World Academy of Science, Engineering and Technology
International Journal of Medical and Health Sciences
Vol:10, No:3, 2016

Properties of Adipose Tissue Derived Mesenchymal Stem Cells with Long-Term Cryopreservation

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***Abstract*—**Adult mesenchymal stem cells (MSCs) have been investigated using preclinical approaches for tissue regeneration. Porcine MSCs (pMSCs) are capable of growing and attaching to plastic with a fibroblast-like morphology and then differentiating into bone, adipose, and cartilage tissues *in vitro*. This study was conducted to investigate the proliferating abilities, differentiation potentials, and multipotency of miniature pig adipose tissue-derived MSCs (mpAD-MSCs) with or without long-term cryopreservation, considering that cryostorage has the potential for use in clinical applications. After confirming the characteristics of the mpAD-MSCs, we examined the effect of long-term cryopreservation (> 2 years) on expression of cell surface markers (CD34, CD90 and CD105), proliferating abilities (cumulative population doubling level, doubling time, colony-forming unit, and MTT assay) and differentiation potentials into mesodermal cell lineages. As a result, the expression of cell surface markers is similar between thawed and fresh mpAD-MSCs. However, long-term cryopreservation significantly lowered the differentiation potentials (adipogenic, chondrogenic, and osteogenic) of mpAD-MSCs. When compared with fresh mpAD-MSCs, thawed mpAD-MSCs exhibited lower expression of mesodermal cell lineage-related genes such as peroxisome proliferator-activated receptor-γ2, lipoprotein lipase, collagen Type II alpha 1, osteonectin, and osteocalcin. Interestingly, long-term cryostoraged mpAD-MSCs exhibited significantly higher cell viability than the fresh mpAD-MSCs. Long-term cryopreservation induced a 30% increase in the cell viability of mpAD-MSCs when compared with the fresh mpAD-MSCs at 5 days after thawing. However, long-term cryopreservation significantly lowered expression of stemness markers such as Oct3/4, Sox2, and Nanog. Furthermore, long-term cryopreservation negatively affected expression of senescence-associated genes such as telomerase reverse transcriptase and heat shock protein 90 of mpAD-MSCs when compared with the fresh mpAD-MSCs. The results from this study might be important for the successful application of MSCs in clinical trials after long-term cryopreservation.

***Keywords*—**Mesenchymal stem cells, Cryopreservation, Stemness, Senescence.

I. Introduction

MESENCHYMAL stem cells (MSCs) have the ability to differentiate into multi-lineage cells, which confers great promise for use in regenerative medicine. In recent years, many researchers have actively sought to establish pig MSC lines from various tissues. To date, pig MSCs have been isolated from bone marrow, adipose tissues, liver tissue, fetal skin, eye tissues, and salivary gland. Similar to human MSCs, the pig MSCs derived from various tissues are capable of attaching to plastic culture dishes and growing with fibroblast-like morphology, after which *in vitro* differentiation into bone, adipose, and cartilage tissues [1], [2]. Miniature pigs in particular are extensively used for medical research as a source of organs for xenotransplantation, as well as in the field of stem cell research [3], [4]. This study was conducted to identify the cell proliferating abilities, mesodermal-lineage differentiation potentials, and stem cell multipotency of miniature pig adipose tissue-derived MSCs (mpAD-MSCs) with long-term cryostorage.

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II. Materials and Methods

A. Cell Isolation and Culture

The mpAD-MSCs isolated from adipose tissues were cultured in DMEM-low complete medium. Briefly, adipose tissues were washed 2-3 times with phosphate buffered saline (PBS, Gibco, CA). Tissue samples were then sliced into 1-2 mm pieces and incubated in 0.1% collagenase type I (Gibco, USA) at 37°C for 30 min. The digested tissues were filtered with a 100 μm cell strainer (Becton Dickinson, USA) and centrifuged at 1500 rpm for 10 min. The cell pellets were then resuspended in growth medium.

B. Cumulative Population Doubling Level (CPDL)

During continuous passaging, population doubling was calculated using the formula: $X = \ln(Nf/Ni)\ln 2$, where Ni and Nf are the initial and final cell numbers, respectively, and ln is the natural log. To yield cumulated doubling level, the population doubling for each passage was calculated and added to the population doubling levels of the previous passages. The cells were seeded at a density of 5×104 cells in 6-well culture plates (n=3) and subcultured 5 days later, with replacement of the media every 3 days.

C. Flow Cytometry Analysis

The cells grown on culture plates were digested with 0.25% trypsin/EDTA and washed with PBS. For identification of surface markers, cells (1×10^5) were labeled with antibodies against the surface markers CD34, CD90 and CD105 from Becton Dickinson (USA) for 1 hr. Labeled cells were washed twice in PBS and analyzed using a FACSCalibur™ flow cytometer (Becton Dickinson, USA) with Cell Quest Pro software for data analysis. The cells were examined cell surface markers (CD34, CD90 and CD105) at passage 3 by FACS analysis.

D. Lineage Differentiation

For adipogenic and osteogenic differentiation, mpAD-MSCs were seeded onto 4-well plates containing differentiation medium for 21 days. Media were changed every 2-3 days. The undifferentiated or differentiated cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min, and then washed with PBS again. For evaluation of adipogenic differentiation, fixed cells were stained with an Oil red O stain kit (IHC World, USA), through which red lipid vacuoles accumulated in the differentiated cells. After osteogenic differentiation, the presence of extracellular calcium was confirmed using an Alizarin red stain kit (IHC World, USA). For chondrogenic differentiation, cells were cultured in 5 µl droplets of the growth media in 4-well plates for 3 hr in the presence of 5% CO_2 and changed with chondrogenic differentiation medium for 21 days. After differentiation, the cells were fixed and stained with Alcian blue (IHC World, USA) to detect the presence of glycosaminoglycan.

E. Quantitative Real-Time RT-PCR (qRT-PCR)

Total RNA was extracted using an RNeasy Mini kit (Qiagen, CA) according to the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm with a spectrophotometer (Thermo, USA), and cDNA was generated using total RNA (2 µg), reverse primers (10 pmol of each) and GoScript™ Reverse Transcriptase (Promega, USA). PCR was performed for 40 cycles, with each cycle consisting of denaturation at 95°C for 45 sec, annealing at 57-64°C for 45 sec, and elongation at 72°C for 45 sec, with additional 5 min incubation at 72°C after completion of the last cycle. Each sample was analyzed in triplicate. The PCR products were then separated on a 2% agarose gel by electrophoresis, stained with Safe DNA Gel Stain (Invitrogen, USA), and visualized under UV light. Images were digitally captured with a CCD camera. The qRT-PCR analysis was carried out in 96-well plates with LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, USA). The following program was used for amplification: predenaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 10 sec at 95°C, annealing for 10 sec at 60°C, and elongation for 10 sec at 72°C. Fold difference in gene expression of the differentiated MSCs compared to the undifferentiated MSCs was calculated using the $2^{-\Delta Ct}$ method, as described by Livak and Schmittgen [5].

The expression levels of multi-potential stemness markers (Oct3/4, Sox2, and Nanog), mesodermal lineage differentiation markers (adipogenic, chondrogenic, and osteogenic genes) and senescence-associated genes (telomerase reverse transcriptase and heat shock protein 90) were examined by qRT-PCR.

F. Statistical Analysis

The relative expression of differentiation marker genes was analyzed by one-way ANOVA (analysis of variance), and differences among two methods were compared using Student's *t*-test (JMP®6.0; SAS Institute Inc., Cary NC). Values of $p < 0.05$ were considered statistically significant.

III. Results

A. The Expression of Cell Surface Markers is Similar between Thawed and Fresh mpAD-MSCs

To investigate the effect of long-term cryostorage in the expression of cell surface markers of mpAD-MSCs under cryopreservation, we examined the effect of long-term cryopreservation (> 2 years) on expression of cell surface markers (CD34, CD90 and CD105). These results showed that the expression of cell surface markers is similar between thawed and fresh mpAD-MSCs.

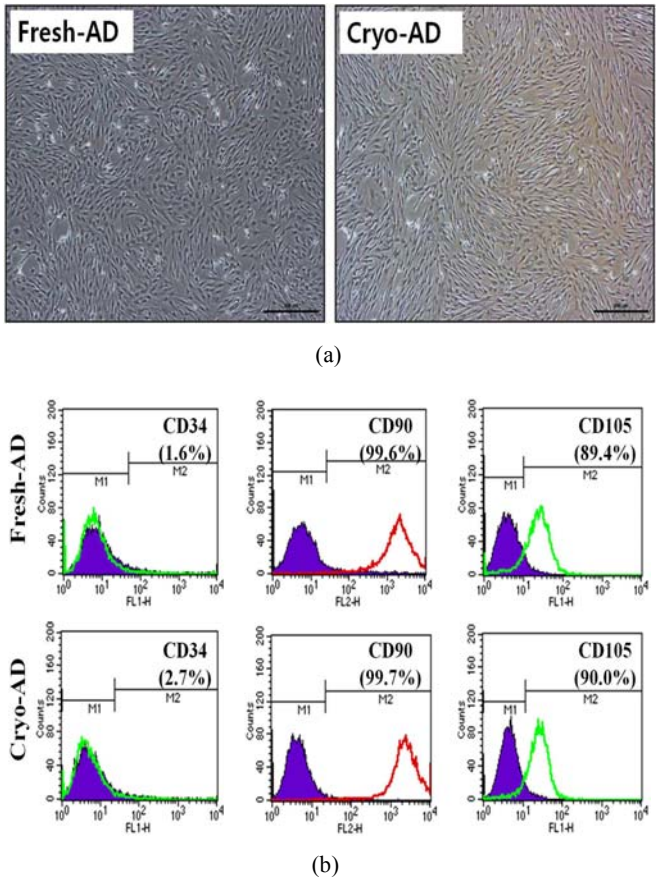
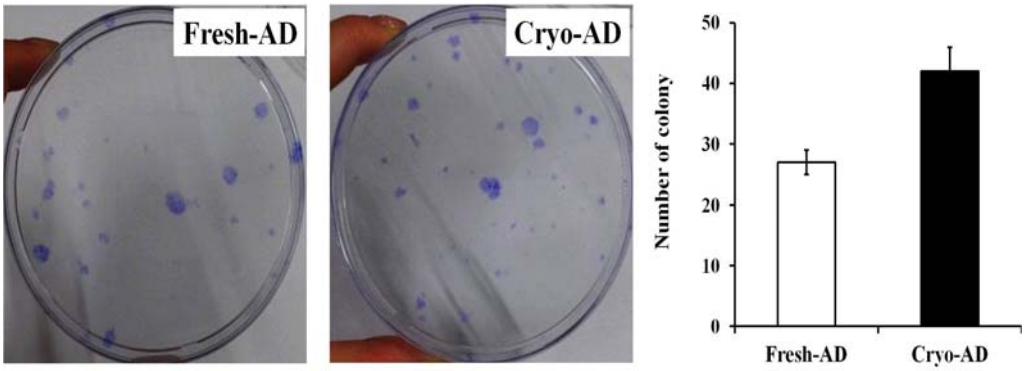


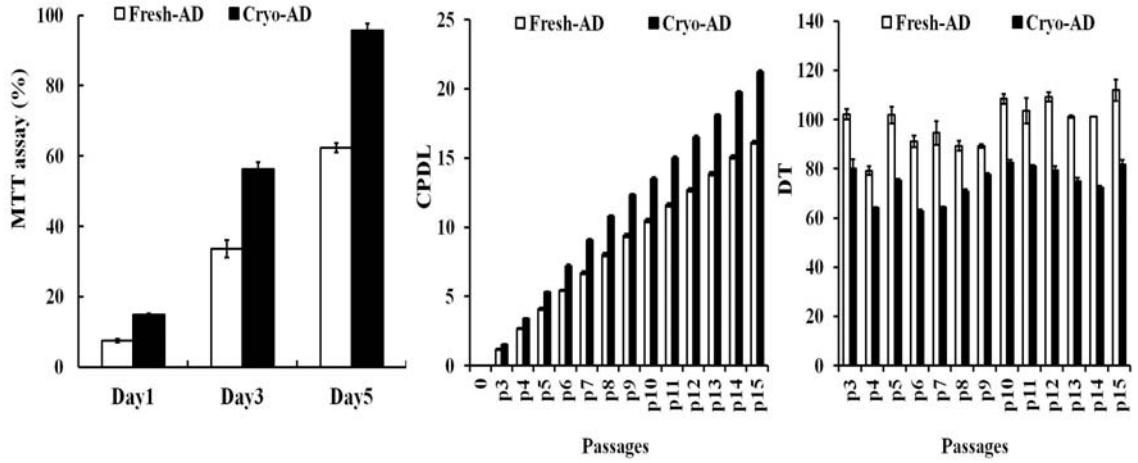
Fig. 1 Isolation and characterization of mpAD-MSCs at passage 3 (P3). (a) Cell morphology showed a fibroblast-like shape (×40, ×100), (b) Flow cytometry histograms show the expression of selected molecules (CD34, CD90 and CD105) by mpAD-MSCs populations compared with controls

B. Long-Term Cryostoraged mpAD-MSCs Exhibited Significantly Higher Cell Viability than Fresh mpAD-MSCs

Next, to investigate the effect of long-term cryostorage in cell proliferating abilities of mpAD-MSCs under cryopreservation, we observed the effect of long-term cryopreservation on cell proliferation. As a result, long-term cryostoraged mpAD-MSCs exhibited higher cell viability than the fresh mpAD-MSCs. Specifically, long-term cryopreservation (> 2 years) induced a 30% increase in the cell viability when compared with the fresh mpAD-MSCs at 5 days after thawing.



(a)



(b)

Fig. 2 Proliferation of mpAD-MSCs at P3: (a) Colony forming unit assay, (b) MTT assay and CPDL of mpAD-MSCs during continuous passages. Data are expressed as means ± standard error of values obtained by three determinations. The relative difference of CPDL was shown as the mean ± standard error of the mean (n=3)

C. Long-Term Cryopreservation Significantly Lowered Expression of Stemness Markers Such as Oct3/4, Sox2, and Nanog

Even though long-term cryostoraged mpAD-MSCs exhibited higher cell viability than the fresh mpAD-MSCs, long-term cryopreservation significantly lowered expression of stemness markers such as Oct3/4, Sox2, and Nanog. Also, long-term cryopreservation negatively affected expression of senescence-associated genes such as telomerase reverse transcriptase (TERT) and heat shock protein 90 (HSP90) of mpAD-MSCs when compared with the fresh mpAD-MSCs.

D. Long-Term Cryostorage Significantly Lowered the Differentiation Potentials of mpAD-MSCs

We examined the effect of long-term cryopreservation (> 2 years) on stem cell differentiation potentials. As a result, thawed mpAD-MSCs exhibited significantly lower expression of mesodermal lineage-related genes such as peroxisome proliferator-activated receptor-γ2 (PPAR-γ2), lipoprotein lipase (LPL), collagen Type II alpha 1 (COL2AII), osteonectin (ON), and osteocalcin (OC) than fresh mpAD-MSCs.

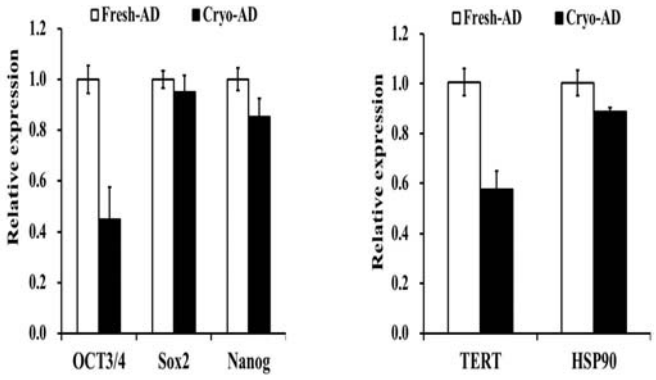


Fig. 3 Expression patterns of pluripotency markers (Oct3/4, Sox2, and Nanog) and senescence-associated markers (TERT and HSP90) in mpAD-MSCs. Expressions of the genes were analyzed by qRT-PCR with GAPDH as a housekeeping control gene. The level of expression was shown as the mean ± standard error of the mean (n=3)

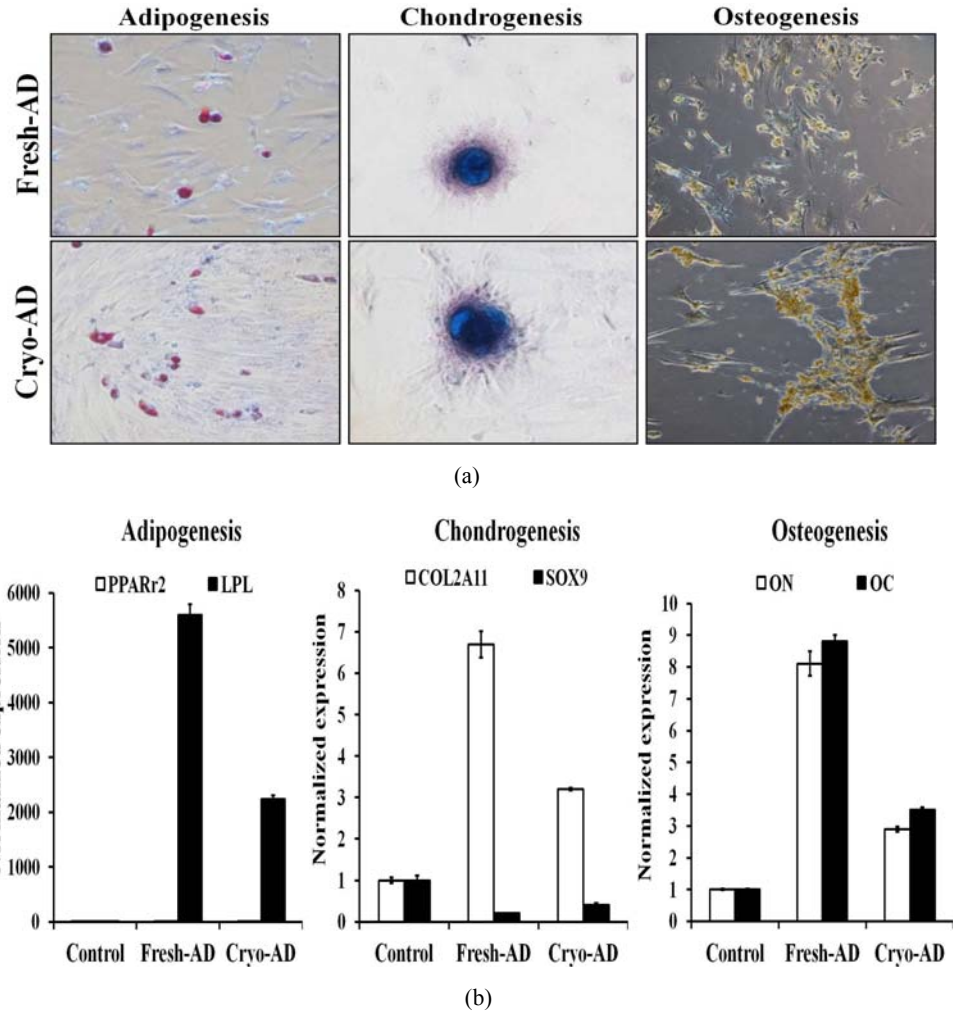


Fig. 4 Mesodermal-lineage differentiation potentials of mpAD-MSCs. (a) Staining were conducted after the cells were grown in differentiation media. (b) Expression level of PPAR-γ2, LPL, COL2AII, Sox9, ON, and OC was analyzed by qRT-PCR and compared between undifferentiated and differentiated cells at P3. The level of expression was shown as the mean ± standard error of the mean (n=3).

IV. Conclusion

The effect of cryostorage on optimization of growth and differentiation of mpAD-MSCs should affect future stem cell-based reparative and regenerative studies. This study was to evaluate the effects of long-term cryostorage on the cell viability, differentiation potentials, and stemness markers between thawed and fresh mpAD-MSCs.

The expression of cell surface markers is similar between thawed and fresh mpAD-MSCs. However, long-term cryostorage significantly lowered the differentiation potentials of mpAD-MSCs. Also, long-term cryopreservation significantly lowered expression of stemness markers such as Oct3/4, Sox2, and Nanog. Furthermore, long-term cryopreservation negatively affected expression of senescence-associated genes such as TERT and HSP90 of mpAD-MSCs when compared with the fresh mpAD-MSCs [6]-[10]. These results might be important for the successful application of stem cells in clinical trials after long-term cryopreservation (> 2 years).

Acknowledgment

This work was funded by the Animal and Plant Quarantine Agency, the Republic of Korea.

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