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Three Dimensional Spheroid Culture of Canine Amniotic Fluid Derived Mesenchymal Stem Cells Enhances Differentiation Efficacycy

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Amniotic fluid derived mesenchymal stem cells (AFS cells) have been considered as an applicable treatment of stem cell therapy in regenerative medicine. AFS cells have similar differentiation ability to embryonic stem cells with less ethical controversy. For applying in regeneration of injured and damaged tissues there are still considerable huddles which need to be overcome. One of the crucial problems is how sustains or even amplifies their multi–potent ability till administration in damaged tissue/organ sites. The 2–D adherent culture method provides different environment compared to those of in vivo. 3–D culture method is an alternative manner to culture cells in in vivo–like cell culture condition. Hanging drop is one of simple and effective 3–D culture systems. Here canine AFS cells were cultured in the hanging drops in which cells form spheroids with micro–networks that allow exchanging cellular signalings similar to in vivo. Canine AFS cells were aggregated in tightly spaced spheres in hanging drops supplemented with 20% FBS. In the study of cellular senescence, AFS cells successfully formed and maintained spheroids regardless of their passages. In addition, the multipotent differentiation capacity of AFS cells in shape of 3–D spheroids was enhanced based on MSC differentiation assay after hanging drop culture. Noticeably, the expression levels of cartilage oligomeric matrix protein (COMP) and aggrecan (AGG) were significantly increased in 3–D spheroid cultured AFS cells compared to 2–D adherent cultured controls. Therefore, induction of spheroid formation by hanging drops is a useful method to provide 3–D in vivo–like culture condition without changes of AFS cells differentiation capacity especially to induce chondrogenic differentiation for regenerating damaged cartilages.

Key words: Amniotic fluid derived mesenchymal stem cells, 3–D spheroid culture, Hanging drop, Multi–potency, Chondrogenic differentiation

INTRODUCTION

Mesenchymal stem cells (MSCs) have a great ability for tissue engineering because of their unlimited in vitro expansion potential, self–renewal capacity and multi–potentiality (Kolf *et al*., 2007; Sacchetti *et al*., 2007). For several years, MSCs have been largely studied and used as a new therapeutic tool for clinical applications. It has been suggested that MSCs could reside in virtually all post–natal organs and tissues (da Silva *et al*., 2006). MSCs were first identified in the bone marrow (Friedenstein *et al*., 1976) but are now described to exist in connective tissues and particularly in adipose tissue (Zuk *et al*., 2002), placenta (Fukuchi *et al*., 2004), umbilical cord (Romanov *et al*., 2003), dental pulp (Gronthos *et al*., 2000), tendon (Bi *et al*., 2007), trabecular bone (Nöth *et al*., 2002) and synovium (De Bari *et al*., 2001). Multi–potent MSCs differentiate into distinctive end–stage cell types such as bone, cartilage, muscle, bone marrow stroma, tendon/ligament, fat, dermis and other connective tissues. Moreover it has been known that MSCs release various soluble factors which are immunoregulatory and support to regenerate microenvironment of tissues. In the damaged tissues, MSCs are especially believed to secrete a broad spectrum of paracrine factors that participate in the regenerative microenvironment and regulate immune infiltration (Fong *et al*., 2011).

Amniotic fluid is comprised of embryonic or fetal cells derived from three germ layers (In't anker *et al*., 2004; De Coppi *et al*., 2007). It has been considered that MSCs in amniotic fluid would be similar to embryonic stem cells as the inherent precursors of all three germ layers. Since the initial identification of human amniotic fluid derived mesenchymal stem cells (hAFS cells), amniotic fluid has been used as a source of stem cells for cell therapy and regenerative medicine (De Coppi *et al*., 2007). Amniotic fluid derived mesenchymal stem cells express CD90, CD105, and CD73 including Oct4 which is an embryonic stem cell marker (In't Anker *et al*., 2004; De Germmis *et al*., 2006; De Coppi *et al*., 2007). Oct4 was expressed over 90% of AFS cells. AFS cells have a self–renewal and differentiation potential toward adipocytes, osteoblasts, chondrocytes (Kolambkar *et al*., 2007), and neurocytes (Rehni *et al*., 2007) indicating that amniotic fluid may be a promising source of regenerative medicine without any ethical concerns unlike

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embryonic stem cells (Prusa AR *et al*., 2003; Chung Y *et al*., 2008).

In general, cell based assay is performed in two–dimensional (2–D) culture system. Monolayer or suspension culture systems (2–D) present a large artificial cellular environment that limits and alter their cellular potential for therapeutic application. It seems that the 2–D culture system provides a non–physiological environment which affects innate cellular functions of cells (Friedrich *et al*., 2007; Pampaloni *et al*., 2010). 3–D systems have been used and applied to culture cells *in vitro* to overcome the limitation. 3–D culture systems mimic in vivo tissue environment and provide micro–networks of cells which allow cells to communicate each other like in vivo that supports them to sustain their function.

The objective of this study was to investigate the efficacy of 3–D culture system using hanging drops to form spheroid of canine AFS (cAFS) cells. Furthermore it was evaluated and compared the multi–linege differentiation potential and morphological distinction of the cAFS cells between 3–D spheroids and 2–D monolayer. It demonstrated that the hanging drop method offered a microgravity circumstance and conserved constant condition with reliable oxygen and nutrient transfer that allow maintaining their multi–potent differentiation capacity which is involved to regenerate various tissues.

MATERIALS AND METHODS

Isolation and characterization of cAFS cells

All animal experiments were performed by the protocols approved by the Research Ethics Committee and the Institutional Animal Care and Use Committee of Chungnam National University. Canine amniotic fluids were collected from healthy pregnant beagles' amniocentesis using ultrasonographic guidance. Isolation and culture of cAFS cells were performed by the published protocols in Choi *et al.* (2013). Briefly, the cells were isolated from the amniotic fluid by centrifugation at 3,000 rpm for 10 min, and washed twice with Phosphate Buffered Saline (PBS, Gibco). Isolated cells were cultured in low glucose Dulbecco's modified eagle medium (L–DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco), 5 ng of fibroblast growth factor (FGF, Sigma), 10 ng of epidermal growth factor (EGF, Sigma) and 1% penicillin–streptomycin (Sigma) at 39°C, 5% CO_2 incubator for 4–5 days.

Spheroid formation and culture in 3–D micro–environment by hanging drop method

cAFS cell spheroids were prepared using the hanging drop method. AFS cells grown as a monolayer were dissociated with trypsin, collected by centrifugation and resuspended in growth medium with 5%, 10% or 20% of fetal bovine serum (FBS, Gibco) at a density of 1×10^6 cells per 1 ml. Hanging drops were formed on the culture dish lid after inverting it using 20 μl of 20,000 cells in growth medium, and allowed to form cell aggregates at 39°C in 5% CO_2 for 3 days. The culture dish was filled with 10 ml PBS to warrant high humidity (Fig. 1A and B). On day 3, the hanging drops were harvested, and the cells were transferred to a new culture dish. The spheroids were cultured for 5 days and culture medium was replenished every 3 days. cAFS cells were also cultured in adherent manner and used as 2–D control group. For differentiation assay, cAFS cells were cultured in hanging drop method for 3 days and then placed into adherent culture. Within 3 days of plating, cells initiated to expand from the edge of the attached spheroids.

Adipogenic differentiation

Adipogenic differentiation was induced as described by Pittenger *et al.* (1999). Briefly, 50,000 cells were seeded per well and cultured until confluence. For 3 days, the cells were treated with the adipogenic induction medium consisting of L–DMEM base medium, 10% FBS, 10 ng EGF, 5 ng bFGF, penicillin/streptomycin solution (10,000 IU/10,000 IU/ 100 ml), 1 μM dexamethasone (Sigma–Alorich, USA), 0.2 mM indomethacin (Sigma–Alorich, USA), 1 mg/ml insulin (Lily, Korea) and 1 mM 3–isobutyl–1–methylxanthin (IBMX) (Sigma–Alorich, USA). Then the medium was changed every 3 days for 21 days. Cells were fixed with formaldehyde solution (Samchun chemical, Korea) (10% v/v in PBS) and washed with 3% (v/v) isopropanol (Amresco, USA). Samples were stained with 0.5% (w/v in 60% isopropanol) Oil Red O (Sigma–Alorich, USA) to determine the presence of oil droplet.

Osteogenic differentiation

40,000 cells were seeded per well in a six–well–plate and cultured until they reach confluence. The osteogenic induction medium was prepared as described by Pittenger *et al.* (1999). The medium is consisted of L–DMEM base medium supplemented with 10% FBS, 10 ng EGF, 5 ng bFGF, penicillin/ streptomycin solution (10,000 IU/10,000 IU/100 ml), 0.1 μM dexamethasone, 10 mM β–glycero–phosphate (Sigma–Alorich, USA) and 50 μM ascorbate–2–phosphate (Sigma–Alorich, USA). Alizarin Red S (Sigma–Alorich, USA) staining was used to determine the presence of calcium deposition. For Alizarin Red S staining, cells were washed with distilled water (D.W.) two times and fixed in a solution of ice–cold 70% ethanol for 1 hr. After carefully washed 7 times with D.W., cells were stained for 10 min with 40 mM Alizarin Red S and then washed again with D.W. for 2 times in room temperature.

Chondrogenic differentiation

50,000 cells were seeded per well in a six–well–plate and cultured until they reach confluence. For 3 days, the cells were treated with the chondrogenic induction medium including L–DMEM base medium, 10 ng EGF, 5 ng bFGF, penicillin /streptomycin solution (10,000 IU/10,000 IU/ 100 ml) and 10 ng/ml transforming growth factor–β1 (TGF–β1) (Sigma–Alorich, USA). And then the cells were cultured in chondrogenic maintenance medium consisting of L–DMEM base medium, penicillin/ streptomycin solution (10,000 IU/10,000 IU/100 ml), TGF–β1 (10 ng/ml), dexamethasone (0.1 μM), ascorbate–2–

phosphate (50 μM), sodium pyruvate (100 μg/ml) (Sigma–Alorich, USA), ITS (50 mg/ml) (Sigma–Alorich, USA), cis–3–Hydroxy–DL–Proline (20 μg/ml) (Sigma–Aldorich, USA) and 0.1 mg/ml insulin for 21 days. This cycle was repeated six times. Alizarin Red S staining was used to determine the presence of calcium mineralization. For Alizarin Red S staining, cells were washed with D.W. two times and fixed in a solution of ice–cold 70% ethanol for 1 hr. After carefully washed 7 times with D.W., cells were stained for 10 min with 40 mM Alizarin Red S and then washed again with D.W. for 2 times in room temperature.

Reverse–transcription polymerase chain reaction (RT–PCR)

Total RNA was extracted by RNA extract kit following the protocol of manufacturer (MACHEREY–NAGEL). Expression of pluripotent genes (Oct4, NANOG and SOX2) and adipogenic (LPL and Leptin), osteogenic (Runx2 and Osteocalcin), and chondrogenic (Comp and AGG) genes were detected from each samples. β–Ⅱ microglobulin was used as an internal control. RNA samples (1 μl of total RNA) were primed with oligo dT primer to synthesize cDNA using iScript reverse transcriptase (BIO–RAD). PCR reactions were consisted of 3 min denaturation at 94°C, followed by 35 cycles at 94°C for 30 sec, 60°C or 58°C for 30 sec and 72°C for 30 sec. A final extension for 5 min was performed at 72°C. β–Ⅱ microglobulin RT–PCR reactions were modified to 25 cycles with annealing at 60°C. Each amplicon of targeted specific genes was confirmed by DNA sequencing. RT–PCR results were evaluated by Image J software. Primer sequences used were listed in Table 1.

RESULTS

Spheroid formation in 3–D micro–environment by hanging drop method

Using hanging drop method, cAFS cells reproducibly were aggregated into 3–D spheroids. To characterize and estimate the self–assembling potential, spheroids were grown in different culture conditions. cAFS spheroids were generated in growth medium supplemented with 5%, 10% or 20% of FBS concentration (Fig 1). There were more compacted spheroids found in the medium with 20% FBS compared to (Fig 1. E and H) the medium with 5% (Fig 1. C and F) and 10% (Fig 1. D and G) of FBS, and compacted structure were maintained tightly with 20% FBS than 5% and 10% FBS.

Under the phase contrast microscope, cAFS cells were aggregated in the time dependent manner in hanging drops. Initially, cAFS cells formed a loose connection and then several small aggregates that progressively combined into a single spheroid (Fig 2. A). Once aggregated, the size of spheroids was not changed, and gradually compacted tensely between 48h and 96h after culturing (Fig 2. B).

In addition, it has investigated how the impact of senescence onto forming spheroids of cAFS cells is. Within 3 days of cultivation with 5, 10, and 15 passages of cAFS cells, there was no change of morphology and size enlargement observed. Regardless of the passages, cAFS cells were aggregated compactly, and most of spheroids were generated fairly (Fig 3. C–H).

Table 1. Reverse–transcription polymerase chain reaction primers for adipogenic–, osteogenic–, and chondrogenic–specific genes

Genes	Primer sequences (5'–3')	Product size
LPL	F : AAAACCATCGTGGGCAATTA	204 bp
	R : ACAATTTGGATTCCCAGCAA	
Leptin	F : TTCCACCATCCTGCCACTAT	200 bp
	R : ACCATCTGGAATGCAAGGTC	
Runx2	F : CCCAACTTCCTGTGCTCTGT	197 bp
	R : TCGTTGAACCTTGCTACTTGG	
Osteocalcin	F : AGGGAAGTATGCGAGCTCAA	198 bp
	R : GATGACAAGGACCCCACACT	
COMP	F : AGACATACTGGCAGGCGAAT	197 bp
	R : CCAGCCGTAGGATGTCTTGT	
Aggrecan	F : ATCAACAGTGCTTACCAAGACA	130 bp
	R : ATAACCTCACAGCGATAGATCC	

cAFS cells differentiation

When cultured in adipogenic induction medium for 21 days, spheroid–derived cAFS cells (3–D spheroids in adherent culture) showed morphological changes and appeared as round shaped cells with lipid droplets in their cytoplasm more than those of in 2–D culture (monolayer culture) (Fig 3. A and B). cAFS cells from 3–D spheroids had large number of Oil Red O positive lipid droplets in their cytoplasm and had higher detachment rate than those from 2–D culture during the adipogenic induction (not shown). After induction of osteogenic differentiation cAFS cells were changed their morphology from spindle shape to cuboidal shape (Fig 3. C and D). Deposition of extracellular calcified matrix was increased in cAFS cells from 3–D spheroids compared to that of 2–D adherent culture. Chondrogenic differentiation of 3–D spheroid derived cAFS cells induced rapid proliferation and cAFS cells reached to 90% confluence before cAFS cells from 2–D culture (Fig 3. E and F). Moreover 3–D spheroids showed morphologically more chondrogenic differentiated than 2–D culture .

RT–PCR analysis of cAFS cell differentiation

After induction of MSC differentiation, the specific gene expressions of adipogenic–, osteogenic– and chondrogenic lineages were examined by RT–PCR. The expression of LPL, Leptin (specific markers of adipocytes), Runx2, and Osteocalcin (specific markers of osteoblasts) were detected in both 2–D and 3–D cultured cAFS cells (Fig. 4). Interestingly the expression of COMP and AGG (specific markers of chondrocytes) were barely detectable in 2–D culture. However, under 3–D culture condition COMPp and AGG expression were significantly increased (Fig 4. A and B). The 3–D spheroid derived

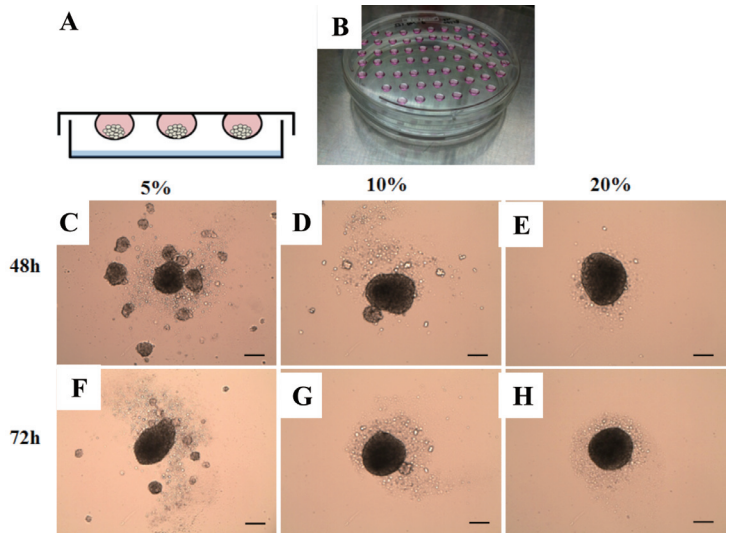

Fig. 1. Illustration (A) and macroscopic view (B) of hanging drop culture method. Cell suspension drops were deposited onto the underside of the lid of the culture dish. When the lid is inverted, drops are held in place by surface tension and gradually precipitated. Formation of cAFS spheroids with 20,000 cells/20 μl with FBS concentration of 5% (C and F), 10% (D and G) and 20% (E and H) following 48 hr and 72 hr of hanging drop culture. (Scale bar, 300 μm), Magnification : X100

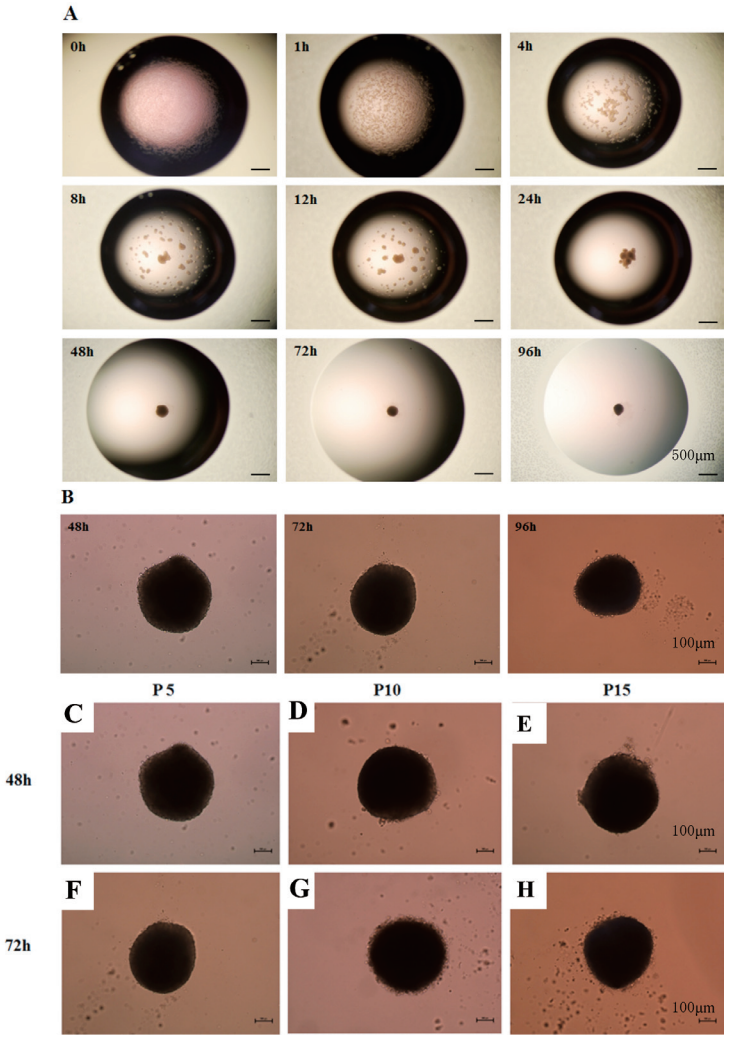

Fig. 2. Time courses of the aggregation of cAFS into a spheroid in a hanging drop (A). Spheroids progressively compacted between 48 and 96 h. (B) Formation of spheroids with cAFS at passage 5 (C and F), 10 (D and G), and 15 (E and H) after 48hr and 72 hr.

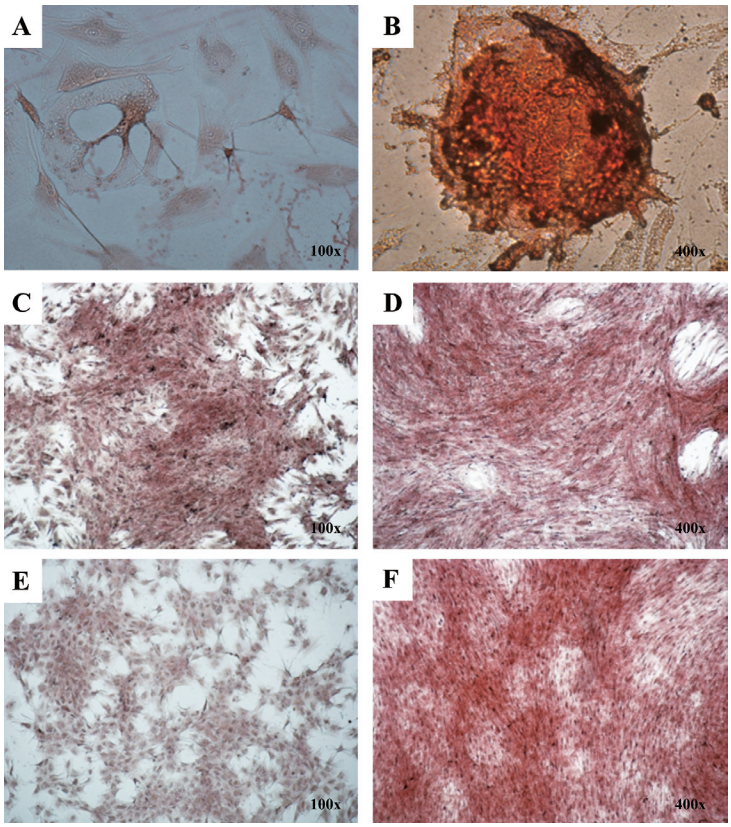

Fig. 3. Cytochemical differentiation assay. cAFS differentiation into adipocytes detected by Oil Red O staining (A and B), osteoblasts (C and D), and chondrocytes (E and F) detected by Alizarin Red staining. Adherent cultured (A, C and E) and haning drop cultured (B, D and F).

cAFS cells expressed 200% and 600% higher levels of COMP and AGG than those of the 2–D cultured cells (Fig 4. B).

DISCUSSION

Mesenchymal stem cells (MSCs; also called mesenchymal stromal cells) are defined as adherent cells which are relatively easy to isolate from donors and patients, and highly proliferate and differentiate into several different cell types in vitro and in vivo (Pittenger *et al*., 1999; Kolf *et al*., 2007). A study in 1996 was the first to suggest the possibility of multilineage potential of non–hematopoetic cells present in the amniotic fluid, by showing myogenic conversion of amniocytes (Streubel *et al*., 1996). Human amniotic cells have been shown their multipotent potential that is able to differentiate at least into neural glial cells and hepatocyte precursors (Sakuragawa *et al*., 1996; Elwan *et al*., 1997; Saulnier *et al*., 2009). The primary advantage of AFS cells is the easiness of harvest, isolation, and expansion in culture with highly advanced differentiation capacity into multi cell lineages that allow them to be a useful source of the stem cell therapy in regenerative medicine.

Most adult stem cell base assays were applied in monolayer or suspension cultures which provide a non–physiological environment to the cells that alters cell–cell and cell–ECM interaction unlike in vivo. For that reason, it has been accepted that mesenchymal stem cells lose their innate potentials including morphology, proliferation, gene expression, and cellular function after passages in vitro. On the other hand 3–D culture system has been used in several different cell types including tumor cells and human primary cells (Bjerkvig *et al*., 1997; Kunz–Schughart *et al*., 1999; Kelm *et al*., 2004). These studies demonstrated that 3–D cell culture system is reproducible, and would be a definitive method for the formation of 3–D spheroids like the hanging drop method that provides in vivo–like environment with many potential advantages. It seems that 3–D culture provides in vivo–like microenvironment with higher similarity to in vivo conditions in many aspects for tissue engineering and regenerative medicine. For examples, previous studies reported that the spheroid formation method resulted in a beneficial culture environment for reconstruction of liver (Landry *et al*., 1985), pancreas (Matta *et al*., 1994), blood vessel (Korff *et al*., 1998) and bone tissue (Akiyama *et al*., 2006). Spheroids have also offered as biological models for avascular tumor as drug delivery assays and toxicology. Recently they have gained attention for their potential to serve as a building block for organ reconstruction.

In this study, cAFS cells were induced to form spheroids from aggregates by hanging drop method. After forming spheres, spheroids become gradually compact and were sustained their spheroid structure without dis-

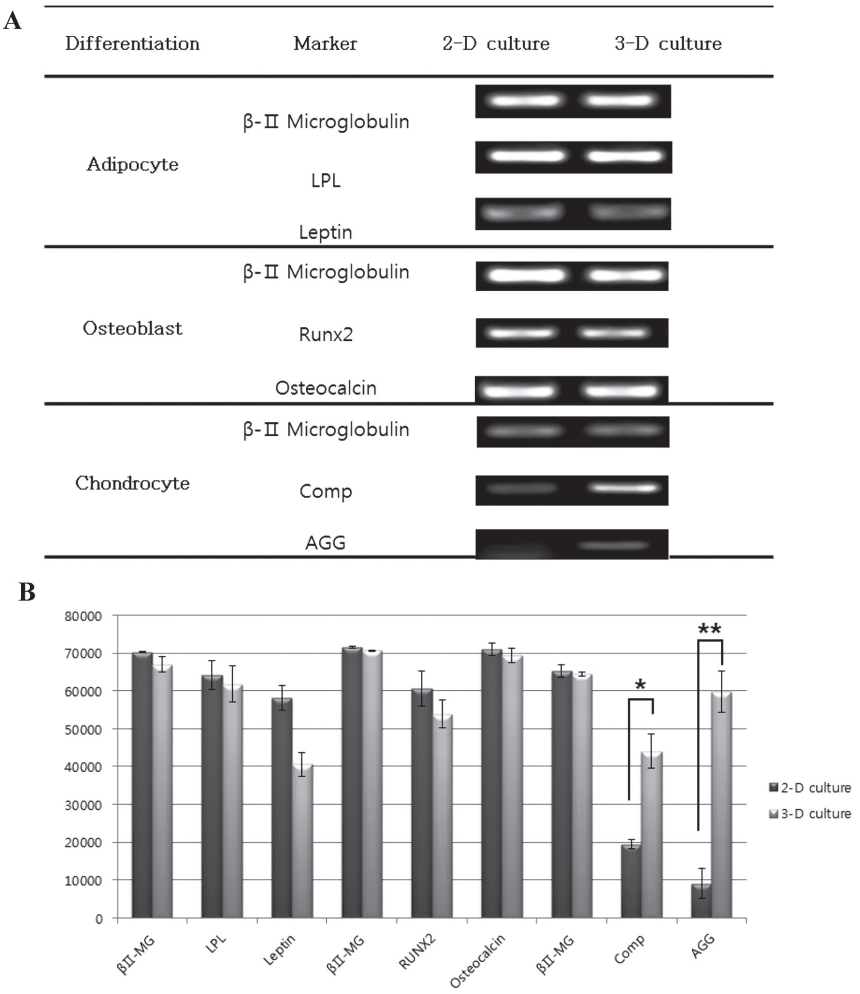

Fig. 4. RT–PCR anaylsis of linage specific gene expression for adipocytes, osteoblasts and chondrocytes. Total RNA was extracted from the cells under each differentiation condition. (A) RT–PCR amplicon images and (B) statistical analysis of the relative gene expression. 2–D culture; monolayer culture, 3–D culture; Hanging drop culture, *β*–Ⅱ Microglobulin ; the internal control.

assembly. To identify the multi–lineage differentiation potential, spheroid derived cAFS cells were induced to differentiate into adipocytes, osteoblasts, and chondrocytes. In MSC differentiation assay, the spheroid–derived cAFS cells acquired adipogenic, osteogenic and chondrogenic morphological changes with typical staining patterns. The spheroid–derived cAFS cells had a higher ratio of positive patterns with Oil–Red–O or Alizarin Red S staining after each differentiation assay compared with 2–D culture system. The cells cultured in 3–D spheroids had remarkable detachment during differentiation compared to cells cultured in 2–D system.

In addition, lineage specific gene expressions were confirmed by RT–PCR analysis. Adipocyte (LPL and Leptin), Osteoblast (Runx2 and Osteocalcin), and chondrocytes (COMP and AGG) specific genes were abundantly expressed in both groups. However, in case of chondrogenic differentiation, AGG was not detected and COMP was barely expressed in the 2–D cultured cells. Under 3–D culture condition COMP and AGG expression were significantly increased.

These results indicated that hanging drop culture method supports to maintain multi–lineage differentiation ability of cAFS cells, and even amplified the differentiation potential of cAFS cells into chondorgenic lineage. The beneficial effect of 3–D spheroids culture system for chondrogenic differentiation has been reported (Kii *et al*., 2004; Arufe *et al*., 2009). In the hanging drop culture, cadherin causes cells to adhere intensively each other through homophilic calcium–dependent interaction through their extracellular domains. With that, it is possible to form tight cadherin–mediated cell–cell interactions and spherical cell aggregates. In addition 3–D spheroid formation has a positive effect on cartilaginous matrix production of cells (Sekiya *et al*., 2002; Shirasawa *et al*., 2006).

To regenerate osteochondral defects, tissue transplantations were previously performed (Amiel *et al*., 1985; Yamashita *et al*., 1985; O'Driscoll *et al*., 1986; Matsusue *et al*., 1993). Chondrocytes and mesenchymal stem cells have been applied for transplantations that were contributed in regeneration of hyaline–like cartilage with reduc-

ing clinical symptoms. However, the optimal resource of stem cells for the osteochondral repair has not yet been established. Besides, transplanted single stem cells hardly reside and disappear in the transplanted sites due to joint motion and weight supporting besides the immune cell attack. Studies suggested that the small amounts of survived transplanted 3–D spheroid derived cells could contribute to chondrogenic and osteogenic lineage cells, and they encourage the reconstruction of the defects especially during the beginning of repair process (Kim *et al*., 1998; No Da *et al*., 2012). Therefore, 3–D spheroid would be a suitable cell format to transplant highly concentrated stem cells into damaged tissu sites with less possibility of losing cells unlike transplantation of single cells.

In conclusion, this study demonstrated that the simple and economical hanging drop method provides 3–D in vivo–like culture environment that can effectively maintain multi–lineage differentiation potency and even improved chondrogenic differentiation ability of cAFS cells. The results support that 3–D spheroids derived AFS cells would be a potential and applicable source of stem cell therapy in regeneration medicine especially osteochondral defects.

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AUTHOR DISCLOSURE STATEMENT

None of the authors have any competing financial interests to declare.

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