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Studies on Improvement of Bone Marrow-derived Cell Establishment and Manipulation in Chicken

닭 골수 유래 세포의 확립과 조작기법 향상에 관한 연구

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SUMMARY

Bone marrow-derived cells have an enormous value for the research on mesenchymal stem cell and cell transformation. And also Mesenchymal-derived, multipotent cells have become a valuable resource for cell-to-tissue regeneration and experimental modelling for differentiation and reprogramming. In the chicken, bone marrow-derived cells can induce somatic chimerism and if their pluripotency is confirmed, they could be used as resources to expand the applications of chicken pluripotent cells for various purposes. Bone marrow cells are considered as the basic material for clinical application to injuries and degenerative diseases. 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. And also no information on cell culture properties have been reported because of their heterogeneous phenotype and mixed cell population. In this study, optimized and improved manipulation system of chicken bone marrow derived cells was established

and by evaluating the effect of addition of growth factors on the growth and maintenance of chicken bone marrow derived cells.

First, I observed two different populations were maintained: a spindle shape dominant population consisting mainly of fibroblast-like cells and a cuboidal shape dominant population. So, I investigated A significant correlation affecting cell retrieval was detected between the parameters of body weight and leg length in the spindle shape cell-dominant and cuboidal shape cell-dominant populations. Additionally, analysis of cell kinetics, protein marker expression of the chicken bone marrow cells at passage 5. As the results, based on these observations, we conclude that chicken bone marrow cells possess different cellular characteristics compared with the bone marrow cells of mice and humans, and that a different approach and culture regimen may be necessary for manipulating chicken bone marrow-derived multifunctional cells.

Next, I investigated the necessity of growth factors and different basic media to modify the culture system of chicken

bone marrow cells and the change of cellular properties in chicken bone marrow cells through a suspended culture. Four-day-old white leghorn chicks were employed for experimental animal and the isolated bone marrow derived cells were cultured in different four types media (High glucose DMEM, Low glucose DMEM, F-12/DMEM, αMEM) to which 5 ng/ml bFGF and 500 unit/ml LIF were supplemented or not. To monitor of the effect of growth factors on the capacity of cell maintenance in vitro, primary cell attachment, CFU-F colony number, proliferative activity was evaluated as experimental parameters. As a results, the number of CFU-F-positive colonies was higher ($p<0.05$) after the bFGF and LIF addition than after no addition, which resulted improved primary cell attachment and also better proliferative activity was detected in the growth factor supplementation group.

Additionally, analysis of pluripotency- or differentiation-related gene expression and protein marker expression in the chicken bone marrow derived cells were detected. Different expression profile in the expression of several genes was detected after the supplementation or not,

which differed from that of primordial germ cells and embryonic fibroblasts. And also, flow cytometry analysis data shows more expression rate detected such as CD44, CD105, MCAM in growth factors supplementation group.

In conclusion, the combined addition of bFGF and LIF to culture medium improved the culture efficiency of chicken BMCs of mixed population, which may contain various reprogrammable cells.

Keywords : Bone marrow-derived cells, Cell culture, Chicken, Morphology, Physical profiles, Growth Factors, Proliferation, Cell characteristic

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BHA : butylated hydroxyanisole

BM : bone marrow

BMC : bone marrow derived-adherent cell

BMP : bone morphogenetic protein

CFU-F: colony forming unit-fibroblast

CMBC : chicken bone marrow derived cell

CREB : cyclic AMP-responsive element binding protein

DMEM : dulbecco's modified Eagle's medium

DMSO : dimethyl sulfoxide

DPBS : dubecco' s phosphate buffer saline

FACS : fluorescence activated cell sorter

FBS : fetal bovine serum

FGF-2 : fibroblast growth factor-2

GMP : good manufacturing practices

GP130 : glycoprotein 130

HPSGs : heparin sulfate proteoglycans

IF : immunofluorescence

IL-6 : interleukin-6

ITS : insulin, transferrin and selenium

JAK : janus kinase

LIF : leukemia inhibitory factor

MACS : magnetic-activated cell sorting

MAPKs : mitogen-activated protein kinases

MHC : major histocompatibility complex

MSC : mesenchymal stem cell

PCR : polymerase chain reaction

PI3K : phosphatidylinositol-3-OH kinase

RBC : red blood cell

SAS : statistical analysis system

SH2 : src homology-2

SMA : smooth muscle actin

SOP : standard operating procedures

STAT : signal transducer and activator of transcription

CHAPTER 1.

GENERAL INTRODUCTION

Bone marrow (BM) 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 constitutes 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). 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). Mesenchymal-derived cells are crucial to the development of novel biotechnologies for stem cells. 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.

In the chicken, bone marrow-derived cells can induce somatic chimerism (Ishii and Mikawa, 2005; Heo et al., 2011; Heo et al., 2012) and if their pluripotency is 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 have not been established. No information on donor selection and cell 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; Hudson et al., 2011; Dai et al., 2014). Although many reports on the isolation and culture of bone marrow-derived cells from mammalian (e.g., feline, canine, and porcine) species exist, little information is available on bone marrow-derived cells from avian species.(Martin et al., 2002; Zhang et al., 2011b; Munoz et al., 2012; Do et al., 2015; Paknejad et al., 2015) Furthermore, several other method of bone marrow-derived cells manipulation, including optimization of culture density and frequent medium change, have also been proposed but these protocols are not standardized.

Mesenchymal-derived, multipotent cells are crucial to the development of novel biotechnologies for stem cells. 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 protocol of

chicken bone marrow cells and the large-scale expanded culture system has been used to high reproducibility of bone marrow cells. In this study, the necessity of growth factors and different basic media were investigated to modify the culture system of chicken bone marrow cells and the change of cellular properties in chicken bone marrow cells was observed through a suspended culture. 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. Knowing the response of chicken bone marrow cells to optimized medium will directly contribute to developing a culture system. It improves the efficiency of chicken bone marrow cells culture system.

The hypothesis of this study is to harvest a large amount of reliable mesenchymal stem cells-like cells in chicken

bone marrow, different four types of basic media and with or without growth factors were optimized for promoting cell attachment and proliferation of mesenchymal stem cells-like cells to tissue culture plate. By using these optimized culture media, the effects of on the proliferation and differentiation potential capacity of chicken bone marrow cells were examined. Given these observations, we provide important clues about the optimized culture medium of chicken bone marrow cells for the cultivation in different media with or without growth factors and the feasibility of large-scale expansion.

CHAPTER 2.

LITERATURE REVIEW

1. Bone marrow derived cells

Bone marrow mononuclear cells are a mixed population of single nucleus cells that include diverse type of cells sush as monocytes, lymphocytes, hematopoietic stem cells, mesenchymal stem cells and progenitor cells (Alvarez-Viejo et al., 2013). Bone marrow mononuclear cells are invaluable for research studying the genetic and tissue engineering such as regenerative disease study (Henrich et al., 2015b, a). Bone marrow is the most common source of mesenchymal stem cells and progenitor cells (Hu et al., 2015; Huang and Yao, 2015). Mesenchymal stem cells and bone marrow derived stromal cells are adherent to tissue culture plastic dish within 24 to 48 hours (Ramakrishnan et al., 2013). But red blood cells and hematopoietic stem cells are not adherent to culture plate (Kern et al., 2006; Jin et al., 2013). Bone marrow derived mesenchymal stem cell showing fibroblast like morphology and it can be take a colony-forming unit-fibroblast (CFU-F) assay (Horwitz et al., 2005). Furthermore, bone marrow derived mesenchymal stem cells have multi-lineage differentiate ability like osteogenic, adipogenic, chondrogenic differentiation in vitro

(Jaiswal et al., 1997; Mackay et al., 1998; Jaiswal et al., 2000; Zavan et al., 2007; Wechsler et al., 2015). 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. Osteoprogenitor cell, preosteoblast, osteoblast and osteocytes (Hofstetter et al., 1991; Aubin, 2001). Osteoblast is generally accepted that derived from a multipotent mesenchymal stem cells. It can be differentiated into adipogenic, osteoblastic,

chondroblastic, myoblastic, reticular and fibroblastic lineages (Friedenstein et al., 1966; Orlic et al., 2001; Toma et al., 2002; Morikawa et al., 2009).

2. Mesenchymal stem cells differentiation pathways

Bone marrow derived mesenchymal stem cells can differentiate into multi lineages of mesodermal, ectodermal and endodermal (Woodbury et al., 2002). It can be differentiate such as bone, fat, chondrocyte, 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 to 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).

2.1 Mesoderm differentiation

Generally mesenchymal stem cells are easily differentiated into mesodermal lineage because of the same embryonic origin. Mesenchymal stem cells can differentiate into osteogenic, adipogenic and chondrogenic lineage. 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 adipogenic differentiation, differentiation medium consisting of culture media supplemented with isobutylmethylxanthine, indomethacin, dexamethasone, human insulin (Choi et al., 2011; Yu et al., 2011; Fu et al., 2014; Naderi et al., 2014). And it can be demonstrated by formation of lipid droplet using Oil Red O staining solution (Ramirez-Zacarias et al., 1992). 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). Furthermore, gene expression regulates lineage repression. PPAR−γ and C/EBP are involved in adipogenesis and SOX9 induce chondrogenesis (Hu et al., 1995; Wu et al., 1995). And also PPAR−γ and Cbfa−1 gene have correlation. Overexpression of the PPAR−γ gene in adipogenesis also represses Cbfa−1 gene expression in osteogenic cells (Heim et al., 2004; Lian et al., 2004).

2.2 Ectoderm differentiation

Mesenchymal stem cells can differentiate into ectoderm lineage such as neuron. Notch−1 and protein kinase A are involved in regulate lineage repression (Wu et al., 2007). In neuronalgenic differentiation, differentiation medium consisting of culture media supplemented with β−mercaptoethanol, Dimethyl sulfoxide(DMSO), butylated hydroxyanisole(BHA), Kcl, vaporic acid, forskolin, hydrocortisone, insulin. And it can be demonstrated by formation of neuron or synapse using by

specific detection antibody (Patterson and Nawa, 1993).

3. Mesenchymal stem cells isolation

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). Identification of mesenchymal stem cells are difficult to detection of surface marker. Because mesenchymal stem cells lack clearly defined surface marker. For the purified isolation of mesenchymal stem cells, whole bone marrow are counted and resuspended in culture medium, and plate in culture dish plate appropriate cell number. Nonadherent cells like blood cells are removed after 24hr later by changing medium. After 3days or later, remained cells are attached on bottom of culture dish plate (Ohgushi et al., 1996; Bennaceur-Griscelli et al., 1999; Bernardo et al., 2007). These

cells are generally referred to bone marrow stromal cells. Continuous cultured with medium change per 3 days and removal of nonadherent cells, stromal cells are morphologically homogeneous and characteristic similar mesenchymal stem cells.

Mesenchymal stem cells can various culture media depending on species and organ of origin. Generally used media is Dulbecco's modified Eagle's medium (DMEM) and α-minimum essential medium (Beyer Nardi and da Silva Meirelles, 2006). Ideal culture condition may influence functional characteristic of cells and similar phenotype. The fetal bovine serum used to these cell that influence the selection and expansion of these cells (Bieback et al., 2009). In mesenchymal stem cell culture, addition of specific growth factors are also important and guide to final characteristic of mesenchymal stem cells culture. For instance, insertion of Leukemia inhibitory factor (LIF) and use of fibronectin-coated surface influence on growth of murine bone marrow derived multipotent adult progenitor cells (Dowsing et al., 2000). And supplementation of fibroblast growth factors-2 (FGF-2) increase the lifespan of

human mesenchymal stem cells (Bianchi et al., 2003).

Cell seeding density various depending on cell types and influences expansion capacity of mesenchymal stem cells. Long-term culture and appropriate cell density are also decision factors of loss of differentiation potential for cell and self-renewal capacity (Kolbe et al., 2011).

3.1 Mesenchymal stem cell isolation methods

Recent days few established protocols for the bone marrow derived mesenchymal stem cells are suggested (Soleimani and Nadri, 2009). The most and popular method for the isolation of bone marrow derived mesenchymal stem cells are whole bone marrow adherent assay (Dobson et al., 1999). This protocol is whole bone marrow was seeded in appropriate culture dish that from tibia, femur and limb such as on of experimental animals. This method is cutting edge of the bone and flushing out whole bone marrow using appropriate size of syringe and niddle. And then, few hours or days later non-adherent cells are removed during media changes. After several

times of media changes, adherent cells like mesenchymal stromal cells are homogenous and proliferated rapidly.

It can be possible isolate mesenchymal stem cells from tissue like cartilage and fat (Pittenger et al., 2000). In order to isolate mesenchymal stem cells from these tissue, enzymatic treat assay like a collagenase also needed. Using this protocol, it is possible get more purified and homogenous and can isolate 100-fold more mesenchymal stem cells compared to whole bone marrow flushing method (Sakaguchi et al., 2004). And also isolated mesenchymal stem cells using enzyme treated possess higher metabolic cell activity compared to whole bone marrow flushing method.

Another isolation method of mesenchymal stem cells is using density gradient of bone marrow (Tondreau et al., 2004). This protocol involves the high density with low osmotic and viscosity to isolate mononuclear cells fraction of whole bone marrow. It can be separated by density gradient and obtained mesenchymal stem cells fraction. Also to overcome of the hematopoietic cell contamination, cell surface binding technique

is developed such as MACS and flow cytometry assay (Chang et al., 2009; Insausti et al., 2012; Xia et al., 2013). MACS is using magnetic beads that binding on cell surface labelled with antibody-coated magnetic beads (Amiri et al., 2015). And flow cytometry assay can sort cell fraction using intensity of fluorescence that emit while they passing through a nozzle of detection machine.

3.2 Leukemia inhibitory factor (LIF) signaling

Leukemia inhibitory factors is generally addition on culture medium and it typically reduce spontaneous differentiation. In mouse embryonic stem cells, it was discovered that their 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).

JAK-STAT signaling is controlled primarily interaction of the family of the cytokine interleukin-6 (IL-6) that signal

cascade via receptors (Liu et al., 1998; Taylor et al., 2016). LIF is first bind to its signal receptor LIF-R and recruits another signaling receptor, glycoprotein 130 (GP130), to form heterodimer that mediates downstream signal transduction (Smith and Treutlein, 1998; Song and Lim, 2006; Skiniotis et al., 2008). The formation of this complex expresses activation of the receptor-accociated Janus kinase(JAKs), in the phosphorylation of receptor docking sites, and finally in the recruitment of Src homology-2 (SH2) domain containing proteins such as signal transducer and activator of transcription 3 (STAT3) (David et al., 1995; Wakioka et al., 1999; Borges et al., 2008). After binding to receptor, STAT3 are phosphorylated on tyrosine 705 (Tyr705) residues and dimerize with another phosphorylated STAT3 (Petterino et al., 2007; Wakahara et al., 2012; Lin et al., 2014). Then dimers translocated to the nucleus in a regulated promoter and enhancer regions of their target genes. Cytokine signaling 3 (SOCS3) is an important negative regulator of the LIF/STAT3 pathway (Tamiya et al., 2011; Collins et al., 2013). SOCS3 plays a critical role in many cell types and tissue where the LIF pathway is active (Tamiya et al., 2011; Elsaeidi et al., 2014).

LIF signaling also activates mitogen-activated protein kinases (MAPKs), cyclic AMP-responsive element binding protein (CREB) and ribosomal s6 kinase, src family kinases and phosphatidylinositol-3-OH kinase (PI3K) (Zhou et al., 2014; Chien et al., 2015).

3.3 Fibroblast growth factor-2 (FGF-2) signaling

Fibroblast growth factors (FGFs) regulate fundamental developmental pathways like mesoderm patterning in 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). FGFs are controlled by secretion of glycoproteins that are sequestered to the extracellular matrix and also heparin sulfate proteoglycans (HPSGs) (Pomin, 2016). The specificity of the FGF-FGFR interaction is mediated by the tissue-specific expression of particular ligands and receptors, coupled with several cell surface or secreted proteins that facilitate the FGF-FGFR

interaction (Kato and Sekine, 1999; Flippot et al., 2015).

In mouse study, FGFs activation mechanism revealed more widely how influence proliferation. In mouse prostate stromal compartment, FGF10 is concomitant with androgen receptor 115 and resulted in epithelial hyperproliferation (Pu et al., 2007). Excessive cell proliferation is one of the most common character of cancer cells. FGF10 signaling also involved in cancer cell development (Turner and Grose, 2010). It is dependent on FGF receptor 1 and so attenuate cancer cell development, meanwhile also inhibit FGFR2 signaling through receptor heterodimerization. Also epithelium, FGF10 signaling combine with AKT promotes more tumorigenesis (Memarzadeh et al., 2007). Another example in prostate epithelium, Pten knockout cells are prostate adenocarcinoma that synergized with overexpression of FGF8 (Mulholland et al., 2008). So FGF signaling is important in cancer development and also suggest that PI3K−AKT pathway.

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). The FGFs is dependent pattern of expression of FGF1 and FGF2 that are located in mesenchymal cells prior accumulation of bone matrix at long bone development. FGFR3 are found in preosteoblasts and osteoblasts. 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 regulate cell proliferation and osteoblast precursor cells replication into osteoblasts and controlled apoptosis of immature osteoblasts (Mansukhani et al., 2000). FGFs signaling interact with other growth factors signaling and regulate 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.

4. Mesenchymal stem cells characterization

Multiple type CD marker is used distinguish mesenchymal stem cell from hematopoietic cells. Generally hematopoietic cells are expressed CD34, CD45, CD14 and HLA-DR whereas mesenchymal stem cells are expressed CD29, CD44, CD73, CD105, CD166 and Stro-1 (Spangrude et al., 1988; Lee et al., 2004; Pereira et al., 2016). CD29 is also known as integrin beta-1 is a protein that in cardiac muscle and skeletal muscle, the integrin beta-1D isoform is expressed and have been found in limb girdle muscular dystrophy and polyneuropathy (Krampera et al., 2006). CD44 is cell-surface glycoprotein that involved on cell to cell interaction. It functions as a bone homing receptor, so directing migration of human hematopoietic stem cells and mesenchymal stem cells to bone marrow (Bajorath, 2000). Specific inhibition of CD73/CD29 demonstrated their substrate dependent involvement in MSC migration after loading (Ode et al., 2011). MSC migration is controlled by CD73/CD29, which in turn are regulated by mechanical stimulation of cells. CD105 also called endoglin is type I membrane glycoprotein and crucial role in angiogenesis (Duff et al., 2003; Fonsatti et al., 2003). So it is expressed on

endothelial cells in particular on proliferating cells and on mesenchymal cells (Minhajat et al., 2006; Tachezy et al., 2010). Stro-1 involved in mesenchymal stem cells differentiates into multi lineage like adipose, cartilage and bone cells (Ahdjoudj et al., 2001). Vimentin, laminin, fibronectin and osteopontin can be synthesized by bone marrow derived mesenchymal stem cells and also expressed myofibroblasts, smooth muscle actin, nestin and transforming growth factor-β receptor (Puche et al., 2013). Fibroblast colony forming units (CFU-F) are distinct feature of the mesenchymal stem cells (Kolf et al., 2007). Isolated from bone marrow cells were selected from attached cell and non-attached cell. Attached cells are forming colony and some growth factors like basic fibroblast growth factor, epidermal growth factor, insulin growth factor can accelerate proliferation of CFU-F. CFU-F assay also distinguishable from colony sizes, aggregated cell number, cell morphology and differentiation ability.

5. Mesenchymal stem cell clinical application

Stem cell therapy means autologous and allogenic cell

transplantation into patients, using local transplantation or systemic infusion. Mesenchymal stem cells can adapt in various type of clinical application. Many researchers have been studied on 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). But it is different expression pattern by species, so cell type and culture condition may effect on mesenchymal stem cells immunogenicity.

Mesenchymal stem cells have multipotent ability and it can be differentiated into mesodermal lineage. And based on stem cells characteristic, immunosuppressive properties and some possible cell transformation can be suggested potential risk (Flores-Figueroa et al., 2006; Merino-Gonzalez et al., 2016). So using of mesenchymal stem cells in therapeutic application has potential risk. For example, recent study revealed mesenchymal stem cells injected into rat heart were differentiated into bone like cells and driven to calcification of

heart tissue (Lv et al., 2016). Mesenchymal stem cells have differentiation and cell transformation ability during long time culture. So it is important sustain normal character and never undergo cell transformation.

Mesenchymal stem cells can differentiate into osteoblast and chondrocyte. The osteoblastic progenitor cells are developed into osteoblast and then deposit into outer layer of bones during intramembranous ossification (Shapiro, 2008). Meanwhile chondrocytes are proliferated and hypertrophy and deposited into growth plate of each end of bone. This process called endochondral ossification (Zhang et al., 2011a). Mesenchymal stem cells important function during bone healing as stem cells are precursor of osteoblasts and chondrocytes. Also variety of signaling pathways are involved in mesenchymal stem cells differentiation. BMP, Wnt, Notch signaling are important on extracellular mediators of differentiation (Lin and Hankenson, 2011).

Mesenchymal stem cells have potential clinical application into heart and vessels (Pittenger and Martin, 2004).

Bone marrow derived mesenchymal stem cells can differentiate cardiomyocytes when treated supplement chemical like 5-azazystidine (Shake et al., 2002). Differentiated into cardiomyocytes fully functioned responding to physiological stimuli. They also differentiate into endothelial cells in vitro and some researchers implanted into vascular prostheses and confirmed these cells contribute to make a vascular wall (Griese et al., 2003). Also bone marrow derived mesenchymal stem cells are not clearly demonstrated possess neural regenerative. Adipose derived stem cells are more adequative in differentiated into neuronal potential than bone marrow derived cells (Anghileri et al., 2008). But MSCs can differentiate into neuronal related cell by treated diverse chemicals and co-culture with neuronal cells (Anghileri et al., 2008). The main problem of the application in therapeutic approach is immunosuppressive properties and transformation during long term culture.

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.

CHAPTER 3.

Culture and Subsequent Characterization of Bone Marrow Cells Retrieved from Neonatal White Leg Horn of Different Physical Profiles

1. Introduction

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). In the chicken, bone marrow-derived cells can induce somatic chimerism (Ishii and Mikawa, 2005; Heo et al., 2011; Heo et al., 2012) and if their pluripotency is 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 have not been established. No information on donor selection and cell 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; Hudson et al., 2011; Dai et al., 2014). Although many reports on the isolation and culture of bone marrow-derived cells from mammalian (e.g., feline, canine, and porcine) species exist, little information is available on bone marrow-derived cells from avian species.(Martin et al., 2002; Zhang et al., 2011b; Munoz et al., 2012; Do et al., 2015; Paknejad et al., 2015) Furthermore, several other method of bone marrow-derived cells manipulation, including optimization of culture density and frequent medium change, have also been proposed but these protocols are not standardized.

Given these observations, we presumed that chicken bone marrow contains various types of cells, and we conducted the present study to establish basic protocols for chicken bone marrow cell retrieval and initial culture. From this study, we obtained information on the cell donor profile and various aspects of primary cell culture. And also we found from this study, cell surface marker expression of each different cell types. Randomized, controlled trials with an even distribution were conducted. Therefore, it is necessary to develop a reliable and easy system to isolate and expand a homogeneous

population chicken bone marrow cells.

2. Materials and Methods

Experimental design

General experimental procedure was depicted in Fig 1. Two series of experimentation was conducted to evaluate donor effect on bone marrow cell retrieval and the capacity of retrieved cells for in vitro expansion following subculture. In Exp 1, physical profile of the donor such as body weight, height and the length of legs were measured and to examine their relationship with the cell number retrieved, relation coefficient was calculated. In Exp 2, the bone marrow cell collected were subsequently cultured in our standard medium and the subculture was conducted up to three times. Cell number and accumulated number of cultured cells were counted at the end of each subpassge and doubling time of each subpassage was calculated. Morphology of cultured cells were monitored at the end of the third passage.

Experimental animals

Four-day-old white leghorn (*Gallus gallus*) were employed as the donor of bone marrow cell donors. The animals were maintained at the University Animal Farm, Seoul National University, Korea, using our standard management program. All procedures for animal management, breeding and euthanasia were performed according to the standard protocols of Seoul National University and institutional animal care and unit committee (SNU-140912-4) approved all relevant procedures of our experiments. The procedures used for animal management followed the standard operating procedure of our laboratory.

Measurement of physical characteristics

Measurement of body weight, height and length of leg was conducted before euthanasia. In the case of height, the length from the top of the head to the bottom of the feet was measured, and the length from the pelvic arch to the epicondyle of the tibia was measured to know the length of leg.

Isolation and culture of bone marrow-derived primary cells

The femurs and tibia separated from both legs of euthanized donors were washed with 1% (v/v) antibiotic-antimycotic solution (Gibco Invitogen, Grand Island, NY) and 2% (v/v) fetal bovine serum (FBS; Welgene Inc., Daegu, Korea) containing Dubecco's phosphate buffer saline (DPBS; Welgene Inc., Daegu, Korea). Muscle tissues attached to the bones were subsequently removed and the marrow cavity was exposed by excising the spongious part of each bone. Bone marrow cells were retrieved by flushing of bone marrow tissue with 2% (v/v) FBS containing DPBS. Whole bone marrow-derived cells including red blood cells were spread on culture dish.

Culture of bone marrow-derived cells

One million bone marrow cells were spread in 100 mm tissue culture dishes and cultured in nutrient mixture F-12 (DMEM/F-12; Gibco Invitogen) supplemented with 10% (v/v) FBS (FBS; Welgene Inc., Daegu, Korea) at 37 ℃, 5% CO2 in a

humidified atmosphere. Three days after culture, red blood cell and non-adherent cells were removed and medium change was performed every three days interval. When bone marrow derived cells reached to 80% confluence, the cells cultured were dissociated by 0.25% trypsin-EDTA (Gibco Invitogen) and counted total cell number by hemocytometer. Two-hundreads throusands cells were reseeded and subsequently cultured up to the third passage. At the end of culture, morphology of cultured cells were observed under an inverted microscope (TS 100-F, Nikon, Tokyo, Japan).

Assessing of cell proliferation during in vitro-culture of cells

The number of cultured cells at the end of each passage and the initial number of cells seeded for each passage were counted. Accumulated number of cells throughout whole subpassage, the number of cells collected at the end of each passage and doubling time were counted or calculated for monitoring cell proliferation during in vitro culture. Doubling time of bone marrow derived cells in each passage was calculated by $t\log 2/(\log N_t - \log N_0)$, where t is time to

confluence, N_t is the number of cells at the end of each passage and N_0 is the number of seeded cells (Schoene and Kamara, 1999).

Statistical analysis

All experiments were replicated more than three times. A correlation analysis between number of retrieved cells and physical profiles was undertaken with the use of the Pearson product-moment correlation coefficient and correlation was admitted when the coefficient calculated as r value was either less than -0.5 or higher than 0.5. 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 except for correlation test. When a significant model effect was detected, comparisons among groups were subsequently conducted the Duncan methods. A p-value of less than 0.05 indicated a significant difference.

3. Result

Effects of the donor on the retrieval of bone marrow cells

In total, 19 chicks were euthanized to collect bone marrow cells. As shown in Fig 2, two different morphologies among the retrieved cells were detected following in vitro culture: a stromal cell-dominant population consisting of fibroblasts or fibroblast-like cells and a cuboidal cell-dominant population consisting of amorphous cuboidal cells. These differences in morphology were detected at the time of cell retrieval but became obvious by the end of the third passage.

The physical profile of the cell donors was as follows: body weight, 29.5 to 45.5 g; height, 7.5 to 11.0 cm; and leg length, 1.7 to 3.8 cm. A reverse analysis of the physical profile of the donors based on the culture outcome was performed subsequently (Fig 2). As shown in Table 1, 12 of 19 cases (63%) yielded a cuboidal cell-dominant population, whereas a stromal cell-dominant population was observed in 5 of 19 cases (27%). There were no significant differences in the physical parameters of the donors (body weight, 39.4 vs. 34.6 g,

p = 0.1738; height, 9.1 vs. 9.3 cm, p = 0.7807; and leg length, 3.4 vs. 3.1 cm; p = 0.1812) or in the number of retrieved cells at the time of red blood cell removal (1.61 × 108 vs. 2.03 × 108 cells; p = 0.3353) between the cuboidal and stromal cell-dominant populations.

The calculation of correlation coefficients revealed differences in the stromal and cuboidal cell-dominant populations (Fig 3). A significant correlation affecting cell retrieval was detected for the parameters of body weight and leg length in both the stromal cell-dominant (-0.857 to -0.809) and cuboidal cell-dominant populations (0.316 to 0.536). Negative coefficients were detected for body weight (r = -0.809) and leg length (p = -0.857) in the stromal cell-dominant population, whereas a positive coefficient was detected only for the relationship with height (r = 0.303). In the case of the cuboidal cell-dominant population, however, all profiles were related positively to the cell number (r > 0.316).

Aspects of bone marrow cell culture

A significant difference (passage 2, p = 0.0119; passage 3, p = 0.0033) in the total number of bone marrow cells cultured was detected between the stromal and cuboidal cell-dominant populations of the second subculture. As shown in Fig 4, cultures yielding a cuboidal cell-dominant population supported cell proliferation more efficiently than cultures yielding a stromal cell-dominant population. The cells retrieved at each passage (passage 0, p = 0.4539; passage 1, p = 0.3956; passage 2, p = 0.5775; passage 3, p = 0.7444) and the doubling time of each culture (passage 1, p = 0.4851; passage 2, p = 0.7978; passage 3, p = 0.6671) were constant throughout the subculture period, although these results did not reach statistical significance.

Figure 1. General experimental design

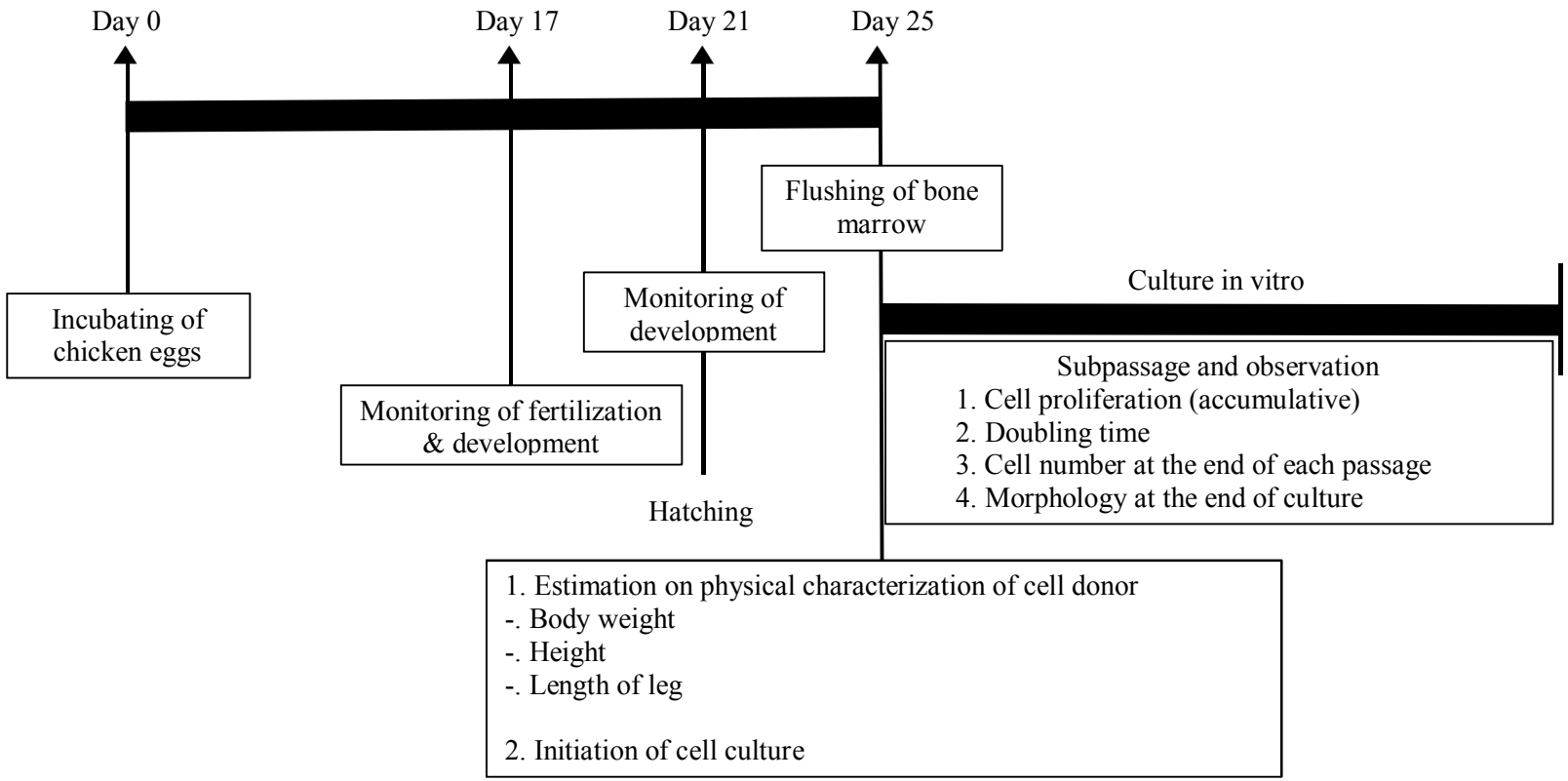


Figure 2. Morphology of chicken bone marrow-retrieved cells. After being collected from the femur and tibia, the cells were seeded to a 100 mm tissue culture dish and subsequently cultured in nutrient mixture F-12 (DMEM/F-12; Gibco Invitogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Welgene Inc., Daegu, Korea). Morphology of the bone marrow-retrieved cells were observed at the third passage. (A) Cultured cells formed fibroblast-like morphology determining as a stromal cell-dominant population. (B) Cultured cells formed amorphous morphology like cuboidal cells, which formed differently from the morphology at the time of cell retrieval. Bar=200㎛

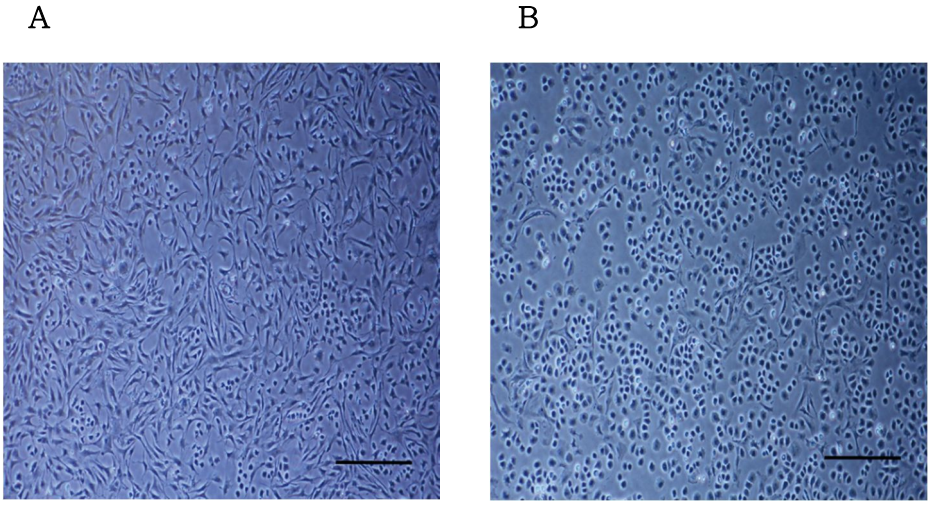
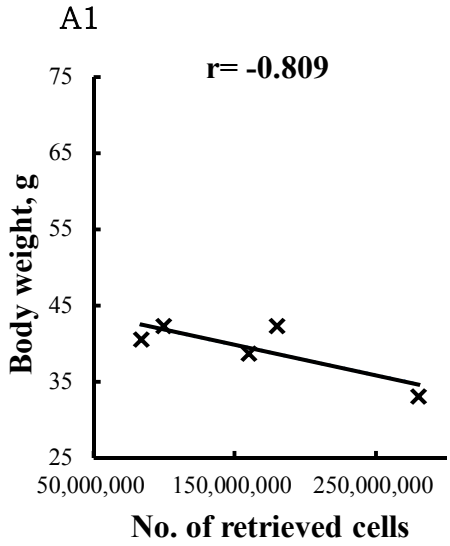
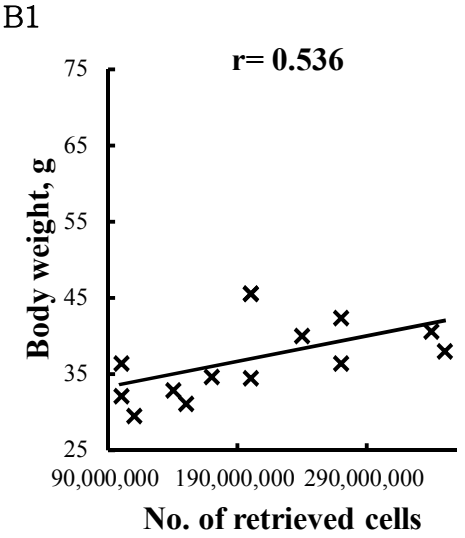
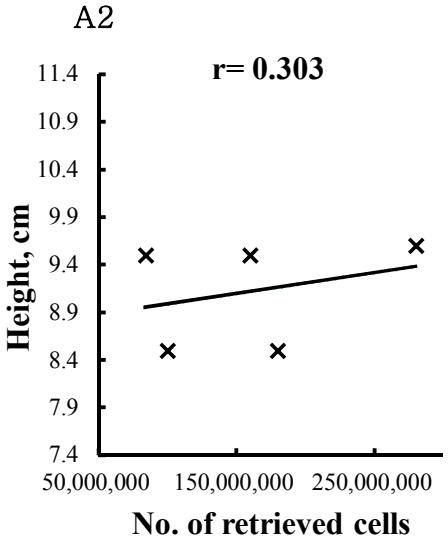
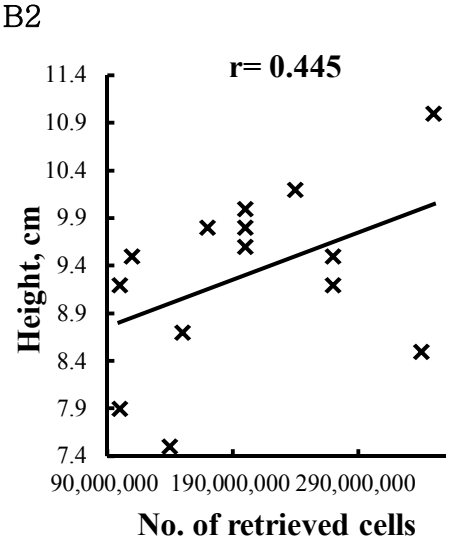


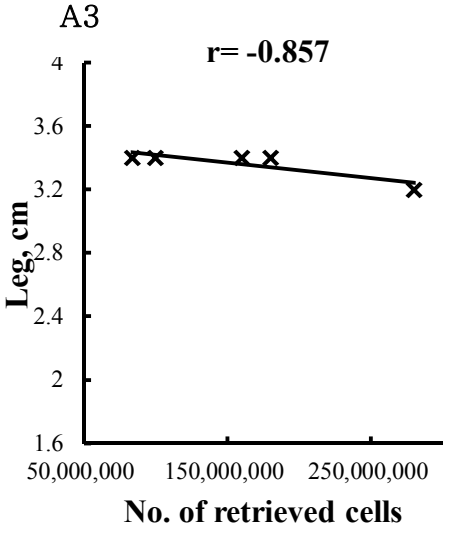
Figure 3. Relation co-efficient between the number of bone marrow-retrieved cells and physical profiles of chick donors yielding different types bone marrow cells. (A1-A3) Correlation rate in the donor yielding the stromal cell-dominant population. (B1-B3) Correlation rate in the donor yielding the cuboidal cell-dominant population.


A1
r= -0.809
Body weight, g
75
65
55
45
35
25
50,000,000
150,000,000
250,000,000
No. of retrieved cells


B1
r= 0.536
Body weight, g
75
65
55
45
35
25
90,000,000
190,000,000
290,000,000
No. of retrieved cells


A2
r= 0.303
Height, cm
11.4
10.9
10.4
9.9
9.4
8.9
8.4
7.9
7.4
50,000,000
150,000,000
250,000,000
No. of retrieved cells


B2
r= 0.445
Height, cm
11.4
10.9
10.4
9.9
9.4
8.9
8.4
7.9
7.4
90,000,000
190,000,000
290,000,000
No. of retrieved cells


A3
r= -0.857
Leg, cm
4
3.6
3.2
2.8
2.4
2
1.6
50,000,000
150,000,000
250,000,000
No. of retrieved cells

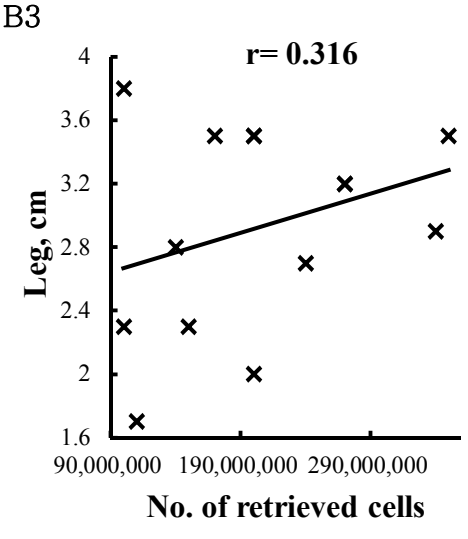
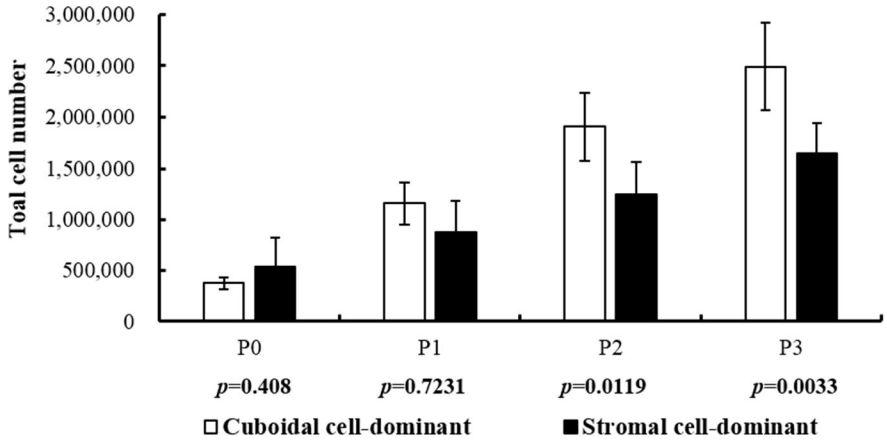
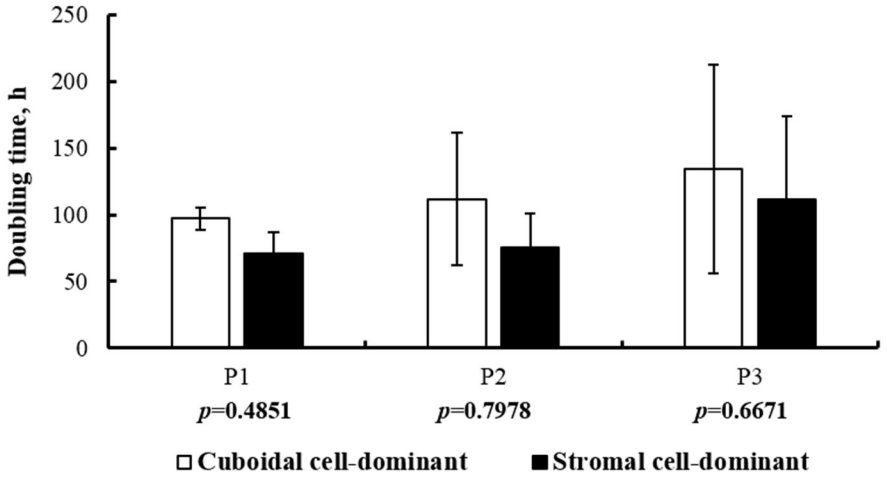

B3
r= 0.316
Leg, cm
4
3.6
3.2
2.8
2.4
2
1.6
90,000,000
190,000,000
290,000,000
No. of retrieved cells

Figure 4. Proliferation of bone marrow-retrieved cells up to the third passage. Cultured cells were retrieved from the femur and tibia of four-day-old white leghorn and cell number was counted at the end of each passage. A nutrient mixture F-12 (DMEM/F-12; Gibco Invitogen) supplemented with 10% (v/v) fetal bovine serum(FBS; Welgene Inc., Daegu, Korea) was used as the based medium for the culture of bone marrow-retrieved cells (A) Better proliferation was detected in cuboidal cell-dominant batches than in stromal cell-dominant batches and significant difference was detected. (B) Comparison of cell number at the end of each passages. (C) Comparison of doubling time. Data were indicated as Mean±SE and p value indicate model effect. *$p < 0.05$ within each passage.

A


Toal cell number
3,000,000
2,500,000
2,000,000
1,500,000
1,000,000
500,000
0
P0
P1
P2
P3
p=0.408
p=0.7231
p=0.0119
p=0.0033
Cuboidal cell-dominant
Stromal cell-dominant

B


Doubling time, h
250
200
150
100
50
0
P1
P2
P3
p=0.4851
p=0.7978
p=0.6671
Cuboidal cell-dominant
Stromal cell-dominant

C

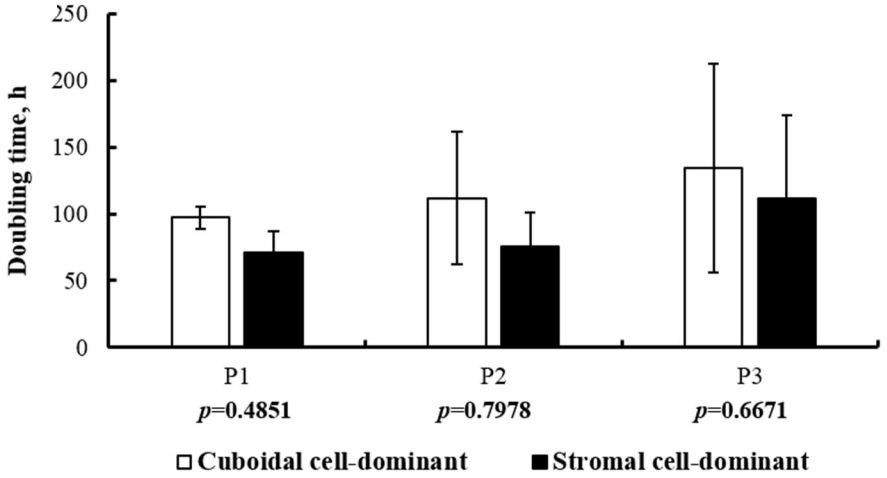

Doubling time, h
250
200
150
100
50
0
P1
P2
P3
p=0.4851
p=0.7978
p=0.6671
Cuboidal cell-dominant
Stromal cell-dominant

Table 1. Physical characterization of white leghorn chicks employed as the donor of bone marrow-retrieved cells.

Heads	Physical characterization			Mean no. of cells retrieved	Morphology of dominant cells
	Body weight (g)	Height (cm)	Length of leg (cm)		
130415-1	42.34	8.5	3.4	$1.0X10^{8}$	Stromal cell[b]
130415-2	40.61	9.5	3.4	$8.4X10^{7}$	Stromal cell[c]
130415-3	38.70	9.5	3.4	$1.6X10^{8}$	Stromal cell[b]
130419-1	42.34	8.5	3.4	$1.8X10^{8}$	Stromal cell[c]
130510-1	33.11	9.6	3.2	$2.8X10^{8}$	Stromal cell[c]
Mean±SE	39.42±1.9	9.12±0.3	3.36	$1.61±0.4X10^{8}$	-
130806-1	40.00	10.2	2.7	$2.4X10^{8}$	Cuboidal cell[c]
130806-2	38.00	11.0	3.5	$3.5X10^{8}$	Cuboidal cell[c]
138006-3	45.50	9.6	3.5	$2.0X10^{8}$	Cuboidal cell[c]
130809-1	42.34	9.5	3.2	$2.7X10^{8}$	Cuboidal cell[c]
130809-2	40.61	8.5	2.9	$3.4X10^{8}$	Cuboidal cell[c]
130826-1	45.50	10.0	2.0	$2.0X10^{8}$	Cuboidal cell[c]
130909-1	32.80	7.5	2.8	$1.4X10^{8}$	Cuboidal cell[c]
130909-2	29.50	9.5	1.7	$1.1X10^{8}$	Cuboidal cell[c]
130909-3	36.40	9.2	3.2	$2.7X10^{8}$	Cuboidal cell[c]

140124-1	31.10	8.7	2.3	$1.5X10^8$	Cuboidal cell[c]
140201-1	32.10	7.9	2.3	$1.0X10^8$	Cuboidal cell[c]
140617-1	36.40	9.2	3.8	$1.0X10^8$	Cuboidal cell[c]
140617-2	34.59	9.8	3.5	$1.7X10^8$	Cuboidal cell[c]
140617-3	34.40	9.8	3.5	$2.0X10^8$	Cuboidal cell[c]
Mean±SE	34.61±1.2	9.27±0.2	3.09±0.1	$2.03±0.2X10^8$	-
Model effect[a]	0.1738	0.7807	0.1812	0.3353	-

[a]Indicated as p value between the values of stromal cell and those of mixed cell morphologies.

[b]Cell morphology observed at passage 2.

[c]Cell morphology observed at passage 3.

Table 2. 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')		
GAPDH	NM_204305.1	ATCACAGCCACACAGAAGA	AGGTCAGGTCAACAACAGAG	199	55
VEGFR-2	AJ884687.1	AAGTTGAGCTGGCGGTAGGAGAA	TCATGTTCATGCGACCACTGGATG	218	60
E-cadherin	NM_001039258.2	TGTACGAAGGTGTGGTGGAA	AGCGGCTCTTGGTCTCATAA	216	53
EpCAM	NM_001012564.1	TTGAAATGAGGCATGCTGAG	CAGGGGTTTTGTCTGAGGAA	177	53
CD29	NM_001039254	CCAAGAAAGGGCAAATGA	ACATCTGCATGCACCACATT	171	60
CD31	XM_004946203	CAGGCAAAGGAGACGCACGAT	CTTCTGGCAGCTCACAACGT	221	60
CD44	NM_204860.2	GGTTTTATAGTGGGGCATATTGTTATCCC	TTAACCGCGATGCACACGGC	700	58
CD45	NM_204417.2	CACTGGGAATCGAGAGGAAA	CTGGTCTGGATGGCACTTTT	574	55
CD90	NM_204381.2	GGTCTACATGTGCGAGCTGA	AAAGCTAAGGGGTGGGAGAA	471	56
CD105	NM_001080887.1	ACGGATGACACCATGGAAAT	ATGAGGAAGGCTCCAAAGGT	704	56
CD133	NM_001291653.1	CTGCCAACCAACACTTAACTAGCCA	TTCTCTGATTGCTCCTGCCATTGTC	187	60

4. Discussion

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; Roson-Burgo et al., 2014). The retrieved cells were maintained up to at least the third passage, and a number of differences in cell morphology were observed compared with cultured mammalian cells. Characterization of retrieved cells that shows different morphology 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.

In this study, a significant correlation was detected between the physical parameters and number of bone marrow cells retrieved from each individual, and different correlations

were detected between the cuboidal and stromal cell-dominant populations. These results may be due to differences in cell proliferation dynamics according to the maturity of the individuals between the two cell populations. On the other hand, no significant difference in cell number was detected between the stromal and cuboidal cell-dominant populations. The use of four-day-old, neonatal chicks had little effect on the increase in the experimental population. Several studies using chicken bone marrow cells have relied on donor chicks that were older than ours or sexually mature chickens (Khatri et al., 2009; Wu et al., 2010; Bai et al., 2012; Kocamaz et al., 2012; Bai et al., 2013).

Nevertheless, regardless of cell type or the retrieved cell number, chicken bone marrow cells of various morphologies can be cultured and maintained *in vitro* to a certain extent. The cuboidal cell-dominant population exhibited better proliferation than the stromal cell-dominant population due to its rapid outgrowth and doubling time during culture. Chicken bone marrow cells have different characteristics compared with bone marrow cells of mammalian species, which usually yield a

fibroblast-dominant population.(Larsen et al., 2010; Ward et al., 2015) 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.(Kolf et al., 2007)

CHAPTER 4.

OPTIMIZATION OF MEDIUM COMPOSITION FOR THE EXPANSION OF BONE MARROW-DERIVED CELLS IN CHICKEN

1. Introduction

Mesenchymal-derived, multipotent cells are crucial to the development of novel biotechnologies for stem cells. And also it become a valuable resource for cell-to-cell tissue regeneration and experimental modelling for differentiation and reprogramming. Bone marrow cells are considered as the basic material for clinical application to injuries and degenerative diseases (Hay et al., 1988; Staal et al., 2011; Catacchio et al., 2013). 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. In this study, the necessity of different basic media were investigated to modify the culture system of chicken bone marrow cells and the change of cellular properties in chicken bone marrow cells was observed through a suspended culture. Several culture systems have been developed for effectively maintaining chicken bone marrow cells (Foot, 1913; Kudriavtsev and Kudriavtseva, 1966; Cormier and Dieterlen-Lievre, 1990; Lee et al., 2016). 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. Knowing the response of chicken bone marrow cells to optimized medium will directly contribute to developing a culture system. It improves the efficiency of chicken bone marrow cells culture system. In this study, to harvest a large amount of reliable mesenchymal stem cells-like cells in chicken bone marrow, the High-glucose DMEM was optimized for promoting cell attachment and proliferation of mesenchymal stem cells-like cells to tissue culture plate.

Given these observations, we provide important clues about the optimized culture medium of chicken bone marrow cells for the cultivation in different media and the feasibility of large-scale expansion.

2. Material and Method

Experimental design

This study was designed to know culture and optimization culture media of bone marrow cells retrieved from neonatal white leg horns. General experimental procedure was depicted in Figure 9. Some series of experimentation was conducted to understand retrieved bone marrow cells and to evaluate their capacity for in vitro expansion following subculture. In experiment 1, whole bone marrow cells from white leg horn at passage 0 were seeded into culture plate dish to evaluate their capacity for in vitro expansion. In experiment 2, collected bone marrow cells were subsequently cultured in a designated each different medium and subcultures were conducted up to 12 times. Cell number and accumulated number of cultured cells were counted at the end of each subpassage and doubling time of each subpassage was calculated. In experience 3, collected bone marrow cells were detected cell surface marker related mesenchymal stem cells using flow cytometry analysis. And gene expression was conducted on

different types of mesenchymal stem cells related marker. Also protein expression was confirmed by immunofluorescence. Finally collected bone marrow cells were differentiated into multi-lineage like adipogenic and osteogenic differentiation.

Experimental animals

Four-day-old chickens (White leg horn) were employed as the donor of bone marrow cell donors. The animal was maintained at the University Animal Farm, Seoul National University, Korea, using our standard management program, which were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU 140912-4).

Isolation and culture of bone marrow-derived primary cells

The femurs and tibia separated from both legs of euthanized donors were washed with 1% (v/v) antibiotic-antimycotic solution and 2% (v/v) fetal bovine serum containing Dubecco's phosphate buffer saline. Muscle tissues attached to

the bones were subsequently removed and the marrow cavity was exposed by excising the spongious part of each bone. Bone marrow cells were retrieved by flushing of bone marrow tissue with 2% (v/v) FBS containing DPBS. Whole bone marrow-derived cells including red blood cells were spread on culture dish.

Maintenance of chicken bone marrow cells

Chicken bone marrow cells were culture on the monolayer and subcultured when they reached 70-80%. Employed for chicken bone marrow cells culture media is different four type of basic media, High-glucose DMEM, Low-glucose DMEM, F-12 and α-MEM, with or without growth factors like LIF and bFGF and supplemented with 10% (v/v) FBS, 1% (v/v) solution of antibiotic-antimycotic at 37℃, 5% CO2 in a humidified atmosphere.

Colony forming unit fibroblast (CFU-F) assay

To quantify the frequency of stromal progenitors,

mono-nuclear cells were resuspended in each different media and plated at a density of One million cells on 100mm culture plate. The medium was changed after 3 days to wash non adherent cells and cultured 21 days. After 21 days, cells were washed with DPBS and fixed 4% (v/v) paraformaldehyde for 15 minutes at room temperature. Subsequently, the fixed cells were stained with 1% crystal violet in 4% (v/v) paraformaldehyde for 5 minutes at room temperature. After several washes, colonies formed by more than 55 cells were counted under a light microscope at low magnification. Results were expressed as total number of colonies on 100mm culture plate.

Assessing of cell proliferation during in vitro-culture of cells

The number of cultured cells at the end of each passage and the initial number of cells seeded for each passage were counted. Accumulated number of cells throughout whole subpassage, the number of cells collected at the end of each passage and doubling time were counted or calculated for monitoring cell proliferation during in vitro culture. Doubling time of bone

marrow derived cells in each passage was calculated by tlog2/(log Nt – log N0), where t is time to confluence, Nt is the number of cells at the end of each passage and N0 is the number of seeded cells.

Analysis of the relative mRNA levels using real-time PCR

Chicken bone marrow cells maintained in four different medium containing with or without growth factors were transferred into RNAlater, and subsequently stored at −80℃ until qPCR 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 Revers Transcriptase. The expression of specific chicken bone marrow cells genes was quantified by real-time PCR using iQTM SYBR® Green Supermix. The ß-actin primer was included in every treatment group for standardization and the mRNA level of each gene was normalized to that of ß-actin. The primer sequences are listed in Table 1.

Characterization of chicken bone marrow cells by FACS

To immunophenotypically characterize the chicken bone marrow cells, 1x106 cells were suspended in PBS and incubated with the following FITC- or PE- or APC-conjugated or unconjugated antibodies: anti-CD45-APC, anti-CD44-PE, anti-MHC class II-PE, anti-MHC class I-FITC, anti-MCAM-FITC. Then the cells were washed with PBS and analyzed by fluorescence-activated cell sorting. The data were analyzed using BD Cell/Quest Pro Software.

Assessment of chicken bone marrow cells senescence assay

Chicken bone marrow cells survival and senescence were evaluated using a Senescence ß-Galactosidase Staining Kit. Both passage 3 and passage 10 chicken bone marrow cells cultured in different types of media were employed as senescence assay. Number of senescence cell was counted using inverted microscope.

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 indicated a significant difference.

3. Result

Isolation and proliferation of bone marrow derived cells

After flushing from tibia and femur at each side of legs, one million cells were seeded into culture dish counted cell number at the end of the first subculture. The cells formed a monolayer of heterogenous morphology in culture plate. Bipolar spindle cells like fibroblast cell with a cuboidal morphology cells within 2 weeks. The morphology of the bone marrow derived cells was consistent with regardless of culture medium compositions. As the results, seeding efficiency of chicken bone marrow derived-adherent cells (BMCs) at the end of the first passage is no significant difference detected ($p=0.2164$) (Figure 6). Seeding efficiency between four different media have no effects on attachment and proliferations of bone marrow derived primary cells.

Proliferation of bone marrow derived cells in different four 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. Cumulative number of cells at each passages are significant difference detected in each media. (Figure 8-1-A) Except for passage 0, until passage 12 significant difference are detected in each media. Bone marrow derived cells are cultured in DMEM/F-12 have better proliferation ability than other types of medium (Figure 8-1-B). Furthermore, significant increase of proliferation activity was detected in the bone marrow derived cells cultured on DMEM/F12 medium. (Figure 8-2)

Comparisons of colony forming ability of bone marrow-derived primary cells in different type of media

Isolated bone marrow cells from chicken tibia and femurs were cultured in vitro more than twelve passages with mixed population morphology. To evaluate of bone marrow derived primary cells attachment, colony forming unit-fibroblast analysis and primary cell attachment calculation were conducted. One million bone marrow cells retrieved from primary passage were seeded into 100mm cell culture plate and

crystal violet staining was performed to detect single cell-derived clones during 21 days of culture. Positive staining was defined as a colony that contained 50 positive cells on average. As the results, in different four types of media no significant difference were detected (p=0.2541) (Figure 7). It shows similar pattern between four different media but slightly increased pattern in DMEM/F12, αMEM medium. In DMEM/F12 and αMEM medium, there are morphological similarity but different size and number of colony was observed.

Comparisons of morphology of chicken bone marrow derived cell cultured on different four types of basic medium

To observe morphology of chicken bone marrow derived cells, isolated primary cells were subpassaged until homogeneous morphology. At passage 5, cells were showed still mixed cell shape. Stromal cell and cuboidal-like cells are mixed and relatively much cuboidal-like cells are represented in DMEM/F12 medium (Figure 9).

Assessment gene expression on established chicken bone marrow derived cells at different passages

Gene expression of pluripotency, differentiation and bone marrow derived stromal cell markers in chicken bone marrow derived-adherent cells (BMCs) were assessed at passage 5 and passage 10 (Figure 10). RT-PCR analysis showing that pluripotency related gene such as *pouV* is slightly expressed in αMEM group at passage 5. But pluripotency related gene such as *pouV* is expressed in all types of media at passage 10. But other pluripotency related marker such as *Nanog, Sox2* were not expressed all of the different types of medium regardless of passages. And mesenchymal stem cells related genes such as *CD29, CD44, CD90, CD105, SMA* also expressed regardless of passages. But importantly *CD45* as a hematopoietic marker also expressed on different passages. And *CD31* as an endothelial cell maker also expressed on isolated cells. other differentiation related genes such as *Desmin, Nestin* also expressed. (Figure 10). Through the results, there were still heterogeneous cell population and composed of different cell types.

Comparisons of Immunophenotypic expression of chicken bone marrow derived cells cultured on different medium

The detection of expression of cell surface marker of the chicken bone marrow derived cells were cultured at passage 5. Various types of cell surface marker such as mesenchymal lineage, hematopoietic makers were detected by using flow cytometry analysis. Representative flow cytometry histogram show the expression (violet peaks) of selected molecules (CD45, CD44, MCAM, MHC I, MHC II, CD105) by different MSC populations compared with isotype controls (green peaks) (Figure 11). As a result, the chicken bone marrow derived cells were negative expression for all of the marker except for CD45. CD45 as a hematopoietic lineage maker is regardless of types of cultured medium.

Assessment of cell surface maker expression of chicken bone marrow derived cells cultured on different medium

Immunocytochemistry analysis of chicken bone marrow

derived-adherent cells. Surface antigen characterization of mesenchymal stem cell related marker such as MCAM, CD44, CD105 was non-expressed (Figure 12-A). Surface antigen characterization of endothelial cell related marker such as CD31 were also non expressed (Figure 12-B). Although, these results were non matched between gene expression data, it is truly isolated bone marrow cells were composed of mixed population.

Assessment of cell apoptosis of chicken bone marrow derived cells cultured on different medium

Senescence of chicken bone marrow derived-adherent cells (BMCs) during subculture in vitro. β-galactosidase (SA-β-Gal) was used for the senescence assay of CBMCs subcultured performed on each type of cultures and the number of SA-β-Gal positive cells was counted. Comparison after cultured in different media under different four types of culture media. Cell number was counted at the end of passage 10. Regardless of culture media types, there was no significant difference between types of media (Figure 13).

Figure 5. General experimental procedures.

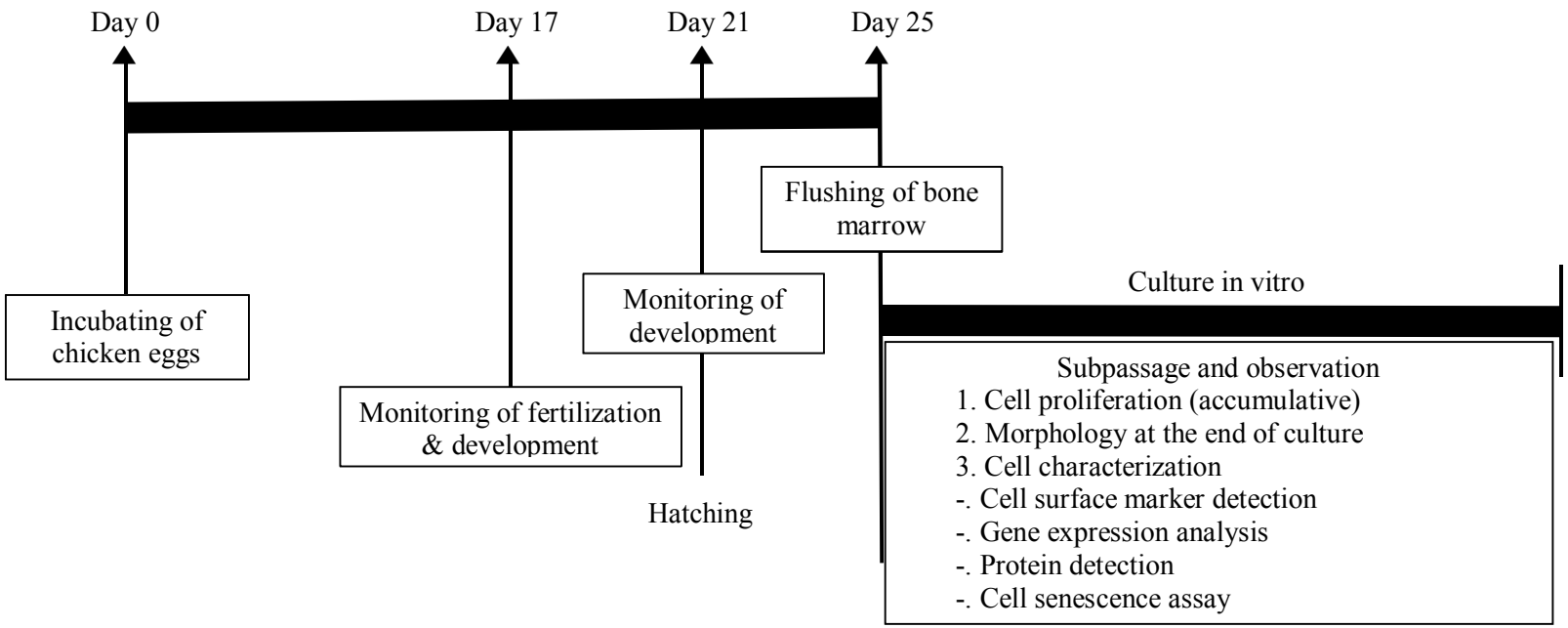


Figure 6. Comparisons number of chicken bone marrow derived cells (CBMCs) seeding efficiency at the end of the first passage. One million chicken BMCs retrieved from primary culture were seeded into one well of 6-well plate and counted cell number at the end of the first subculture. (A) Comparison of cell number after cultured in the media without growth factors. Data were indicated as Mean±SE.

A

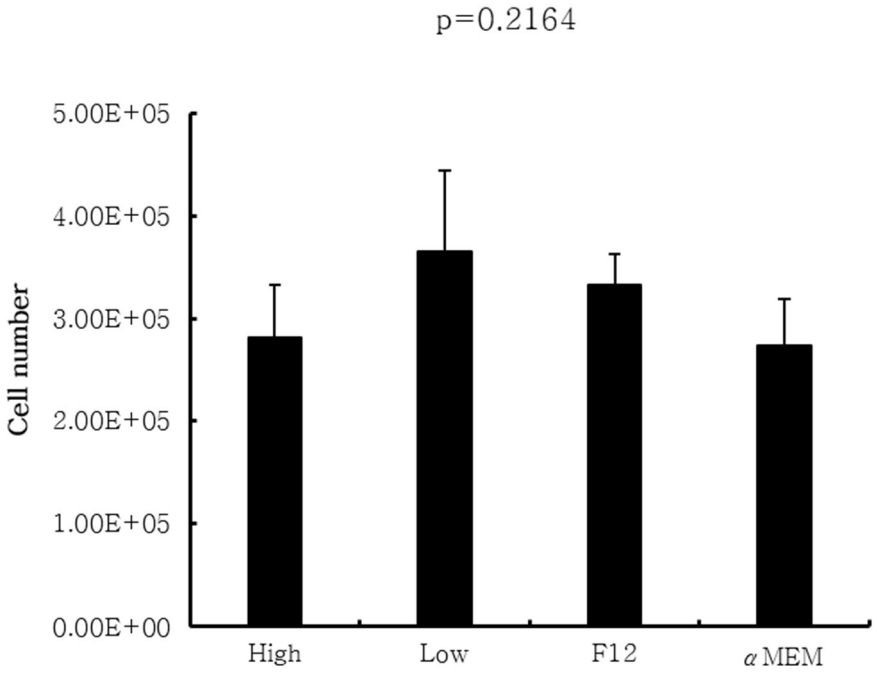


Figure 7. Colony-forming activity of chicken bone marrow derived-adherent cells (CBMCs) by colony form unit (CFU) analysis. Positive staining was defined as a colony that contained 50 positive cells on average. (A) Comparison of CFU level among growth factor-free media. (B) Number of colonies after cultured in growth factors-free media. Data were indicated as Mean±SE.

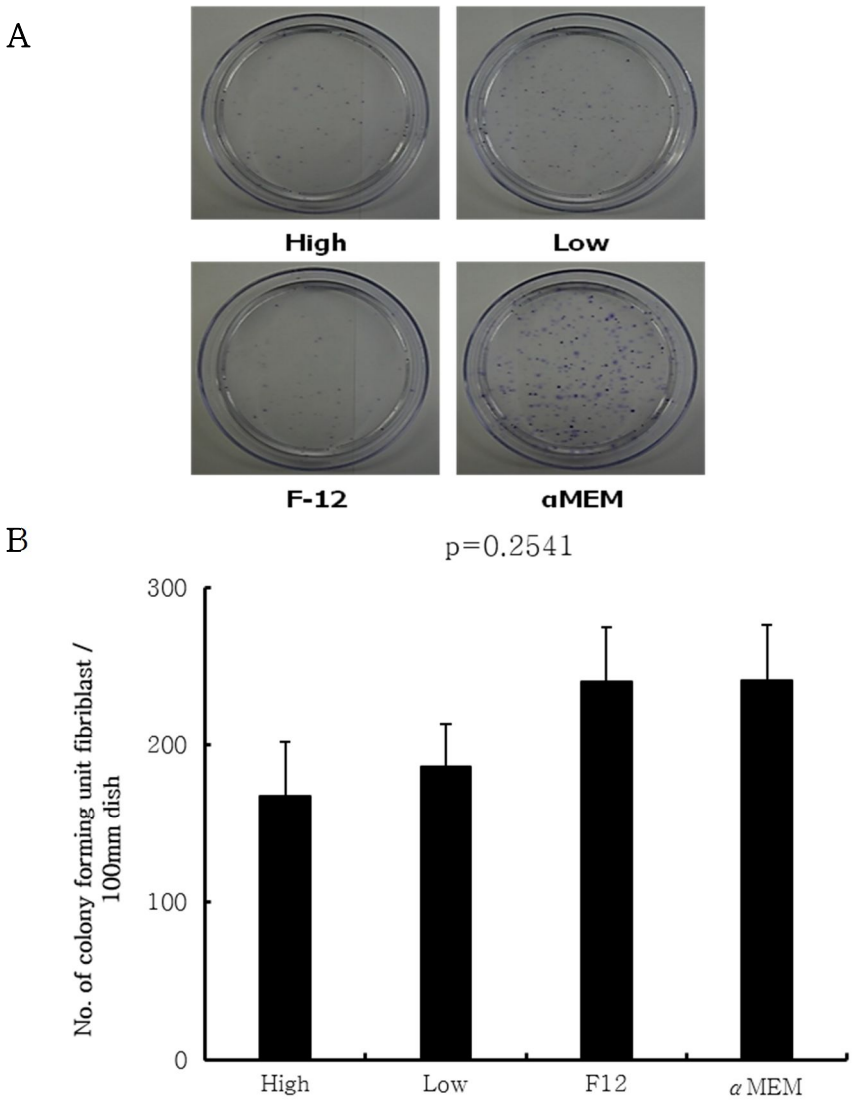
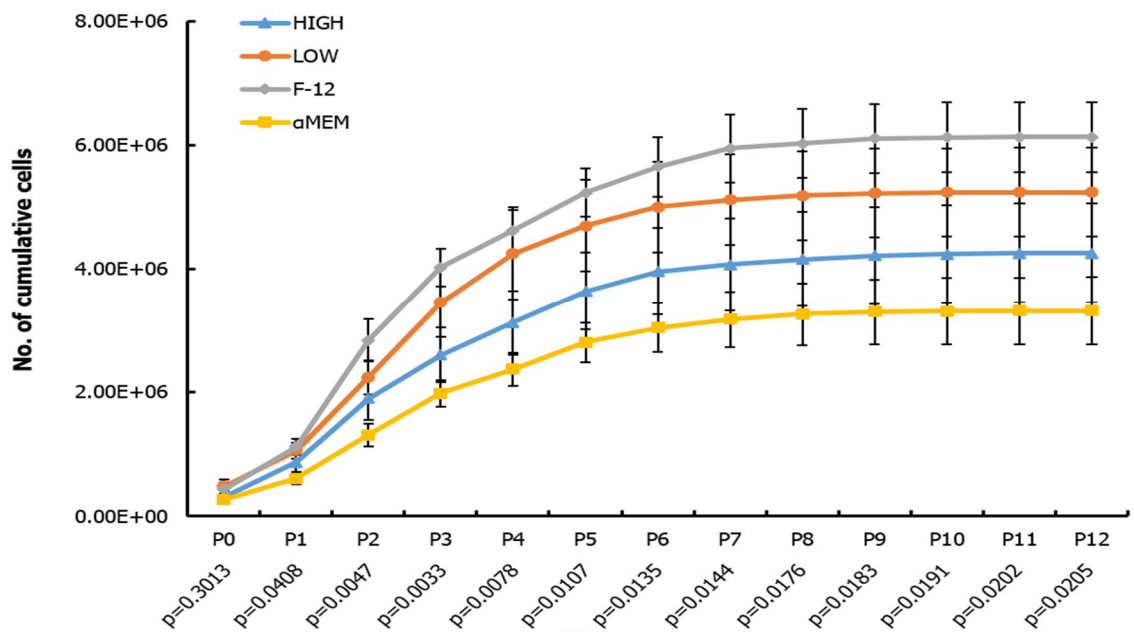
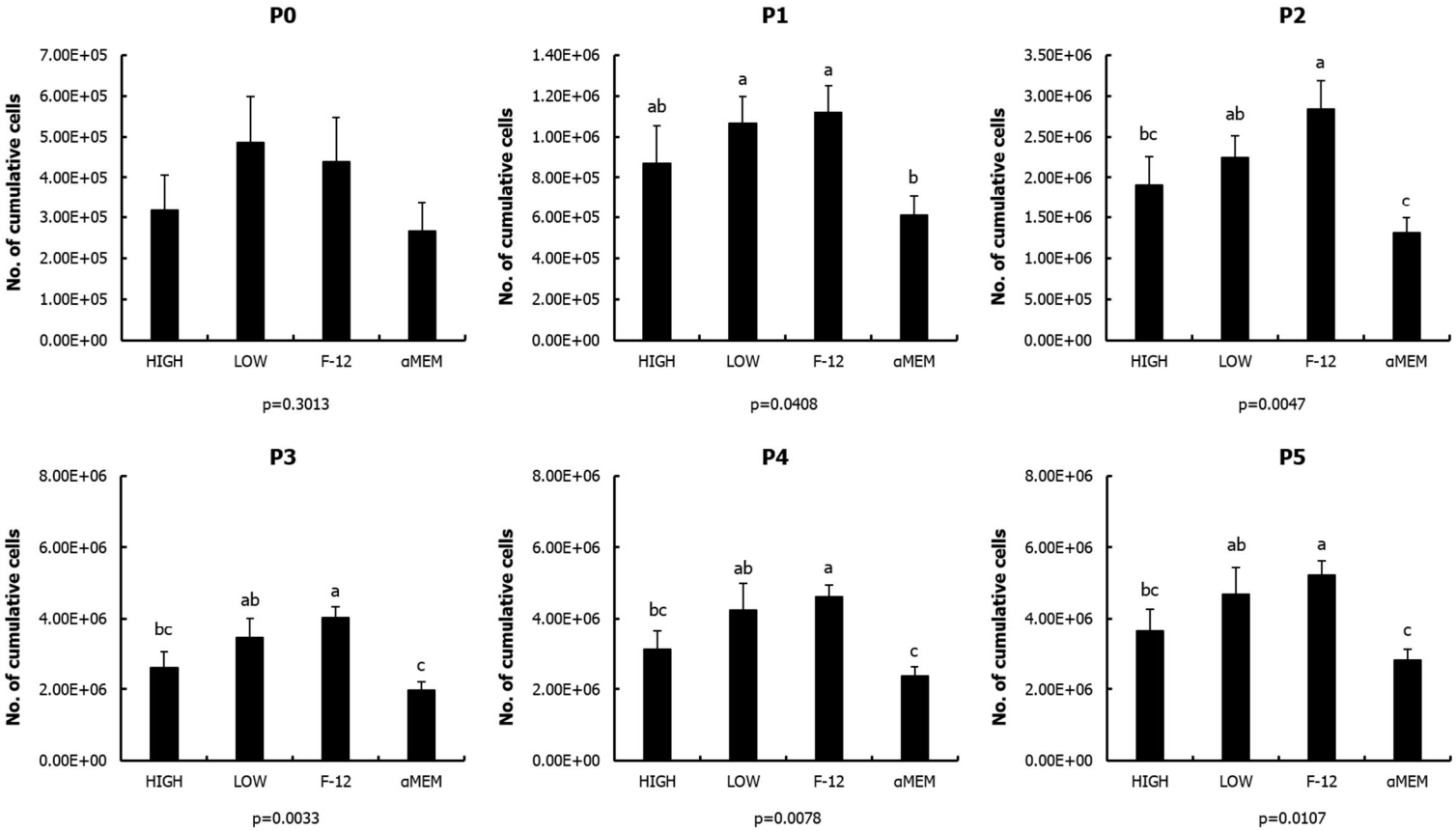


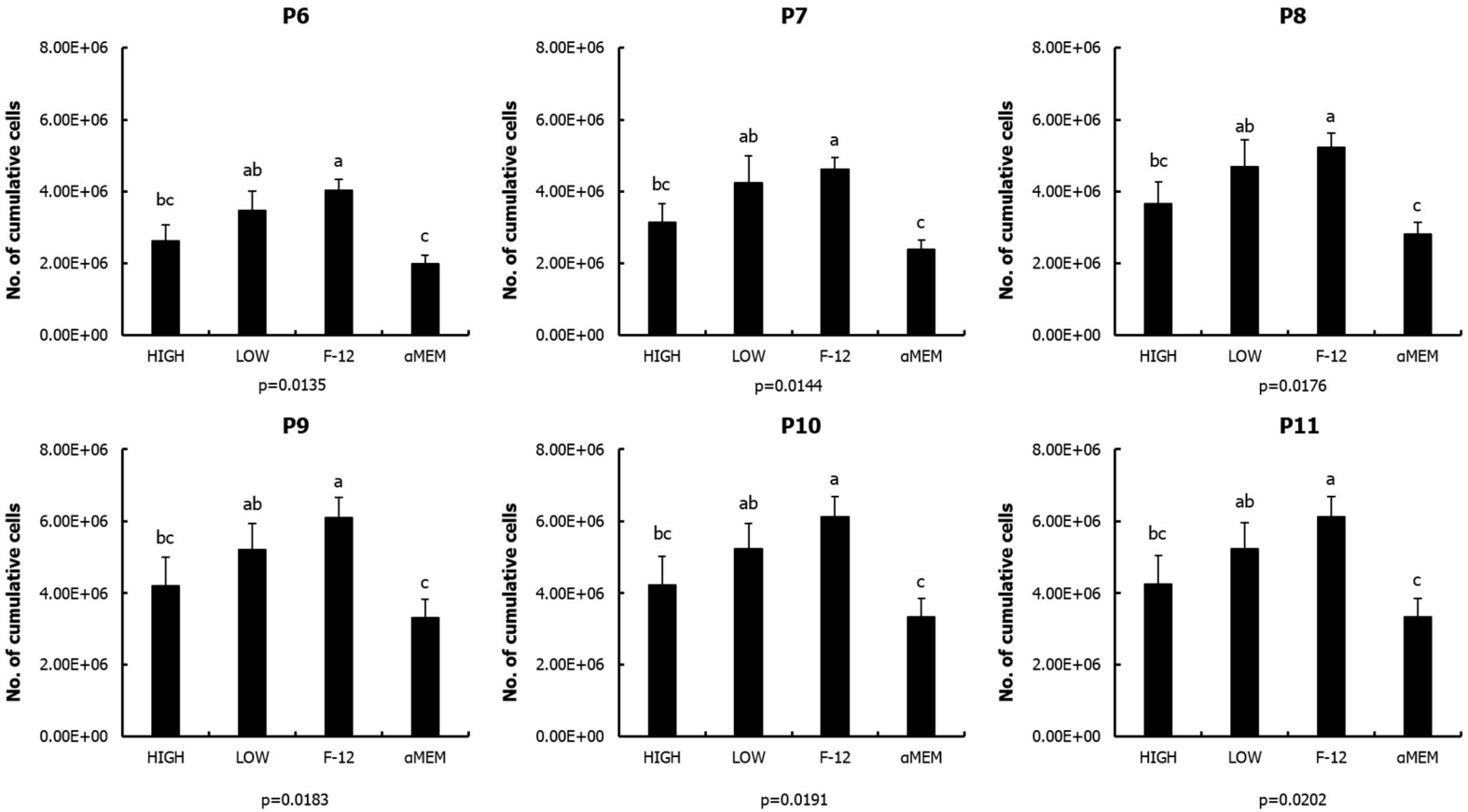
Figure 8-1. Retrieval number of chicken bone marrow derived-adherent cells (CBMCs) and their proliferation following subculture in vitro. Chicken BMCs are retrieved from the white leghorn of 4-day-old chickens and the number of cultured cells was counted at the end of each passage. (A) Number of cumulative cells in each passage. Significant difference was detected in each media. Data were indicated as Mean±SE.

A



B


P0
No. of cumulative cells
7.00E+05
6.00E+05
5.00E+05
4.00E+05
3.00E+05
2.00E+05
1.00E+05
0.00E+00
HIGH
LOW
F-12
αMEM
p=0.3013
P1
1.40E+06
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
ab
a
a
b
p=0.0408
P2
3.50E+06
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
bc
ab
a
c
p=0.0047
P3
8.00E+06
6.00E+06
4.00E+06
2.00E+06
bc
ab
a
c
p=0.0033
P4
bc
ab
a
c
p=0.0078
P5
bc
ab
a
c
p=0.0107


P6
P7
P8
P9
P10
P11
No. of cumulative cells
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
bc
ab
a
c
HIGH
LOW
F-12
αMEM
p=0.0135
p=0.0144
p=0.0176
p=0.0183
p=0.0191
p=0.0202

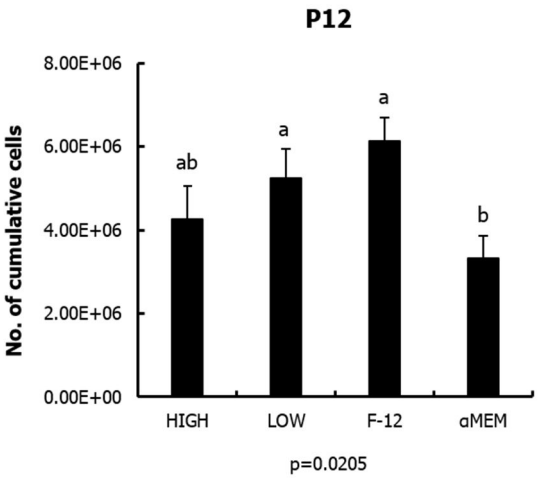

P12
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
No. of cumulative cells
ab
a
a
b
HIGH
LOW
F-12
αMEM
p=0.0205

Figure 8-2. Proliferation activity was evaluated using the Cell Counting Kit (CCK-8). Ten-thousands cells were seeded on one well of 96-well plates and 100㎕ medium was subsequently incubated with the mixture of 10㎕ CCK-8 at 37℃ with 5% CO_2 in air atmosphere for 2h and CCK values were measured at each observation time. Absorbance was measured at 450nm using a microplate reader. All data were shown as mean±SE and PLOC-GLM in SAS program was employed for statistical analysis. Values in parentheses indicate model effect of treatment and bars with different letters differ significantly ($p<0.05$).

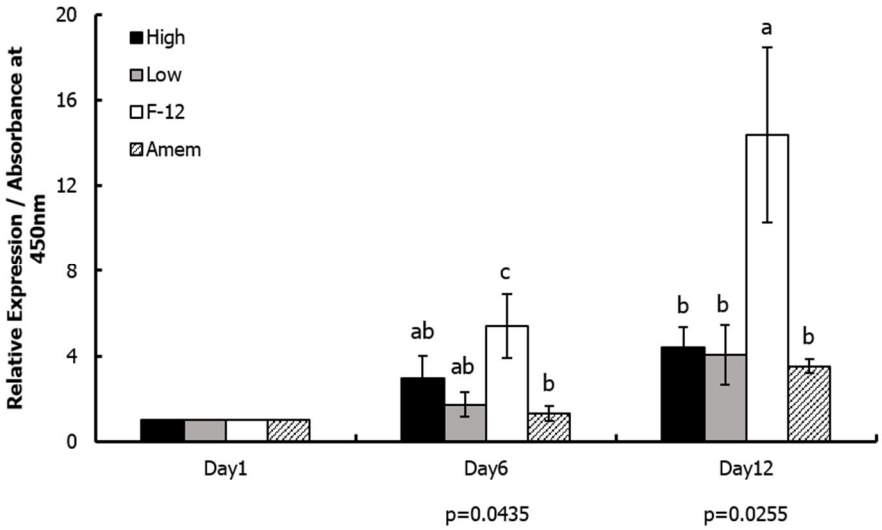


Figure 9. Morphology of primary and sub-cultured chicken bone marrow derived-adherent cells (CBMCs). Primary cells after whole bone marrow culture. Passage 5 CBMCs were purified, and cuboidal-like cells dominant. There were no obviously morphological differences among different four types of media. Scale bar=100㎛.

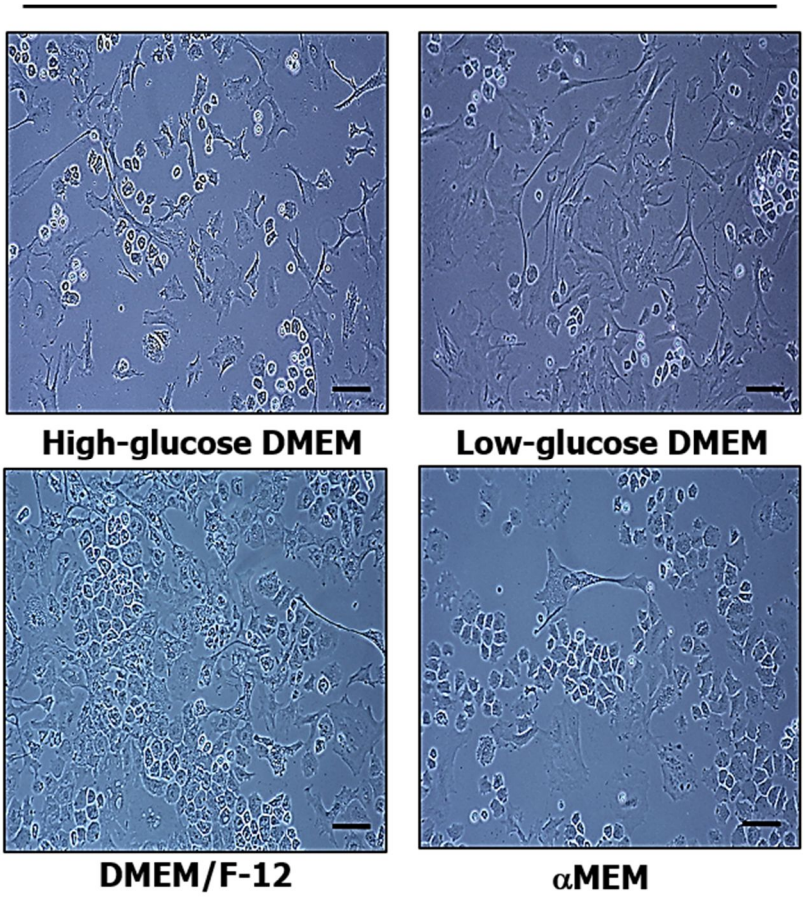


Table 3. Oligonucleotide primer sequences and thermal cycling conditions used in real-time and reverse transcriptase PCR.

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	DQ867024.1	TCAATGAGGCAGAGAACACG	GGACTGGGCTTCACACATTT	154	60
Nanog	NM_001146142.1	CAGCAGACCTCTCCTTGACC	TTCCTTGTCCCACTCTCACC	187	60
Sox2	AB092842.1	AGGCTATGGGATGATGCAAG	GTAGGTAGGCGATCCGTTCA	163	60
Desmin	XM_003641642.2	AGGTGGTGGAGTCCAAGGAG	CTTTGTTGGCAATGACGCTT	204	60
SMA	M13756.1	CCTGTATGCTTCTGGGCGTA	TGGCCATCTCGTTTTCAAAG	263	60
Nestin	NM_205033.1	ACAGGACTGGGAAGGGACAG	TCTCCCTCATCCTCACCCTC	177	60
CD29	NM_001039254.2	GAACGGACAGATATGCAACGG	TAGAACCAGCAGTCACCAACG	300	60
CD31	XM_004946203	CAGGCAAAGGAGACGCACGAT	CTTCTGGCAGCTCACAACGT	221	60
CD34	XM_417984.4	GTGCCACAACATCAAAGACG	GGAGCACATCCGTAGCAGGA	239	60
CD44	NM_204860.2	GGTTTTATAGTGGGGCATATTGTTATCCC	TTAACCGCGATGCACACGGC	700	58
CD45	NM_204417.2	CACTGGGAATCGAGAGGAAA	CTGGTCTGGATGGCACTTTT	574	55
CD90	NM_204381.2	GGTCTACATGTGCGAGCTGA	AAAGCTAAGGGGTGGGAGAA	471	56
CD105	NM_001080887.1	ACGGATGACACCATGGAAAT	ATGAGGAAGGCTCCAAAGGT	704	56

Figure 10. Assessment of expression of pluripotency, differentiation and bone marrow derived stromal cell markers in chicken bone marrow derived-adherent cells (CBMCs) at passage 5 and passage 10. (A) RT-PCR analysis showing that pluripotency related gene such as pouV is slightly expressed in αMEM group at passage 5. And other differentiation related genes such as endoderm and mesoderm marker also expressed. (B) RT-PCR analysis showing that pluripotency related gene such as pouV is expressed in all types of media at passage 10. And other differentiation related genes such as endoderm and mesoderm marker also expressed. β-actin is positive control.

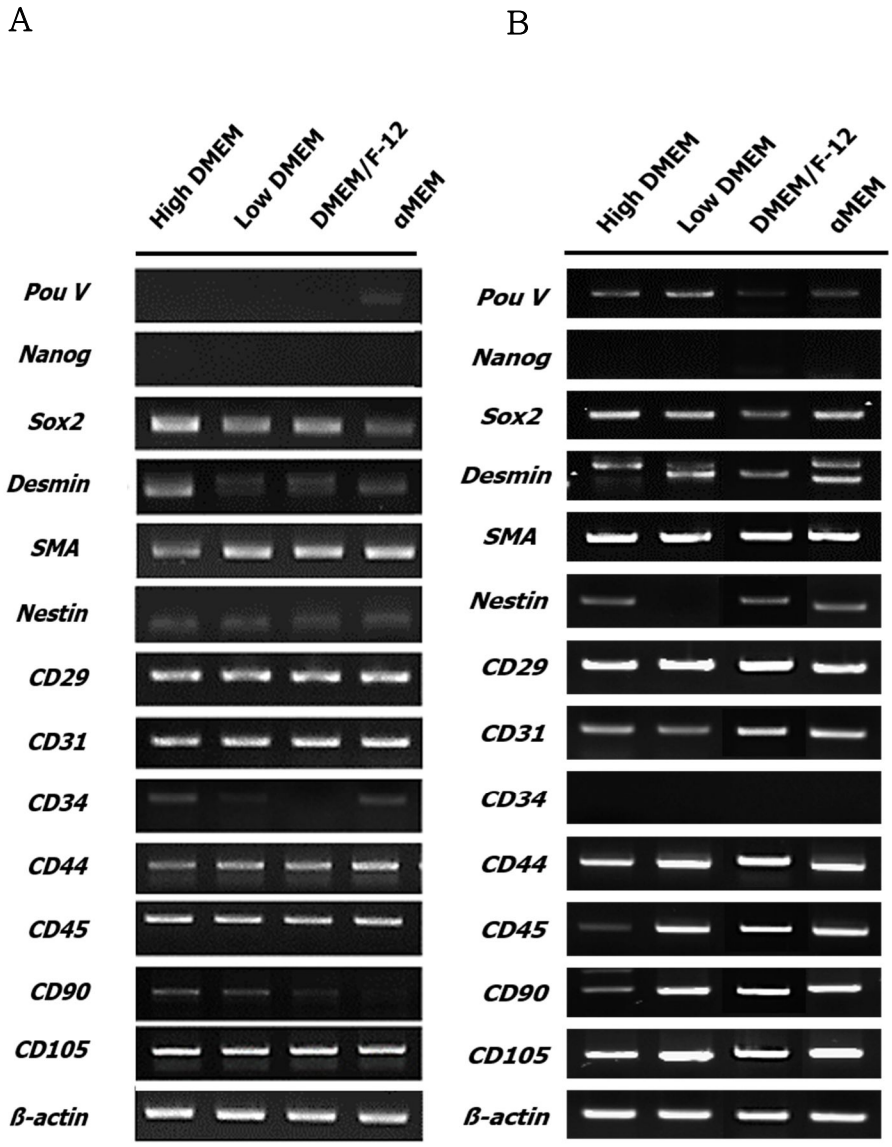

A
B
High DMEM
Low DMEM
DMEM/F-12
αMEM
Pou V
Nanog
Sox2
Desmin
SMA
Nestin
CD29
CD31
CD34
CD44
CD45
CD90
CD105
ß-actin

Figure 11. Immunophenotypic profile of chicken bone marrow derived-adherent cells (CBMCs) at passage 5. Representative flow cytometry histogram show the expression (violet peaks) of selected molecules (CD45, CD44, MCAM, MHC I, MHC II, CD105) by different MSC populations compared with isotype controls (green peaks). BMSCs cultured in non-addition of growth factors media were stained with surface antibodies and analyzed by FACS.

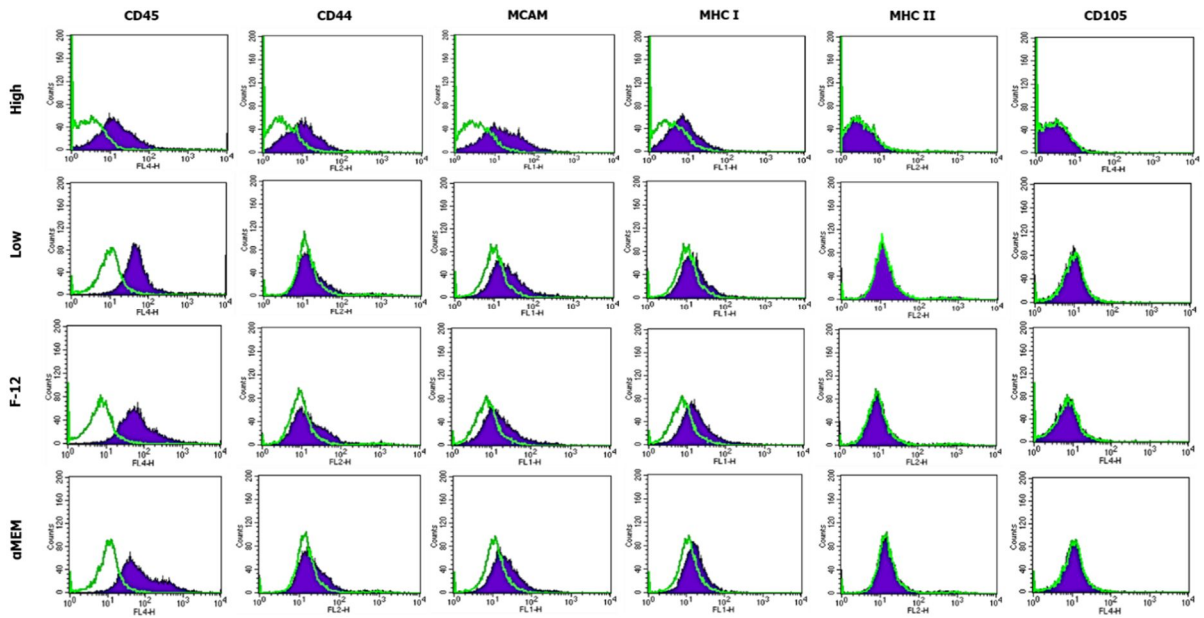


Figure 12. Immunocytochemistry analysis of chicken bone marrow derived-adherent cells (CBMCs). (A) Surface antigen characterization of mesenchymal stem cell and epithelial cell related marker such as MCAM, CD44, CD105, Vimentin and E-cadherin expression. (B) Surface antigen characterization of endothelial cell related marker such as CD31 expression.

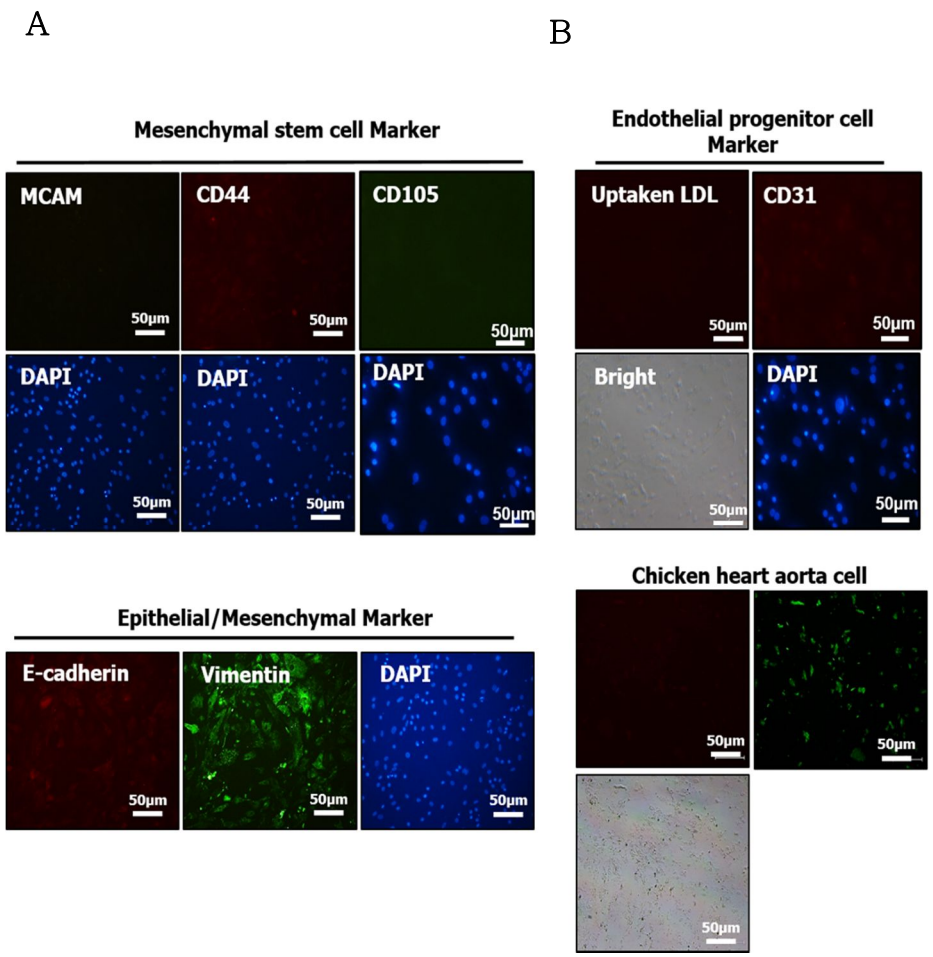
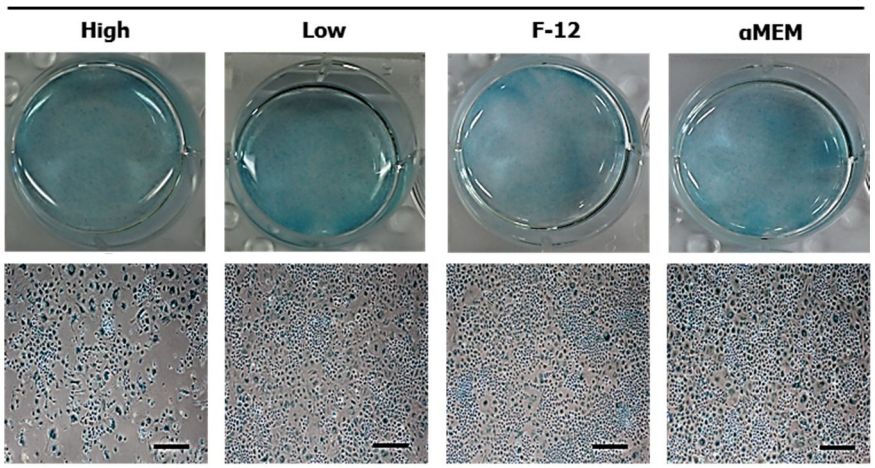
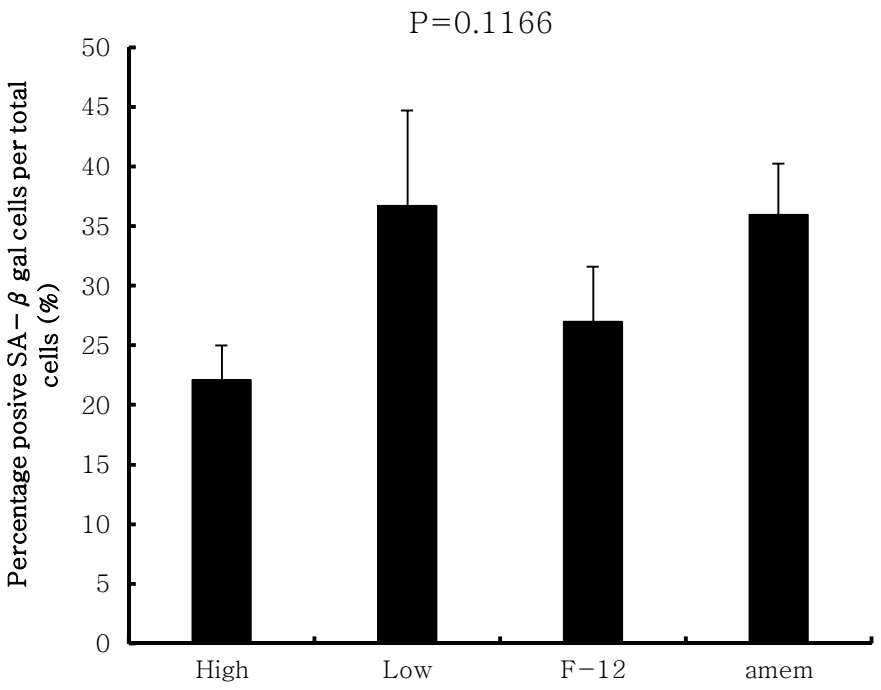


Figure 13. Senescence of chicken bone marrow derived-adherent cells (CBMCs) during subculture in vitro. β-galactosidase (SA-β-Gal) was used for the senescence assay of BMCs subcultured performed on each type of cultures and the number of SA-β-Gal positive cells was counted. (A) Images showed the cells positive for SA-β-Gal or not. Scale bars =200㎛. (B) Comparison after cultured in different media under without growth factors. Cell number was counted at the end of passage 10. Data were indicated as Mean±SE.

A


High
Low
F-12
αMEM

B


P=0.1166
Percentage posive SA-β gal cells per total cells (%)
50
45
40
35
30
25
20
15
10
5
0
High
Low
F-12
amem

4. Discussion

The above results establish a reliable and optimized methods to isolate and culturing bone marrow derived cells. Actually many research teams were studied and established methods for maintenance of chicken bone marrow derived cells (Khatri et al., 2009; Heo et al., 2011; Heo et al., 2012; Bai et al., 2013). However, it remains necessary to develop optimized and modifying culture conditions due to using different age at birth chicken in this paper. Since many researchers were using 11 or more older chicken for experimental animals, there was no coincidence culture condition in this research (Khatri et al., 2009; Bai et al., 2013; Liang et al., 2015; Yasmin et al., 2015). And also in this research, only Low glucose DMEM was used on isolated and expand a cell population of chicken bone marrow derived cells (Khatri et al., 2009; Bai et al., 2013).

In this paper, 4-day-old chicken were used for experimental animals and four different types of media were used for optimized chicken bone marrow cells culture condition. To investigate the effect of different types of media on chicken bone marrow derived cells expansion and proliferation, primary

cell attachment ability and total cell proliferation activity was detected. From this results, there was no significantly difference in primary cell attachment. But in number of cumulative cell assay, CBMCs cultured on DMEM/F12 were shown significant increase of cell proliferation detected. And also in CCK analysis to detect cell growth kinetics, shown significantly increase on DMEM/F12 media.

Adult stem/progenitor cells derived from bone marrow were originally referred to as fibroblastoid colony-forming-cells (Baksh et al., 2003). Because one of their characteristic features is adherence to tissue culture plastic and generation of colonies when plated at low densities. In colony forming unit fibroblast (CFU-F) assay, colonies were derived from a single precursor cells (Latsinik et al., 1986). The efficiency with which they form colonies were important assay for the indication the effect of culture condition on proliferation potential of CBMCs. From the results of CFU-F assay, there were no significantly difference between different types of media.

Morphology of chicken bone marrow derived cells (CBMCs) purified and shown cuboidal-like cells dominant. But

there were no obviously morphological differences among different four types of media. Adult stem/progenitor cells derived from bone marrow in other species like human and mouse were originally showed like fibroblast-like morphology (Tropel et al., 2004; Miao et al., 2006; Jones and McGonagle, 2008; Soleimani and Nadri, 2009). So, it is need to study in cell characterization like molecular biological investigations such as gene expression and protein expression and also focused cell differentiation into multi-lineages.

In this paper's results explain that chicken bone marrow derived cells (CBMCs) at passage 5 were no coincidence in mesenchymal stem cell related marker expression. Compare to bone marrow derived cells from other species, CBMSs were expressed mesenchymal stem cell related marker such as CD29, CD44, CD90, CD105. But interestingly endothelial and hematopoietic related marker CD31, CD45 also expressed on CBMCs in gene level. It seems little different appearance comparing to other species like human and mouse. In addition, in surface maker detection results using FACs analysis shows only CD45 expressed on CBMCs. And protein expression such as mesenchymal stem cell and endothelial cell related marker

shows no coincidence with bone marrow derived cell's from other species.

Finally, senescence assay also shows regardless of culture media types, there was no significant difference between types of media.

Through these results, we can conclude that chicken bone marrow derived cells from 4-day-old chick has different cell characterization and it should be adapted different culture conditions. DMEM/F12 is optimized in proliferation of chicken bone marrow derived cells.

CHAPTER 5.

IMPROVEMENT OF MEDIUM COMPOSITION BY THE ADDITION OF GROWTH FACTORS FOR THE EXPANSION OF BONE MARROW-DERIVED CELLS IN 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 is 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 have not been established.

This study was conducted to enhance the efficiency of culture medium for expanding chicken bone marrow-derived cells (CBMCs). Four-day-old white leghorn chicks were employed for CBMC donor and the isolated CBMCs were cultured in DMEM, to which 5 ng/ml bFGF and 500 unit/ml LIF were supplemented or not. To monitor the capacity of cell maintenance in vitro, primary cell attachment, CFU-F colony number, proliferative capacity, cell morphology, senescence

assay and gene expression were evaluated as experimental parameters. PLOC-GLM model in SAS package was employed for statistical analysis of these experimentations. The number of CFU-F positive colonies was higher ($p<0.05$) after the bFGF and LIF addition than after no addition, which resulted improved primary cell attachment. In total cell population, better proliferative capacity was detected in the growth factor supplementation, whereas in senescence assay, a significant increase in the number of SA-β-Gal positive cells were also counted in the growth factor-containing than in growth factor-free condition. Different expression profile in the expression of several pluripotency- or differentiation-related genes was detected after the supplementation or not, which differed from that of primordial germ cells and embryonic fibroblasts.

In conclusion, the combined addition of bFGF and LIF to culture medium improved the culture efficiency of chicken BMCs of mixed population, which may contain various reprogrammable cells.

2. Material & Method

Experimental design

This study was designed to know effects of growth factors on bone marrow cells retrieved from neonatal white leg horns. General experimental procedure was depicted in Figure 22. Some series of experimentation was conducted to understand retrieved bone marrow cells and to evaluate their capacity for in vitro expansion following subculture. In experiment 1, whole bone marrow cells from white leg horn at passage 0 were seeded

into culture plate dish to evaluate their capacity for in vitro expansion. In experiment 2, collected bone marrow cells were subsequently cultured with or without growth factors and subcultures were conducted up to 12 times. Cell number and accumulated number of cultured cells were counted at the end of each subpassage and doubling time of each subpassage was calculated. In experience 3, collected bone marrow cells were detected cell surface marker related mesenchymal stem cells using flow cytometry analysis. And gene expression was

conducted on different types of mesenchymal stem cells related marker. Finally collected bone marrow cells were differentiated into multi-lineage like adipogenic and osteogenic differentiation.

Experimental animals

Four-day-old chickens (White leg horn) were employed as the donor of bone marrow cell donors. The animal was maintained at the University Animal Farm, Seoul National University, Korea, using our standard management program, which were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU 140912-4).

Isolation and culture of bone marrow-derived primary cells

The femurs and tibia separated from both legs of euthanized donors were washed with 1% (v/v) antibiotic-antimycotic solution and 2% (v/v) fetal bovine serum containing Dubecco's phosphate buffer saline. Muscle tissues attached to the bones were subsequently removed and the marrow cavity

was exposed by excising the spongious part of each bone. Bone marrow cells were retrieved by flushing of bone marrow tissue with 2% (v/v) FBS containing DPBS. Whole bone marrow-derived cells including red blood cells were spread on culture dish.

Maintenance of chicken bone marrow cells

Chicken bone marrow cells were culture on the monolayer and subcultured when they reached 70-80%. Employed for chicken bone marrow cells culture media is with or without growth factors like LIF and bFGF and supplemented with 10% (v/v) FBS, 1% (v/v) solution of antibiotic-antimycotic at 37℃, 5% CO2 in a humidified atmosphere.

Colony forming unit fibroblast (CFU-F) assay

To quantify the frequency of stromal progenitors, mono-nuclear cells were resuspended in each media and plated at a density of One million cells on 100mm culture plate. The medium was changed after 3 days to wash non adherent cells

and cultured 21 days. After 21 days, cells were washed with DPBS and fixed 4% (v/v) paraformaldehyde for 15 minutes at room temperature. Subsequently, the fixed cells were stained with 1% crystal violet in 4% (v/v) paraformaldehyde for 5 minutes at room temperature. After several washes, colonies formed by more than 55 cells were counted under a light microscope at low magnification. Results were expressed as total number of colonies on 100mm culture plate.

Analysis of the relative mRNA levels using real-time PCR

Chicken bone marrow cells maintained in containing with or without growth factors were transferred into RNAlater, and subsequently stored at −80℃ until qPCR 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 Revers Transcriptase. The expression of specific chicken bone marrow cells genes was quantified by real-time PCR using iQTM SYBR® Green Supermix. The ß-actin primer was included in every treatment group for standardization and the mRNA level of each gene was

normalized to that of ß-actin.

Characterization of chicken bone marrow cells by FACS

To immunophenotypically characterize the chicken bone marrow cells, 1x106 cells were suspended in PBS and incubated with the following FITC- or PE- or APC-conjugated or unconjugated antibodies: anti-CD45-APC, anti-CD44-PE, anti-MHC class II-PE, anti-MHC class I-FITC, anti-MCAM-FITC. Then the cells were washed with PBS and analyzed by fluorescence-activated cell sorting. The data were analyzed using BD Cell/Quest Pro Software.

Assessment of chicken bone marrow cells senescence assay

Chicken bone marrow cells survival and senescence were evaluated using a Senescence ß-Galactosidase Staining Kit. Both passage 10 chicken bone marrow cells cultured in with or without growth factors media were employed as senescence assay. Number of senescence cell was counted using inverted microscope.

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 indicated a significant difference.

3. Result

Isolation and proliferation of bone marrow derived cells

After flushing from tibia and femur at each side of legs, one million cells were seeded into culture dish counted cell number at the end of the first subculture. The cells formed a monolayer of heterogenous morphology in culture plate. Bipolar spindle cells like fibroblast cell with a cuboidal morphology cells within 2 weeks. The morphology of the bone marrow derived cells was consistent with regardless of insertion of growth factors. Proliferation of bone marrow derived cells in different four 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. Cumulative number of cells at each passages are significant difference detected in each media. (Figure 15) Until passage 12 significant difference are detected in two different media. Bone marrow derived cells are cultured insertion of growth factors media have better proliferation ability than without growth factors.

And also, seeding efficiency of chicken bone marrow derived-adherent cells (CBMCs) at the end of the first passage is significant difference detected ($p<0.0001$) (Figure 16). Seeding efficiency between two different media have effects on attachment and proliferations of bone marrow derived primary cells.

Comparisons of colony forming ability of bone marrow-derived primary cells with or without growth factors

Isolated bone marrow cells from chicken tibia and femurs were cultured in vitro more than twelve passages with mixed population morphology. To evaluate of bone marrow derived primary cells attachment, colony forming unit-fibroblast analysis and primary cell attachment calculation were conducted. One million bone marrow cells retrieved from primary passage were seeded into 100mm cell culture plate and crystal violet staining was performed to detect single cell-derived clones during 21 days of culture. Positive staining was defined as a colony that contained 50 positive cells on average. As the results, more colonies were detected in growth factors

with media ($p<0.0001$) (Figure 18).

Comparisons of morphology of chicken bone marrow derived cell cultured on different four types of basic medium

To observe morphology of chicken bone marrow derived cells, isolated primary cells were subpassaged until homogeneous morphology. At passage 5, cells were relatively much cuboidal-like cells are represented in insertion of growth factors media (Figure 19).

Assessment gene expression on established chicken bone marrow derived cells at different passages

Gene expression of pluripotency, differentiation and bone marrow derived stromal cell related markers in chicken bone marrow derived-adherent cells (CBMCs) were assessed at passage 5 (Figure 20). RT-PCR analysis showing that except for Sox2, pluripotency related gene such as *pouV* and Nanog is not expressed in both groups at passage 5. And mesenchymal stem cells related genes such as *CD29, CD44,*

CD90, CD105, SMA also expressed regardless of growth factors. But importantly *CD45* as a hematopoietic marker and endothelial cell maker also expressed on both media. other differentiation related genes such as *Desmin, Nestin* also expressed. Through the results, there were still heterogeneous cell population and composed of different cell types.

Comparisons of Immunophenotypic expression of chicken bone marrow derived cells cultured on different medium

The detection of expression of cell surface marker of the chicken bone marrow derived cells were cultured at passage 5. Various types of cell surface marker such as mesenchymal lineage, hematopoietic makers were detected by using flow cytometry analysis. Representative flow cytometry histogram show the expression (red peaks) of selected molecules (CD45, CD44, MCAM, MHC I, MHC II, CD105) by different MSC populations compared with isotype controls (black peaks) (Figure 21). As a result, the chicken bone marrow derived cells were slightly expressed only CD44. CD45 as a hematopoietic lineage maker is only expressed on without

growth factors media.

Assessment of cell apoptosis of chicken bone marrow derived cells cultured on different medium

Senescence of chicken bone marrow derived-adherent cells (BMCs) during subculture in vitro. β-galactosidase (SA-β-Gal) was used for the senescence assay of CBMCs subcultured performed on each type of cultures and the number of SA-β-Gal positive cells was counted. Comparison after cultured in different media under different four types of culture media. Cell number was counted at the end of passage 10. Regardless of culture media types, there was no significant difference between types of media (Figure 22).

Figure 14. General experimental procedures.

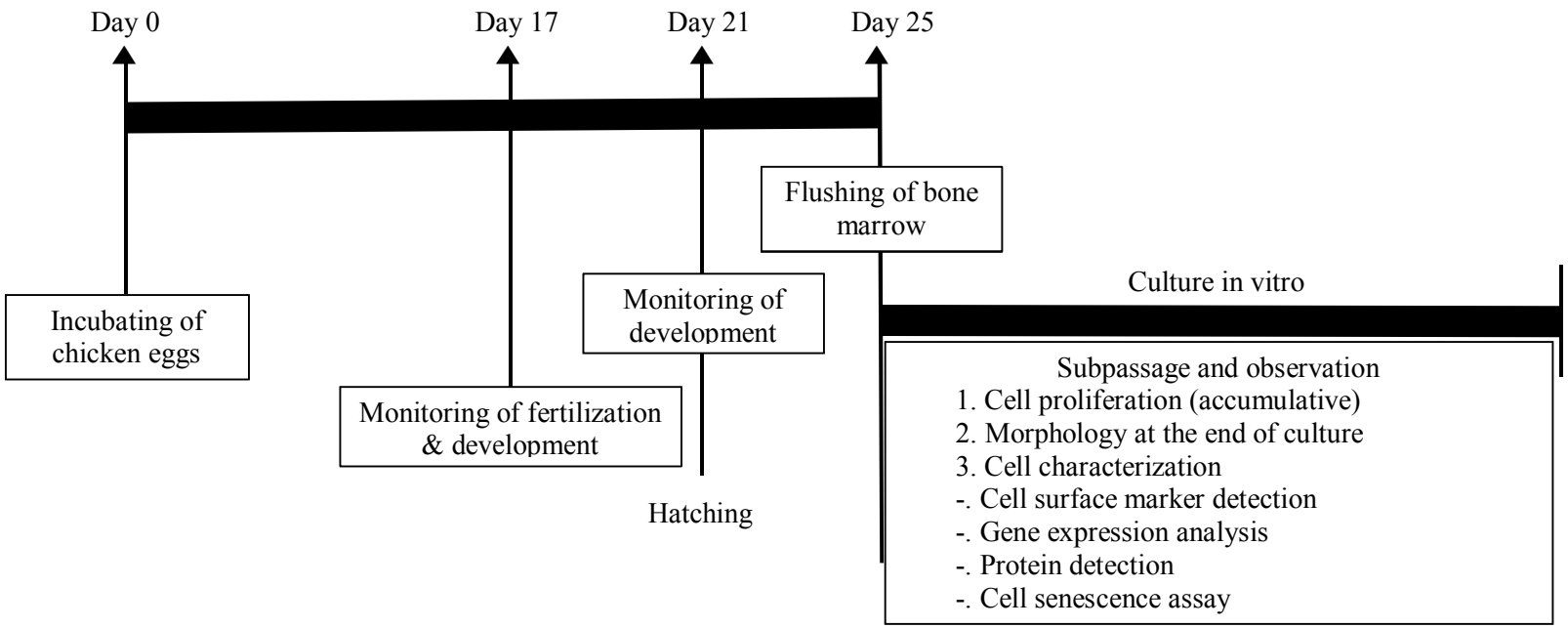


Figure 15. Retrieval number of chicken bone marrow derived-adherent cells (CBMCs) and their proliferation following subculture in vitro. Chicken BMCs are retrieved from the white leghorn of 4-day-old chickens and the number of cultured cells was counted at the end of each passage. The collected CBMCs were cultured in with or without growth factors. Data were indicated as Mean±SE.

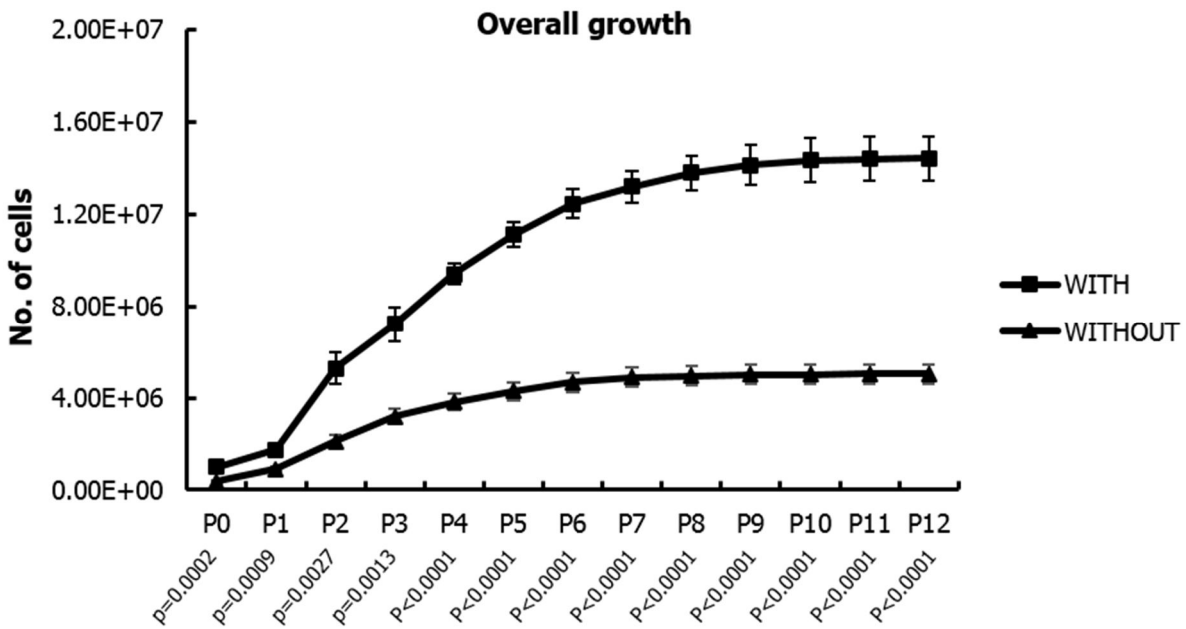


Figure 16. Comparisons number of chicken bone marrow derived cells (CBMCs) seeding efficiency at the end of the first passage. One million chicken BMCs retrieved from primary culture were seeded into one well of 6-well plate and counted cell number at the end of the first subculture. Comparison of cell number after cultured in the media with or without growth factors. Data were indicated as Mean±SE and p value indicate model effect. ***p<0.0001 within each passage.

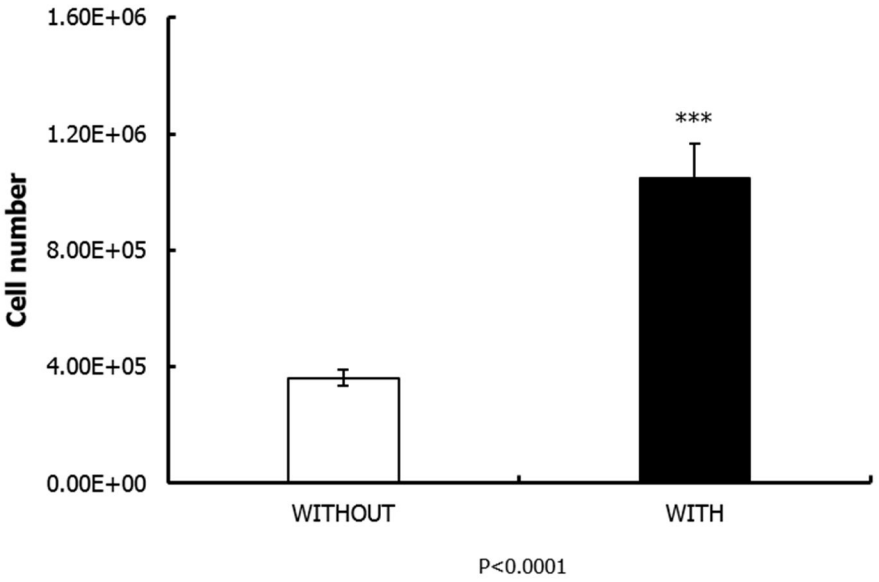


Figure 17. Proliferation activity was evaluated using the Cell Counting Kit (CCK-8) according to with or without growth factors. Ten-thousands cells were seeded on one well of 96-well plates and 100㎕ medium was subsequently incubated with the mixture of 10㎕ CCK-8 at 37℃ with 5% CO_2 in air atmosphere for 2h and CCK values were measured at each observation time. Absorbance was measured at 450nm using a microplate reader. All data were shown as mean±SE and PLOC-GLM in SAS program was employed for statistical analysis. Values in parentheses indicate model effect of treatment and bars with different letters differ significantly ($p<0.05$).

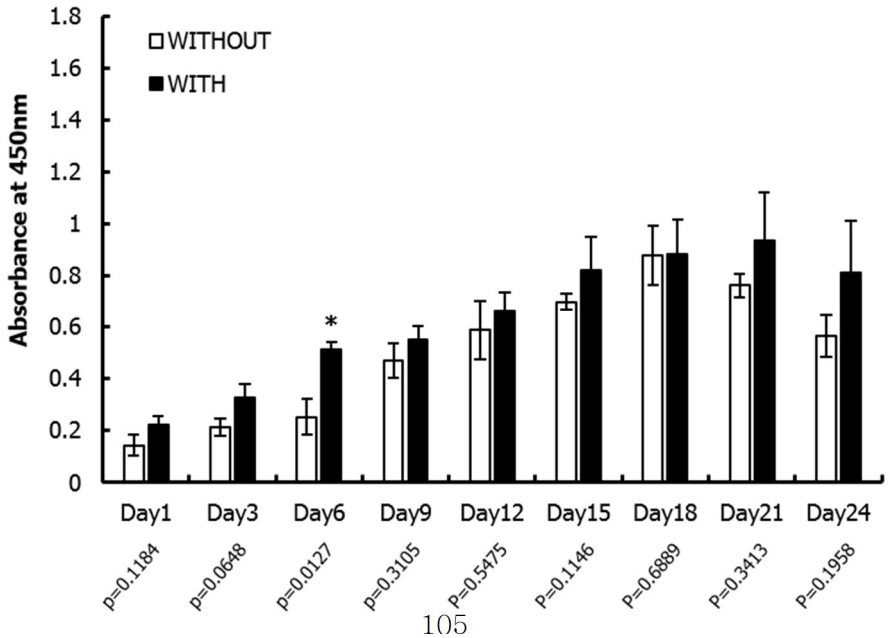
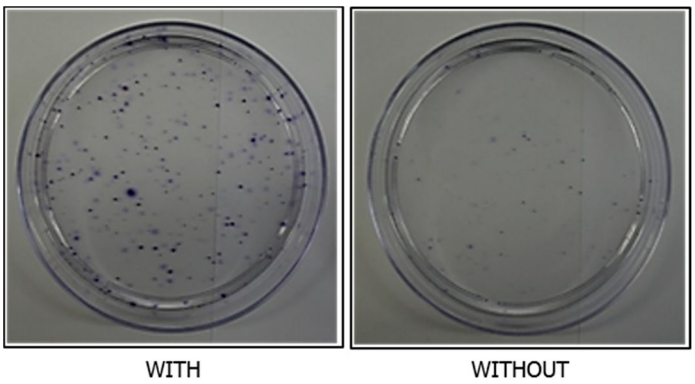


Figure 18. Colony-forming activity of chicken bone marrow derived-adherent cells (CBMCs) by colony form unit (CFU) analysis. (A) Comparison of CFU level among growth factors with or without media. (B) Number of colonies after cultured in growth factors-free media. Data were indicated as Mean±SE and p value indicate model effect. ***p<0.0001 within each passage.

A



B

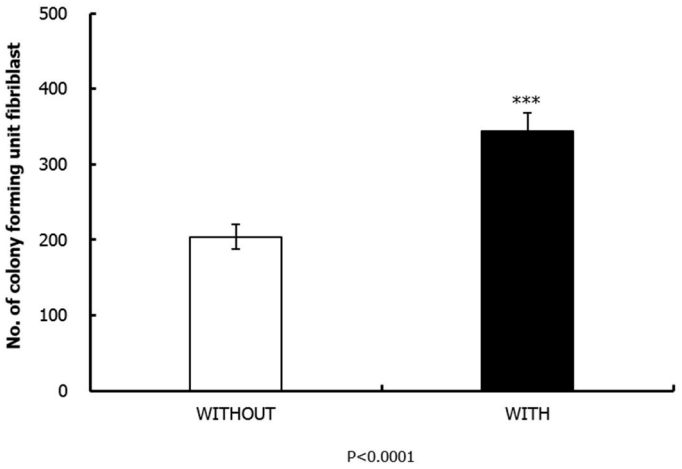


Figure 19. Morphology of primary and sub-cultured chicken bone marrow derived-adherent cells (CBMCs). Primary cells after whole bone marrow culture. Passage 5 CBMCs were purified, and cuboidal-like cells dominant. (A-B) There were no obviously morphological differences among with or without growth factors. Scale bar=100㎛.

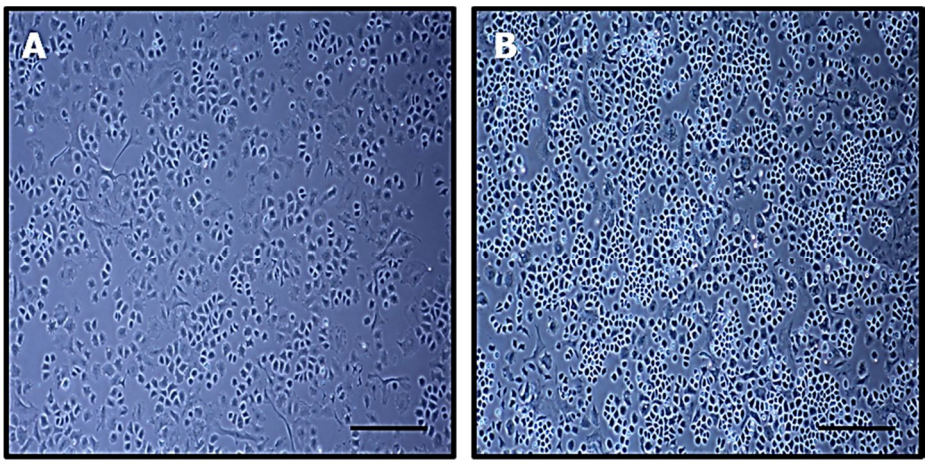


Table 4. Oligonucleotide primer sequences and thermal cycling conditions used in real-time and reverse transcriptase PCR.

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	DQ867024.1	TCAATGAGGCAGAGAACACG	GGACTGGGCTTCACACATTT	154	60
Nanog	NM_001146142.1	CAGCAGACCTCTCCTTGACC	TTCCTTGTCCCACTCTCACC	187	60
Sox2	AB092842.1	AGGCTATGGGATGATGCAAG	GTAGGTAGGCGATCCGTTCA	163	60
Desmin	XM_003641642.2	AGGTGGTGGAGTCCAAGGAG	CTTTGTTGGCAATGACGCTT	204	60
SMA	M13756.1	CCTGTATGCTTCTGGGCGTA	TGGCCATCTCGTTTTCAAAG	263	60
Nestin	NM_205033.1	ACAGGACTGGGAAGGGACAG	TCTCCCTCATCCTCACCCTC	177	60
CD29	NM_001039254.2	GAACGGACAGATATGCAACGG	TAGAACCAGCAGTCACCAACG	300	60
CD31	XM_004946203	CAGGCAAAGGAGACGCACGAT	CTTCTGGCAGCTCACAACGT	221	60
CD34	XM_417984.4	GTGCCACAACATCAAAGACG	GGAGCACATCCGTAGCAGGA	239	60
CD44	NM_204860.2	GGTTTTATAGTGGGGCATATTGTTATCCC	TTAACCGCGATGCACACGGC	700	58
CD45	NM_204417.2	CACTGGGAATCGAGAGGAAA	CTGGTCTGGATGGCACTTTT	574	55
CD90	NM_204381.2	GGTCTACATGTGCGAGCTGA	AAAGCTAAGGGGTGGGAGAA	471	56
CD105	NM_001080887.1	ACGGATGACACCATGGAAAT	ATGAGGAAGGCTCCAAAGGT	704	56

Figure 20. Assessment of expression of pluripotency, differentiation and bone marrow derived stromal cell markers in chicken bone marrow derived-adherent cells (CBMCs) at passage 5. RT-PCR analysis showing that pluripotency related gene is not expressed in regardless of growth factors. And other differentiation related genes such as endoderm and mesoderm marker also expressed. Also insertion of growth factors has effects on more strongly expressed all the cell types. β-actin is positive control.

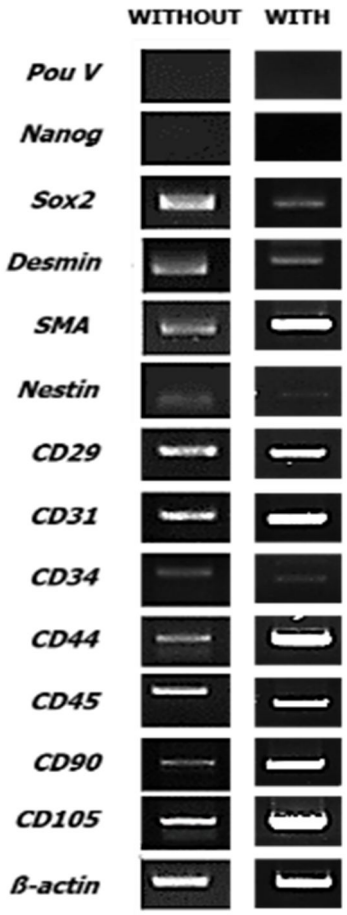


Figure 21. Immunophenotypic profile of chicken bone marrow derived-adherent cells (CBMCs) at passage 5. Representative flow cytometry histogram show the expression (red peaks) of selected molecules (CD45, CD44, MCAM, MHC I, MHC II, CD105) by different MSC populations compared with isotype controls (black peaks).

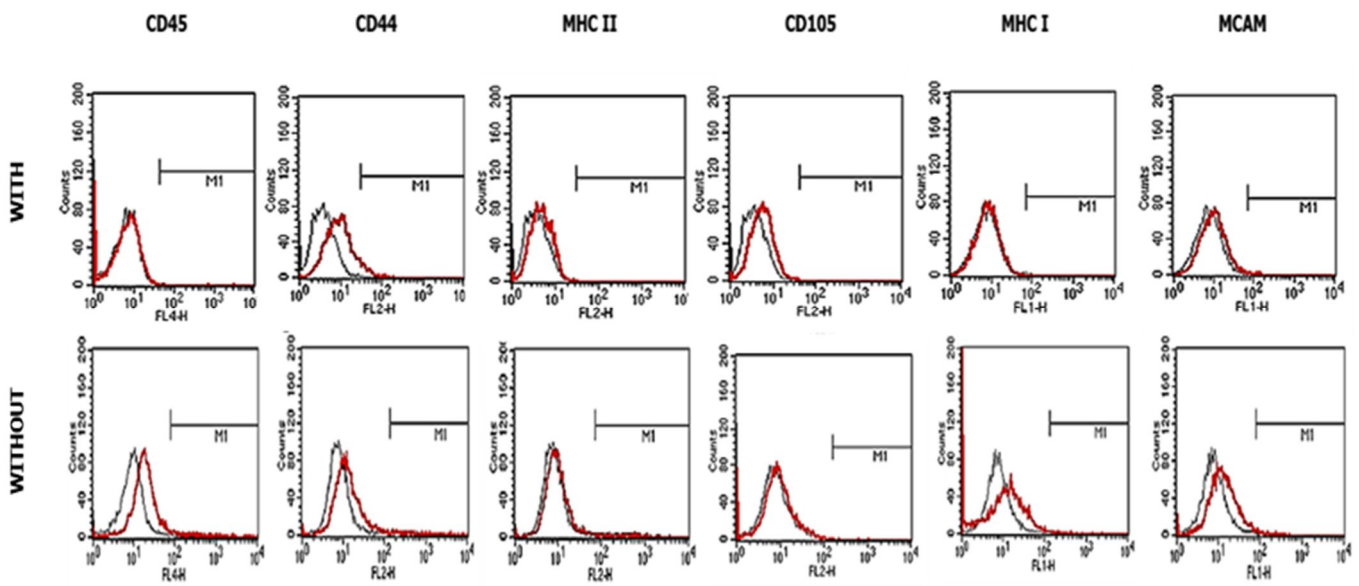
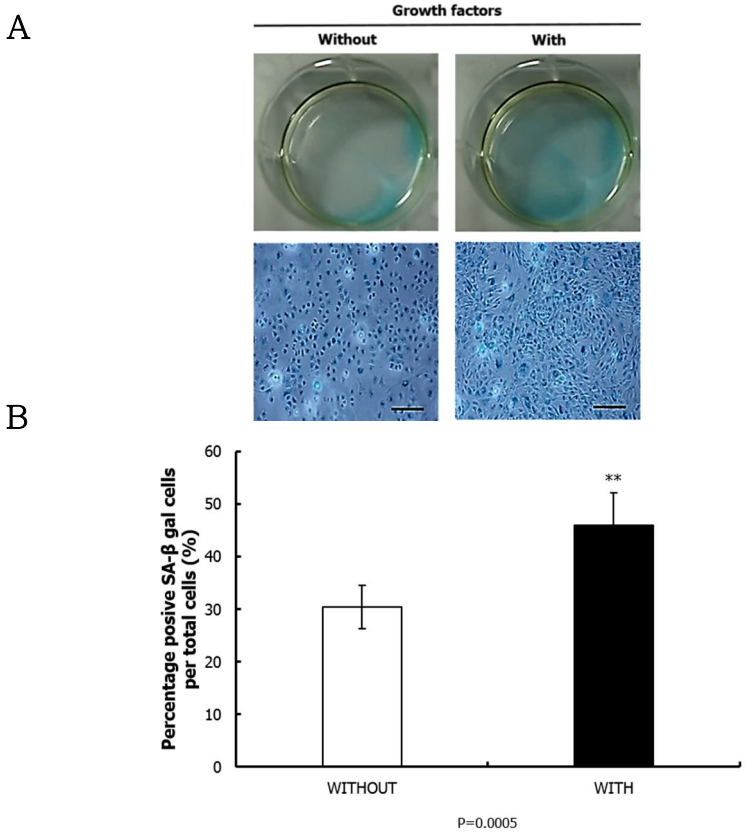


Figure 22. Senescence of chicken bone marrow derived-adherent cells (CBMCs) during subculture in vitro. β-galactosidase (SA-β-Gal) was used for the senescence assay of CBMCs subcultured performed on each type of cultures and the number of SA-β-Gal positive cells was counted. (A) Images showed the cells positive for SA-β-Gal or not. Scale bars =200㎛. (B) Comparison after cultured in different media under with or without growth factors. Cell number was counted at the end of passage 10. Data were indicated as Mean±SE.



4. Discussion

This study was conducted to improvement of medium composition by the addition of growth factors like LIF and bFGF for the expanstion of bone marrow derived cells in chicken. Leukemia inhibitory factors (LIF) is generally addition on culture medium and it typically reduce spontaneous differentiation (Murray and Edgar, 2001; Lee et al., 2009). Fibroblast growth factors (FGFs) regulate fundamental developmental pathways like mesoderm patterning in the early embryo through to the development of multiple organ systems (Yamaguchi et al., 1994; Turner and Grose, 2010). In mouse study, FGFs activation mechanism revealed more widely how influence proliferation.

In this paper, 4-day-old chicken were used for experimental animals and four different types of media were used for optimized chicken bone marrow cells culture condition. To investigate the effect of growth factors in media on chicken bone marrow derived cells expansion and proliferation, primary cell attachment ability and total cell proliferation activity was detected. From this results, there was significantly difference in

primary cell attachment. Also in number of cumulative cell assay, CBMCs cultured on with growth factors were shown significant increase of cell proliferation detected. In colony forming unit fibroblast (CFU-F) assay, there was significantly difference in types of with or without growth factors. Morphology of chicken bone marrow derived cells (CBMCs) purified and shown cuboidal-like cells dominant regardless of growth factors. In this paper's results explain that chicken bone marrow derived cells (CBMCs) at passage 5 were no coincidence in mesenchymal stem cell related marker expression in PCR and FACs analysis. Finally, senescence assement also shows insertion of growth factors significantly effects on chicken bone marrow derived cell's senescence.

Through these results, we can conclude that growth factors like LIF and bFGF are effects on chicken bone marrow derived cells proliferating and differentiation ability.

CHAPTER 6.

GENERAL DISCUSSION AND CONCLUSION

In this study, we investigated a reliable and optimized methods to isolate and culturing bone marrow derived cells. 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; Roson-Burgo et al., 2014). We sought to develop a method for using chicken bone marrow derived cells (CBMCs) for the establishment and culture systems, based on the ease of manipulation and their expansion.

As described in chapter 3, significant correlation was detected between the physical parameters and number of bone marrow cells retrieved from each individual, and different correlations were detected between the cuboidal and stromal cell-dominant populations. These results may be due to differences in cell proliferation dynamics according to the maturity of the individuals between the two cell populations.

Chicken bone marrow cells have different characteristics compared with bone marrow cells of mammalian species, which usually yield a fibroblast-dominant population.(Larsen et al., 2010; Ward et al., 2015) 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(Kolf et al., 2007). Therefore, further studies are required to determine aspects of the different properties of CBMCs culture conditions.

In Chapter 4 and 5, we focused the optimized culture conditions of chicken bone marrow derived cells. We used 4-day-old chicken for experimental animals and different types of media and with or without growth factors were used for optimized chicken bone marrow cells culture condition. Many research teams were studied and established methods for

maintenance of chicken bone marrow derived cells (Khatri et al., 2009; Heo et al., 2011; Heo et al., 2012; Bai et al., 2013). However, it remains necessary to develop optimized and modifying culture conditions due to using different age at birth chicken in this paper. Since many researchers were using 11 or more older chicken for experimental animals, there was no coincidence culture condition in this research. Through in Chapter 4 results, we can conclude that chicken bone marrow derived cells from 4-day-old chick has different cell characterization and it should be adapted different culture conditions. DMEM/F12 is optimized in proliferation of chicken bone marrow derived cells.

In the results of Chapter 5, we conducted to improvement of medium composition by the addition of growth factors like LIF and bFGF for the expanstion of bone marrow derived cells in chicken. Leukemia inhibitory factors (LIF) and Basic Fibroblast growth factors (bFGF) were used for chicken bone marrow derived cells expansion, proliferation and cell sustaining. Through these results, we can conclude that growth factors like LIF and bFGF are effects on chicken bone marrow

derived cells proliferating ability.

Future studies are needed to reveal the precise cell characterizations by which osteochondro progenitor cell's target gene expression for detect and cell localized position in bone marrow. Through these future studies, new knowledge will be gained to aid not only the development of growth plate mechanisms, but also for the treatment of growth related diseases such as dwarfism, which are associated with growth plated formations.

In conclusion, we have verified that chicken bone marrow derived cells are different morphology and also cell proliferation ability and characterizations. And we success culture condition of chicken bone marrow derived cells related cell proliferation and differentiation. The results obtained from this study have implications for the understanding of chicken bone marrow derived cells and their difference suggests relevance in study of inner bone cell's characterization.

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SUMMARY IN KOREAN

본 논문은 닭 골수 유래 세포 (Chicken bone marrow derived cell) 의 배양 환경 및 조건을 확립함으로써 닭 골수 유래 세포의 효율적인 조작 기법 시스템을 구축하였다. 기존에 수행되어 왔던 연구팀의 주령 수와는 달리 본 연구진에서는 4일령의 닭을 실험 모델 동물로 사용 하였으며, 4일령 닭의 골수에서 회수된 세포의 양상이 기존에 보고된 결과와는 다름을 확인하였다. 포유류를 포함한 다른 모델 동물에서 회수된 골수 유래 세포는 형태학적으로 기질세포 (Stromal cell)와 유사하지만 본 연구에서 회수된 골수 유래 세포는 기질세포와 입방상피세포 (Cuboidal epithelial cell)가 혼재되어 있는 양상을 확인하였다. 따라서 이러한 차이점을 기반으로 본 연구를 통해 닭 골수 유래 세포의 효율적인 배양 조건의 정립과 세포주 확립을 진행 하였다.

먼저 Chapter 3 에서, 4일령 병아리의 개체 특성 분석을 통해 기질세포와 입방상피세포 중 지배적인(dominant) 세포에 따라 각 개체와 회수된 세포간의 상관관계를 추적하였다. 회수된 세포 중 cuboidal dominant population에서 회수된 세포의 양과 각 개체 별 특성분석에서만 정의 상관 관계만이 있음을 확인하였고, 회수된 세포에서 outgrowth 하는 배양 조건의 확립을 통해 4일령 닭의 특성

분석을 진행하였다.

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. 해외 몇 연구진을 통해 확립된 골수 유래 세포의 경우 본 논문에서 사용된 닭의 일령 수와 다르며 세포의 특성 및 형태학적 부분에서도 큰 차이가 있음을 확인하고 세포 배양 조건 확립 실험을 진행하였다. 회수된 닭 골수 유래 세포를 상용화 된 배지 4종류 (High glucose DMEM. Low glucose DMEM, DMEM/F12, aMEM)에서 세포의 성장능 및 특성분석을 진행하였다. 그 결과, 닭 골수 유래세포의 성장에서는 DMEM/F12 이 유의적으로 성장능이 뛰어난 것을 확인하였고 이에 따라 닭 골수 유래 세포의 최적화된 배양 환경을 구축 할 수 있었다.

또한 이 결과를 토대로 닭 골수 유래 세포에 필요한 성장인자 (LIF, bFGF) 첨가를 통해 성장인자의 첨가 유무에 따른 세포의 성장능 및 세포 노화에 관련 된 실험을 진행 하였다. 그 결과 성장인자의 첨가는 닭 골수 유래세포의 세포 성장능을 포함한 전반적인 세포 지지에 유의미적인 효과를 얻음을 확인 할 수 있었다. 본 연구에서 진행된 실험만으로는 알 수 없지만 후속 실험들을 통해 일령 수 비

교를 통한 닭 골수 내 함유 세포의 추가적인 세포 특성 분석과 기존에 보고된 영장류 및 포유류에서의 골세포 발달 과정의 차이점을 토대로 성장판 구성 세포 및 확립 기작에 관련된 연구가 이루어 질 수 있으며, 세포를 이용한 치료 접목 분야에 관한 가능성을 밝혀 낼 수 있을 것이다.

본 논문에서 수행한 닭 골수 유래 세포의 배양 조건 확립과 체외 배양 기술은 골수 내 줄기세포 및 골수 구성 세포들을 활용한 치료기술에 접목 될 수 있으며, 성장관련 질병 및 세포 치료를 이용한 다양한 임상 분야에 기여 할 수 있는 중요한 기술이 될 것으로 사료된다.