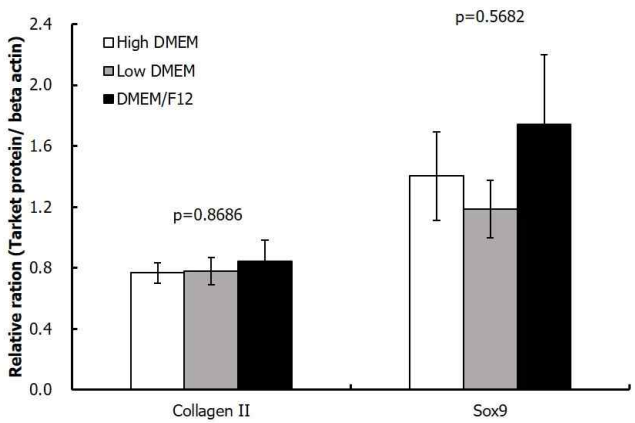
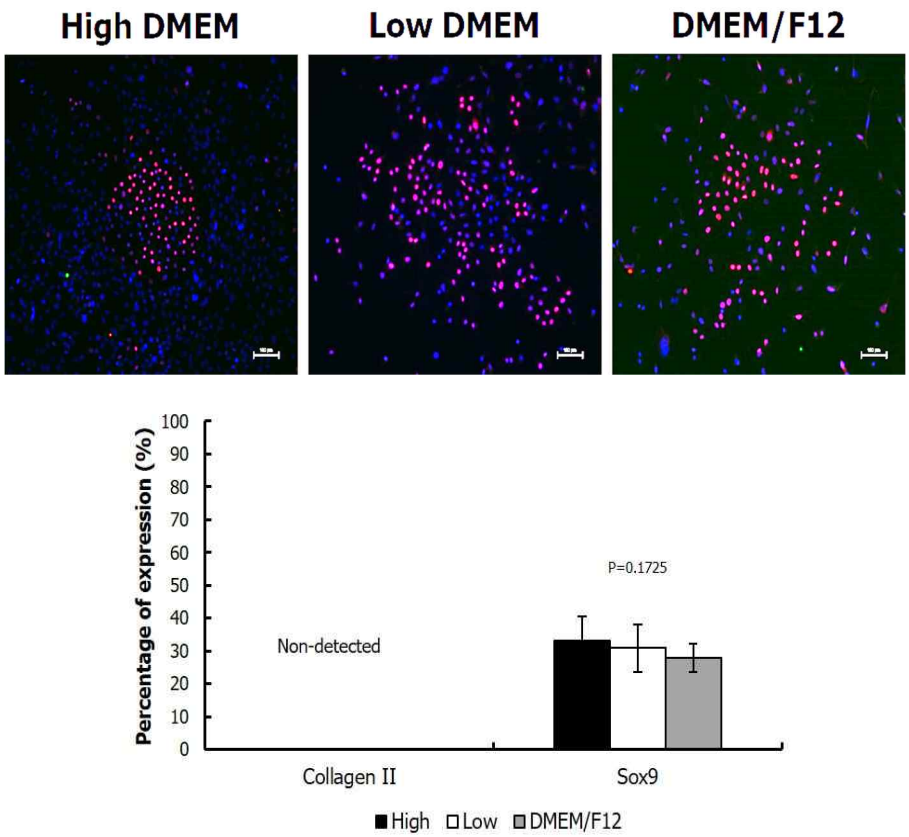
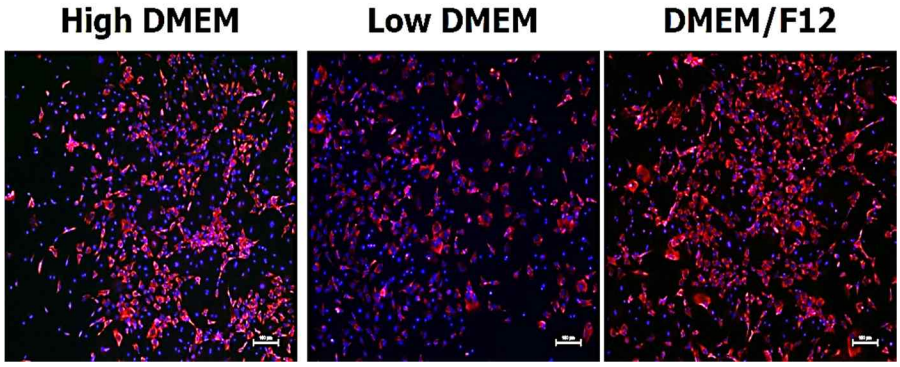


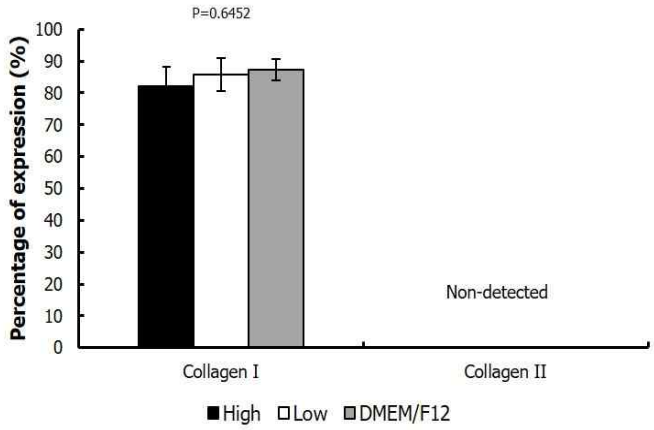
Figure 14. Immunocytochemistry analysis of the bone marrow cells retrieved from 4-day-old chick femur at passage 0 and cultured in different media until passage 5 (P5). (A) Surface antigen characterization of osteochondrogenic related marker such as Sox9, Col I, Col II expression. (B) Percentage of expression results show the bone marrow cells at passage 0 are expressed early osteochondrogenesis-related markers Sox9 and Col I. Col II, a marker of advanced stage of development, was not expressed at passage 0. Statistically significant different were not observed among the groups. (C) Surface antigen characterization of osteochondrogenic related marker such as Sox9, Col I and Col II expressions. Model effect of media on the expression was not detected ($p>0.1119$). (D) Percentage of expression results show in the P5 cells were expressed early osteochondrogenic related markers of Sox9 and Col I. Col II, a marker of an advance stage of chondrogenesis was also expressed in the P5 cells. Significant ($p=0.0042$) model effect of media on the marker expression was only detected in Col I. Data were indicated as Mean±SE.

A

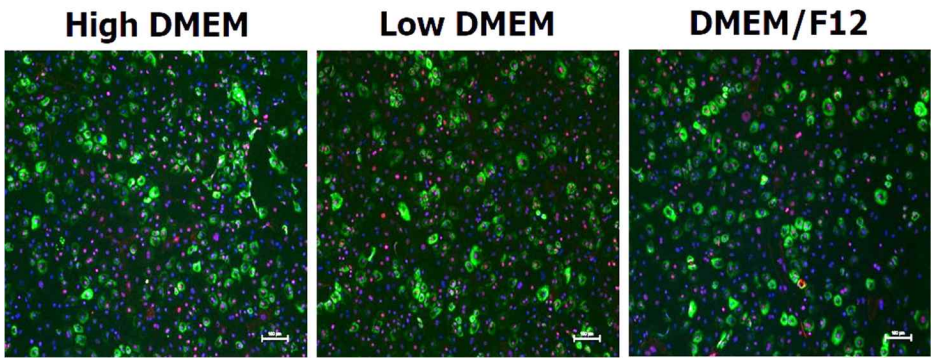

High DMEM
Low DMEM
DMEM/F12
Percentage of expression (%)
100
90
80
70
60
50
40
30
20
10
0
P=0.1725
Non-detected
Collagen II
Sox9
High
Low
DMEM/F12

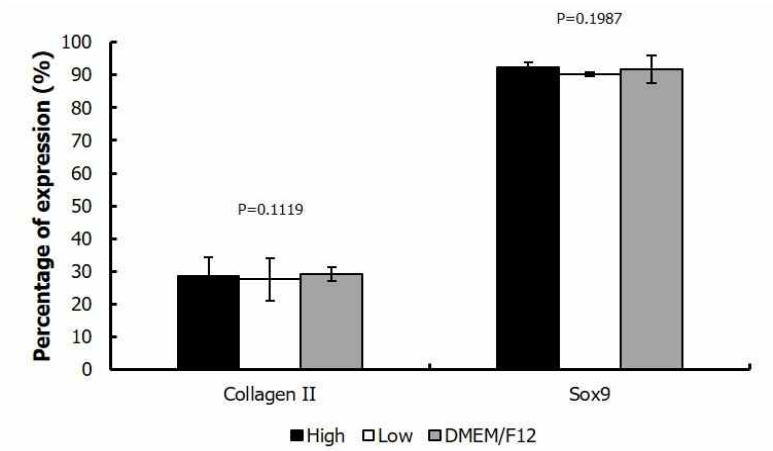
B


High DMEM
Low DMEM
DMEM/F12

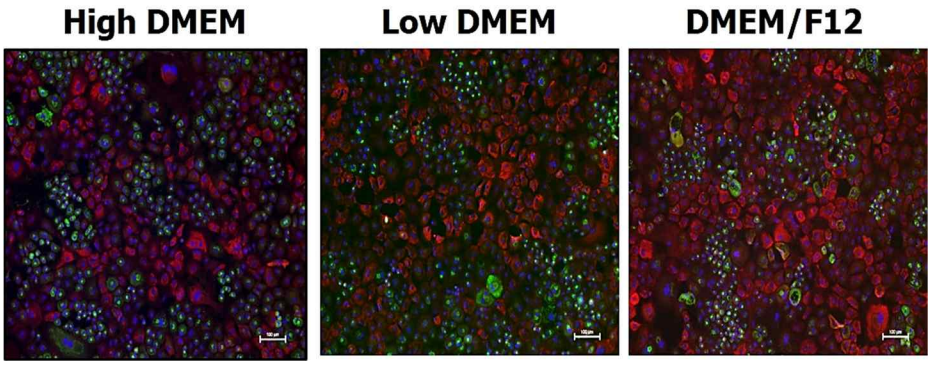

P=0.6452
Percentage of expression (%)
100
90
80
70
60
50
40
30
20
10
0
Non-detected
Collagen I
Collagen II
High
Low
DMEM/F12

C


High DMEM
Low DMEM
DMEM/F12


P=0.1987
P=0.1119
Percentage of expression (%)
100
90
80
70
60
50
40
30
20
10
0
Collagen II
Sox9
High
Low
DMEM/F12

D


High DMEM
Low DMEM
DMEM/F12

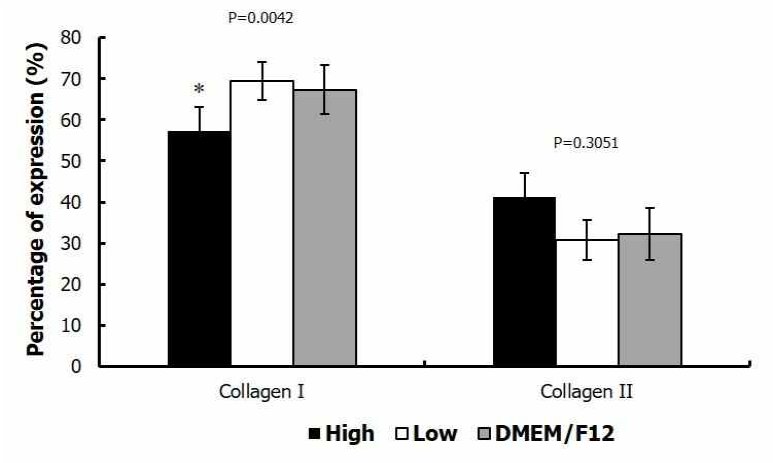

P=0.0042
P=0.3051
*
Percentage of expression (%)
80
70
60
50
40
30
20
10
0
Collagen I
Collagen II
High
Low
DMEM/F12

Figure 15. Differentiation of the bone marrow cells cultured in different media until passage 5 (P5). The bone marrow cells retrieved from 4-day-old chick femur were cultured in different media, high glucose Dulbecco's minimal essential medium (high DMEM), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12). (A) Osteogenic differentiation. Total $3x10^5$ cells at passage 5 were seeded into 6-well plate containing an osteogenesis induction medium and were subsequently cultured for 21 days. Alizarin Red S dye was used for the detection of differentiation. The morphology of the cultured cells was monitored under an inverted microscope. Significant ($p=0.0058$) model effect was detected and DMEM/F12 induced more osteogenic differentiation than the others. Data are presented as Mean±SE. Scale bars=200 μm. (B) Chondrogenic differentiation. The $2.5x10^5$ P5 were seeded into 15ml conical tube and pellet culture in a chondrogenic induction. Twenty-one days after culture, the cells were stained with Alcian blue dye. More induction was observed in DMEM/F12 than high or low DMEM. Scale bars =200 μm.

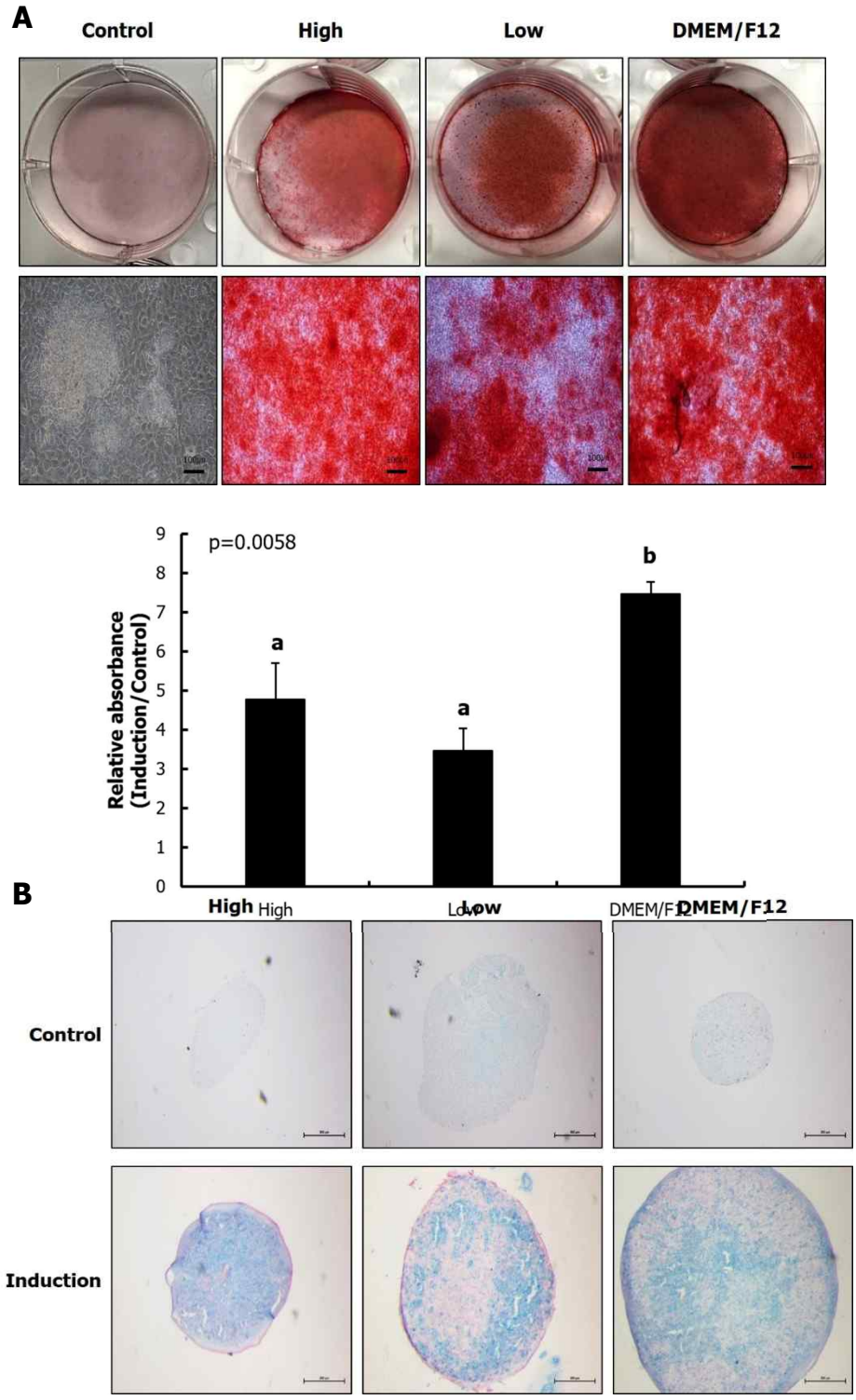
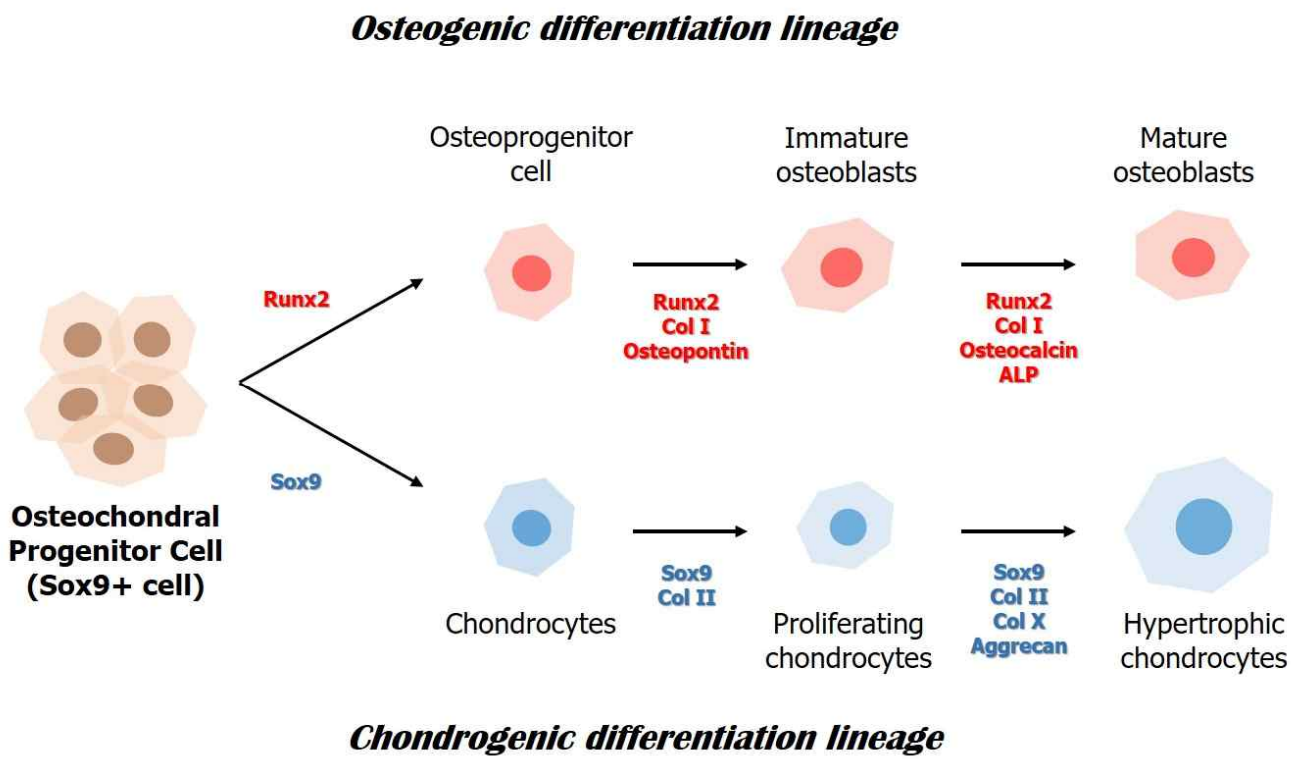

A
Control
High
Low
DMEM/F12
p=0.0058
Relative absorbance
(Induction/Control)
a
a
b
B
Control
Induction

Figure 16. Development of Chicken 4-day-old Sox9 positive cells. Schematic representation of Sox9 positive cells development and expression main molecular markers at each stage.



4. Discussion

In chicken, various methods for culturing bone marrow cells have been studied. It has been reported in above 11weeks old chicken that culture environment and isolated cell characterization. In this study, use of 4-day-old chicken bone marrow derived cell compared with previous studies confirmed the applicability of the culture system. Since these two different age chickens are showed different cell morphology and characteristics, my results further expanded the feasibility of the 4-day-old chicken bone marrow progenitor cells culture system for the research of various purpose.

Endochondral ossification began with the deposition of collagen type I by cells derived from bone marrow on the surface of the eroded cartilage. The transcription factor Sox9 is essential for early chondrogenesis, and abnormal Sox9 expression causes severe abnormal regulation of endochondral ossification (Hata et al., 2017). SOX9 is involved in the control of the activation of Collagen II in chondrocytes, an essential signaling of the differentiation program (Lefebvre et al., 1997) (Figure 16).

Two major findings were derived from the results of this

study. First, the subculture of neonatal bone marrow cells specifically amplifies both Sox9− and Col II−positive cells, which resulted Sox9−positive cells became dominant in the P5 cells, switching from the Col I dominant in nBMCs at the P0. Intact nBMCs contained considerable number of Sox9−positive cells (30% of total), but no Col II−positive cells were detected. Second, use of DMEM/F12 medium could optimize the outcome of nBMC subculture, which further contributed to customizing nBMC−mediated, differentiation protocol for both osteogenesis and chondrogenesis

The nBMCs maintained by subculture were cuboidal, which appeared as early as from the primary culture. Considering the results of histological observation, the morphology, as well as cell fraction, of nBMCs might different from that of adult BMCs. Culture of adult BMCs usually yielded stromal cell−dominant population, while not in this study employing neonatal chicken. However, the results of this study demonstrated that media and culture protocols did not affect the morphology of nBMC maintained in vitro and cuboidal cells were dominant throughout the culture. Changing in basic culture media may majorly influence cellular activity.

Apparent difference between the P0 cells and the P5 cells was osteochondrogenesis-related protein expression, which demonstrated that in vitro-manipulation of nBMCs might be beneficial for activating (or inhibiting) specific cell function of nBMCs. From the results of this study, nBMC culture can be used for preparing Sox9-positive fraction. Sox9-positive cells are a pivotal role in osteochondrogenesis, which is a progenitor cell of Col I-positive cells and Col II-positive cells. The co-expression of Sox9 and Col II was detected with that of Col I and Col II in the P5 cells which supported our finding that nBMCs maintained in vitro preferentially induced both osteogenic and chondrogenic differentiation, not adipogenic differentiation.

Use of DMEM/F12 as a basic medium for nBMC culture can be recommended from the results of this study. Noting no changing of gene expression profiles among the media tested in this study, changing of basic medium from high DMEM to DMEM/F12 mainly serves as the activator of cell proliferation. However, this beneficial function directly to enhance osteogenic or chondrogenic differentiations of nBMCs, although they were mixed population of the cells involving endochondral ossification and mesenchymal cells. Tissue regeneration requires lots of the

cells from different lineages and biomaterials for completion of matrix formation and function of the tissue engineered, and our results may suggest the contribution of nBMCs from different viewpoints.

CHAPTER 6.

Effects of Basic Fibroblast Growth Factor (bFGF) on the Proliferation and Differentiation of Sox9 Positive Cells in 4-Day-Old Chicken

1. Introduction

Fibroblast growth factors (FGFs) involved in developmental pathways like mesoderm patterning during the early embryo through to the development of multiple organ systems (Nutt et al., 2001; Deimling and Drysdale, 2011). Also FGF signaling extends on many physiological roles in the adult organism, including the regulation of the angiogenesis and wound repair (Ishihara et al., 2006). The major function of FGF signaling in bone, FGF2 and FGF9 transcripts are found in mesenchymal cells and osteoblasts (Marie, 2003). FGF18 is expressed in mesenchymal cells, in differentiating osteoblasts during bone development and in the long bones (Ohbayashi et al., 2002).

Regenerative medicine is important as it can stop or slow the progression of disease. Biologic and cellular therapies help improve the efficiency of traditional tools and reduce the need for invasive surgeries. Sox9 positive cells in bone marrow, which are also known as osteochondrogenic progenitor cells, are considered as the basic material for clinical application to injuries and degenerative diseases. In osteo and chondro progenitor cells,

Sox9 as a transcriptional factor is a key regulator signaling that responsible for expression of Runx2, Collagen type I, Collagen type II, Collagen type X, aggrecan and the other multiple ECM related markers according to each differentiation lineage. For clinical implementation of sox9 positive cells, their more homogeneous isolation and abundant and stable maintenance is important for optimizing manipulation protocol of chicken sox9 positive cells and the large-scale expanded culture system has been used to high reproducibility of cells. And also it become a valuable resource for cell-to-cell tissue regeneration and experimental modelling for differentiation and reprogramming.

In mouse bone formation, overexpressed FGF2 signaling causes abnormal bone development, but its inhibition of this signal leads to malformation of bones (Iseki et al., 1997). Also FGF signaling regulates cell proliferation and osteoblast precursor cells replication into osteoblasts and controlled apoptosis of immature osteoblasts (Mansukhani et al., 2000). FGFs signaling interacts with other growth factors signaling and regulates osteoblast function. FGF2 and FGFR2 resulting in increased BMP4 activity, therefore can control cranial suture

fusion through BMP signaling (Kim et al., 1998). Activation of FGF signaling can regulate genes involved at full steps of osteogenesis. However, optimal concentration and role of the bFGF in promoting the proliferation of sox9 positive cells derived from chicken bone marrow has not been reported. In this study, I conducted to suggest the optimal concentration of bFGF on the multi cellular activity of chicken bone marrow derived sox9 positive cells.

2. Materials and Methods

Experimental design

In experiment 1, the sox9 positive cells retrieved from 4-day-old chicks were subcultured and cell morphology and proliferation profile were monitored insertion on different concentrations of bFGF. In experiment 2, According to insertion of bFGF, the reactivity of the specific markers like sox9 and osteogenic and chondrogenic related gene expression of the P0 and P5 cells were evaluated. The expression of specific genes for osteo-chondrogenesis (Sox9, Col I, Col II, Col X and Aggrecan) was subsequently monitored. In experiment 3, osteogenic and chondrogenic differentiation of the P5 cells was induced. And finally, I conducted on effect of bFGF to functional ability of sox9 positive cells.

Assessing of cell proliferation during in vitro-culture of cells

Cell number was monitored at the end of each passage up to 12 passages according to different concentration of bFGF. Both the

cell number accumulated from primary passage were counted. Cell counting kit-8(CCK-8) analysis of the sox9 positive cells was detected at day 1, 3, 5, 7, 10.

Scratch Wound healing assay

The cells were seeded onto six-well plates and cultured at 37℃ in a 5% CO2 cell incubator. After the cells reached 70%-80% confluence, scratch assay conducted upper to down direction carefully using a 200-μL sterile pipette tip. Then cell culture plates were washed three times with sterile PBS to remove the scratched cells. The cells were continuously cultured in serum-free culture medium. After 18 h, the cells were photographed and detects the size of scratch recovery. Cell migration distance = distance at start time - distance at end of after 18 h.

Invasion assay

The retrieved cell suspension is placed in upper chamber. Then after 24hours, invasive cell pass through basement membrane layer and cling to the bottom of the insert membrane. Non-

invasive cells stay in the upper chamber. After removal of non-invasive cells, invasive cells are stained using crystal violet and photographed and count of the cells.

3. Results

bFGF stimulates the proliferation of sox9 positive cells in vitro

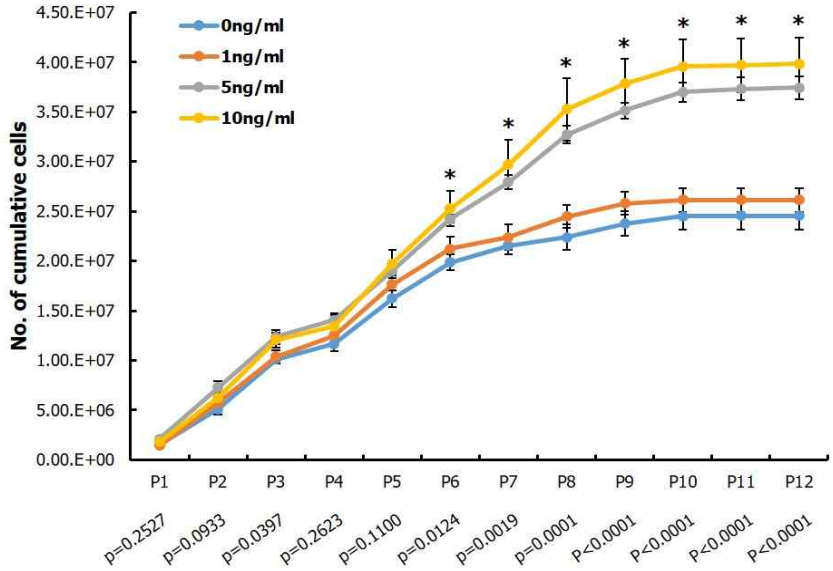
To investigate the effect and optimal concentration of bFGF on the proliferation of sox9 positive cells, I seeded the cells into 6-well plates with culture medium supplemented with the different concentrations of bFGF (0, 1, 5 and 10 ng/mL) (Fig 17-A). Cumulative cell number at each passage for up to 12 passage was employed for statistical analysis. A significant model effect was detected from passage 6 and better proliferation was detected at 5 or 10 ng/ml than 0 or 1 ng/ml. Asterisk indicate significant model effect ($p<0.05$).

Furthermore, the CCK-8 data showed that, passage 5 bone marrow cells exposed continuously to 0, 1, 5 or 10 ng/ml bFGF were provided for CCK-8 assay on day 1, 3, 5, 7 and 10 of culture. Significant model effect was detected from day 7 of culture (Fig 17-B).

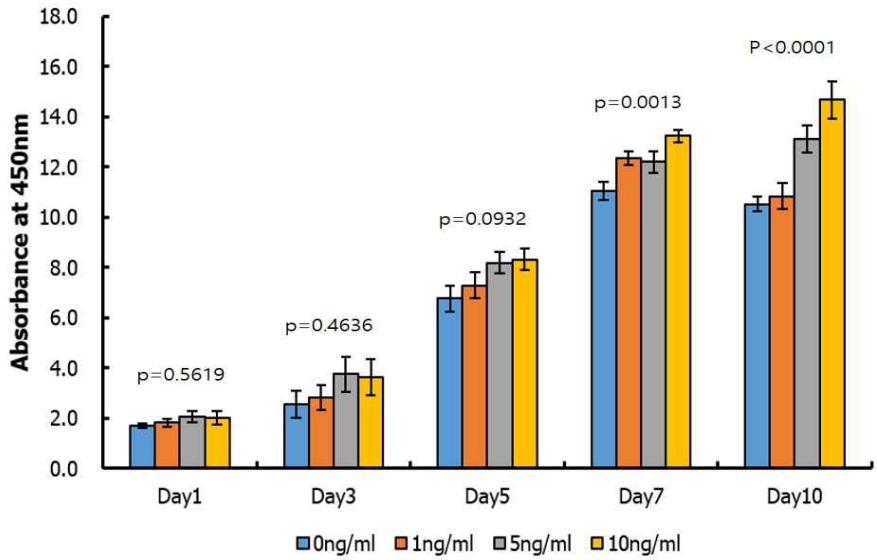
Morphology of 4-day-old, chick sox9 positive cells exposed to bFGF

Figure 17. Proliferation profile for up to passage 12 and cellular activity at passage 5 of 4-day-old, chick bone marrow cells. The bone marrow cells were cultured in nutrient mixture of DMEM/F12 medium, to which 0, 1, 5 or 10 ng/ml basic fibroblast growth factor (bFGF) was added, and the number of cells was counted at the end of each passage. (A) Cumulative cell number at each passage for up to 12 passage was employed for statistical analysis. A significant model effect was detected from passage 6 and better proliferation was detected at 5 or 10 ng/ml than 0 or 1 ng/ml. Asterisk indicate significant model effect ($p<0.05$) (B) The passage 5 bone marrow cells exposed continuously to 0, 1, 5 or 10 ng/ml bFGF were provided for CCK-8 assay on day 1, 3, 5, 7 and 10 of culture. Significant model effect was detected from day 7 of culture.

A


No. of cumulative cells
4.50.E+07
4.00.E+07
3.50.E+07
3.00.E+07
2.50.E+07
2.00.E+07
1.50.E+07
1.00.E+07
5.00.E+06
0.00.E+00
0ng/ml
1ng/ml
5ng/ml
10ng/ml
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
p=0.2527
p=0.0933
p=0.0397
p=0.2623
p=0.1100
p=0.0124
p=0.0019
p=0.0001
P<0.0001
P<0.0001
P<0.0001
P<0.0001

B


Absorbance at 450nm
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
p=0.5619
p=0.4636
p=0.0932
p=0.0013
P<0.0001
Day1
Day3
Day5
Day7
Day10
0ng/ml
1ng/ml
5ng/ml
10ng/ml

Morphology of the bone marrow cells at the end of passage 0 and 5 was observed under an inverted microscope. Increased density of cuboidal cells was noticed after 1 or 5 ng/ml bFGF exposure compared with no exposure (Fig 18).

bFGF effects on the Sox9 and osteogenic and chondrogenic related gene expression of cultured sox9 positive cells

Expression of osteogenesis-related or chondrogenesis-related genes in sox9 positive cells. Quantitative RT-PCR was conducted to detect the expression of Sox9, Runx2, Collagen type I, Collagen type II, Collagen type X and Aggrecan. Significant model effect was detected in all genes evaluated and exposure to bFGF increased gene expression. However, the optimal level of exposure is different (Fig 19). Detection of Sox9 expression in cultured cells at the end of passage 5 culture by immunocytochemistry. 0, 1, 5 or 10 ng/ml basic fibroblast growth factor (bFGF) was added and Sox9 expression were monitored. There were no significant model effects of different concentration of bFGF on each expression ($p=0.4361$) (Fig 20).

Figure 18. Morphology of 4-day-old, chick bone marrow cells exposed to basic fibroblast growth factor (bFGF) after being retrieved from the long bone. The bone marrow cells were seeded to a 100-mm culture dish and subsequently cultured in nutrient mixture DMEM/F12 medium, to which 0, 1, 5 or 10 ng/ml bFGF was added. Morphology of the bone marrow cells at the end of passage 0 and 5 was observed under an inverted microscope. Increased density of cuboidal cells was noticed after 1 or 5 ng/ml bFGF exposure compared with no exposure. Scale bar=250 μm.

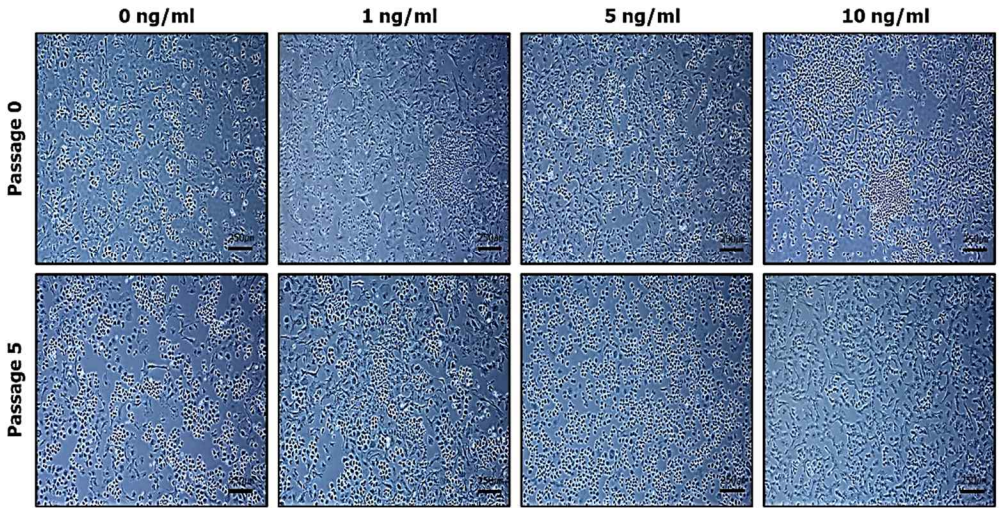
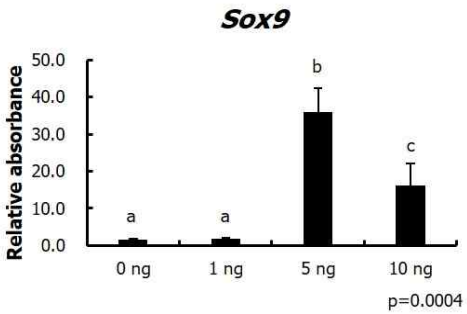
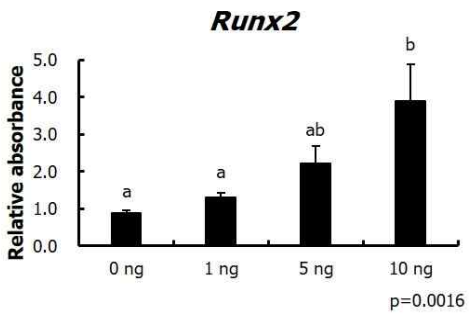
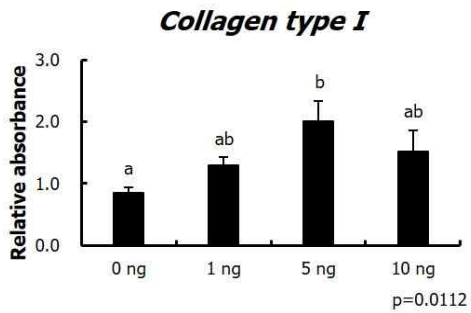
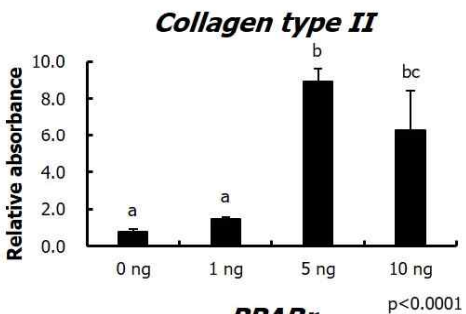


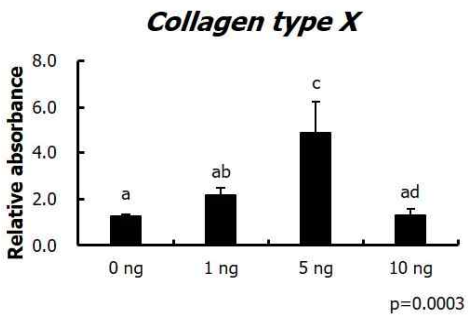
Figure 19. Expression of osteogenesis-related or chondrogenesis-related genes in 4-day-old chick bone marrow cells. The bone marrow cells were cultured for up to 5 passage in nutrient mixture of DMEM/F12 medium to which 0, 1, 5 or 10 ng/ml basic fibroblast growth factor (bFGF) was added and quantitative RT-PCR was conducted to detect the expression of Sox9, Runx2, Collagen type I, Collagen type II, Collagen type X and Aggrecan. Significant model effect was detected in all genes evaluated and exposure to bFGF increased gene expression. However, the optimal level of exposure wass different.

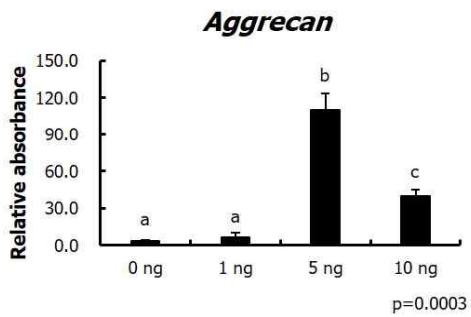

Sox9
Relative absorbance
50.0
40.0
30.0
20.0
10.0
0.0
a
a
b
c
0 ng
1 ng
5 ng
10 ng
p=0.0004


Runx2
Relative absorbance
5.0
4.0
3.0
2.0
1.0
0.0
a
a
ab
b
0 ng
1 ng
5 ng
10 ng
p=0.0016


Collagen type I
Relative absorbance
3.0
2.0
1.0
0.0
a
ab
b
ab
0 ng
1 ng
5 ng
10 ng
p=0.0112


Collagen type II
Relative absorbance
10.0
8.0
6.0
4.0
2.0
0.0
a
a
b
bc
0 ng
1 ng
5 ng
10 ng
p<0.0001


Collagen type X
Relative absorbance
8.0
6.0
4.0
2.0
0.0
a
ab
c
ad
0 ng
1 ng
5 ng
10 ng
p=0.0003


Aggrecan
Relative absorbance
150.0
120.0
90.0
60.0
30.0
0.0
a
a
b
c
0 ng
1 ng
5 ng
10 ng
p=0.0003

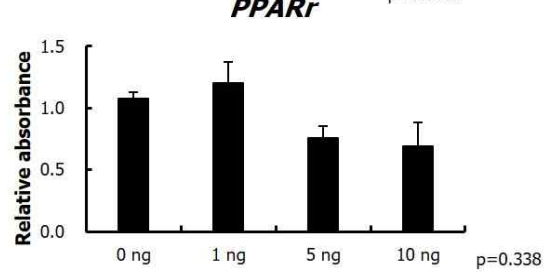
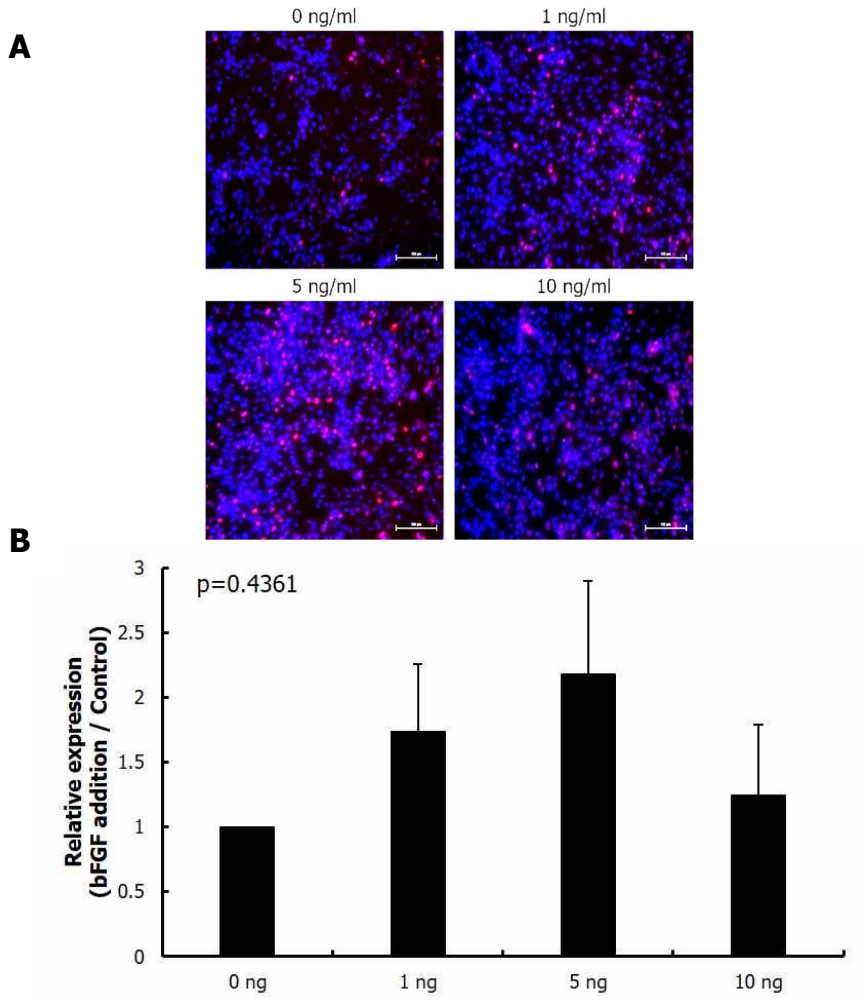

PPARr
Relative absorbance
1.5
1.0
0.5
0.0
0 ng
1 ng
5 ng
10 ng
p=0.338

Figure 20. Detection of Sox9 expression in 4-day-old, chick bone marrow cells at the end of passage 5 culture by immunocytochemistry. The bone marrow cells were cultured in nutrient mixture of DMEM/F12 medium to which 0, 1, 5 or 10 ng/ml basic fibroblast growth factor (bFGF) was added and Sox9 expression were monitored. There were no significant model effects of different concentration of bFGF on each expression. Data were indicated as Mean±SE.



bFGF stimulates the differentiation ability of sox9 positive cells in vitro

In osteogenic differentiation, calcium deposition and mineralization were detected in monolayers. More differentiation was detected at 5 ng/ml and 10ng/ml than at other levels (Fig 21). And also chondrogenic differentiation, signals on differentiation showing blue color was weak, but noticeable at certain level of bFGF (Fig 22).

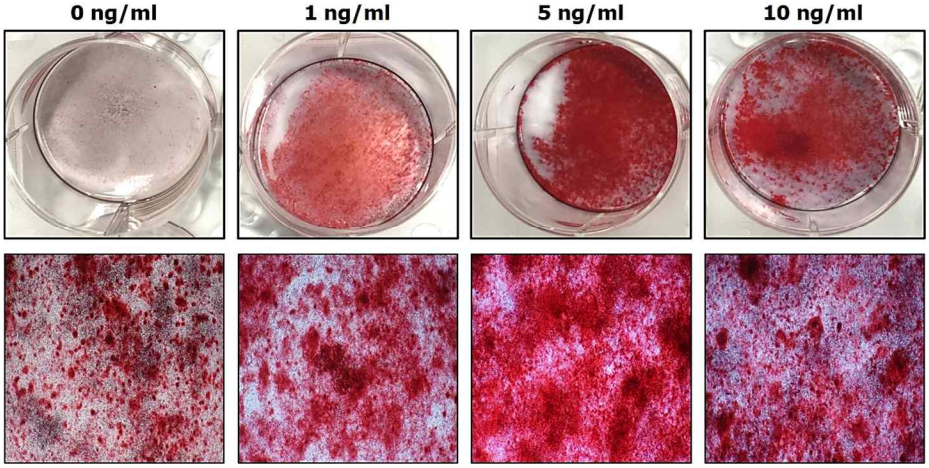
bFGF effects on the invasion assay and transwell cell migration *of cultured sox9 positive cells*

The invasion assay and transwell cell migration provides thorough analysis of the ability of cells to sense a particular chemo-attractant and migrate through a physical barrier toward it (Justus et al., 2014). Exposure of 1 and 5 ng/ml bFGF to the bone marrow cells subcultured improved migration ability, which was evaluated by the response to scratch 18 hours after damage ($p=0.0084$) (Fig 23-A). And quantification of the bone marrow cell invasion through matrigel and transwell membrane. Exposure

of 5ng/ml bFGF improved invasion activity of sox9 positive cells ($p<0.0001$) (Fig 23-B).

Figure 21. Osteogenic differentiation of 4-day-old, chick bone marrow cells after being exposed to basic fibroblast growth factor (bFGF) throughout subculture. The bone marrow cells were cultured in nutrient mixture of DMEM/F12 medium, to which 0, 1, 5 or 10 ng/ml bFGF and the passage 5 cells were provided for osteogenic differentiation. (A) Osteogenic differentiation. The $3x10^5$ bone marrow cells were seeded into 6-well plate containing an osteogenic induction medium and subsequently cultured for 21 days. Alizarin Red S staining was conducted for the differentiation, while morphogenic transformation was observed under an inverted microscope. Calcium deposition and mineralization were detected in monolayers. More differentiation was detected at 5 ng/ml and 10ng/ml than at other levels. Scale bars =200 μm. (B) Numerical comparison by using of densitometry showed a significant model effect. Either 5 or 10 ng/ml bGFG was optimal. Data are presented as Mean±SE and different script indicate significant difference.

A


0 ng/ml
1 ng/ml
5 ng/ml
10 ng/ml

B

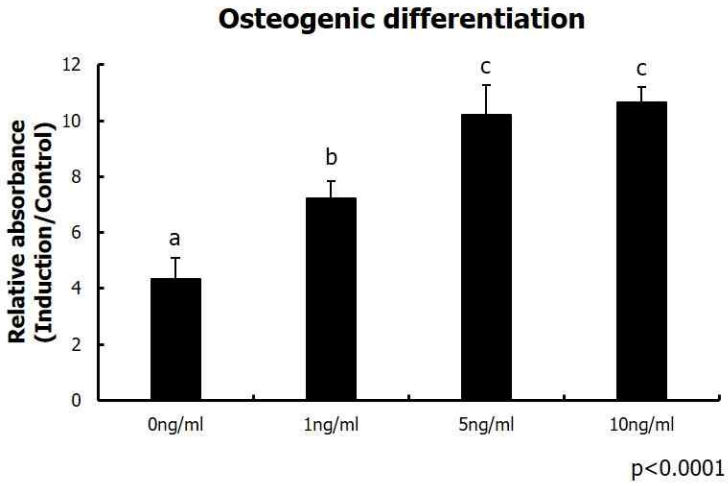
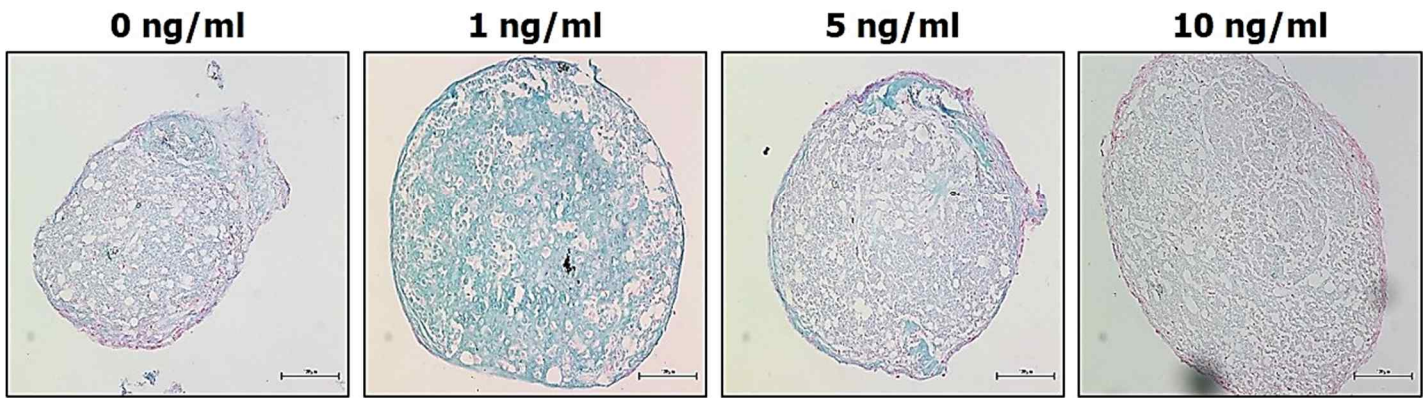

Osteogenic differentiation
Relative absorbance (Induction/Control)
12
10
8
6
4
2
0
a
b
c
c
0ng/ml
1ng/ml
5ng/ml
10ng/ml
p<0.0001

Figure 22. Chondrogenic differentiation of 4-day-old, chick bone marrow cells after being exposed to basic fibroblast growth factor (bFGF) throughout subculture. The bone marrow cells were cultured in nutrient mixture of DMEM/F12 medium, to which 0, 1, 5 or 10 ng/ml bFGF and the passage 5 cells were provided for osteogenic differentiation. (A) Chondrogenic differentiation. The $2.5x10^5$ bone marrow cells were seeded into 15-ml conical tube for pellet culture using a chondrogenesis induction medium for 21 days. Alcian blue staining was used for glycosaminoglycan, a secretory product of chondrocyte, detection. Morphogenic transformation was monitored under an inverted microscope. Signals on differentiation showing blue color was weak, but noticeable at certain level of bFGF. (B) Numerical comparison by using of densitometry showed a significant model effect. One ng/ml bGFG showed prominent differentiation than other bFGF level. Data are presented as Mean ±SE and different script indicate significant difference.

A


0 ng/ml
1 ng/ml
5 ng/ml
10 ng/ml

B

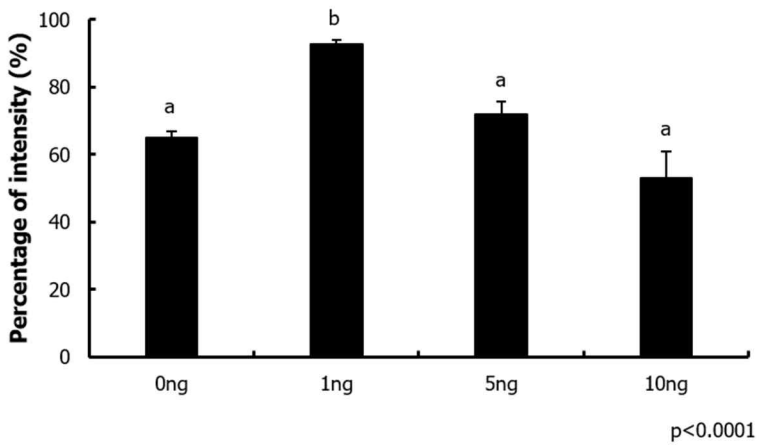
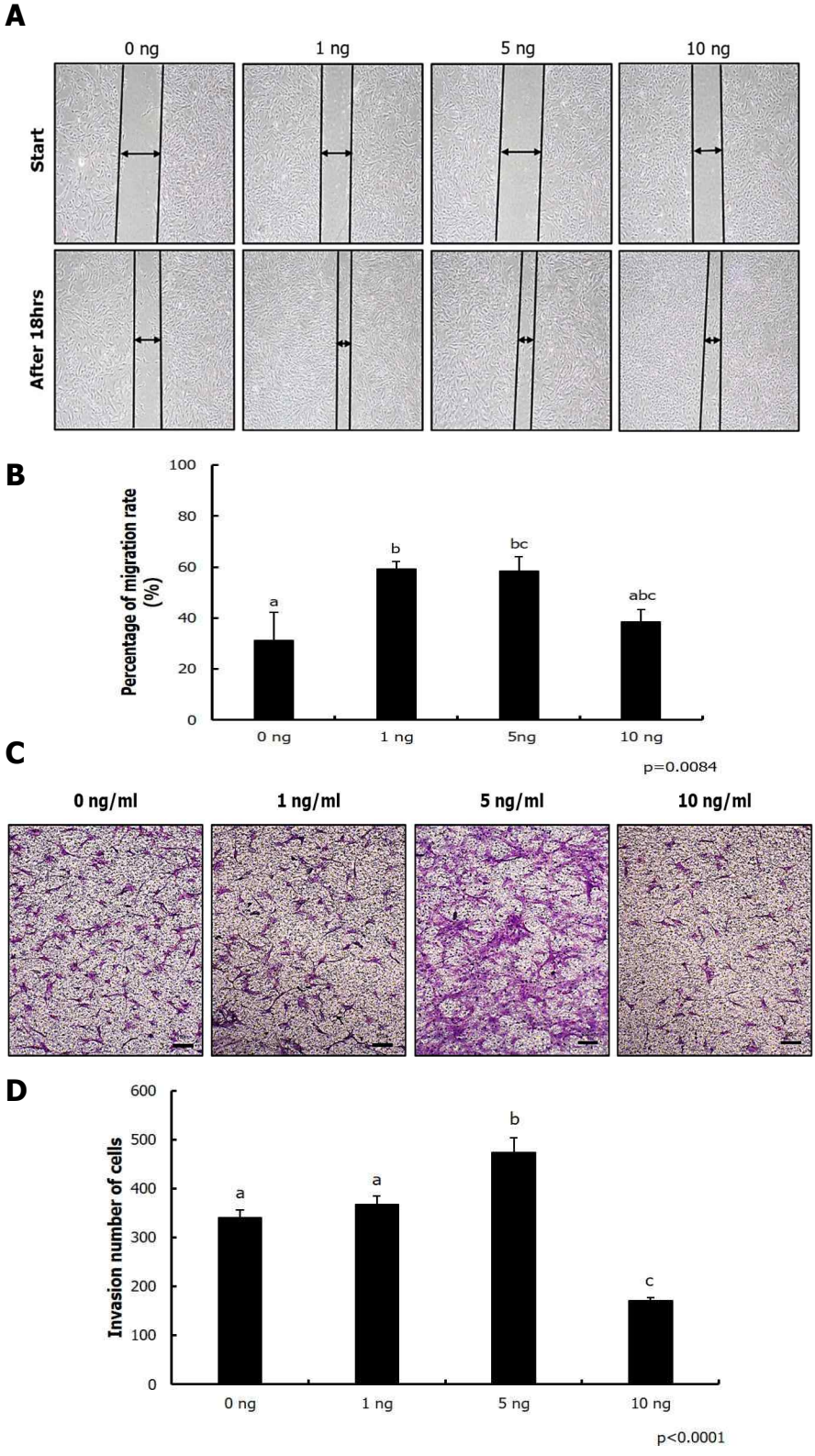

Percentage of intensity (%)
100
80
60
40
20
0
a
b
a
a
0ng
1ng
5ng
10ng
p<0.0001

Figure 23. The ability of cell migration and invasion in 4-day-old, chick bone marrow cells after being exposed to basic fibroblast growth factor (bFGF) throughout subculture. The bone marrow cells were cultured in nutrient mixture of DMEM/F12 medium, to which 0, 1, 5 or 10 ng/ml bFGF and the passage 5 cells were provided for the analysis. (A) Wound healing assay of chicken OCPCs for assessing cell migration ability. (B) Quantification of cell migration activity. Exposure of 1 and 5 ng/ml bFGF to the bone marrow cells subcultured improved migration ability, which was evaluated by the response to scratch 18 hours after damage. (C) Transwell invasion assay of the bone marrow cells being exposed to bFGF. (D) Quantification of the bone marrow cell invasion through matrigel and transwell membrane. Exposure of 5ng/ml bFGF improved invasion activity of bone marrow cells. Data are presented as Mean±SE and different script indicate significant difference.


A
0 ng
1 ng
5 ng
10 ng
Start
After 18hrs
B
Percentage of migration rate (%)
100
80
60
40
20
0
a
b
bc
abc
0 ng
1 ng
5ng
10 ng
p=0.0084
C
0 ng/ml
1 ng/ml
5 ng/ml
10 ng/ml
D
Invasion number of cells
600
500
400
300
200
100
0
a
a
b
c
0 ng
1 ng
5 ng
10 ng
p<0.0001

4. Discussion

In recent years, bone marrow cells have been investigated as promising candidates for use in cell-based therapeutic strategies such as disease and regenerative medicine. This study was conducted to confirm the effectiveness of basic fibroblast growth factor (bFGF) for expanding chicken bone marrow-derived cells (BMCs). Basic fibroblast growth factor (bFGF) is a member of the FGF family and is a critical component of stem cell culture field(Sotiropoulou et al., 2006; Colenci et al., 2014). However, the role and optimized concentration of bFGF on promoting the proliferation of MSCs derived from chicken bone marrow (BM) has not been reported. This study aimed to investigate the optimal concentration and the role of bFGF on the cellular activity of bone marrow cells derived from chicken, which may provide prominent guideline for addictiveness and possibility of the cell applications in regenerative medicine. bFGF related research has studied the optimal concentration on proliferation of different origin of the cells. Like dental pulp cells and apical papilla stem cells were concluded to be 100 ng/mL and

5 ng/mL respectively (Shimabukuro et al., 2009; Wu et al., 2012). Like these results and this study using chicken as an experimental animal suggest that an optimal concentration of bFGF on cellular activity must be optimized in each different types of cell line (Lee et al., 2015).

To monitor the influence of bFGF effects on retrieved cells in vitro, proliferative capacity, cell morphology, gene expression, immunophenotype and differentiation potential and functional test like cell migration and invasion assay were evaluated as experimental parameters. During the prolonged subculture, cumulative cell number significantly incensement detected from passage 6 in the insertion of above 5ng/ml bFGF DMEM/F12 group ($p<0.05$). In CCK-8 assay, significant model effect was detected from day 7 of culture ($p<0.05$). From this results, bFGF is responsible for the proliferation of chicken bone marrow cells but the underlying molecular regulatory mechanism are still not well understood and thus need further study about the proliferation related specific antibodies. However, from now on no specific antibodies against chicken are commercially available at the present, this research was limited. In gene

expression analysis, osteogenesis and chondrogenesis related genes were more expressed in exposure of above 5 ng/ml bFGF.

In example of study using MSCs, bFGF treatment will induce condrogenesis and osteogenic differentiation (Chiou et al., 2006; Ito et al., 2008; Kim et al., 2011). Osteogenic differentiation was significantly incensement ($p<0.0001$) detected on insertion of more 5 ng/ml bFGF and chondrogenic differentiation was apparently detected on addition of 1 ng/ml bFGF. In migration and invasion assay, exposure of 5 ng/ml bFGF improved each activity. In conclusion, this study suggests that addition of 5 ng/ml bFGF in vitro on chick osteochondro progenitor cells has effectiveness on proliferation, differentiation, migration and invasion ability.

CHAPTER 7.

Effects of Stem Cell Culture Environments on the Cellular Characteristics of Bone Marrow Cells in 4-Day-Old Chicken

1. Introduction

Mesenchymal-derived, multipotent cells have become a valuable resource for cell-to-tissue regeneration and experimental modelling for differentiation and reprogramming (Bianco et al., 2001; Anbari et al., 2014; Yan et al., 2014). In the chicken, bone marrow-derived cells can induce somatic chimerism (Heo et al., 2011; Heo et al., 2012) and if their pluripotency was confirmed, they could be used as resources to expand the applications of chicken pluripotent cells for various purposes(Jiang et al., 2002; Bhuvanalakshmi et al., 2014). To date, however, basic guidelines for the manipulation of bone marrow-derived cells has not been established. No information on culture properties have been reported because of their heterogeneous phenotype and mixed cell population (Ratajczak et al., 2004).

Based on information obtained from research in mammals (Muraglia et al., 2000; Conget et al., 2001; Bosnakovski et al., 2005), chicken bone marrow cells are comprised of population

may contain various undifferentiated progenitors and precursors, which could provide enormous information on cell differentiation using an *ex vivo* system (Friedenstein et al., 1987; Pittenger et al., 1999; Csaki et al., 2007).

In this study, primordial germ cell media (PGC) as embryonic stem cell culture environment was introduced to increase the number of cells of different characteristics among the mixed population of primary bone marrow cells and the expression of the pluripotent and germness related genes. Embryonic stem cell culture environment contains various promoting factors for cell viability, which may help to maintain stem cell related characteristics or to facilitate of progenitor cells.

Given these observations, I presumed that chicken bone marrow contains various types of cells, and I conducted the present study to expansion of stem cell-like cellular properties in primary bone marrow cells through using stem cell culture medium. From this study, I obtained information on various aspects of primary cell culture.

2. Materials and Methods

Experimental design

In experiment 1, the bone marrow cells retrieved from 4-day-old chicks were subcultured and colony forming ability and proliferation profile were monitored. Actual colony forming time period reduced 7days. The time of seeding and actual cell number counted at the end of each subpassage were employed using each different culture medium. In experiment 2, the reactivity of the P5 cells to the specific markers for representative stromal cell marker (CD44) and osteochondrogenesis (Sox9, Col I, Col II and AP) and gene expression were evaluated. The expression of specific genes for pluripotency (pouV, Nanog and Sox2) and osteo-chondrogenesis (Sox9, Col I, Col II, Col X and Aggrecan) was subsequently monitored. In experiment 4, osteogenic, adipogenic differentiation of the P5 cells was induced.

Western blotting

Western blot analysis was conducted to monitor the expression of specific proteins for CD44 and Collagen type II. Cellular proteins were extracted using lysis buffer [10mM Tris-HCl (PH7.4), 150Mm NaCl, 2.5Mm EDTA, and 0.125% Nodidet P-40(v/v)] supplemented with both a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Twenty μg protein lysates were electrophoresed with SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membrane was blocked with Casein (0.5% w/v) prior to exposure to each antibody. Immnoreactivity was visualized by incubation with horseradish peroxidase-linked secondary antibody followed by exposure to Electrochemiluminescence reagent according to manufacturer's instructions (GE Healthcare Biosciences, Piscataway, NJ).

3. Results

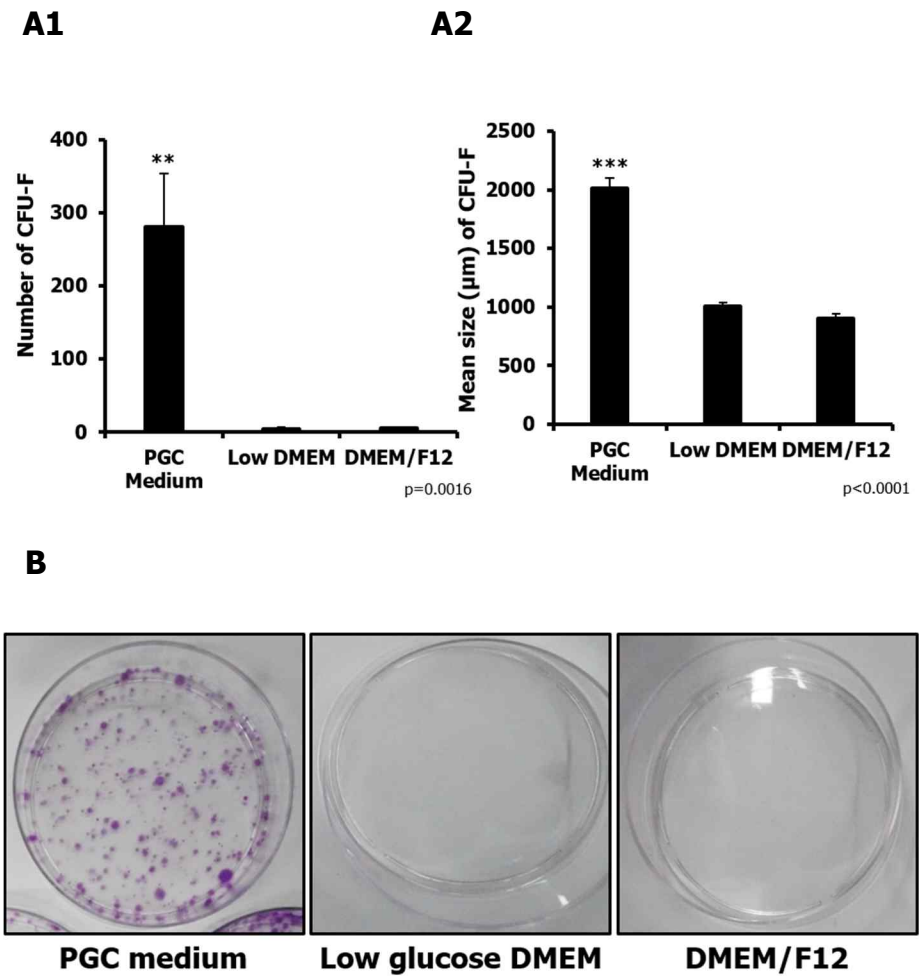
Comparisons of colony forming ability of nBMCs in various media

To evaluate of bone marrow derived primary cells attachment, colony forming unit-fibroblast analysis were conducted. One million bone marrow cells retrieved from primary passage were seeded into 100 mm cell culture plate and crystal violet staining was performed to detect single cell-derived clones during 7 days of culture. Positive staining was defined as a colony that contained 55 positive cells on average. As the results, in different three types of media significant difference were detected ($p=0.0016$, $p<0.0001$) (Figure 24 A 1-2).

Proliferation of nBMCs

Proliferation of nBMCs in different three types of medium are detected by continuous subculture in vitro. Total number of subcultured cells are counted at the end of each passages. Total number of counted cells are cumulative in each passages.

Figure 24. Colony-forming activity of chick bone marrow cells. The number and size of the CFU colonies were significantly difference among the media ($p=0.0016$, $p<0.0001$) (A1-2). The bone marrow cells were cultured in different media, Primordial germ cell medium (PGC medium), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12) (B).

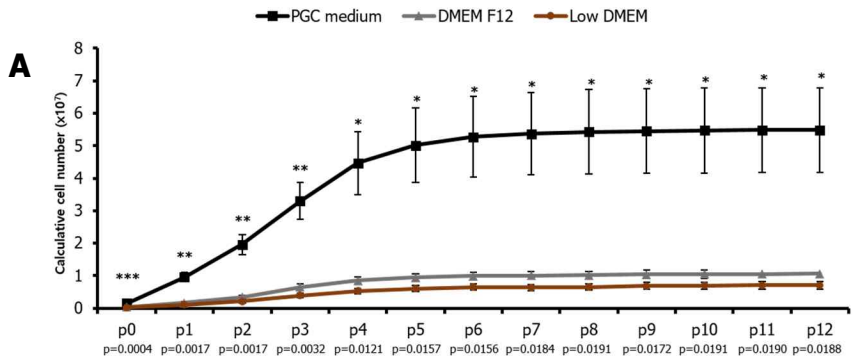


Cumulative number of cells at each passages are significant difference detected in each media. From passage 0, until passage 12 significant difference are detected in each media. Bone marrow derived cells are cultured in PGC medium have better proliferation ability than other types of medium (Figure 25 A). Actual cell number detects significant ($p<0.0001$) model effect in each parameters and vigorous proliferation was gradually decreased from passage 4 or 5 (Figure 25 B 1-3).

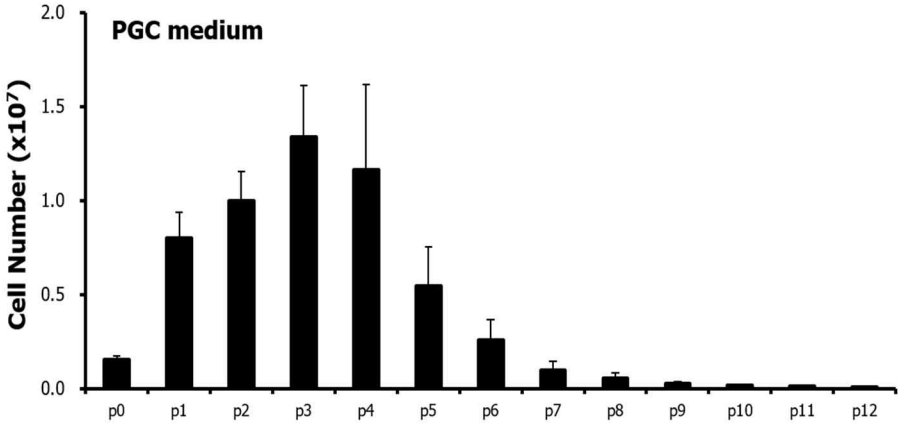
Assessment of gene expression and specific marker proteins for stromal cell marker (CD44) and osteo and chondrogenic related marker (Collagent type II) in the nBMCs cultured on different medium

Gene expression of nBMCs were assessed at passage 0, 4 (Figure 26). RT-PCR analysis showing that pluripotent related marker (Pou V, Nanog, Sox2) and osteogenic and chondrogenic related genes such as Sox9, Runx2, Col I, Col II, Col X, Aggrecan were expressed in regardless of culture media. Immunocytochemistry and western blot analysis of chicken

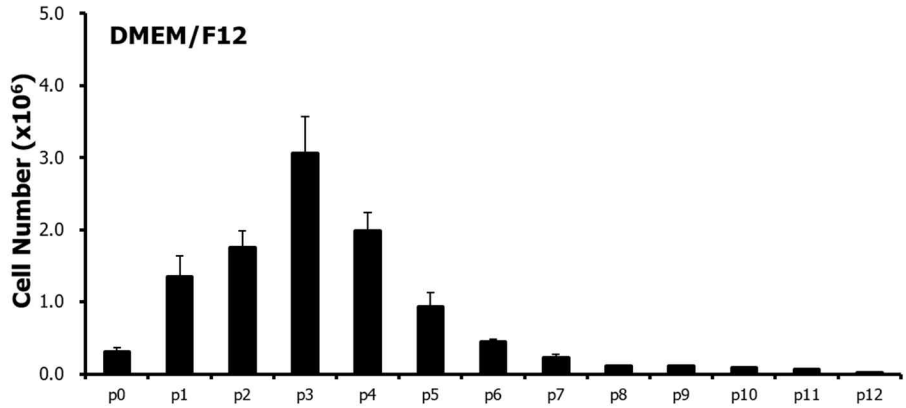
Figure 25. Proliferation of the bone marrow cells retrieved from the femur of 4-day-old chicks following subculture in vitro. The bone marrow cells were cultured in different media, primordial germ cell medium (PGC medium), low glucose DMEM (low DMEM) or a nutrient mixture of DMEM or Ham's F-12 medium (DMEM/F12). Total cell number was counted at the end of each passage up to 12 and either cumulative cell number since P0 culture (A) or actual cell number at the end of each passage (B) was compared among the media. (A) Significant ($p<0.0191$) model effects of the media were detected from P0 and PGC medium yielded the largest cells. Actual cell number after culture in PGC medium (B1), low DMEM (B2) and DMEM/F12 (B3). Greater proliferation was detected in P3 than in other passages, regardless of the kinds of medium, while incensement of cell number by subpassage was not detected from P11 and DMEM/F12.



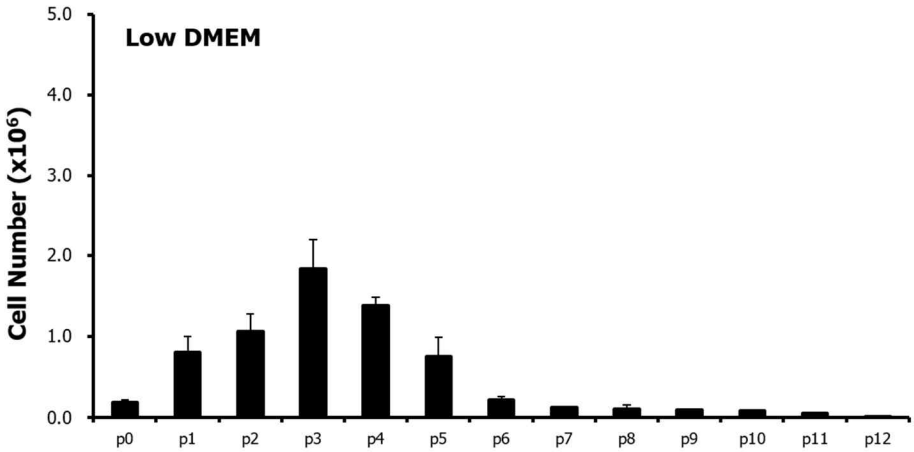
B1


PGC medium
Cell Number (x10⁷)
2.0
1.5
1.0
0.5
0.0
p0
p1
p2
p3
p4
p5
p6
p7
p8
p9
p10
p11
p12

B2


DMEM/F12
Cell Number (x10⁶)
5.0
4.0
3.0
2.0
1.0
0.0
p0
p1
p2
p3
p4
p5
p6
p7
p8
p9
p10
p11
p12

B3


Low DMEM
Cell Number (x10⁶)
5.0
4.0
3.0
2.0
1.0
0.0
p0
p1
p2
p3
p4
p5
p6
p7
p8
p9
p10
p11
p12

nBMCs at passage 0 and passage 3. Expression of CD44 (stromal cell marker) and collagen type II (proliferative chondrocyte marker) proteins in 4-day-old chick bone marrow cells collected from passage 0 and passage 3 (Figure 27 A-1, B-1). Immunofluorescence staining of CD44 and collagen type II at passage 0. Collagen type II-positive cells (stained red) expressed on cultured in DMEM/F12 and Low DMEM. CD44-positive cells (stained green) only expressed in PGC medium cultured cells. (Figure 27 A-2, B-2) In western blot analysis results, collagen type II was not expressed on cultured cells using PGC medium and CD44 as expressed regardless of culture media.

Comparison of Differentiation activity of the bone marrow cells cultured in different media until passage 5 (P5)

As shown in Figures 28 and 29, both osteogenic and adipogenic differentiation were detected. Differentiation of 4-day-old chick bone marrow-derived cells collected from passage 5 using PGC media and Low glucose DMEM. Osteogenic

differentiation. Passage 5 cells of $3x10^5$ were seeded into 6-well plate and subsequently cultured in an osteogenic induction media for 21 days. Alizarin Red S staining was conducted for the differentiation, while morphogenic transformation was detected under an inverted microscope. Calcium deposition and mineralization were detected in monolayer observation. After three weeks of osteogenic differentiation, nBMCs induced using PGC medium and Low DMEM media showed a significantly similar calcium deposit and the results effects on Alizarin Red S staining ($p=0.0862$). And in adipogenic differentiation, same $3x10^5$ cells were seeded and cultured in an adipogenic induction media supplemented with insulin for 21 days. After three weeks of adipogenic differentiation, nBMCs induced using PGC medium showed a significantly more lipid deposit than Low DMEM media and the results effects on Oil Red O staining ($p=0.0225$).

Figure 26. Expression of pluripotency- and osteogenic or chondrogenic-related genes in 4-day-old chick bone marrow-derived cells collected from the femur and the tibia. The cells collected at the end of passage 0 (A) and 4 (B) were provided for RT-PCR. (A) RT-PCR analysis showing that pluripotency-related genes such as pouV, Nanog, Sox2 were not expressed. Osteo-chondrogenic genes of *Sox9* and its downstream genes such as *Collagen type I, II, X* were concomitantly expressed. *β-actin* was positive control.

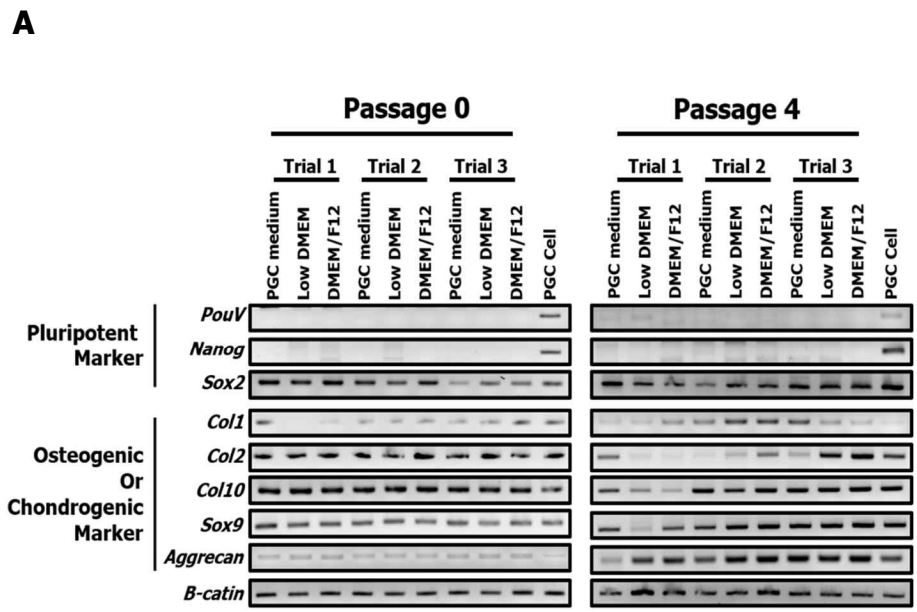


Figure 27. Expression of CD44 (stromal cell marker) and collagen type II (proliferative chondrocyte marker) proteins in 4-day-old chick bone marrow cells collected from passage 0 and passage 3. **(A-1, B-1)** Immunofluorescence staining of CD44 and collagen type II at passage 0. Collagen type II-positive cells (stained red) expressed on cultured in DMEM/F12 and Low DMEM. CD44-positive cells (stained green) only expressed in PGC medium cultured cells. **(A-2, B-2)** In western blot analysis results, collagen type II was not expressed on cultured cells using PGC medium and CD44 expression were expressed regardless of culture media.

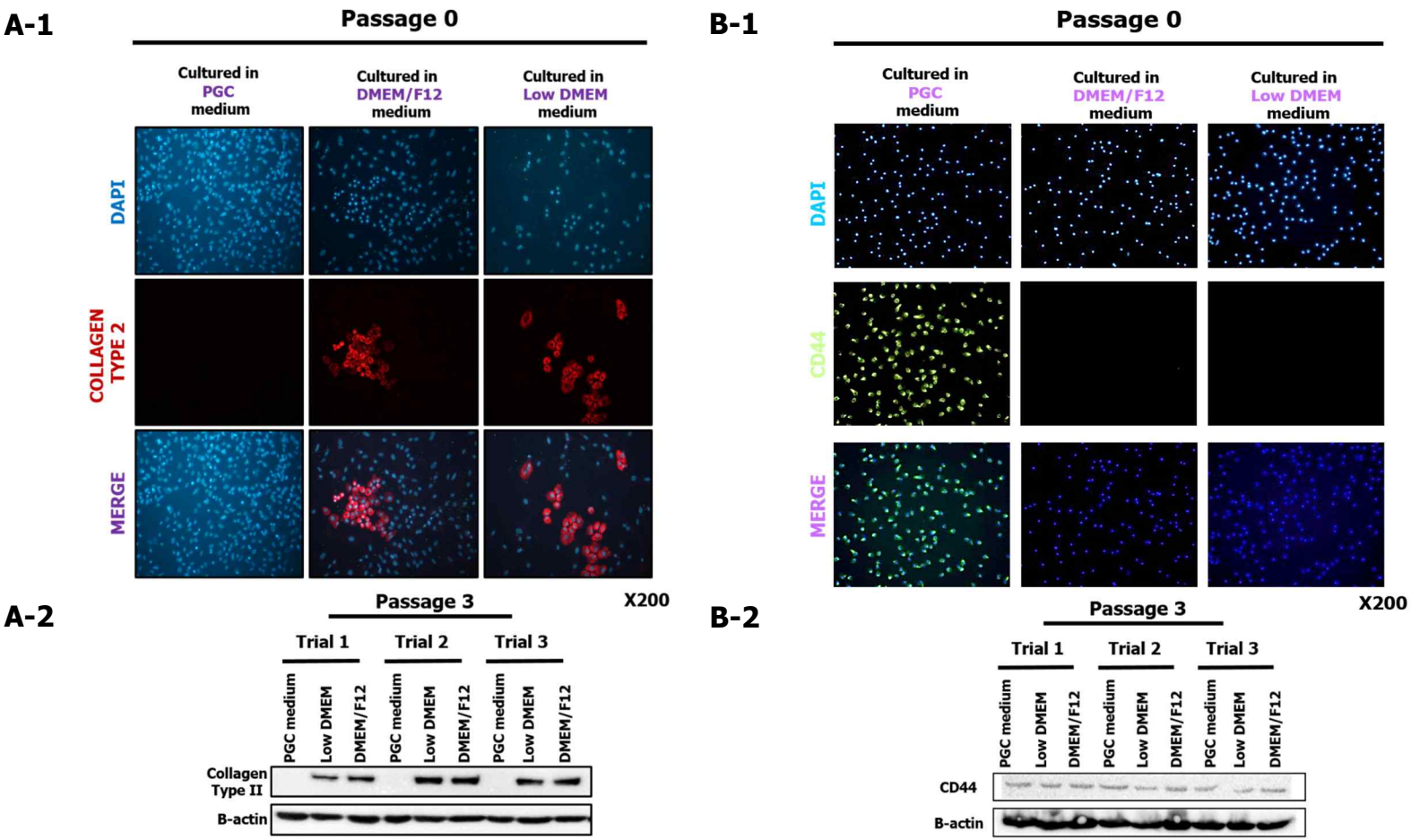

A-1
Passage 0
Cultured in PGC medium
Cultured in DMEM/F12 medium
Cultured in Low DMEM medium
DAPI
COLLAGEN TYPE 2
MERGE
X200
B-1
Passage 0
Cultured in PGC medium
Cultured in DMEM/F12 medium
Cultured in Low DMEM medium
DAPI
CD44
MERGE
X200
A-2
Passage 3
Trial 1
Trial 2
Trial 3
PGC medium
Low DMEM
DMEM/F12
Collagen Type II
B-actin
B-2
Passage 3
Trial 1
Trial 2
Trial 3
PGC medium
Low DMEM
DMEM/F12
CD44
B-actin

Figure 28. Differentiation of 4-day-old chick bone marrow-derived cells collected from passage 5 using PGC media and Low DMEM. Osteogenic differentiation. Passage 5 cells of $3x10^5$ were seeded into 6-well plate and subsequently cultured in an osteogenic induction media for 21 days. Alizarin Red S staining was conducted for the differentiation, while morphogenic transformation was detected under an inverted microscope. Calcium deposition and mineralization were detected in monolayer observation. Scale bars =250 μm.

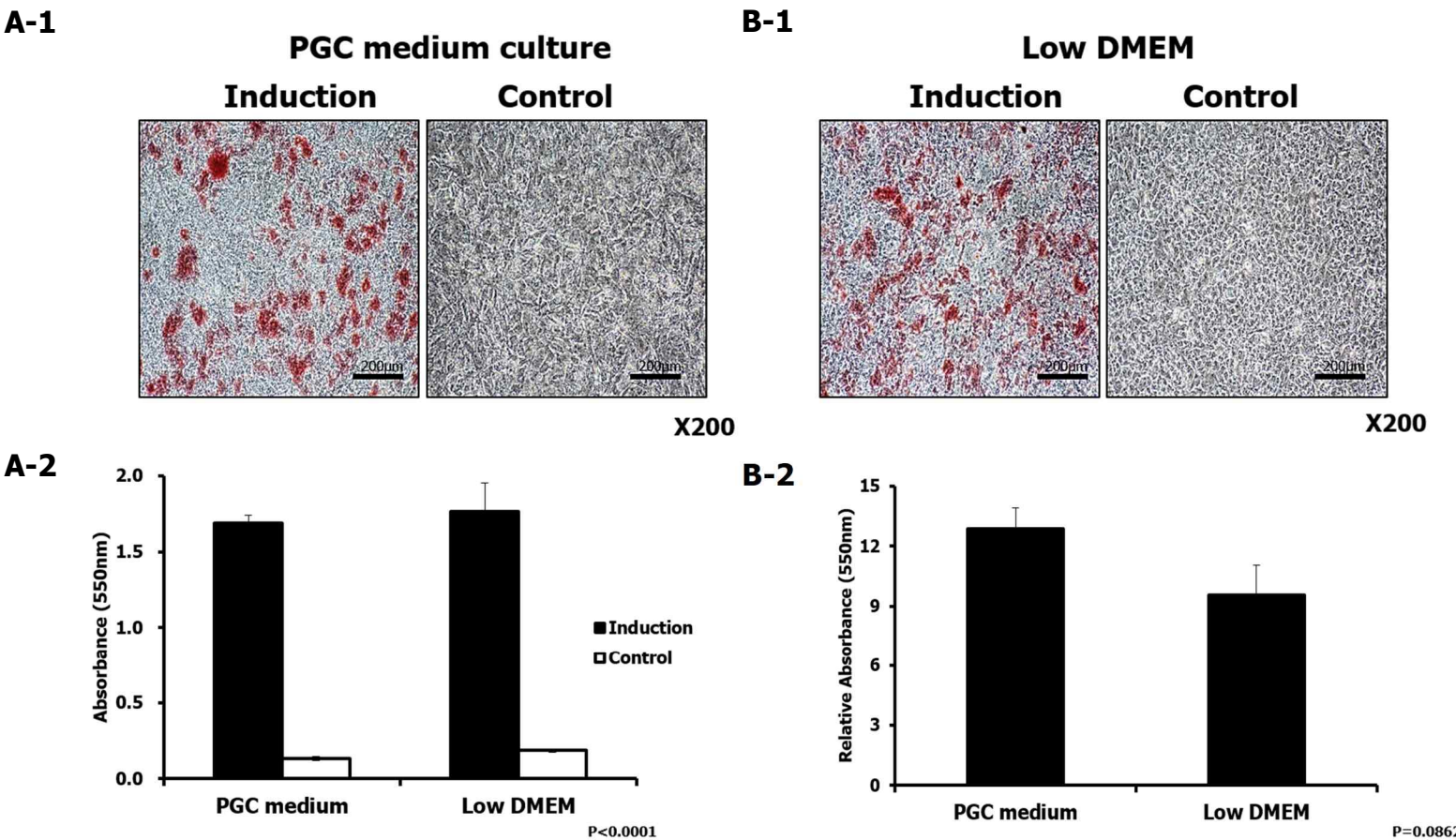
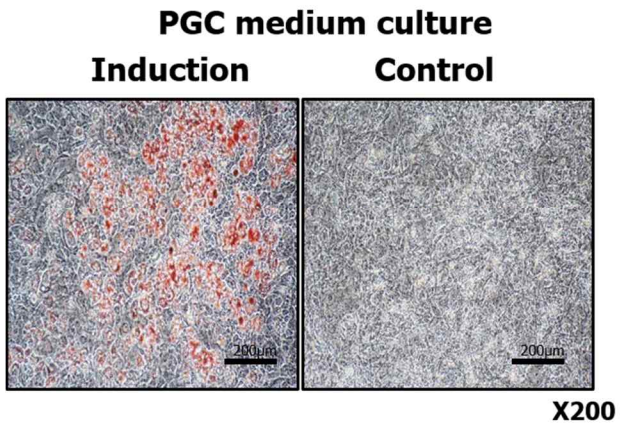
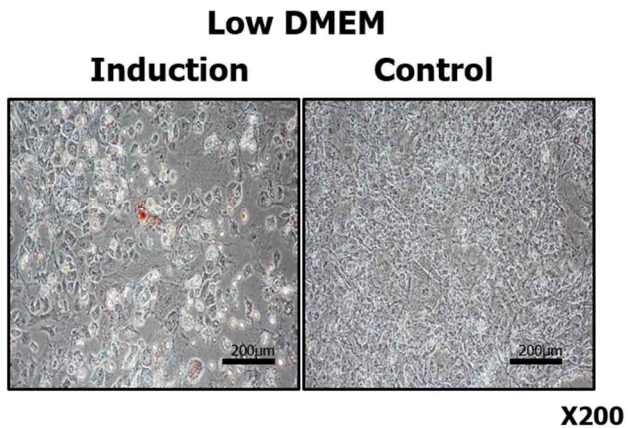

A-1
PGC medium culture
Induction
Control
200μm
200μm
X200
B-1
Low DMEM
Induction
Control
200μm
200μm
X200
A-2
Absorbance (550nm)
2.0
1.5
1.0
0.5
0.0
Induction
Control
PGC medium
Low DMEM
P<0.0001
B-2
Relative Absorbance (550nm)
15
12
9
6
3
0
PGC medium
Low DMEM
P=0.0862

Figure 29. Differentiation of 4-day-old chick bone marrow-derived cells collected from passage 5 PGC media and Low DMEM. Adipogenic differentiation. The same $3x10^5$ cells were seeded and cultured in an adipogenic induction media supplemented with insulin for 21 days. Oil Red O staining for the differentiation was negative and lipid accumulation was not detected in monolayer, while morphogenic transformation was noticed. Scale bars =250 μm.

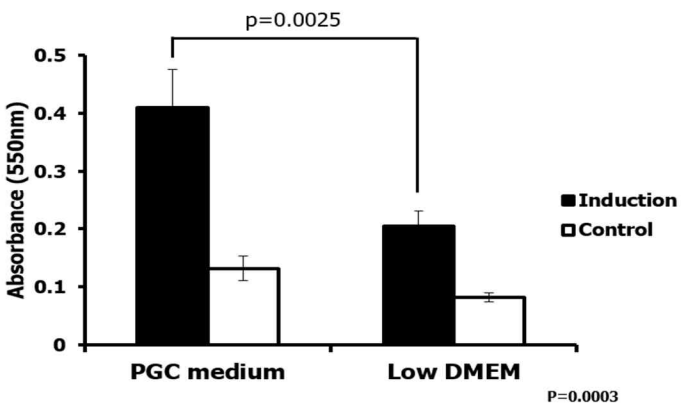
A-1


PGC medium culture
Induction
Control
200μm
200μm
X200

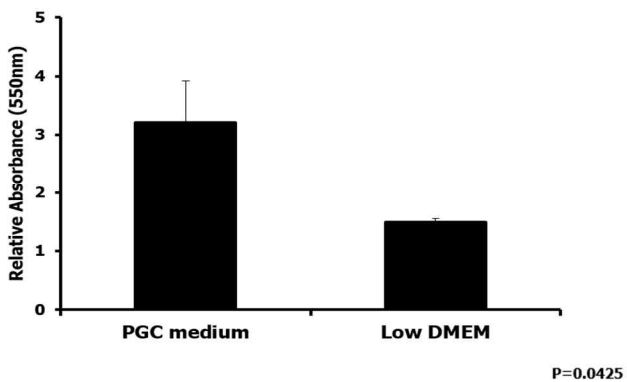
B-1


Low DMEM
Induction
Control
200μm
200μm
X200

A-2


p=0.0025
Absorbance (550nm)
0.5
0.4
0.3
0.2
0.1
0
Induction
Control
PGC medium
Low DMEM
P=0.0003

B-2


Relative Absorbance (550nm)
5
4
3
2
1
0
PGC medium
Low DMEM
P=0.0425

4. Discussion

In previous study found that 4-day-old WL are different developmental stage and so it contains a variety of cell. I explored cell identification and a suitable culture environment based on the analysis of cell characteristics of sox 9 positive cells. However, based on the differences in the genes and cell specific characteristics related to the pluripotency of cells from the common bone marrow origin, the research was carried out by introducing substances that are widely used in stem cell culture. In this study, I compared the growth function and cell characteristics of cells derived from the bone marrow of the day 4 chicken with primordial germ cell culture medium similar to the composition of stem cell culture media. In stem PGC medium, they contain various promoting factors for cell viability and cellular properties such as chicken serum and bFGF.

In chicken stem cell culture, Knockout Dulbecco's modified Eagle's medium (Knockout DMEM or KO/DMEM) is a basic culture medium which can support the growth ability in vitro. Although DMEM based culture medium can support the

growth of various type of stem cells, the composition of KO/DMEM is suitable for all kinds of mammalian and non-mammalian stem cells and optimized culture environment of their growth rate (van de Lavoir and Mather-Love 2006; Amit and Itskovitz-Eldor 2016). Traditionally, the presence of fetal bovine serum (FBS) in KO/DMEM has been commonly used in cell proliferation due to the presence of growth factors. The fetal bovine serum routinely used for mouse embryonic stem cell culture may not be optimized for chicken cell culture environment; because of the fetal environment from which the FBS is derived is rich in growth factors (Horiuchi et al. 2006). One of the fundamental problems with using FBS or chicken serum is that they are both very complex because they contain unknown compounds, including self-reproduction and differentiation factors. (Skottman and Hovatta 2006).

The above results clearly demonstrate that under the regimen used in the present study, the culture of newborn chicken bone marrow tissue yielded both stromal and cuboidal cell- dominant populations. The culture of chicken bone marrow cells differs in many ways from that of mammalian bone marrow

cells, which usually exhibit a fibroblast-like, stromal cell morphology (Toma et al., 2001; Bosnakovski et al., 2005). The retrieved cells using PGC medium were maintained up to at least the third passage, and a number of differences in cell specific protein expressions were observed compared with sox9 positive cells optimized cultured environments. Characterization of retrieved cells that shows different cellular properties also needed. Optimization of the culture system and an analysis of the unique properties of chicken bone marrow cells will be essential to promote the use of chicken cells collected from the bone marrow.

This results may be due to differences in cell proliferation dynamics according to the maturity of the individuals between the two cell populations. Nevertheless, regardless of cell type, chicken bone marrow cells of various morphologies can be cultured and maintained *in vitro* to a certain extent. The PGC culture environment acts as exhibited better proliferation than the optimal compositions of sox9 positive population during culture. The various growth factors in the PGC culture medium and the culture environment suitable for growth of stromal cells

in the bone marrow can be seen to produce these results. And stromal cell marker like CD44 is expressed on only PGC medium culture environment. Chicken bone marrow cells have different characteristics compared with bone marrow cells of mammalian species, which usually yield a fibroblast-dominant population. Considering the mixed population of bone marrow cells, a different regimen may be needed for the outgrowth of chicken bone marrow-derived fibroblasts. Modification of the regimen used for the culture of bone marrow cells from mammals is necessary for the outgrowth of fibroblasts. In any case, the results of this study will make it possible to characterize chicken bone marrow cells of various types further and will increase the feasibility of using chicken bone marrow cells for different applications.

CHAPTER 8.

Effect of Bioactive Substances Derived from Chick Bone Marrow Cells on the Differentiations of Human Adipose-Derived Mesenchymal Stromal Cell

1. Introduction

Cell niche can maintain healthy condition and regulate the mechanisms that stem cell self-renewal or differentiation abilities (Blanpain et al., 2004). Some cell microenvironment or morphogenetic factors can be influenced cell characteristics. Recently, many studies have focused on using natural or synthetic biomaterials to create niches or micro-environments to control the behavior of the target cells and their cell characteristics like proliferation and differentiation (Lutolf and Hubbell, 2005; Peerani and Zandstra, 2010).

Over the past few years, many study groups have studied using conditioned media to evaluate efficiency as a generative biomarker. Released protein from conditioned media by cultured cells is a rich source of materials for biomarker as an application of novel treatment. Recent studies suggested that morphogenetic signals from chondrocytes regulate differentiation into chondrogenic lineages and controlling the endochondral ossification during skeletal formation process. Conditioned media refers to the soluble factors like proteins that contain

morphogenic signals and are processed via the endoplasmic reticulum and Golgi apparatus through the cell secretion pathway. More generally, secretrome contains proteins that fall off the surface of cells and proteins released through exosomes. These secreted proteins include numerous enzymes, growth factors, cytokines and hormones or other soluble mediators.

Adipose derived mesenchymal stromal cells (ADSCs) are a potential cell source for tissue engineering applications with bone or cartilages related diseases and regenerative medicine field. However, controlling differentiation potency and wanted target lineage formation in vivo remains a challenge. And tissue engineering as a clinical application has limitation and inefficiency by proliferation and differentiation sustaining. In these reasons, there is strong necessity for well-defined and improved protocols for directing cell characteristics in vivo.

In these experiments, I hypothesized that morphogenetic signals from chicken sox9 positive cells would regulate ADCSs differentiation ability. From these results, I demonstrate that morphogenetic signals from chicken bone marrow cells, in the

form of soluble factors, induce as osteogenic differentiation enhancer of human ADSCs during in vitro differentiation. This results suggest possibility of the conditioned media as the morphogenetic factors from bone marrow cells to differentiation induction agents or reprogram cells for in vivo bone tissue engineering applications. ADSCs expanded in chicken bone marrow derived osteogenic differentiation conditioned medium enhanced osteogenic differentiation compared by routine protocol method. In addition, incubation of ADSCs with conditioned medium supplemented with osteogenic factors or by oneself induced more osteogenesis and accumulation of calcium and increased ALP activity. These findings reveal that chicken bone marrow cell derived secretion factors promote osteogenesis of ADSCs during in vitro differentiation culture.

Given the current inefficient in vitro human cell differentiation protocols, this method and results develop a simple and innovative differentiation method for generating in vivo bone tissue from adult stem cells.

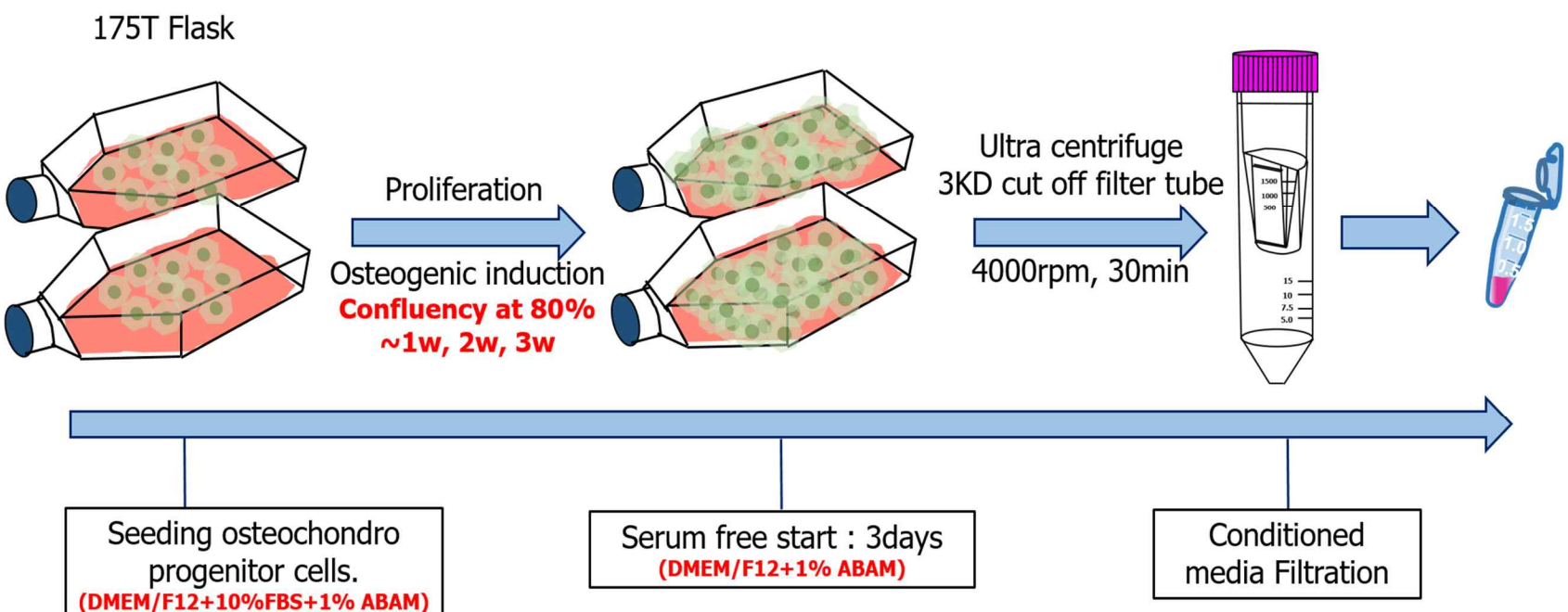
2. Materials and Methods

Experimental design

The sox9 positive cells retrieved from 4-day-old chicks were subcultured and at passage 5 cells were seeded into 175T flask. At 80% cell confluences, these cell were inducted into osteogenic lineages during 3weeks. At the end of the differentiation culture media changed by serum free environment. After 3 days incubation, total media collected and filtered under 3Kd protein using ultra centrifuge. Then total protein was detected using Elisa-kit (Figure 30).

In experiment 1, Checking protein levels in conditioned media using Elisa detection kit. Detection of TGF- β and hyaluronic acid (HA) and Bone morphogenetic protein 4 (BMP-4) using ELIZA. In experiment 2, Proliferation activity was evaluated in each different type of conditioned media using the Ki-67 antibody. In experiment 3, the effect of bone deformation using conditioned media was explored by creating various differentiation induction environments.

Figure 30. General experimental procedures.



Cell culture and conditioned medium

At passage 5 cells seeded into 175T flask. When bone marrow derived cells reached to more than 80% confluence (at the end of P5), induction of osteogenic differentiation begins for each 1 and 2 weeks. End of each differentiation period, chicken bone marrow cells culture media changed with serum-free DMEM for 3days. Then bone marrow cells-conditioned medium was filtered through 3Kd cut off filter tube to concentrate and prevent any cell contamination.

Protein detection using elisa Kit analysis

TGF-β detection

Chicken TGF-β detection assay use double-sandwich elisa technique. Supplied plates were Pre-coated with chicken TGF-β monoclonal antibody and the detecting antibody is biotin labeled polyclonal antibody. Samples and biotin labeling antibody are added into ELISA plate wells and washed out with PBS or TBS. Then Avidin-peroxidase conjugates are added to ELISA wells in

order. Use 3,3',5,5' – tetramethylbenzidine (TMB substrate) for coloring after reactant thoroughly washed out by PBS or TBS. TMB turns into blue in peroxidase catalytic and finally turns into yellow. Then color depth reads optical density(OD) at 450nm within 10min.

Bone morphogenic protein-4 (BMP-4) detection

BMP-4 ELISA kit use also sandwich enzyme immunoassay technique. The assay plate has been pre-coated with a monoclonal antibody specific for BMP-4. In order to quantitatively determine the amount of BMP-4 present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody. The enzyme-substrate reaction is terminated by addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm.

Hyaluronic acid (HA) detection

HA ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-HA antibody and an HA-HRP conjugate. The assay sample and buffer are incubated

together with HA-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution is added to stop the reaction, which will then turn the solution yellow. The intensity of color is measured spectrophotometrically at 450nm in a microplate reader.

Ki-67 Immunocytochemistry

ADSCs were cultured with each different types of conditioned media during 3days. Then washed with PBS, fixed with 4% (v/v) paraformaldehyde and subsequently permeabilized with 0.1% (v/v) of Triton X-100. After washing, the cells were blocked with 10% (v/v) normal goat serum and treated with Ki-67 antibody overnight at 4℃. After being treated with secondary antibody for 2 hours at room temperature, the fixed cells were stained in DAPI staining solution. Images were captured via fluorescent microscopy using software from NIS Elements, Inc.

3. Results

Protein levels in conditioned media using Elisa detection kit.

We collected morphogenetic factors from chicken bone marrow cells differentiation stage cells with serum-free DMEM for 3days. Bone marrow cells-conditioned medium was filtered through 3Kd cut off filter tube to concentrate and prevent any cell contamination. Checking protein levels in proliferation and differentiation conditioned media using Elisa detection kit. Protein detection result of TGF- β, HA and BMP-4 show conditioned media contained small scale of protein (Table 2).

Effect of conditioned media on proliferation and Osteogenic differentiation

First, to determine if conditioned media would also be induced cell proliferation ability, morphogenetic factors produced from bone marrow cells were supplemented with cell proliferation assay using ki-67 antibody (Fig. 31).

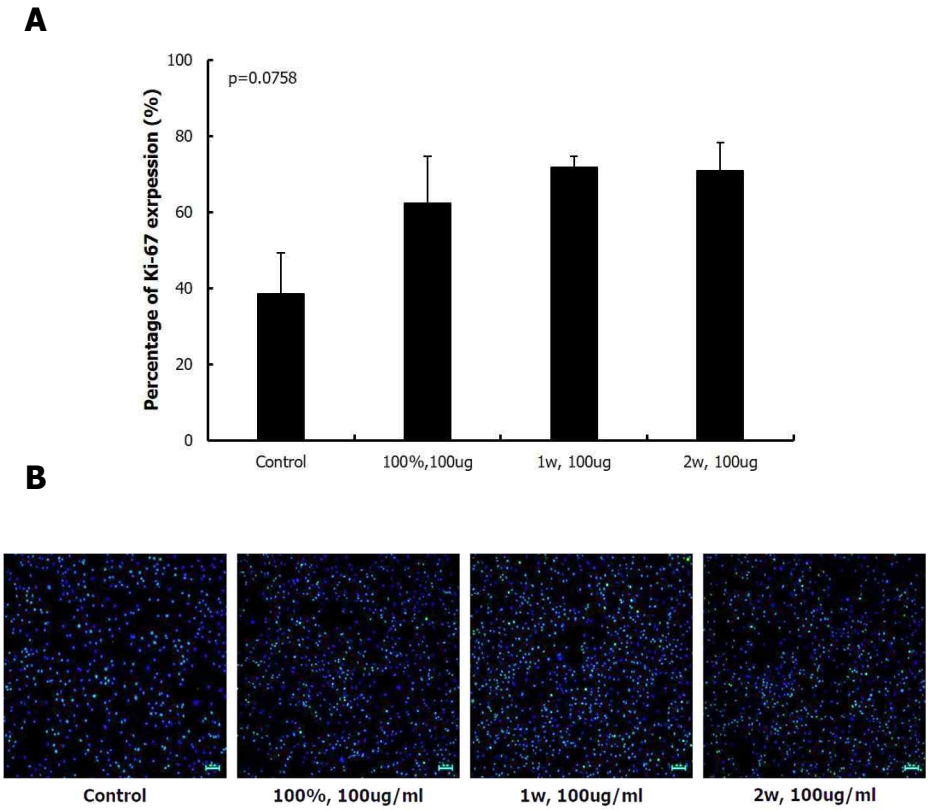
Second, to determine the effects of cell-secreted

Table 2. Checking protein levels in proliferation and differentiation conditioned media using Elisa detection kit. Detection of TGF− β and hyaluronic acid (HA) and Bone morphogenetic protein 4 (BMP−4) using ELIZA.

I. TGF−beta detection	
Types of Conditioned Media	Quantity of Detection (pg/ml)
Proliferation Conditioned Media	None
	None
	None
Mean	−
1 Week Conditioned Media	184.81 pg/ml
	167.76 pg/ml
Mean	
2 Weeks Conditioned Media	51.84 pg/ml
	47.86 pg/ml
	41.04 pg/ml
	57.52 pg/ml
	62.63 pg/ml
Mean±SE	52.18 pg/ml
II. Hyaluronic Acid (HA) detection	
Types of Conditioned Media	Quantity of Detection (pg/ml)
Proliferation Conditioned Media	6.78 pg/ml
	65.97 pg/ml
	None
Mean	
1 Week Conditioned Media	None
	None
Mean	−
2 Weeks Conditioned Media	102.2 pg/ml
	100.3 pg/ml
	103.3 pg/ml
	99.3 pg/ml
	98.4 pg/ml
Mean	99.9 pg/ml
III. BMP−4 detectiion	
Types of Conditioned Media	Quantity of Detection (pg/ml)
Proliferation Conditioned Media	6.78 pg/ml

	65.97 pg/ml
	None
Mean±SE	
1 Week Conditioned Media	None
	None
Mean	–
2 Weeks Conditioned Media	None
	None
	None
	None
	None
Mean	–

Figure 31. Proliferation activity was evaluated in each different type of conditioned media using the Ki-67 antibody. 100 μg/ml of total protein used in this assay. The percentage of ki-67 expression was in cultured cells monitored under an immunofluorescence microscope. (A-B) Significant difference was not detected (p=0.0758) in all of the groups, but 1week, 2week conditioned media induced more osteogenic differentiation trend than the control. Data are presented as Mean ±SE.



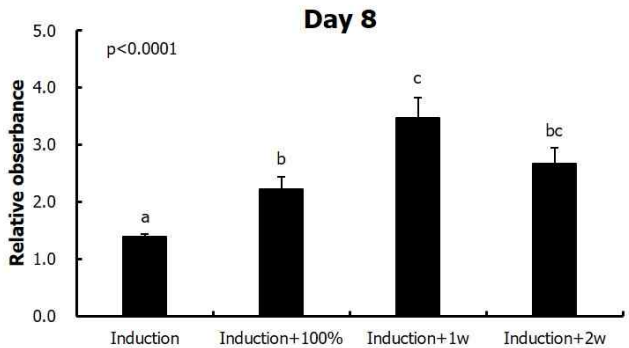
morphogenetic factors on ADSCs osteogenesis, ADSCs were cultured with conditioned DMEM supplemented with 10% FBS. Medium without morphogenetic factors was used as control. At day 8, ADSCs cultured in conditioned medium detected more calcium deposits compared to control group (Fig. 32 A and B). Similarly, when differentiated into 2weeks, ADSCs cultured in conditioned medium detected also more calcium deposits compared to control group (Fig. 33, 34). Osteogenic activity of the morphogenetic factors secreted by chondrocytes was confirmed by strong ARS staining. Taken together, the above results indicated that 100ug/ml conditioned medium acts as a positive enhancer of osteogenesis during in vitro differentiation.

Effect of conditioned media by the modified protocol which is without Dexamethasone on Osteogenic differentiation

Human ADSCs osteogenesis was induced by routine protocol which contains components including beta-glycerophosphate, dexamethasone, and ascorbic acid. ADSCs osteogenic differentiation method modified which is without dexamethasone and adding 100 μg/ml of each type of conditioned

Figure 32. Differentiation of the human ADSCs using different types of conditioned media. Four-day-old white leghorn chicks were employed as the donor of bone marrow cells and the isolated cells collected from the femurs were subsequently cultured in 10%(v/v) fetal bovine serum-containing, DMEM/F12-based media and differentiated into osteogenic 3weeks. End of the differentiation period, the cells were newly cultured in serum-free DMEM/F12 medium and the media used for serum-free culture were collected 3 days after culture. These Collected media were concentrated until total protein concentration reached to 100 μg/ml. Then human ADSCs osteogenesis was induced either by routine protocol or by the protocol using 100 μg/ml of total protein until 8days. **(A)** Significant ($p<0.0001$) model effect was detected and 1week conditioned media induced more osteogenic differentiation than the others. **(B)** The morphology of the cultured cells was monitored under an inverted microscope. Data are presented as Mean±SE. Scale bars=200 μm.

A


Day 8
p<0.0001
Relative oberbance
5.0
4.0
3.0
2.0
1.0
0.0
a
b
c
bc
Induction
Induction+100%
Induction+1w
Induction+2w

B

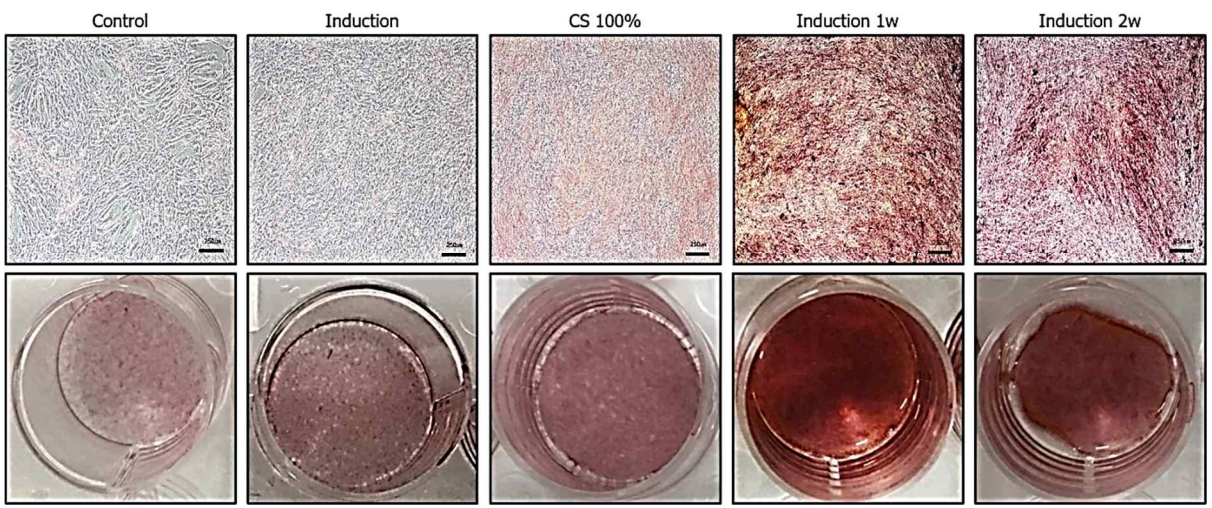

Control
Induction
CS 100%
Induction 1w
Induction 2w

Figure 33. Human ADSCs osteogenesis was induced either by routine protocol or by the protocol using 100 μg/ml of total protein until 2 weeks. (A) Significant ($p<0.0001$) model effect was detected and 1week, 2week conditioned media induced 40 fold more osteogenic differentiation than the others. (B) The morphology of the cultured cells was monitored under an inverted microscope. Data are presented as Mean±SE. Scale bars=200 μm.

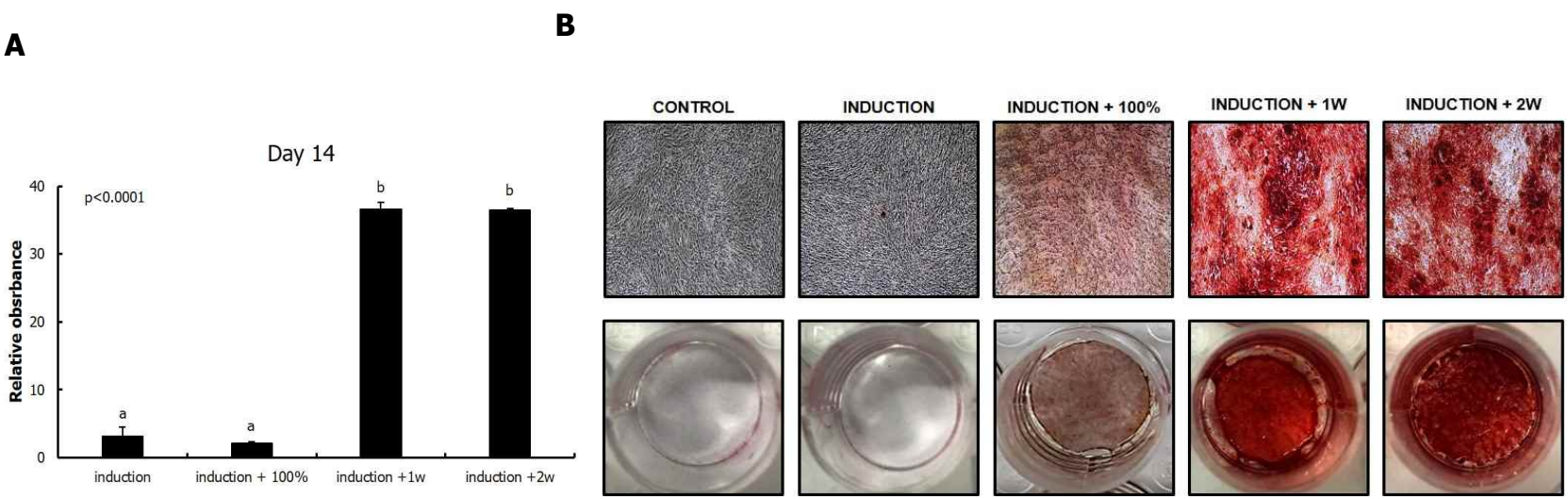
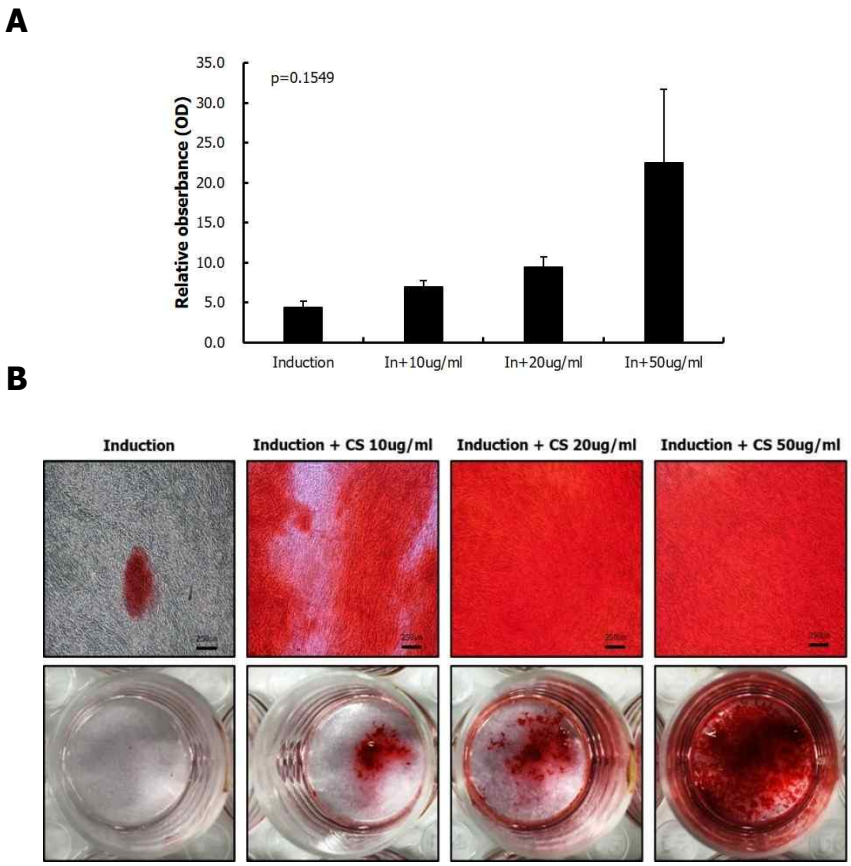


Figure 34. Human ADSCs osteogenesis was induced either by routine protocol or by the protocol using under 100 μg/ml of 2weeks conditioned media total protein (10 μg/ml, 20 μg/ml, 50 μg/ml) until 2 weeks. (A) Significant model effect was not detected among the group (p=0.1549), but induced more osteogenic differentiation trend in 50 μg/ml conditioned media adding group. (B) The morphology of the cultured cells was monitored under an inverted microscope. Data are presented as Mean±SE. Scale bars=200 μm.



media total protein until 9 days and 2 weeks. At 9 days differentiation, significant model effect was not detected among the group (p=0.1314), but induced more osteogenic differentiation trend in differentiation conditioned media adding group. And 2 weeks differentiation assay, significant model effect was detected (p=0.0241) (Fig. 35, 36). These observations indicate that morphogenetic factors also enhance osteogenic differentiation of ADSCs and can be substituted role of dexamethasone.

Only effect of conditioned media by the modified protocol on Osteogenic differentiation

Directing osteogenic differentiation without any exogenous cytokines, growth factors, and only insertion of conditioned media shows significant incensement (p=0.0142) on 50ug/ml conditioned media only group (Fig. 37).

This results indicated that the microenvironment produced during chicken bone marrow cells osteogenesis regulates the subsequent osteogenic differentiation of ADSCs, suggesting that soluble factors of cell secretion from bone marrow cells can

alternative significant roles in differentiation methods. Therefore, it can be utilized the morphogenetic signals from bone marrow cells during ADSCs reprograming into bone related tissue.

Figure 35. Human ADSCs osteogenesis was induced either by routine protocol or by the modified protocol which is without Dexamethasone and adding 100 μg/ml of each type of conditioned media total protein until 9days. (A) Significant model effect was not detected among the group (p=0.1314), but induced more osteogenic differentiation trend in differentiation conditioned media adding group. (B) The morphology of the cultured cells was monitored under an inverted microscope. Data are presented as Mean±SE. Scale bars=200 μm.

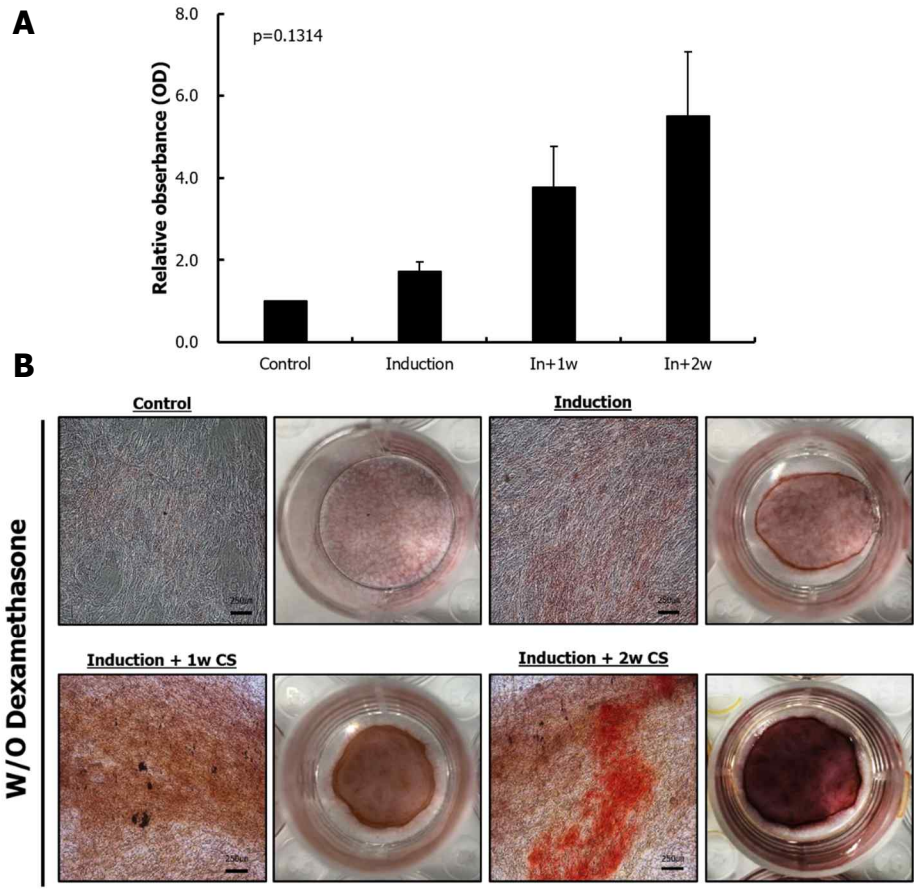


Figure 36. Human ADSCs osteogenesis was induced either by routine protocol or by the modified protocol which is without Dexamethasone and adding 100 μg/ml of 2weeks differentiation conditioned media total protein until 14days. (A) Significant model effect was detected (p=0.0241). (B) The morphology of the cultured cells was monitored under an inverted microscope. Data are presented as Mean±SE. Scale bars=200 μm.

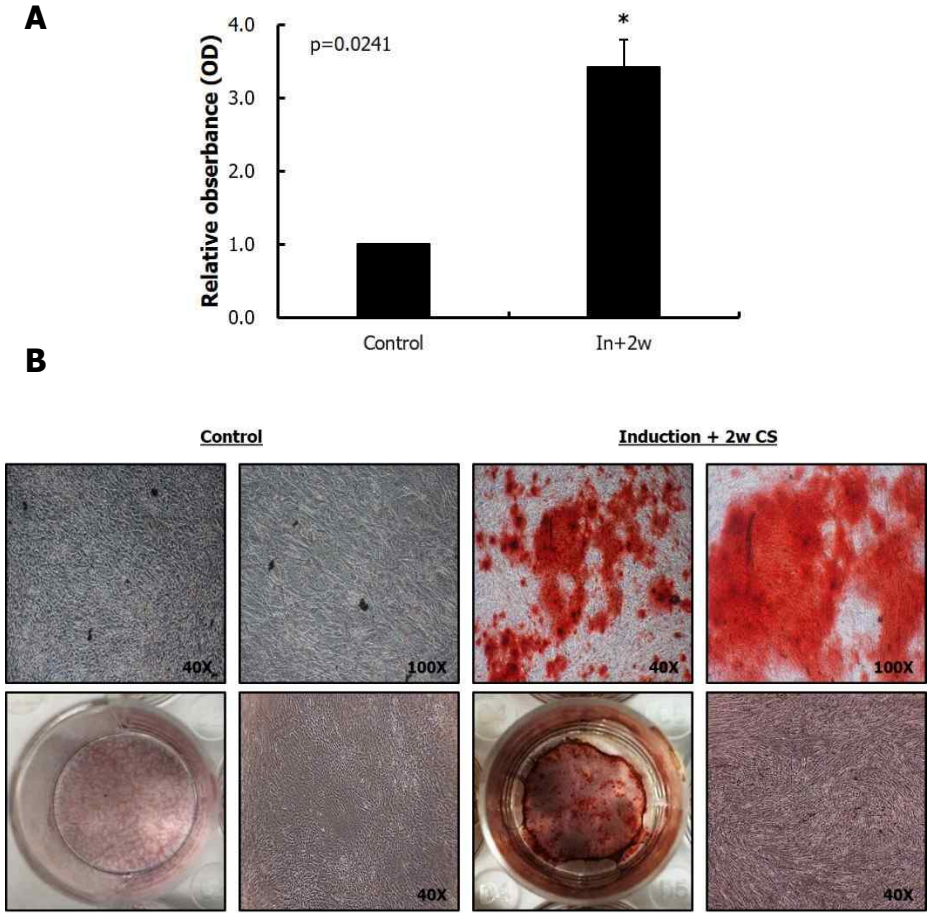
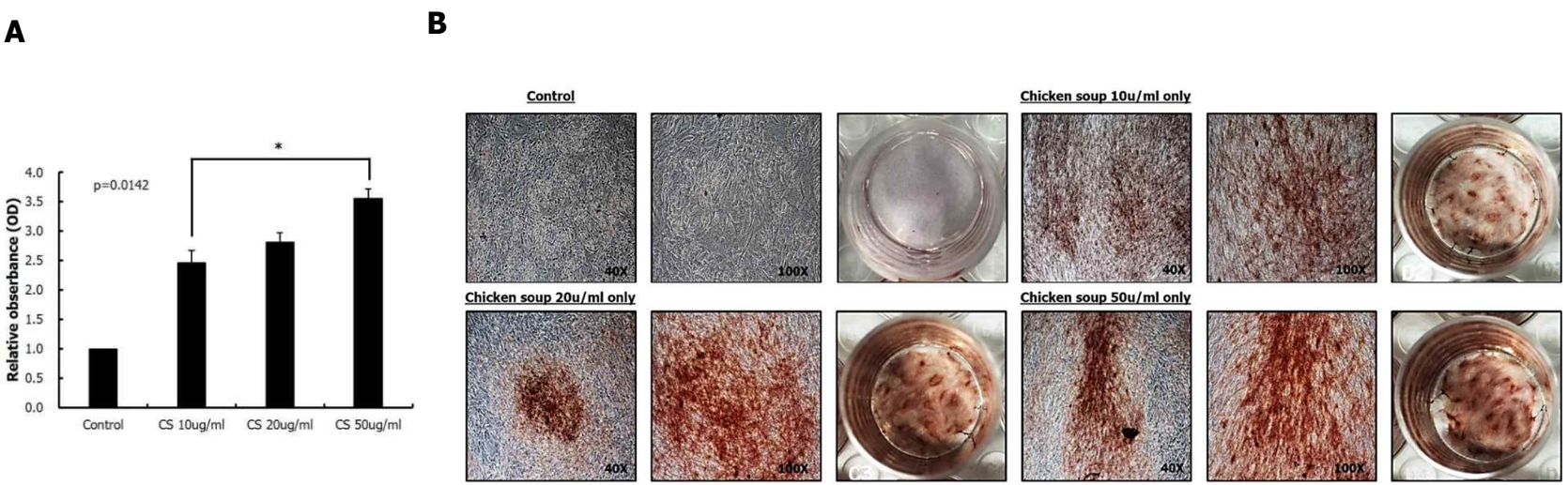


Figure 37. Human ADSCs osteogenesis was induced either by routine protocol or by the modified protocol which is without any type of inducing chemicals and only adding 2weeks differentiation conditioned media total protein as 10ug/ml, 20ug/ml, 50ug/ml until 14days. (A) Significant model effect was detected (p=0.0142) on 50ug/ml conditioned media only group. (B) The morphology of the cultured cells was monitored under an inverted microscope. Data are presented as Mean±SE. Scale bars=200 μm.



4. Discussion

This study was conducted to examine the effect of chicken bone marrow cell-derived, bioactive substances on osteogenic differentiation of human adipose tissue-derived stromal cells (hADSCs).

Over the past few years, many study groups have studied using conditioned media to evaluate efficiency as a generative biomarker. Released protein from conditioned media by cultured cells is a rich source of materials for biomarker as an application of novel treatment (Xue et al., 2008). Useful proteins released from cells culture medium can be used as novel bio-active substances to apply for the disease or regenerative treatment effects. This technique provides a useful, easy and effective method for discovering new biomarkers. The point here is that protein analysis is enhanced due to finding identification of biomarker candidate in which grown serum-free culture conditions (Righetti et al., 2003). Protein found in the medium controlled by the cell lines will come from cells through many different channels. The development of mass spectroscopy (MS),

unlabeled techniques, LC systems, and biometrics approaches had a profound impact on protein dynamics (Wong et al., 2009). These advances, combined with analysis of conditioned media, will undoubtedly facilitate identification of candidate biomarkers in cell culture environments.

The standard procedure for the osteogenic differentiation of mesenchymal stem cells is addition with dexamethasone (Dex), ascorbic acid (Asc) and β-glycerophosphate (β-Gly). Osteogenic differentiation protocols using these three chemicals are frequently used for many experimental studies, including tissue engineering or detection of differentiation capabilities of particular cell types. These complex chemical mechanisms involved in osteogenic differentiation of stem cell cultures under the influence of Dex, Asc and β-Gly. Dex, Asc and β-Gly orchestrate several regulatory mechanisms during the differentiation process and does not start with an initial differentiation that if any insufficient. In some reports, Dex prevents apoptosis of BMSCs in confluent cultures (Song et al., 2009) and promotes mesenchymal stem cell proliferation (Wang et al., 2012). In osteogenic differentiation, Dexamethasone acts

as a key molecule to differentiate into osteogenic lineage. FHL2 signaling is a critical signal in the osteogenic differentiation of BMSCs and that Dexamethasone induces osteogenesis through the upregulation of FHL2 expression (Hamidouche et al., 2008). Then FHL2 induces the upregulation of the key transcription factor Runx2. Furthermore, Dexamethasone induces upregulation of TAZ, which binds to Runx2 (Hong et al., 2009). Additionally, Dexamethasone further induces osteogenic differentiation is the modulation of Runx2 phosphorylation through MKP-1 (Langenbach and Handschel, 2013). Ascorbic acid facilitates osteogenic differentiation by increasing the secretion of Collagen type I, as the binding increases integrin $\alpha 2\beta 1$ to Col1 (Kishimoto et al., 2013). This signal pathway increased phosphorylation of ERK1/2 in the MAPK signaling pathway and then PERK1/2 moved into the nucleus, where it binds to Runx2 and increase the osteogenic related lower signals then make expression of osteogenic proteins (Chaudhary and Avioli, 2000). β-Glycerophosphate increases osteogenic differentiation by the phosphorylation of ERK1/2 (Langenbach and Handschel, 2013).

The results of this experimentation suggests that certain substrates secreted by chick sox9 positive cells stimulate osteogenic differentiation of hADSCs. But in this results have the unresolved questions of the identity of key morphogenetic factors influencing osteogenic differentiation of ADSCs. Identification of specific an key morphogenetic factors promoting ADSCs differentiation may elucidate mechanisms of leading to skeletal development.

CHAPTER 9.

GENERAL DISCUSSION AND CONCLUSION

In this study, I investigated an establishment of scale-up culture environment of chicken sox9 positive cell and the characterization of putative bioactive substance of cell secretion. The above results clearly demonstrated under the established culture regimen used in the present study, the culture of neonatal chicken bone marrow tissue yielded cuboidal cell- dominant populations. The isolated bone marrow cells characterization and cellular activities differs in many ways from that of mammalian bone marrow cells, which usually exhibit a fibroblast cell – dominant morphology (Toma et al., 2001; Bosnakovski et al., 2005; Roson-Burgo et al., 2014). Then examine the effect of chicken sox9 positive cell -derived, bioactive substances on osteogenic differentiation of human adipose tissue-derived stromal cells (hADSCs). I sought certain substrates secreted by chick sox9 positive cell stimulate osteogenic differentiation of hADSCs using the chicken sox9 positive cell and their culture systems, based on the ease of manipulation and their expansion.

As described in chapter 3-4, The results of this study clearly demonstrate that bone marrow tissue of neonatal chicks was premature at least up to 4 days after hatching. This tissue

contains a variety of cells involved in endochondral ossification as well as mature bone marrow cells. The majority of these cells became cuboidal until the end of primary culture, and more than 80% of cells were Sox9-positive. The results of the differentiation induction suggest the feasibility of Sox9 for regenerating bone marrow tissues.

Despite a mixed cell population, most cells collected at the end of P5 were Sox9-positive. Subculture of chick bone marrow cells may enable the vigorous proliferation of Sox9-positive cells. Although a quantitative analysis was not performed, RT-PCR results demonstrated that Sox9 expression in P0 cells was weaker than that in P5 cells. Otherwise, a mixed culture of Sox9-positive cells with other cells may increase their action when they are co-cultured at least up to five times, and the culture of premature bone marrow cells can trigger certain cells with a specific function. These hypotheses are supported by this finding that the proliferation of endothelial cell- and macrophage-specific CD31-positive cells was strongly enhanced, and that all osteogenesis- and chondrogenesis-related markers were expressed until P5. And two major findings were derived from the results of this study. First, the subculture

of neonatal bone marrow cells specifically amplifies both Sox9-and Col II-positive cells, which resulted Sox9-positive cells became dominant in the P5 cells, switching from the Col I dominant in nBMCs at the P0. Intact nBMCs contained considerable number of Sox9-positive cells (30% of total), but no Col II-positive cells were detected. Second, use of DMEM/F12 medium could optimize the outcome of nBMC subculture, which further contributed to customizing nBMC-mediated, differentiation protocol for both osteogenesis and chondrogenesis

Different culture systems may be specific to the proliferation of a specific cell type. The positive role of the culture system employed in this study in amplifying osteochondrognic cells was confirmed. The chick sox9 positive cell established in this study directed their differentiation towards osteogenesis and chondrogenesis, not adipogenesis, which confirmed their specific expression for osteochondrogenesis-related genes and markers. Taken together with the results of histological, molecular, and cytological analyses, these results suggest the potential use of chick sox9 positive cell as a basic biomaterial for clinical

applications for injuries and degenerative diseases (Barry and Murphy, 2004; Dezawa et al., 2005; Nandoe Tewarie et al., 2006; Kassem and Abdallah, 2008).

In Chapter 5 and 6, I focused the confirm the effectiveness of basic fibroblast growth factor (bFGF) and different culture systems for expanding chicken bone marrow-derived cells (BMCs). Basic fibroblast growth factor (bFGF) is a member of the FGF family and is a critical component of stem cell culture field(Sotiropoulou et al., 2006; Colenci et al., 2014). However, the role and optimized concentration of bFGF on promoting the proliferation of MSCs derived from chicken bone marrow (BM) has not been reported. Then the above results clearly demonstrate that under the regimen used in the present study, the culture of newborn chicken bone marrow tissue yielded both stromal and cuboidal cell- dominant populations. The culture of chicken bone marrow cells differs in many ways from that of mammalian bone marrow cells, which usually exhibit a fibroblast-like, stromal cell morphology (Toma et al., 2001; Bosnakovski et al., 2005). The retrieved cells using PGC medium were maintained up to at least the third passage, and a number

of differences in cell specific protein expressions were observed compared with our optimized cultured environments.

From this results, bFGF is responsible for the proliferation of chicken sox9 positive cell but the underlying molecular regulatory mechanism are still not well understood and thus need further study about the proliferation related specific antibodies. In example of study using MSCs, bFGF treatment will induce condrogenesis and osteogenic differentiation (Chiou et al., 2006; Ito et al., 2008; Kim et al., 2011). Osteogenic differentiation was significantly incensement ($p<0.0001$) detected on insertion of more 5ng/ml bFGF and chondrogenic differentiation was apparently detected on addition of 1mg/ml bFGF. In migration and invasion assay, exposure of 5ng/ml bFGF improved each activity. In conclusion, this study suggests that addition of 5 ng/ml bFGF in vitro on chick sox9 positive cell have effectiveness on proliferation, differentiation, migration and invasion ability. And the PGC culture environment acts as exhibited better proliferation than the optimal compositions of sox9 positive population during culture. And stromal cell marker like CD44 is expressed on only PGC medium culture environment. Chicken bone marrow cells have different characteristics

compared with bone marrow cells of mammalian species, which usually yield a fibroblast-dominant population. Considering the mixed population of bone marrow cells, a different regimen may be needed for the outgrowth of chicken bone marrow-derived fibroblasts. Modification of the regimen used for the culture of bone marrow cells from mammals is necessary for the outgrowth of fibroblasts. In any case, the results of this study will make it possible to characterize chicken bone marrow cells of various types further and will increase the feasibility of using chicken bone marrow cells for different applications.

In chapter 7, I was conducted to examine the effect of chicken sox9 positive cell -derived, bioactive substances on osteogenic differentiation of human adipose tissue-derived stromal cells (hADSCs). In this study, I demonstrate that morphogenetic signals from chicken bone marrow cells, in the form of soluble factors, induce as osteogenic differentiation enhancer of human ADSCs during in vitro differentiation. This results suggest possibility of the conditioned media as the morphogenetic factors from sox9 positive cell to differentiation induction agents or reprogram cells for in vivo

bone tissue engineering applications. ADSCs expanded in chicken sox9 positive cell derived osteogenic differentiation conditioned medium enhanced osteogenic differentiation compared by routine protocol method. In addition, incubation of ADSCs with conditioned medium supplemented with osteogenic factors or by oneself induced more osteogenesis and accumulation of calcium and increased ALP activity. These findings reveal that chicken sox9 positive cell derived secretion factors promote osteogenesis of ADSCs during in vitro differentiation culture.
Given the current inefficient in vitro human cell differentiation protocols, this method and results develop a simple and innovative differentiation method for generating in vivo bone tissue from adult stem cells.

Future studies are needed to reveal the unresolved questions of the identity of key morphogenetic factors influencing osteogenic differentiation of ADSCs. Identification of specific a key morphogenetic factors promoting ADSCs differentiation may elucidate mechanisms of leading to skeletal development. Through these future studies, new knowledge will be gained to overcome shortcomings growth factor or gene

deliveries for instructive signals or other efficient factors. Given the current inefficient in vitro human cell differentiation protocols, this method and results develop a simple and innovative differentiation method for generating in vivo bone tissue from adult stem cells.

In conclusion, I have verified that chicken bone marrow derived cells are different morphology and also cell characterizations, differentiation ability. And I establish culture condition of chicken bone marrow derived cells related cell proliferation and differentiation using DMEM/F12 + 5ng/ml bFGF. Then I explored the effect of chicken sox9 positive cell-derived, bioactive substances on osteogenic differentiation of human adipose tissue-derived stromal cells (hADSCs). The results obtained from this study have implications for the understanding of chicken sox9 positive cell and their difference suggests how it could be a newly alternative medicines. And it brings advances in therapy of several chronic and degenerative diseases.

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SUMMARY IN KOREAN

본 논문에서는 닭 골 및 연골 전구세포 (Chicken sox9 positive cell) 의 최적화된 배양 환경 및 조건을 확립함으로써 회수된 세포의 효율적인 조작 기법 및 대량 배양 시스템을 구축하였다. 포유류 골수 유래 세포의 형태학적 및 세포 특성과 뼈의 성장능에서의 차이를 토대로 닭 장골 유래 세포에 대한 배양 환경 탐색 및 이를 통한 세포 이용 재생의학 분야로의 응용 방안에 대하여 고찰하였다.

먼저 Chapter 4 에서, 4일령 병아리에서 회수된 세포에 대한 특성 검증을 실시하였다. 일련의 실험 결과를 토대로 4일령 병아리에서 회수된 골수 유래 세포는 전반적으로 입방상피세포의 형태를 나타내고 있으며, 4일령 병아리의 장골의 단면을 염색한 결과 성장판 및 제 2차 골 중심 부위가 형성 되지 않음을 확인 할 수 있었다. 또한 골/연골 전구 세포의 활발한 증식 및 포유류와 비교하여 장골 발달 단계에서의 차이를 확인 할 수 있었다. 다양한 세포 특이 마커 발현 테스트를 통하여 회수된 세포는 골/연골 전구 세포인 sox9 positive 세포임을 확인 하였다. 또한 Chapter 5, 6에서, 회수된 세포에 대한 골 및 연골 세포로의 성장 및 발달 그리고 분화능에 따른 배양 환경을 탐색하였다. 이 결과를 토대로 DMEM/F12를

이용한 기본 배양 조건에 5 ng/ml의 bFGF 첨가는 세포 특이 마커 발현 및 성장능, 분화능이 우수함을 확인할 수 있었다. Chapter 7에서는 4일령 골수 유래 세포에서의 전능성 및 줄기세포 관련 유전자의 증폭 및 줄기세포 유사 세포의 회수를 위하여 배야 줄기세포 배양 환경과 유사한 PGC 배양 배지를 도입하였다. 그 결과, CD44를 포함한 기질 세포 마커가 발현 됨을 확인하였으며, 이 결과를 토대로 4일령 병아리 골수에서 줄기세포 관련 특성을 갖는 세포의 배양을 성공하였고 최적화된 배양 환경을 구축하였다. 마지막으로 Chapter 8에서 sox9 positive 세포를 이용한 세포 분비 유래 유용물질 탐색을 실시 하였다. 회수된 닭 sox9 positive 세포를 골 분화를 유도한 후 배양 배지에서 세포 분비 유래 물질을 농축 후 인간 지방 유래 중간엽 기질 세포에 분화유도제로 사용하였다. 그 결과, 일반적인 분화 유도 환경에서 보다 유의적인 차이를 보였으며 분화 유도에 관련된 물질을 대체 함과 동시에 분화 유도에 단독 처리만으로도 골 분화능이 있음을 확인하였다.

골수 유래 세포는 중간엽 줄기세포를 포함한 다양한 여러 세포들을 함유 하고 있으며, 우리 인체의 면역계 관련 세포를 함유하는 중요한 세포로써 치료에 접목 가능한 보건 의료적, 산업적 가치가 큰 세포이다. 본 논문에서 수행한 닭 장골 유래 sox9 positive 세포의 배양 조건 확립과 체외 배양 기술은 골수 내 기능성 세포

및 골수 구성 세포들을 활용한 치료 기술에 접목 될 수 있으며, 성장 관련 질병 및 세포 치료를 이용한 다양한 임상 분야에 기여 할 수 있는 중요한 선도 기술이 될 것으로 사료된다.

앞으로의 추가 연구를 통해 회수된 세포에서 분비되는 물질에 대한 정확한 양적 동정 및 유용 물질에 대한 선별된 동정 과정을 통해 추가적인 분비 물질을 이용한 치료 접목 분야에 관한 가능성을 밝혀 낼 수 있을 것이다.