Effects of Serial Passage on the Characteristics and Chondrogenic Differentiation of Canine Umbilical Cord Matrix Derived Mesenchymal Stem Cells

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ABSTRACT: Mesenchymal stem cells (MSCs) are often known to have a therapeutic potential in the cell-mediated repair for fatal or incurable diseases. In this study, canine umbilical cord MSCs (cUC-MSCs) were isolated from umbilical cord matrix (n = 3) and subjected to proliferative culture for 5 consecutive passages. The cells at each passage were characterized for multipotent MSC properties such as proliferation kinetics, expression patterns of MSC surface markers and self-renewal associated markers, and chondrogenic differentiation. In results, the proliferation of the cells as determined by the cumulative population doubling level was observed at its peak on passage 3 and stopped after passage 5, whereas cell doubling time dramatically increased after passage 4. Expression of MSC surface markers (CD44, CD54, CD61, CD80, CD90 and Flk-1), molecule (HMGA2) and pluripotent markers (sox2, nanog) associated with self-renewal was negatively correlated with the number of passages. However, MSC surface marker (CD105) and pluripotent marker (Oct3/4) decreased with increasing the number of subpassage. cUC-MSCs at passage 1 to 5 underwent chondrogenesis under specific culture conditions, but percentage of chondrogenic differentiation decreased with increasing the number of subpassage. Collectively, the present study suggested that sequential subpassage could affect multipotent properties of cUC-MSCs and needs to be addressed before clinical applications. (**Key Words:** Mesenchymal Stem Cell, Umbilical Cord Matrix, Canine, Multipotent, Differentiation)

INTRODUCTION

A significant number of mesenchymal stem cells (MSCs) are needed for clinical applications which require extensive MSC expansion in culture. However, differentiation potential of bone marrow (BM) and adipose-derived MSCs at later passage is often low. In addition, there are several negative reports on MSC senescence (Bonab et al., 2006; Izadpanah et al., 2008; Gruber et al., 2011) or tumor transformation (Rubio et al., 2005; Røsland et al., 2009).

Canine derived multipotent MSCs have been established from umbilical cord (Romanov et al., 2003; Secco et al., 2008; Schugar et al., 2009), umbilical cord vein (Zucconi et al., 2010), and umbilical cord blood (Seo et al., 2009). Interestingly, recent research has demonstrated that adipose derived stem cells are capable of long-term self-renewal in culture, while maintaining multipotency in several species, such as human, mouse, canine and bovine (Zuk et al., 2001; Zheng et al., 2006; Neupane et al., 2008; Zhao et al., 2011). However, the presence of a multipotent stem cell population in canine tissues has not yet been fully investigated. Therefore, it is considered important to isolate and characterize canine derived MSCs. For this purpose, we isolated umbilical cord-MSCs (cUC-MSCs) from umbilical cord matrix and subjected to proliferative culture for 5 consecutive passages. The MSCs at each passage were characterized by monitoring the transcriptional patterns of MSC-specific pluripotent and surface markers. Finally, chondrogenic differentiation potentials of cUC-MSCs were also tested.

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MATERIAL AND METHODS

Isolation of canine umbilical cord matrix (cUCM) cell and cell culture

For this study, whole cUCMs were obtained from three female dogs (n = 3) during parturition. The whole cUCMs were first separated from amnion and fetuses. The cUCMs were washed with phosphate buffered saline (PBS) to remove blood. Amniotic epithelium surrounding umbilical cord and blood vessel were dissociated by sterile scissors and forceps, and incubated in 20 ml PBS containing 1X Pen Strep (penicillin 100 unit/ml streptomycin 100 μg/ml, GIBCO, USA) and 0.05% trypsin/EDTA at 37°C for 1 h to remove remnant amniotic epithelium. Trypsin was inactivated by adding Hank's buffered salt solution supplemented with 10% fetal bovine serum (FBS). The processed cUCMs were cut into pieces of 1 to 2 mm size with scissors, and incubated in PBS containing 0.1% collagenase type I (Sigma) at 37°C for 30 min. The digested tissues were passed through cell strainer (BD, USA) (100 μm) to remove undigested tissues and centrifuged at 1,500 rpm for 10 min. The cell pellet was resuspended in a growth media, Dulbecco's Modified Eagles Medium (Invitrogen, USA) containing 1X Pen Strep (penicillin 100 unit/ml streptomycin 100 μg/ml, GIBCO, USA) and 10% FBS, and cultured in tissue culture flask at 37°C in 5% CO_2 incubator. After 2 d incubation, cell culture medium was changed with the fresh medium, and cells were passaged every 5 d. Cells at each passage were trypsinized to the single cell and further seeded to contain 5×10^4 per well on a 6-well tissue culture plate and maintained for 5 d until 80 to 90% confluence was reached.

Cumulative population doubling level (CPDL)

During continuous passages, the number of cUC-MSCs at both seeding and harvesting time were counted to calculate CPDL based on the formula "x = {log10(N_H)-log10(N_I)} log10" where N_I is the initial cell number and N_H is the harvest cell number. The cumulative doubling level was obtained by adding doubling level of each passage to that of the previous passage (Cristofalo et al., 1998).

Cell doubling time calculation

Cell doubling times (DT) and numbers (CD) were calculated from hemocytometer counts and cell culture time (CT) for each passage by the formula CD = ln(N_f/N_i)ln2 and DT = CT/CD where N_i is the initial cell number and N_f is the harvest cell number (Vidal et al., 2006).

Reverse transcriptase-polymerase chain reaction (RT-PCR) gene expression analysis

RT-PCR primers for pluripotency (Oct3/4, Nanog, Sox2), MSC surface markers (CD4, CD8A, CD25, CD33, CD34, CD44, CD54, CD61, CD80, CD105, CD117, Flk-1 and HMGA2) and GAPDH internal control were shown in Table 1. Total RNA was extracted from the cUCM-MSCs using TRIzol (Invitrogen, USA). 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 per each) and Superscript II reverse transcriptase (Invitrogen, USA). For pluripotency marker analysis, target genes were amplified in 35 cycles of 94°C (30 s), 55°C to 60°C (30 s), 72°C (20 s), followed with 72°C for 10 min. For MSC CD marker analysis, the target genes were amplified in 35 cycles of 94°C (30 s), 57°C to 60°C (30 s), 72°C (30 s), followed with 72°C for 10 min. For differentiation marker analysis, target genes were amplified in 25 cycles of 94°C (30 s), 58°C to 64°C (30 s), 72°C (30 s), followed with 72°C for 10 min. Each sample was analyzed in triplicate. PCR products were separated on 2% agarose gel by electrophoresis, stained with Red Safe (iNtRon Biotechnology, Korea), and visualized under UV light. Images were digitally captured with a CCD camera (Gel Doc., USA).

Quantitative real-time PCR

Primers for differentiation markers (Sox9, Aggrecan precursor, Collagen type 2 alpha (COL2A)) and GAPDH internal control were shown in Table 2. Total RNA was extracted from the differentiated cells and cDNA (5 μl) of reverse polymerase transcription was used for PCR analysis. The qRT-PCR analysis was carried out in 96-well plates with using LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Germany). The following program was used for amplification: predenaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 10 s at 61°C, and elongation for 10 s at 72°C. Melting curve analysis was performed from 65°C to 97°C to assess the specificity of the qRT-PCR products. Reference genes (GAPDH) that are stably expressed at 61°C were used for quantification. Based on the experiment analysis, we calculated qRT-PCR result by using Ct value.

In vitro differentiation

For chondrogenic differentiation, 1.6×10^7 cells in 5 μl droplets were cultured to maintain hypoxia condition in the growth media at 37°C in 4-well tissue culture plate under the presence of 5% CO_2 and then subjected to chondrogenic differentiation medium containing transforming growth factor beta 3 (TGF-β3; PT-3003, PT-4124 Lonza Walkersville, USA) for 21 d. All the differentiation media were changed every 2 to 3 d, and the differentiation was evaluated after 21 d.

Table 1. Primer sequences used for RT-PCR analyses in this study

Markers/gene	Primer sequence (5'-3')	Amplification size (bp)	Reference
Pluripotency			
OCT3/4	Forward TGACGACAACAAAAATCT	128	Vaags et al.
	Reverse CAGGCATGTGTTCTCCAG		
NANOG	Forward CCTGCATCCTTGCCAATGTC	98	Vaags et al.
	Reverse TCCGGGCTGTCCTGAGTAAG		
SOX2	Forward AACCCCAAGATGCACAACTC	152	Vaags et al.
	Reverse CGGGGCCGGTATTTATAATC		
MSC makers			
CD4	Forward TGCTCCCAGCGGTCACTCCT	381	XM534906
	Reverse GCCCTTGCAGCAGGCGGATA		
CD8A	Forward GAGGAATGAGCCAGCCGCCC	303	NM001002935.2
	Reverse GGAGACACCGGCTTGGACGC		
CD25	Forward GGCAGCTTATCCCACGTGCCAG	364	AB501356.1
	Reverse ATGGGCGGCGTTTGGCTCTG		
CD33	Forward TTCGGCACGAGGCCAGGACC	364	DR105786.1
	Reverse GCTGGCTCCACAACTGGGTGG		
CD34	Forward GCCTGCTCAGTCTGCTGCCC	255	Filioli et al.
	Reverse TGGTCCCAGGCGTTAGGGTGA		
CD44	Forward GCCCTGAGCGTGGGCTTTGA	268	Filioli et al.
	Reverse TCTGGCTGTAGCGGGTGCCA		
CD54	Forward TCCGTGGACCCAGCAGAAGC	468	XM542075
	Reverse GCGGCACGAGAAGTTGGCGA		
CD61	Forward GAGGATTGCGCTGCGGCTCC	412	NM001003162.1
	Reverse AAAGCCGCCCTCTGGGGCAT		
CD80	Forward GCCTCACCTCTCCTGGTGGGA	369	AF257653.1
	Reverse TGGCCATACCTGTACAGTGACCTT		
CD105	Forward GGTTCACTGCATCAACATGG	278	XM546483.2
	Reverse AAGCTGAAGCGCACATCACC		
CD117	Forward CTCAGGGGGTGCGAGGGGAA	332	AF099030.1
	Reverse TGTGTCTGTGCATTAGTCTGGCTG		
Flk-I	Forward CTCCGCACCACATCCGCTGG	337	XM539273
	Reverse TCCTGCTCGGTTGGCTGGGT		
HMGA2	Forward AGTCCCTCCAAAGCAGCTCAAAAG	75	NM003484.1
	Reverse GCCATTTCCTAGGTCTGCCTC		
Housekeeping			
GAPDH	Forward AGTCAAGGCTGAGAACGGGAAACT	114	Vieira et al.
	Reverse TCCACAACATACTCAGCACCAGCA		

Table 2. Primer sequences used for quantitative PCR analyses in this study

Markers/Gene	Primer sequence (5'-3')	Amplification size (bp)	Reference
Chondrocytes			
COL2A	Forward ATCGGGCCTGTCTGCTTCTTGTAA	134	Vieira et al.
	Reverse ACATCAGGTCAGGTCAGCCATTCA		
SOX9	Forward ACGACTACACTGACCACCAGAACT	88	Vieira et al.
	Reverse ATGTAGGTGAAGGTGGAGTAGAGGCT		
Aggrecan	Forward ACAATGCCCAAGACTACCAGTGGA	166	Vieira et al.
	Reverse TTCTCGTGCCAGATCATCACCACA		
Housekeeping			
GAPDH	Forward AGTCAAGGCTGAGAACGGGAAACT	114	Vieira et al.
	Reverse TCCACAACATACTCAGCACCAGCA		

Cytochemical staining

The differentiated cells were washed twice with PBS, fixed with 4% formalin for 10 min and were subjected to cytochemical staining to confirm the lineage specific markers according to the manufacturer's protocols. Chondrogenic differentiation was confirmed with Alcian blue stain kit (NovaUltra™, IHC World, USA) by detecting glycosaminoglycan stained in blue color.

Statistical analysis

Cell doubling time and relative expression of differentiation marker genes were analyzed by one-way analysis of variance test or Student's *t* test (JMP® 6.0; SAS Institute Inc., Cary NC, USA). The graphs were prepared using SigmaPlot (ver. 8.0) (Systat Software, Inc., Point Richmond, CA, USA).

RESULTS AND DISCUSSION

Cell growth and proliferation kinetics

cUC-MSCs were isolated from cUCM and grown in plastic tissue culture flasks. The cells were passaged every 5 d and counted for the number of cells grown after trypsinization. The cells were adherent to the plastic culture plates, and had fibroblast-like spindle shape, forming highly homogenous monolayer (Figure 1A). During five consecutive passages, CPDL of the cells was linearly increased until fourth passage (P4), but decreased thereafter (Figure 1B). In accordance with the CPDL, doubling time was not significantly increased in passage 2 or 3 (4.1/4.5 d) and P4 (5.8 d) vs P1 (3.6 d). However, the doubling time was significantly increased in P5 (16.7 d) versus P4 (5.8 d) ($p<0.05$; Figure 1C).

Expression of pluripotency markers

Expression of pluripotency markers was tested using RT-PCR (Oct3/4, Nanog and Sox2; Figure 2). Expression of Oct3/4 decreased with increasing the number of subpassage. However, expression of Nanog and Sox2 was negatively correlated with the number of subpassage.

Immunophenotyping of cUCM-MSCs

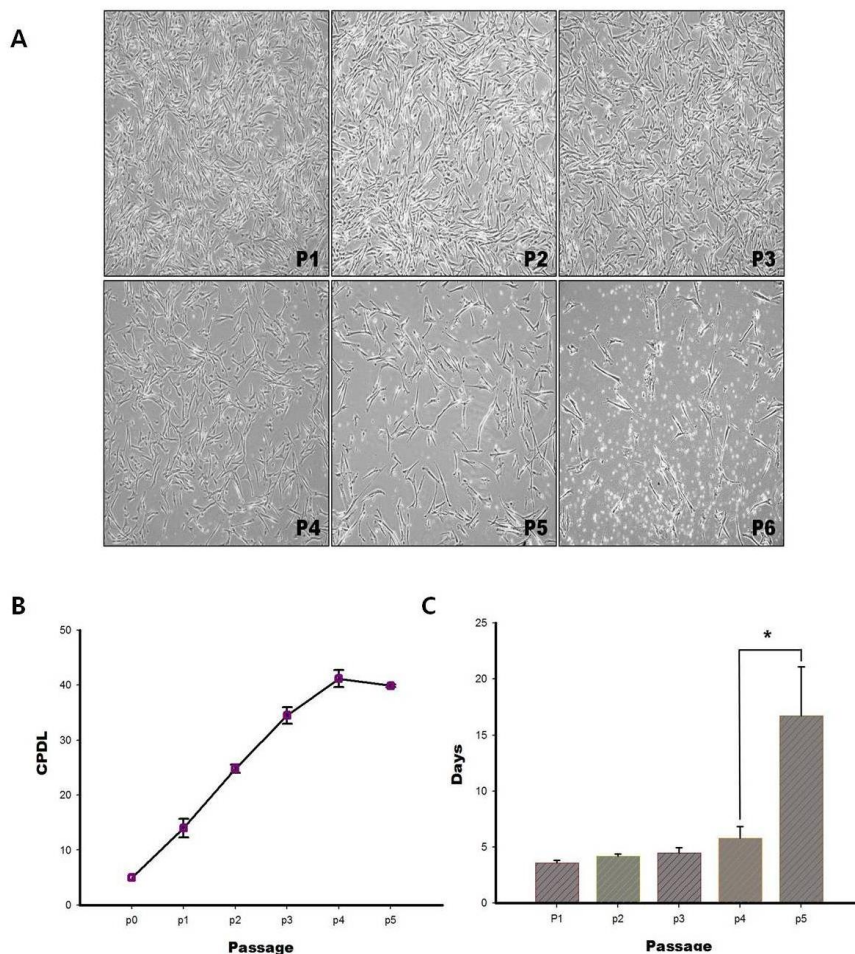Expression of MSC surface markers such as CD4, CD8A, CD25, CD33, CD34, CD44, CD54, CD61, CD80,

Figure 1. Morphology (P1-P6) (40X) (A) and cumulative population doubling level (B) and doubling time (C) of cUC-MSC during continuous passages. Data are expressed as the mean±standard error of values obtained by two determinations. * p<0.05.
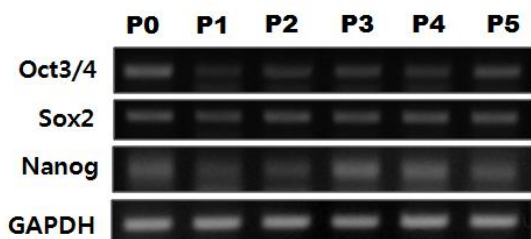
Figure 2. Expression of pluripotency markers (Oct3/4, Nanog and Sox-2) in cUC-MSC during continuous passages by RT-PCR. GAPDH was used as a control. The cells were positive for all markers.

CD90, CD105, CD117, Flk-1 and HMGA2 were conducted using RT-PCR. It was shown that cUC-MSCs were positive for CD44, CD54, CD61, CD80, CD90, CD105, Flk-1 and HMGA2, but negative for CD4, CD8A, CD25, CD33, CD34 and CD117 (Figure 3). In addition, the expression of CD44, CD54, CD61, CD80, CD90, Flk-1 and HMGA2 were negatively correlated with the number of subpassage, but the expression of CD105 decreased with increasing the number of subpassage.

Chondrogenic differentiation

The cUC-MSCs were cultured for chondrogenic differentiation for 21 d in specific media. It was apparent that glycosaminoglycans formed in the differentiated cells were stained in blue color by alcian blue (Figure 4A). It was confirmed by alcian blue staining that all cUCM-MSCs from passage 1 and 5 underwent chondrogenic differentiation, but those in early versus late passage displayed more intense glycosaminoglycan staining. Gene expression of markers associated with chondrogenic differentiation such as COL2A (late chondrogenic marker), Aggrecan precursor and Sox9 (early chondrogenic marker) was evident in differentiated cUC-MSCs compared with the undifferentiated cUC-MSCs (Figure 4B). In real-time RT-PCR, the dramatic increases in Sox9 and Aggrecan precursor mRNA transcripts were observed in cells from passage 4 and 3 which cultured in chondrogenic medium. However, COL2A mRNA transcripts were significantly decreased with increasing the number of subpassage.

It is well known that MSCs have remarkable potential as a cell-mediate repair and regenerative medicine for fatal or incurable diseases, such as spinal cord injury, arthritis, cartilage regeneration and ischemic heart injury, as demonstrated in the field of stem cell therapy (Pittenger and Martin, 2004; Varma et al., 2007; Hatami et al., 2009; Sharp et al., 2010). In the present study, it was aimed to determine the effects of serial passaging (P1 to P5) on the growth, gene expression of MSC CD markers and chondrogenic differentiation potential of cUCM-MSCs.

There was no significant difference in the population doubling time from P1 to P4 (3.6 to 5.8 d), but P5 cells
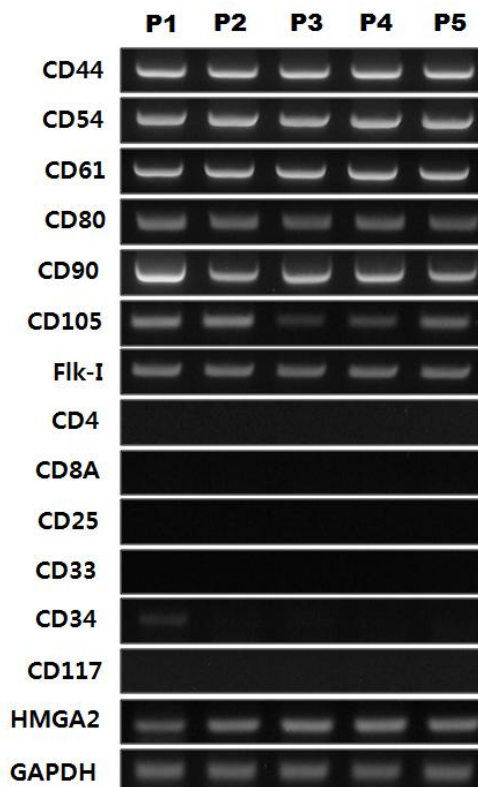

Figure 3. Expression of MSC surface markers in cUC-MSC during continuous passages by RT-PCR. The results indicated that the cUC-MSCs were positive for CD44, CD54, CD61, CD80, CD90, CD105, Flk-1 and HMGA2, while negative for CD4, CD8A, CD25, CD33, CD34 and CD117.

displayed a significantly longer doubling time (16.7 d). This result is consistent with the study by Wall et al. (2007) who found that human ASC doubling times were significantly longer at passage 6 and later. In addition to the slower growth, changes in response to chemical stimuli were observed after passage 4 (Wall et al., 2007). Recent studies reported by Danoviz et al. (2011) and Zhao et al. (2011) showed that increasing doubling times were found in human and bovine adipose stem cells (ASCs) at later passage.

It was found that the cUCM-MSCs expressed pluripotent markers (Oct3/4, Nanog and Sox2) and possessed several surface markers such as CD44, CD54, CD61, CD80, CD90, CD105, Flk-1 and HMGA2. However, expression of Oct3/4 was decreased with increasing the number of subpassage. In addition, the expression of Nanog and Sox2 was negatively correlated with the number of subpassage. It was reported that transcription factors such as Nanog, Rex1, Fzd9, Oct4, Sox2 and Bst1 genes were essential in pluripotency maintenance as well as self-renewal (Safwani et al., 2012). In study with ASCs, long-term cultures from passage 5 to 15 decreased stemness gene expression levels (Zaman et al., 2008), indicating the negative correlation between the differentiation capability
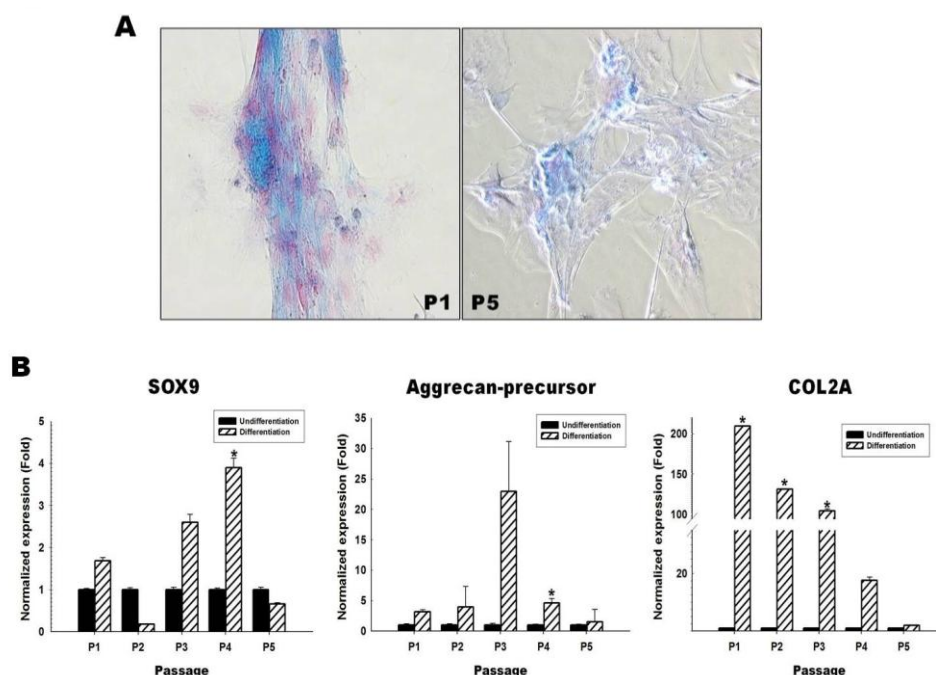
Figure 4. Differentiation potential of cUC-MSC into mesodermal cell lineage, (A) chondrocyte (Alcian blue). The staining was conducted at 21 d after cells were grown in differentiation media. cUC-MSC showed induction of chondrogenic differentiation. Expression levels of chondrogenic (B) differentiation marker genes were compared between undifferentiated and differentiated cells by Real-time PCR. The expression levels were shown as the mean±standard error of the mean (n = 3). *: $p < 0.0001$ vs undifferented cells.

and serial passage of MSCs as seen in this study. Serial passaging has also been found to alter surface marker expression in human adipose-derived MSCs (Mitchell et al., 2006). Mitchell et al. (2006) demonstrated that the percentage of cells expressing stroma cell markers, including CD105 and CD166 increased with subculture up to passage 4. However, we found that the expression of CD105 was decreased at passage 3 to 5.

The key role of CD105 in promoting MSC chondrogenesis may be related to its function as a member of TGF-β receptor superfamily. CD105 is a dimeric membrane glycoprotein expressed at high levels on human vascular endothelial cells. It shares regions of sequence identity with beta-glycan, a major binding protein for TGF-β, and thus plays an important role in modulating cellular responses to TGF-β (Cheifetz et al., 1992; Barry et al., 1999). Since TGF-β is the most crucial factor that directs chondrogenesis and chondrogenic differentiation of MSCs (Johnstone et al., 1998; Ito et al., 2002), CD105 expression is probably linked to TGF-β mediated chondrogenesis in MSCs.

It is well known that alcian blue staining is a better indicator of the differentiation state of the MSC. In this study the intense of glycosaminoglycan was more pronounced in the early versus late passage MSCs, indicating an enhanced chondrogenesis in the early passage cells.

In addition, gene expression of markers associated with

chondrogenic differentiation such as a COL2A, Aggrecan precursor and Sox9 were evident, but the pattern was different between MSCs passages. This observation supports that serial passage also influenced transcriptional levels of differentiation-associated markers in MSCs. Similarly, our gene expression results corroborate with the previous studies that lower expression of Sox9 (Hargus et al., 2008), collagen II and aggrecan were found in the P5-induced pellets (Zhao, 2011).

In conclusion, the present study demonstrated that sequential subpassages affect multipotent properties of cUC-MSCs, which should be considered in the trial of therapeutic application of cUC-MSCs.

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