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Analysis of ROS and Apoptosis of Porcine Skin-derived Stem-like Cells after Differentiation Induction into Mesodermal Cell Types

Hyo-Kyung Bae[1,*], Hwa-Yeon Lee[1,*], Yeo-Reum Park[1], Choon-Keun Park[2], Boo-KeunYang[2] and Hee-Tae Cheong[1,†]

[1]College of Veterinary Medicine, Kangwon National University, Chuncheon 24375, Korea
[2]College of Animal Life Sciences, Kangwon National University, Chuncheon24375, Korea

ABSTRACT

The present study was conduct to examine the H_2O_2 expression level and apoptosis-related gene expression levels inporcineskin-derived stem cell-like cells (pSSCs) after adipogenic, chondrogenic, and osteogenic differentiation induction. The pSSCs were obtained by digestion of porcine ear skin biopsy and cultured in each induction medium for 21 to 26 days to induce adipogenic, chondrogenic, and osteogenic differentiation, respectively. The H_2O_2 levels of pSSCs after induction culture were evaluated by staining with 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). The apoptotic gene expression of pSSCs after induction culture was also estimated by RT-PCR. The pSSCs have a potential to differentiate into three mesodermal cell types (adipocytes, chondrocytes, and osteoblasts). Non-induced control and chondrogenic-induced cells were showed higher H_2DCFDA intensity (P<0.05) than adipogenic- and osteogenic-induced cells. The relative expression of Bax/Bcl-2 level was significantly low (P<0.05) in adipogenic- and osteogenic-induced cells compared to non-induced control. However, there was no difference in the relative expression of Bax/Bcl-2 level among differentiation induction groups. The result of the present study shows that the apoptosis of pSSCs is not detrimentally increased by differentiation induction culture, although chondrogenic-induced pSSCs showed high ROS generation level and apoptotic index similarly to those of non-induced cells.

(Key words : porcine skin-derived stem-like cells (pSSCs), differentiation induction, ROS, apoptosis)

INTRODUCTION

Mesenchymal stem cells (MSCs) have emerged as promising tools for regenerative medicine and tissue engineering (Badylak and Nerem, 2010). MSCs are characterized as undifferentiated cells, able to self-renewal with a high proliferation capacity and supporting cellular therapy (Kern *et al.*, 2006). MSCs have several advantages including their excellent safety and the potential as autologous cells for transplantation into humans (Trounson, 2009). In particular, the quantity and accessibility of subcutaneous adipose tissue in humans makes it an attractive alternative to other tissues as a source of adult stem cells (Fraser *et al.*, 2006; Zuk, 2010). The successful extraction and cultivation of MSCs was achieved from porcine skin (Dick and Scott, 1992; Dyce *et al.*, 2004), adipose tissues (Simon and Maibach, 2000), bone marrow (Kern *et al.*, 2006). The multipotent stem cell component of MSCs isolates is able to differentiate of mesodermal lineage including adipocyte, chondrocyte and osteoblasts (Atashi *et al.*, 2015). Recently, we also reported that successful extraction and differentiation induction into three mesenchymal cell types and further *in vivo* cartilage formation from porcine skin-derived stem cell-like cells (pSSCs) as well as porcine adipose-derived stem cells (Hwang *et al.*, 2015).

It was reported that MSC cells in chondrogenic differentiation induction culture generate hypoxia (Zhu *et al.*, 2006), and undergo apoptosis with increasing cell density (Wang *et al.*, 2009). ROS-derived apoptosis might restrict the differentiation of MSC cells to different cell types as well as chondrogenic differentiation. Exogenous reactive oxygen species (ROS) are one of the important factors that induce apoptosis of MSCs. The high levels of ROS cause cell damage by oxidation and nitration of macromolecules including RNA, DNA and protein (Schwartz *et al.*, 2004). According to the previous study it was clarified that ROS acts as an important signaling molecular pathway in the regulation of cell growth and differentiation

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* These authors contributed equally to this work.
† Correspondence : htcheong@kangwon.ac.kr

(Lambeth, 2004). ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2), and hydroxyl radical (OH－) are generally considered to be damage to cells and tissues. Hypoxia can activate p38 expression and caspase-8 and induce the apoptosis of MSCs via the mitochondrial death pathway and endoplasmic reticulum stress (Wei *et al.*, 2010). Apoptosis is controlled by regulators, Bcl-2 family of apoptosis regulator proteins, which have either an inhibitory effect on programmed cell death or block the protective effect of inhibitors (Tsujimoto *et al.*, 1984; Cleary *et al.*, 1986). Bcl-2, encoded by the *BCL2* gene, is the founding member of the Bcl-2 family and specifically considered an important anti-apoptotic protein. Bax (bcl-2-like protein 4), encoded by the *BAX* gene, is one of the Bcl-2 family member, and functions as an apoptotic activator (pro-apoptotic protein).

In the present study, we examined the H_2O_2 expression level and apoptosis-related gene (Bax/Bcl-2) expression levels in pSSCs after adipogenic, chondrogenic and osteogenic differentiation induction.

MATERIALS AND METHODS

1. Isolation of pSSCs

The pSSCs were isolated according to the method of previous study (Hwang *et al.*, 2015). Briefly, porcine skin samples were obtained from ear tissues of pigs (6 month-old females). Cartilage tissue was completely removed from the ear skin sample, and the epidermis and dermis of the skin tissue were finely chopped and digested by treatment with 10 ml digestion solution containing 0.2% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) and 0.2% collagenase type I in Dulbecco's phosphate buffered saline (DPBS; Gibco, Grand Island, NY, USA) for 30 min at 37℃ with agitation. Cells were recovered and plated in an 100-mm culture dish (BD, San Diego, CA, USA) in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% P/S, in humidified air with 5% (v/v) CO_2 at 37℃, and cultured for more than 10 days. The cells were passaged one or two times with changing the culture medium every 2 days. The MSC characteristics of the pSSCs established in this study were confirmed by fluorescence activated cell sorting (FACS) analysis in our previous study (Hwang *et al.*, 2015).

2. *In Vitro* Differentiation Induction into Three Mesodermal Cell Types

The pSSCs were seeded at a density of 2×10^4 cells/cm^2 for adipogenic and osteogenic differentiation, or 1×10^4 cells/cm^2 for chondrogenic differentiation in 24-well plates (BD) and were grown for 2～3 days in standard DMEM with 10% FBS. At 70～80% confluence of cells, the medium was replaced in test wells by each induction medium. To induce adipogenic differentiation, the cells were cultured in DMEM containing 10 % FBS, 500 μM isobutylmethlxanthine (Sigma-Aldrich), 100 μM indomethacin (Sigma-Aldrich), 1% antibiotics, 1 μM dexamethasone (Sigma-Aldrich), and 10 μg/ml insulin (Sigma-Aldrich) for 21 days. To induce osteogenic differentiation, the cells were cultured in DMEM containing 10% FBS, 50 μM ascorbic acid (Sigma-Aldrich), 10 mM glycerol 2-phosphate (Sigma-Aldrich), 1% antibiotics (Sigma-Aldrich), and 0.1 μM dexamethasone for 26 days. To induce chondrogenic differentiation, the cells were cultured for 26 days in DMEM containing 10% FBS, 1% antibiotics, 1% Insulin-Transferrin-Selenium-A Supplement (Gibco), 50 μM ascorbic acid, 0.1 μM dexamethasone and 10 ng/ml transforming growth factor-beta1(TGFβ 1, ProSpec-Tany TechnoGene, Rehovot, Israel). The induction medium was changed every 2～3 days. Cells cultured in DMEM supplemented with 10% FBS and 1% P/S for 26 days were considered as non-induced controls.

At the end of induction culture, differentiation induced cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature (RT) prior to staining. For adipogenic differentiation, induced cells were stained with 0.6% (w/v) Oil Red O solution (Sigma-Aldrich) for 20 min at RT. For osteogenic differentiation, the cells were stained by von Kossa. The cells were treated with 5% silver nitrate (Sigma-Aldrich) and exposed to strong light for 1 h. The reaction was stopped by treatment with 5% sodium thiosulfate nitrate (Sigma-Aldrich) for 2～3 min. For chondrogenic differentiation, the cells were stained with 0.5% Alcian blue (Sigma-Aldrich) in 0.1 N HCl (pH 1.0) for 30 min at RT. Stained cells were then examined under a light microscope (TE300, Nikon, Tokyo, Japan).

3. Quantification of Intracellular ROS Levels

The ROS levels of induced and control cells were measured using a modified method previously described (Bae *et al.*, 2015). Briefly, the induction media of cells was completely removed and washed with DPBS. Cells were stained in 10 μM 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, Karlsruhe, Germany) dye for 30 min in the dark at 39℃ to measure the H_2O_2 levels. After incubation, the cells were wash-

ed twice with DPBS and immediately examined under a fluorescence microscope (IX71, Olympus, Tokyo, Japan) with filters at 450～480 nm for excitation and at 515 nm for emission. The fluorescent images were obtained at random from at least three clear areas of individual cells per well and recorded as JPEG files using a digital camera (Cellsens entry software 1.6, Olympus, Japan) and analyzed the intensity of fluorescence in each cells using ImageJ software 1.37 (NIH, Bethesda, MD, USA).

4. RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from control and induced cells using Trizol (Invitrogen) and 1 μg total RNA was used for cDNA synthesis with RT-PreMix (Bioneer, Daejeon, Korea). Polymerase chain reaction (PCR) was performed with PCR-PreMix (Bioneer) under standard PCR conditions. The primers used, peroxisome proliferator-activated receptor gamma 2 (PPARγ2), adipocyte fatty acid binding protein 2 (aP2), collagen type I (Col I), runt-related transcription factor 2 (Runx2), collagen type II (Col II), aggrecan (Agg), BCL2-like 1 (BCL2), BCL2-associated X protein (BAX) and glyceraldehyde 3- phosphate dehydrogenase (GAPDH) are shown in Table 1. PCR cycles consisted of an initial denaturation step at 95℃ for 5 min, followed by 34 amplification cycles consisting of 30 s of denaturation at 95℃, 30 s of annealing at 62℃, and 1 min of extension at 72℃. A final extension was performed at 72℃ for 10 min. The PCR products were analyzed by UV irradiation on a 2% agarose gel (Amresco, Solon, OH, USA) stained with ethidium bromide (Sigma-Aldrich). Gels were read using a UV transilluminator (CN-08, Vilber Lourmat, Torcy, France). For apoptotic genes, the relative amount of each mRNA species was calculated by dividing the intensity of target primers by the intensity of the corresponding GAPDH.

5. Statistical Analysis

At least three independent sets of experiments for each condition were performed in triplicate. Statistical analyses were performed using ANOVA and Duncan's multiple range test with the SAS software (SAS Institute, Cary, NC, USA).

RESULTS

Table 1. Nucleotide sequences of primers used in an RT-PCR assay

Gene	Sequence of primers	Length of products (bp)	GenBank accession No.	Annealing temperature (℃) (cycle)
PPARγ2	F-GCGCCCTGGCAAAGCACT R-TCCACGGAGCGAAACTGACAC	238	AF103946	58 (30)
aP2	F-GGCCAAACCCAACCTGA R-GGGCGCCTCCATCTAAG	167	AF102872	58 (30)
Col I	F-CCAAGAGGAGGGCCAAGAAGAAGG R-GGGGCAGACGGGGCAGCACTC	232	AF201723	60 (30)
Runx2	F-CAGACCAGCAGCACTCCATA R-AACGCCATCGTTCTGGTTAG	171	EU668154	58 (30)
Agg	F-TTCCCTGAGGCCGAGAAC R-GGGCGGTAATGGAACACAAC	194	AF201722	67 (42)
Col II	F-CTGGAGCTCCTGGCCTCGTG R-CAGATGCGCCTTTGGGACCAT	138	AF201724	67 (42)
Bcl-2	F-GTTGACTTTCTCTCCTACAAGC R-GGTACCTCAGTTCAAACTCATC	277	NM_214285	55 (30)
Bax	F-ACTGGACAGTAACATGGAGC R-GTCCCAAAGTAGGAGAGGAG	294	XM_003127290	55 (30)
GAPDH	F-GGGCATGAACCATGAGAAGT R-AAGCAGGGATGATGTTCTGG	230	AF017079	58 (30)

1. Multipotency of pSSCs

Following differentiation induction, pSSCs were stained positively by Oil red O with the accumulation of neutral lipid vacuoles for adipocyte induction, von Kossa with the extent of calcium accumulation for osteoblast induction, or Alcian blue with forming cell nodules for chondrocyte induction (Fig. 1). In RT-PCR, the expressions of PPARγ2 and aP2 in adipogenic-induced cells, Col I and Runx2 mRNA level in osteogenic-induced cells, and Col II and Agg in chondrogenic-induced cells were highly expressed from pSSCs (Fig. 1).

2. H_2O_2 Levels of pSSCs after Induction Culture

Each differentiation induced and non-induced control of pSSCs were observed a marked induction of H_2O_2 generation by fluorescence microscopy (Fig. 2). Non-induced control and chondrogenic-induced cells were showed higher H_2DCFDA intensity than adipogenic-andosteogenic-induced cells, meanwhile lowest in osteogenic-induced cells (P<0.05).

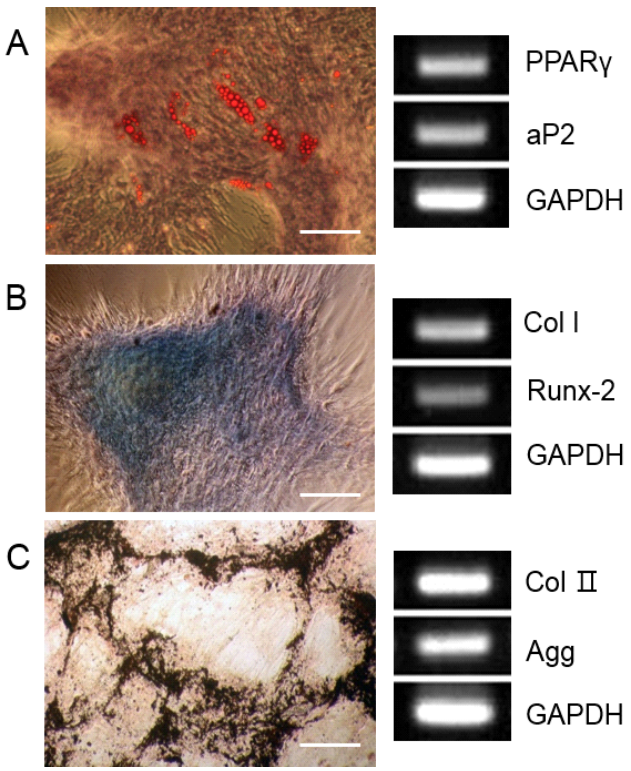

Fig. 1. Morphologies and marker gene expressions of three mesodermal cell types. (A) Adipogenic-induced cells stained by Oil red O, (B) Chondrogenic-induced cells stained by von Kossa, (C) Osteogenic-induced cells stained by Alcian blue. Scale bar: 100 μm (×200).

3. Apoptotic Gene Expression in pSSCs after Induction Culture

The expressions of anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax were detected in all groups (Fig. 3). The relative expression of Bax/Bcl-2 level was significantly low (P<0.05) in adipogenic- and osteogenic-inducted cells compared to non-induced control. However, there was no difference in the relative expression of Bax/Bcl-2 level among differentiation induction groups.

DISCUSSION

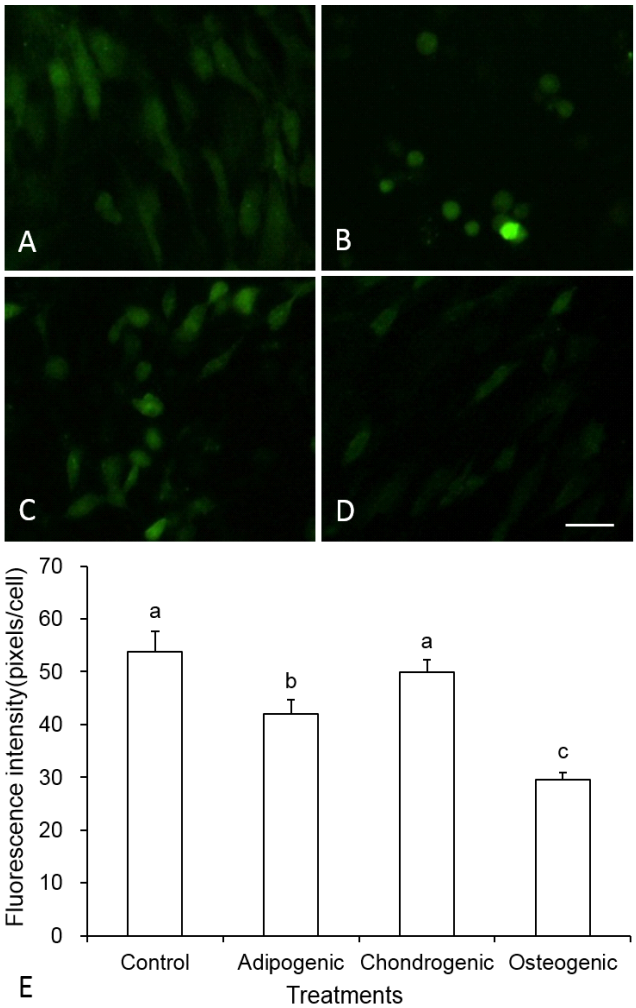

Fig. 2. Fluorescence images and H_2O_2 levels of pSSCs stained with H_2DCFDA after differentiation induction culture. (A) Un-induced control, (B) Adipogenic-induced cells, (C) Chondrogenic-induced cells, (D) Osteogenic-induced cells. Scale bar = 50 μm. (E) H_2O_2 levels of pSSCs were evaluated by the fluorescence intensity in each cells (mean±SEM). $^{a\sim c}$ Values with different letters differ significantly (P<0.05).

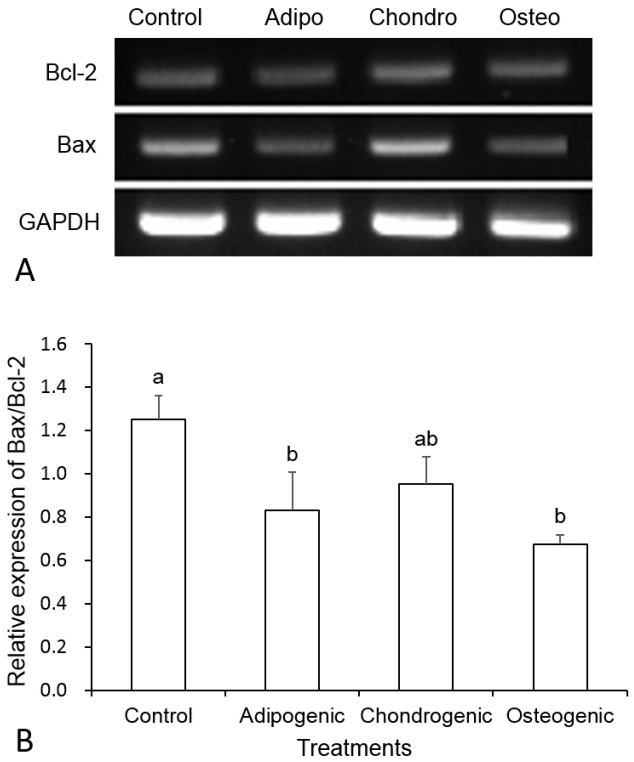

Fig. 3. RT-PCR results of apoptosis-related genes. (A) Expression of apoptosis-related genes in each cell types. Adipo, Adipogenic-induced cells; Chondro, Chondrogenic-induced cells; Osteo, Osteogenic-induced cells. (B) Relative gene expression of Bax/Bcl-2 data are presented as means±SEM, ^{a,b} Values with different letters differ significantly (P<0.05).

Several defining characteristics of MSCs have recently been outlined, including their ability to adhere to plastic, their expression of specific surface antigens, and their multipotent differentiation into adipocytes, chondrocytes, and osteoblasts *in vitro* (Iohara *et al.*, 2006; Kang *et al.*, 2010; Vishnubalaji *et al.*, 2012). MSCs have emerged as an indispensable tool in regenerative medicine. Numerous sources of MSCs have been identified, and cells have been cultured using a variety of methods for use in cell-based therapies. However, there remains a need for an optimal clinical-grade product, and high safety standards must be fulfilled. Recently, it was reported that porcine skin-derived MSC is a potential autologous source for stem cell transplantation in regenerative medicine (Kang *et al.*, 2010). Furthermore, skin-derived cells have a good potential in the cost and time savings. Therefore, skin-derived progenitors/multipotent stem cells are promising resources for the successful development of stem cell therapies.

It was reported that the occurrence of ROS affects the cell growth negatively (Yang *et al.*, 2015). However, an appropriate ROS helps the cells to develop healthy (Stolzing *et al.*, 2007). Cellular aging including oxidative damage, ROS levels increased suggesting a progressive loss of MSC function with age, leading to a reduction in MSC numbers and differentiation capacity (Stolzing *et al.*, 2007). Our result showed that the ROS generation level and relative expression level of Bax/Bcl-2 were high in non-induced pSSCs compared to those of differentiation-induced pSSCs excepting chondrogenic-induced pSSCs. It was suggested that cell aging is getting faster with normal growth culture because of long term culture without differentiation induction and increased cell density (Wang *et al.*, 2009; Bork *et al.*, 2010), whereas, differentiation induction into fresh new type of cells and induction factors may delay cell aging.

In our study, chondrogenic-induced cells generated a high level of ROS compared to other induced groups. It was unclear why ROS generation level in chondrogenic-induced cells was high. We suggested that TGF is the reason for high ROS level by promoting cell growth to confluence rapidly (Hwang *et al.*, 2015). Differentiation induction media are differ among adipogenic-, chondrogenic-, and osteogenic- induction cultures. TGF is the key factor for differentiating pSSCs to chondrocytes. It was reported that chondrogenic-induced cells in monolayer culture generate hypoxia (Zhu *et al.*, 2006), and undergoes apoptosis with increasing cell density (Wang *et al.*, 2009). Based on the previous study, there is an evidence for a function of ROS in MSC proliferation, survival and differentiation to adipocytes and osteoblasts (Atashi *et al.*, 2015). Recent reports describe the importance of appropriate oxidants on MSC differentiation into adipocyte (Higuchi, *et al.*, 2013), chondrocyte (Sasaki *et al.*, 2006) and osteoblasts (Higuchi *et al.*, 2007) through the activation of signaling pathway involved in differentiation (Yuan *et al.*, 2012). Different ROS generation level might be due to different signaling pathways depending on each lineage differentiated from pSSCs. Different induction media for each lineage induce distinct pathway, which might affect the expression levels of ROS and relative expression level of Bax/Bcl-2.

In conclusion, the result shows that the apoptosis of pSSCs is not detrimentally increased by differentiation induction culture, although chondrogenic-induced pSSCs showed high ROS generation level and apoptotic index similarly to those of non-

induced cells.

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