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Downregulation of heat shock protein B8 decreases osteogenic differentiation potential of dental pulp stem cells during in vitro proliferation

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Abstract

Objectives: Tissue-derived stem cells, such as dental pulp stem cells (DPSCs), reduce differentiation capability during in vitro culture. We found that cultured DPSCs reduce expression of heat shock protein B8 (HspB8) and GIPC PDZ domain containing family member 2 (Gipc2). Our objectives were to evaluate the changes in DPSC composition during in vitro proliferation and to determine whether HspB8 and Gipc2 have function in differentiation potential of DPSCs.

Materials and Methods: Different passages of rat DPSCs were evaluated for changes in CD90+ and/or CD271+ stem cells and changes in osteogenic potential. Real-time RT-PCR and immunostaining were conducted to determine expression of HspB8 and Gipc2. Expression of the genes in DPSCs was knocked down by siRNA, followed by osteogenic induction to evaluate the function of the genes.

Results: About 90% of cells in the DPSC cultures were CD90+ and/or CD271+ cells without dramatic change during in vitro proliferation. The DPSCs at passages 3 to 5 (P3 to P5) possess strong osteogenic potential, but such potential was greatly reduced at later passages. Expression of HspB8 and Gipc2 was significantly reduced at P11 versus P3. Knock-down of HspB8 expression abolished osteogenic potential of the DPSCs, but knock-down of Gipc2 had no effect.

Conclusions: CD90+ and CD271+ cells are the major components of DPSCs in in vitro culture. High-level expression of HspB8 was critical for maintaining differentiation potential of DPSCs.

1 | INTRODUCTION

Primary stem cells isolated from tissues usually require in vitro expansion to sufficient amount before using them for tissue regeneration or other clinical applications. It is known that tissue-derived stem cells decrease their differentiation potential during in vitro culture or proliferation.[1-3] Like other tissue-derived stem cells, the differentiation capability of dental pulp stem cells (DPSCs) is reduced over time when cultured in vitro. Takeda et al. have shown that, when subjected to osteogenic induction in vitro, human DPSCs are capable of developing into calcium-depositing cells at early passages.[4] Transplantation of early-passage DPSCs in vivo yielded formation of dentin- and pulp-like connective tissue that was surrounded by a thin layer of odontoblast-like connective tissue. However, such differentiation potential was gradually reduced at in vitro culture, and total loss of differentiation was observed around passage 10 (P10).[4] Loss of differentiation mitigates the value of those stem cells. Various studies have been attempted to preserve the differentiation potential of stem cells, such as the addition of growth factors to culture medium and culture cells in extracellular matrix-coated plates[5]; however, the effect has been limited.

Elucidating the mechanism of the loss of differentiation would facilitate development of techniques to preserve the differentiation potential of cultured stem cells. Currently, there is little information

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available on the topic in the literature. Takeda et al. found that, from early to late passage, many genes had their expression levels reduced by half or more.[4] They observed markedly changed expression of Wnt16 with an increasing number of passages, and such changes were suspected to play a role in the loss of differentiation potential in human DPSCs.[4] We hypothesize that intrinsic changes in gene expression are the cause of the reduction in differentiation potential during the culture of the DPSCs. To rule out genes causing the loss of differentiation, we conducted a preliminary study to compare the transcriptomes of early- and late-passage DPSCs derived from rat dental pulp using whole-genome microarray analysis and found that among other genes, expression of heat shock protein B8 (HspB8, also known as *Hsp22*) and GIPC PDZ domain containing family member 2 (Gipc2) was dramatically decreased (more than 10-fold) in the late-passage DPSCs (Table S1) when the cells lose their differentiation potential.

CD90 was reported to be expressed in a variety of stem cells, such as hair follicle stem cells (HFCSs), spermatogonial stem cells, Wharton's jelly MSCs[6] and DPSCs,[7] and is considered as a positive marker for mesenchymal stem cells (MSCs).[8] High-level expression of CD90 was likely associated with the stemness properties of these stem cells, including their growth and differentiation potential.[6] Besides CD90, extensive studies have been conducted to explore other stem cell-specific markers. In a recent review, CD271 has been classified as one of the specific markers for the purification of human bone marrow MSCs.[9] In an experiment to isolate dental pulp stem cells, CD271+ cells were found to possess the greatest odontogenic potential.[10]

The objectives of this study were (a) to evaluate the dynamics of stem cell composition of CD90+ and/or CD271+ cells, and the change in osteogenic differentiation potential during in vitro proliferation (expansion); (b) to verify whether expression of HspB8 and Gipc2 was significantly down regulated in late-passage DPSCs when compared to the early-passage, and (c) to determine whether those genes were involved in regulating or maintaining the osteogenic differentiation potential of DPSCs.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Dental pulp stem cell cultures were established using a protocol modified from the literature for isolation of dental stem cells.[11,12] Briefly, dental pulps were isolated from rat molars and trypsinized to obtain cell suspension. The cells were grown in α-MEM with 20% foetal bovine serum (FBS) in polystyrene tissue culture flasks.[11,13] Cells were then incubated at 37°C and 5% CO_2 in humidified incubators. At 90% confluence, cells were detached using trypsin-EDTA and passaged from one flask to three flasks of the same size. Different passages of cells were cryopreserved in liquid N2, and the cells were recovered from the cryopreservation when needed. The protocol for use of the animals was reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University.

2.2 | Determination of CD90+ and CD271+ cell dynamics in the DPSCs during proliferation

CD90 is considered as a positive marker for mesenchymal stem cells from various tissues including dental pulp.[7,8] CD271 has been used to identify and isolate mesenchymal stem cells.[9] To evaluate the dynamics of cell subpopulations of established DPSCs during proliferation, different passages of DPSCs were analysed for CD90+ and/or CD271+ cells with fluorescence-activated cell sorting (FACS). To do that, different passage cells were cultured in T-75 flasks and collected at about 90% confluency. Next, cells were processed for staining with APC-conjugated CD90 and FITC-conjugated CD271 antibodies (Stem Cell Technologies) with concentrations recommended by the manufacturer (Stem Cell Technologies). Cells then were analysed and sorted by BD Aria flow cytometer (BD Biosciences) for four populations (CD90+, CD271+, CD90+ CD271+, and CD90- CD271-).

2.3 | Differentiation of DPSCs

Cells were seeded either in sterile 6-well or 24-well plates (~5000 cells/cm^2) and cultured until 80-90% confluence for osteogenic differentiation induction. Induction medium was prepared using low-glucose DMEM supplemented with 10% FBS, 50 μg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone and 10 mM β-glycerolphosphate.[14] Cells were incubated in this induction medium for up to 14 days with medium changed every 4 days prior to staining with Alizarin red S (ARS) to reveal calcium deposition.[15] For ARS staining, cells were washed once with PBS and then fixed in 10% neutral-buffered formalin for 5 minutes. Cells were then washed in sterile milli-Q water and stained in a 1% ARS solution for 5 minutes followed by washing in sterile milli-Q water.

In some experiments, cells were collected after 1 and 2 weeks of induction for analysis of alkaline phosphatase (ALP). Briefly, cells were collected and mixed in CytoBuster™ Protein Extraction Reagent (EMD Millipore) and incubated on ice for about 30 minutes. Cell lysate was analysed for ALP activity using QuantiFluo™ ALP assay kit (BioAssay Systems) according to the manufacturer's protocol. ALP activity of the treatment was normalized to the ALP activity of the control reported as relative ALP activity (ie relative ALP activity = treatment ALP reading/control ALP reading). To examine osteogenic gene expression, cells were collected for total RNA extraction using real-time RT-PCR analysis.

2.4 | RNA extraction and Real-Time RT-PCR

Total RNA was either extracted with RNeasy Mini kit (Qiagen) or with Direct-zol™ RNA MiniPrep Kit (Zymo Research). Using RNeasy mini kit, cells were collected by trypsinization and pelleted by centrifugation. The cells were lysed in RLT buffer (Qiagen). Using Direct-zol™ RNA MiniPrep Kit, cells were collected in TRI reagent and total RNA was extracted according to the manufacturers' protocol. DNase I treatment was conducted to remove possible genomic

DNA contamination to ensure RNA purity during RNA isolation. RNA concentration was measured using a Nanodrop 8000 (ThermoFisher Scientific).

Template cDNA was generated from total RNA using M-MLV reverse transcriptase (Invitrogen). Briefly, 1000-2000 ng total RNA was mixed with 250 ng random primer, reverse transcriptase, 1st strand buffer and dNTPs in 20 μL. The reaction was carried out for 50 minutes of incubation at 37°C before deactivation by incubating at 70°C for 15 minutes for generation of cDNA. The PCR was conducted in 25 μL per reaction by mixing 1-2 μl cDNA template with SYBR Green PCR master mix (Applied Biosystems), dNTPs and gene-specific primers (Table 1). The reactions were carried out in an ABI 7300 sequence detector (Applied Biosystems) to acquire C_T values. The C_T value of a given sample was normalized to its actin C_T value to obtain ΔC_T. The $\Delta\Delta C_T$ was the difference in ΔC_T between the two samples subjected to comparison (eg treatment vs. control), and relative gene expression (RGE) was calculated using the following formula: $RGE = 2^{-(\Delta\Delta Ct)}$.[16]

2.5 | Immunostaining

Cells were fixed with 4% paraformaldehyde-PBS (pH 7.4) for 20 minutes at room temperature. Immunostaining was conducted using Abcam mouse- and rabbit-specific ABC Staining kit (ab64264, Abcam). Briefly, after treatment of the cells with hydrogen peroxide block and permeabilization-block solution (0.2% Triton X-100 in protein block solution), mouse anti-rat HspB8 primary antibody (MAB4987, R&D, 1:200 dilution) and rabbit anti-rat Gipc2 antibody (AV41271, Sigma, 1:100 dilution) were incubated with cells overnight at 4°C. Cells without incubation with primary antibody were served as the negative control. Biotinylated goat anti-polyvalent and streptavidin-peroxidase were sequentially applied to the cells and each incubated for 10 minutes, followed by staining with solution containing DAB chromogen and DAB substrate.

2.6 | Gene knock-down experiment

To study gene effect on differentiation, we used siRNA-mediated knock-down to reduce the expression of the candidate genes in early-passage DPSCs. Dicer-substrate siRNA (DsiRNA) oligos were designed to target mRNA of HspB8 and Gipc2 using the online RNAi Design Tool (Integrated DNA Technologies). The DsiRNA sequences used in this experiment are shown in Table 1. A scrambled siRNA (Ambion) was used as a negative control. These siRNAs were reconstituted to a concentration of 100 μM for preparing cell transfection as detailed below.

Dental pulp stem cells of passages 3 to 5 were seeded in 6- or 24-well plates and cultured until 85% to 90% confluence. Approximately 30 minutes prior to transfection, culture medium

TABLE 1 Sequences of real-time PCR primers and DsiRNA used in this study

Gene		Sequences
Gipc2	PCR primers	Forward: 5′-CACTTGGACTCACCATCACG-3′ Reverse: 5′-ATTCGATATGATCCCCCACA-3′
	DsiRNA	Antisense: 5′-rGrUrCrCrUrArUrUrUrCrArArArUrGrCrCrUrUrCrUrUrGrGrGrUrU-3′ Sense: 5′-rCrCrCrArArGrArArGrGrCrArUrUrUrGrArArArUrArGrGAC-3′
HspB8	PCR primers	Forward: 5′-TCTCCAGAGGGTCTGCTCAT-3′ Reverse: 5′-GCAGGTGACTTCCTGGTTGT-3′
	DsiRNA	Antisense: 5′-rGrGrArGrArCrArArUrCrCrCrArCrCrUrUrCrUrUrGrCrUrGrCrUrU-3′ Sense: 5′-rGrCrArGrCrArArGrArArGrGrUrGrGrGrArUrUrGrUrCrUCC-3′
ALP	PCR primers	Forward: 5′-GACAAGAAGCCCTTCACAGC-3′ Reverse: 5′-ACTGGGCCTGGTAGTTGTTG-3′
BSP	PCR primers	Forward: 5′-ACAGCTGTCCTTCTGAACGG-3′ Reverse: 5′-TTCCCCATACTCAACCGTGC-3′
COL1A1	PCR primers	Forward: 5′-TGGTTATGACTTCAGCTTCCTG -3′ Reverse: 5′- CTCTTGAGGGTAGTGTCCACCT -3′
DCN	PCR primers	Forward: 5′- CCTTGCAGGGAATGAAGGGT -3′ Reverse: 5′- TGTTGCCATCCAGATGCAGT-3′
OCN	PCR primers	Forward: 5′-ACTGCATTCTGCCTCTCTGACCT-3′ Reverse: 5′- TATTCACCACCTTACTGCCCTCCT-3′
RUNX2	PCR primers	Forward: 5′- GCCTTCAAGGTTGTAGCCCT-3′ Reverse: 5′- TGAACCTGGCCACTTGGTTT-3′
Actin	PCR primers	Forward: 5′-CTAAGGCCAACCGTGAAAAGAT-3′ Reverse: 5′-AGAGGCATACAGGGACAACACA-3′ Forward: 5′- CCACCATGTACCCAGGCATT -3′ Reverse: 5′- GAGCCACCAATCCACACAGA -3′

was aspirated from the cultures and replaced with fresh stem cell media in each flask or well. The Lipofectamine RNAiMax transfection reagent (Invitrogen) was mixed with DsiRNAs to prepare the lipofection-siRNA complex using the manufacturer's protocol. The lipofection-siRNA complex was added to the cells at final DsiRNA concentration of 40 nM. Next, the cells were incubated at 37°C for 24 hours for transfection. After incubation, the medium containing lipofection complex was removed and cells were processed for downstream analysis.

To determine knock-down efficiency, transfected cells were cultured in stem cell medium and collected every four days until day 13 post-transfection in a pilot experiment. In another experiment, transfected cells were cultured in stem cell medium and collected only at day 13. Total RNA was isolated from the cells, and real-time SYBR Green RT-PCR was used to determine the relative expression levels of these genes when compared to the transfection control.

To determine whether knock-down of the Gipc2 and HspB8 expression could affect differentiation ability of DPSCs, osteogenic differentiation induction medium was added to the cells after 24 hours of transfection. Cells were incubated in this induction medium with medium changed every 4 days for up to 2 weeks prior to staining with ARS [15] or collected after 1 or 2 weeks of induction for analysis of alkaline phosphatase (ALP) activity using QuantiFluo™ Alkaline Phosphatase Assay method (BioAssay Systems) as described earlier. To further confirm osteogenesis, cells were mixed in TRI reagent after 1 week of osteogenic induction and total RNA was extracted for real-time RT-PCR with the method described earlier to determine expression of ALP, bone sialoprotein (BSP), collagen type-I alpha 1 (COL1A1), decorin (DCN), osteocalcin (OCN) and runt-related transcription factor 2 (RUNX2). ALP, BSP, COL1A1, OCN and RUNX2 are commonly used markers for osteogenic differentiation, and DCN was recently identified as osteogenic signature genes.[17]

2.7 | ImageJ to assessment of Alizarin red S (ARS) staining

ImageJ, an NIH image-processing program (https://imagej.nih.gov/ij/), was used to quantify ARS staining. The photograph scale was set to the pixels per unit for the photographs according to the camera that was used to acquire the images. Next, the program was set at "RGB stack" to split the image into red, green and blue colour components. Selecting the green component heightened the contrast between the stained cells and the background. A red staining threshold was set for the given image into two classes of pixels: "stained" and "not stained," and the percent area of the "stained" pixels was calculated.

2.8 | Statistical analyses

For comparing the means of multiple samples, statistical analyses were performed using SAS program mixed model ANOVA procedure. Statistical significance of the means was determined by Tukey's test at $P \leq .05$. For comparing means of two samples (eg treatment vs. control), Student's *t* test was conducted using an online program. Except the pilot experiment, experiments were repeated at least three times for statistical assessment of treatment effect.

3 | RESULTS

3.1 | Dynamics of CD90 and CD271 cells during culture of DPSCs

Dental pulp stem cells (DPSCs) of passages 3, 5, 9, 11 morphologically showed no notable differences (Figure 1A). FACS indicated that majority of the DPSCs were CD90+ CD271- cells in all passages, which consisted of 60% to 80% of the cells in the population (Figure 1B). Increase in CD90+ CD271- cells was observed as advancement of the culture passage from P3 to P9. CD90+ CD271+ cells were the second largest portion in all passages. A somewhat decrease in this portion was seen in the P9. CD90- CD271- cells were ranked third largest portion consisting of about 10-20% cells in the population. Reduction in this subset was also seen in the late passage. DPSC cultures contained a small portion of CD90- CD271+ cells consisting of only 1-3%. It appeared the number of CD90- CD271+ cells was reduced with progression of cell passages. Overall, there were no major changes in cell compositions (CD90 CD271 subsets) during the proliferation of the established DPSCs based on flow cytometry analysis (Figure 1B).

3.2 | Differentiation potential of DPSCs at different passages

To test the differentiation capability of DPSCs during in vitro expansion or proliferation, different passages of cells were tested to evaluate their osteogenic differentiation capability. Gradual reduction in calcium deposition was observed with progression of serial passaging, as shown by ARS staining and ALP activity assay. The representative images for each passage tested are reported in Figure 2A. ImageJ analysis of the ARS staining showed that P3 DPSCs had strong differentiation potential as more than 90% culture surface was stained by ARS (ie covered by calcium deposition). Differentiation potential was slightly reduced in P5 and significantly reduced in P7 (Figure 2B). In P9, the differentiation potential was dramatically reduced. Generally, no ARS staining could be seen in P11 DPSCs after 2 weeks of induction. Differentiation was also accessed by ALP activity, and no statistically significantly different ALP activity was seen in P3 to P7, but significant reduction in ALP was seen in P9 and P11 (Figure 2C). Based on the results of this experiment, we classified P3 to P5 DPSCs as early-passage DPSCs, which possess strong osteogenic differentiation capability, and P9 to P11 as late passages at which cells greatly reduced or lost differentiation capability. DPSCs at P7 appeared as a transitional passage before significant reduction in differentiation capability.

3.3 | Comparison of gene expression in early and late passages of DPSCs

Real-time RT-PCR was conducted to determine the expression of the Gipc2 and HspB8 in P3 and P11 DPSCs. Relative gene expression

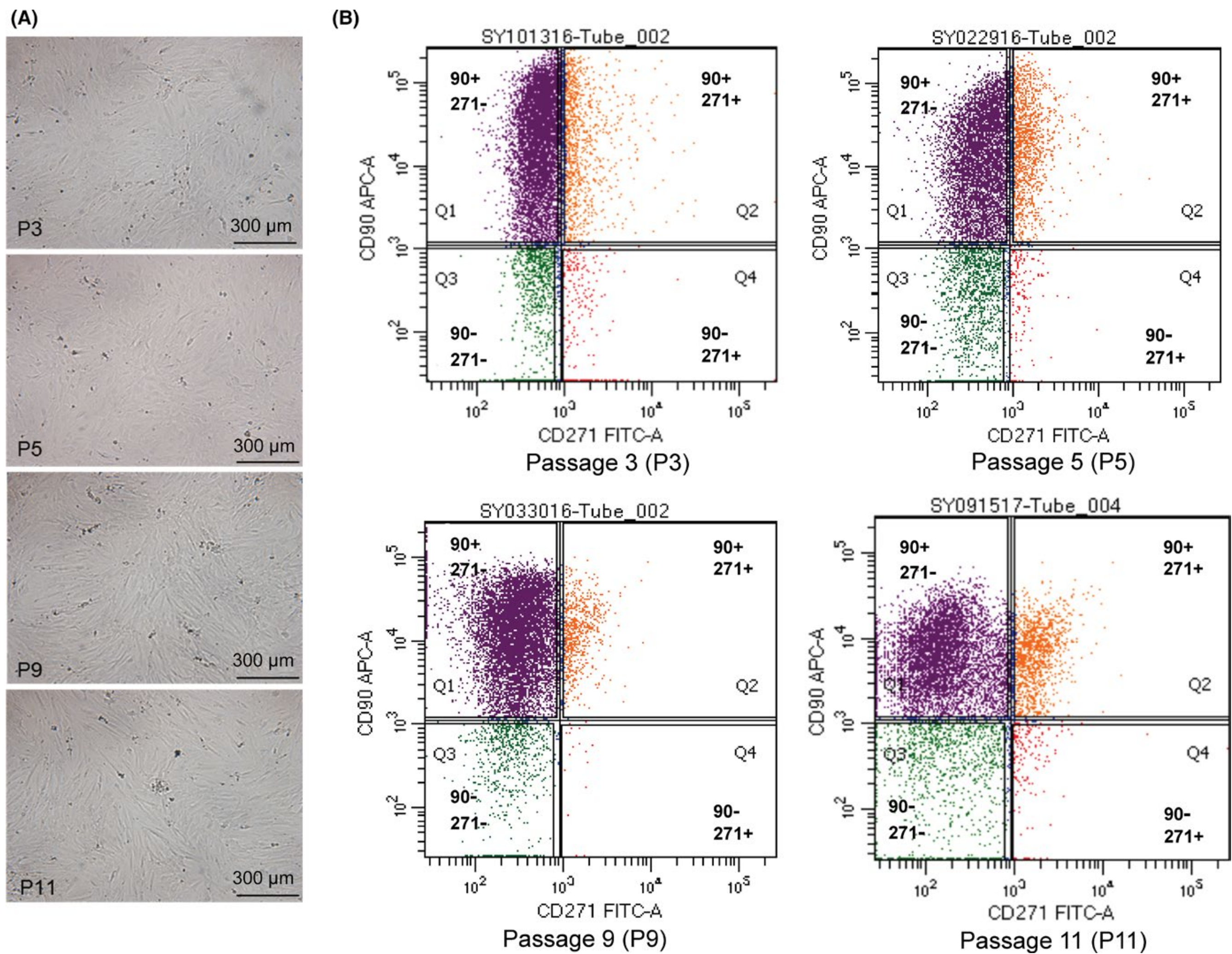

FIGURE 1 Establishment and analysis of different passages of dental pulp stem cell (DPSC) cultures. (A) DPSC cultures showed no morphologically notable difference in the cells at passages 3 to 11. (B) Flow cytometry analysis of different passages of DPSCs with CD90-APC and CD271-FITC showing four portions of cells. Note that CD90+ CD271- and CD90+ CD271+ cells were the major components of the DPSC population in all passages tested

(RGE) was calculated from the C_T values by normalizing P11 to P3. RGE values less than 1.0 indicated decreased expression in P11 cells when compared to P3, whereas values greater than 1.0 indicated increased expression. The results showed that expression of HspB8 and Gipc2 was significantly reduced in P11 as compared to P3 (Figure 3A). Immunostaining of the P3 and P11 DPSCs clearly revealed that P3 cells had stronger staining of HspB8 and Gipc2 than P11 cells confirming higher protein levels in P3 cells (Figure 3B).

3.4 | Effect of gene knock-down on osteogenic differentiation of DPSCs

Dental pulp stem cells were transfected using RNAiMax (Thermo Fisher Scientific) overnight (about 24 hours). Cells appeared to be normal in morphology after transfection (Figure 4A). Because about 2 weeks of induction is required to show in vitro osteogenic differentiation of DPSCs, Gipc2 and HspB8 knock-down efficiencies were checked at different days post-transfection for up to 13 days in a pilot study. As shown in Figure 4B, the post-siRNA transfection knock-down effects appear to last through day 13 for the genes tested. The maximum knock-down appeared to be over 90% at days 1 to 5 post-siRNA transfection for Gipc2 and HspB8 (Figure 4B). Further study showed that overall knock-down efficiency at day 13 post-transfection could be maintained at or greater than 70%, which was statistically significant across multiple transfection replicates (Figure 4C).

With successful knock-down of gene expression following transfection of early-passage DPSCs with siRNA, we tested whether knock-down of the genes would alter differentiation capability. To do that, siRNA-transfected DPSCs and scrambled siRNA transfection (negative control) were subjected to osteogenic differentiation induction for up to 2 weeks (Figure 5A). ARS staining and ImageJ analysis of the staining showed that knock-down of HspB8 resulted in a significant reduction in calcium deposition (Figure 5B,C) as compared to the control. In fact, in some replicates, almost no calcium deposition was

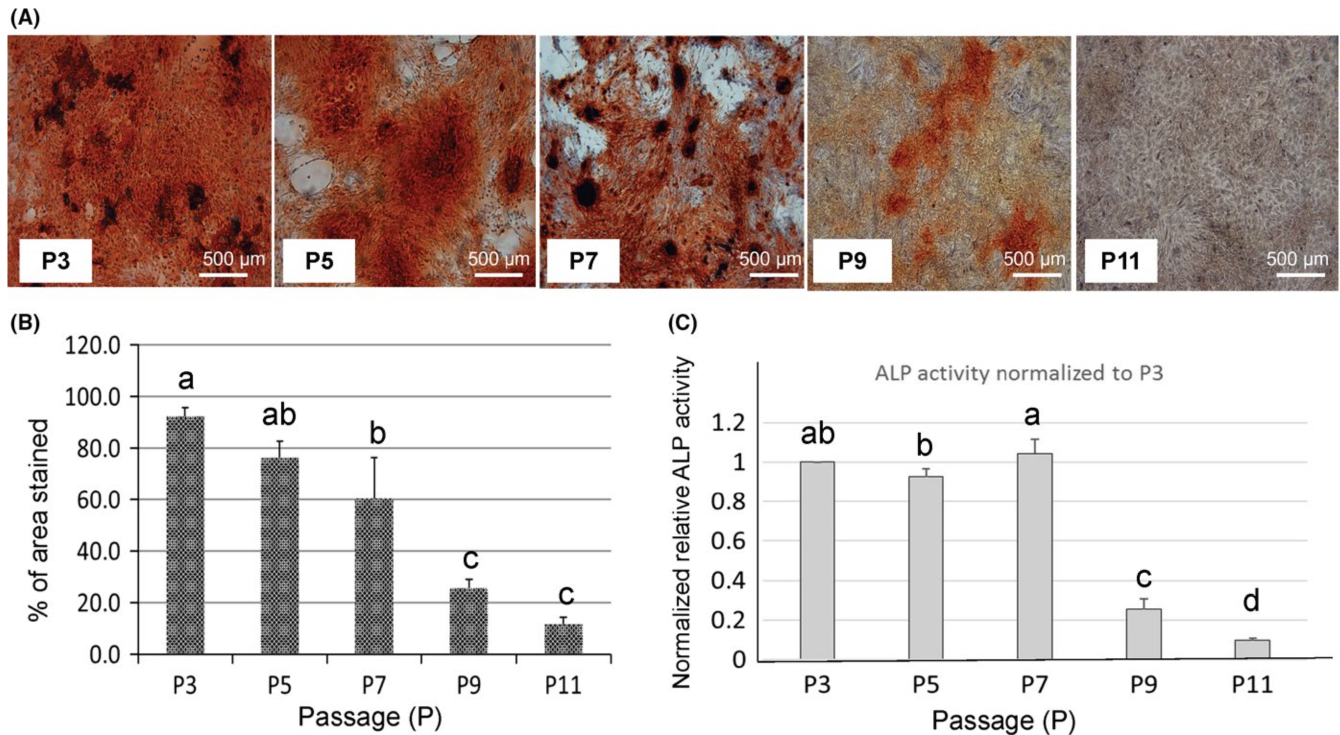

FIGURE 2 Induction of osteogenic differentiation of different passages of DPSCs. (A) ARS staining showed strong calcium deposition in passages 3, 5 and 7 (P3, P5 and P7) and reduced calcium deposition at passages 9 and 11 (P9, P11). (B) ARS staining was quantitatively analysed by ImageJ showing gradually reducing staining with progress of cell passages. (C) ALP activity assay indicated that P9 and P11 DPSCs had significantly lower ALP than P3, P5 and P7 cells. ALP activity was normalized to P3. Note that bars labelled with the same letter indicate no statistically significant difference at $P \leq .05$

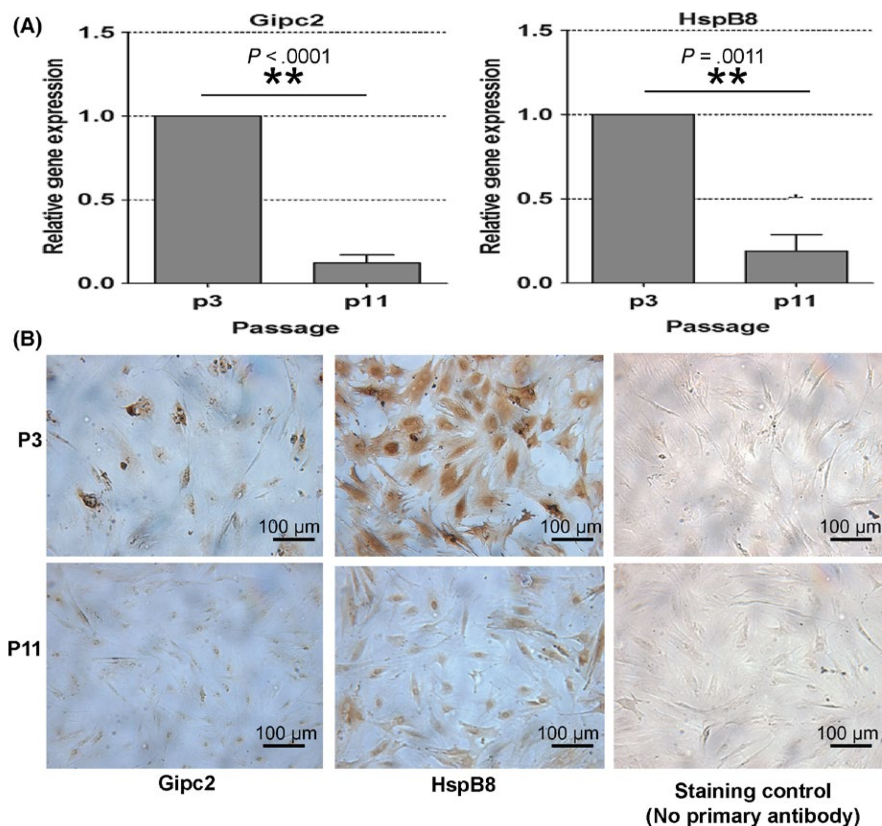

FIGURE 3 Comparison of Gipc2 and HspB8 expression in early passage (passage 3) versus late passage (passage 11) of DPSCs. (A) Relative gene expression of HspB8 and Gipc2 in DPSCs of passage 11 (P11) versus passage 3 (P3) as determined by real-time RT-PCR showing that expression of those genes was highly and significantly decreased in P11 DPSCs at $P \leq .01$ as indicated by asterisks. (B) Immunostaining revealed that the staining for Gipc2 and HspB8 was dramatically weaker in P11 than in P3 cells, indicating that protein expression of Gipc2 and HspB8 was reduced in P11 DPSCs as compared to P3 DPSCs

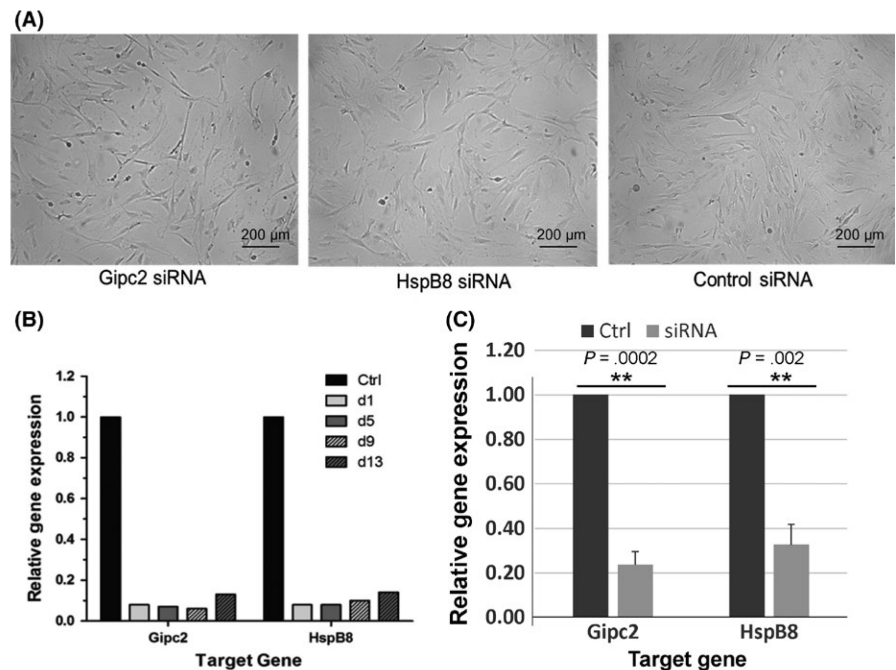

FIGURE 4 Transfection of DPSCs with siRNA to knock-down Gipc2 and HspB8 expression in early-passage DPSCs. (A) No notable change in cell morphology after overnight siRNA transfection. (B) Pilot experiment to check siRNA knock-down efficiency at different days of post-transfection. (C) Knock-down efficiency at day 13 post-transfection to compare Gipc2 and HspB8 siRNA versus control siRNA treatment. Asterisks (**) indicate the difference is highly significant at $P \le .01$

observed when HspB8 expression was inhibited by siRNA (Figure 5B). In contrast, knock-down of Gipc2 appeared to have no effect on calcium deposition as compared to the transfection control (Figure 5B,C). The effect of gene knock-down was confirmed by ALP activity assay showing that knocking down HspB8 significantly reduced ALP activity (Figure 6A).

Expression of osteogenic markers in the Gipc2 and HspB8 knock-down cells was examined at 1 week during osteogenic induction. Significant reduction in ALP, BSP, COL1A1, DCN, OCN and RUNX2 was seen in HspB8 knock-down cells compared to the control (Figure 6B). In contrast, subjecting Gipc2 knock-down DPSCs to osteogenic induction resulted in no significant changes in expression of ALP, COL1A1, OCN and RUNX2 as compared to the siRNA control (Figure 6B). Unexpectedly, increased expression of BSP and DCN was observed in Gipc2 knock-down DPSCs.

4 | DISCUSSION

Loss of differentiation is a common phenomenon in tissue-derived stem cells in vitro, and this is one of the major hurdles in obtaining a large quantity of robust stem cells through expansion of isolated primary stem cells for therapeutic applications. In humans, bone marrow stromal/stem cells (BMSC) demonstrate a change in morphology, from fibroblast-like spindle shape to flat broadened shape after 34-42 cell doublings in vitro.[18] Many studies reported that BMSC largely lose differentiation capability around P6,[19,20] and some studies have shown that differentiation reduction could occur as early as the first [1] or second passage.[2] Wall et al. found that human adipose stem cells lose the ability to differentiate into adipocytes at P10.[21] This cellular ageing has also been observed in hematopoietic stem cells in mice.[22] MSCs isolated from human umbilical cord can be grown in vitro for a longer period than those isolated from bone marrow.[23] Here, we observed that rat DPSCs start to significantly reduce differentiation capability at P7 and completely lose differentiation around P11. Currently, it is unclear why tissue-derived stem cells lose differentiation potential in in vitro at the cellular and molecular level. To begin addressing this issue, we analysed the DPSCs with a classic MSC marker CD90 and novel marker CD271 for MSCs and found CD90+ CD271- cells were the major subpopulation in DPSCs although coexistence of CD271+ cells. This was consistent with reports in the literature; for example, Bonnamain et al. reported that more than 90% cells in human DPSCs at passage 2 were CD90+ cells.[7] Our FACS results indicate that there were some dynamic changes in CD90 and CD271 cells, but overall there were no major changes in stem cell composition during in vitro proliferation of the DPSCs.

To study the intrinsic causes of loss of differentiation for DPSCs, we screened transcriptome of DPSCs. In our preliminary microarray analysis, signal intensity of HspB8 and Gipc2 in P11 DPSCs was at least 10-fold decreased than in P3. Microarray analysis can generate large variability of gene expression data,[24] and other techniques such as RT-PCR to verify microarray results are needed. In this study, we confirmed downregulation of those genes in P11 using real-time RT-PCR and immunostaining. Previously, HspB8 has been studied mainly in cells related to the heart and brain. HspB8 was reported to potentiate BMP signalling in myocytes.[25] HspB8 expression was observed in the neurogenic niche of the hippocampus and in cultured hippocampal neurons.[26] Brain tissue increases HspB8 expression in response to hypoxia stress.[27] It was found that HspB8 plays a role in neuronal survival by inducing macroautophagic removal of misfolded proteins.[28,29]

Hsps have been found to play vital roles in regulating many aspects of stem cells, including self-renewal, differentiation, dormancy and senescence of stem cells.[30] Our laboratory had also shown that dental follicle stem cells (DFSCs) express higher levels of some small

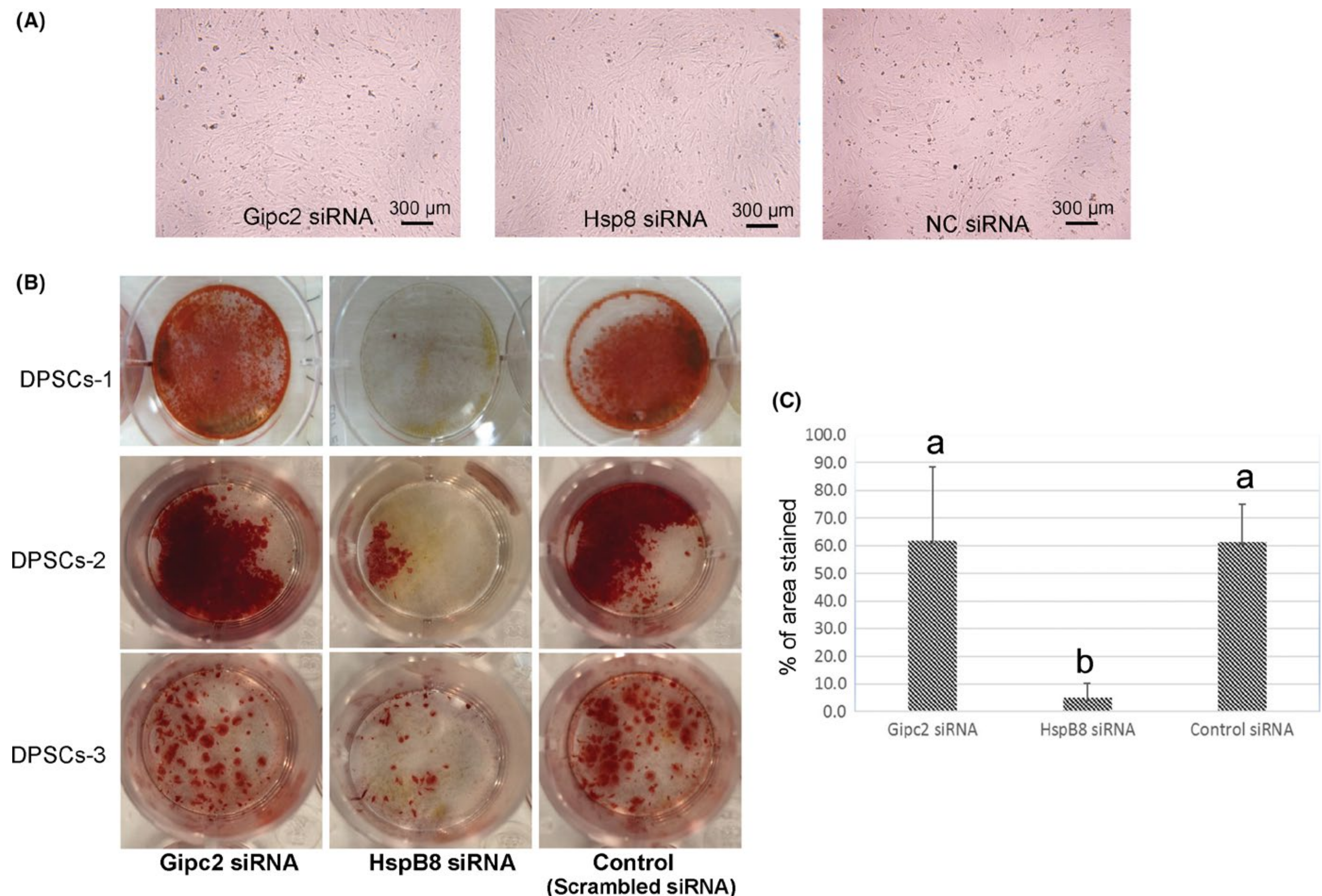

FIGURE 5 Evaluation of osteogenic differentiation after siRNA transfection to knock-down Gipc2 and HspB8 expression. (A) Transfected DPSCs in osteogenic induction medium for one week; note that cells reached full confluence. (B) siRNA transfection and osteogenic induction of three independent cultures of DPSCs derived from different litters of animals as revealed by ARS staining at 2 weeks of osteo-induction. (C) Quantitative analysis of ARS staining using imageJ. Different letters above the bar indicate statistical significance of the means at $P \leq .05$. Note that HspB8-transfected DPSCs resulted in significantly lower staining than the control and Gipc2 siRNA-transfected cells

heat shock proteins (HspB1 and HspB2) than their non-stem counterparts.[31] Previous publications reported that HspB8 could interact with HspB1 (also known as Hsp27) to regulate cell fate [32], and HspB8 was required for proper neurite formation in motor neurons.[33,34] Here, we observed DPSCs reduced expression of HspB8 when the cells lose differentiation potential. Our gene knock-down study showed that downregulation of HspB8 expression indeed resulted in reduction or abolishment of the osteogenic differentiation potential of the early-passage DPSCs, suggesting that HspB8 may exert a function in maintaining or regulating differentiation potential of DPSCs. This is a novel finding regarding the role of HspB8 on stem cells. Given that DPSCs arise from the neural crest,[35] and DPSCs possess the capability to differentiate into neural tissue in vitro,[36] it would not be surprised to see HspB8 expression and function in DPSCs.

Protein structure and cellular location are critical for proper function. Stresses can disturb protein structure and result in loss of function, which in turn would adversely affect cellular metabolism. To protect stress-induced cellular damages, cells often express heat shock proteins (Hsps) in response to many stresses, including thermal stress and environmental stresses. Hsps play an important role in the folding and translocation of polypeptides. They have long been known to function as chaperones to stabilize proteins by ensuring correct folding or to repair proteins by refolding proteins that were damaged by the stresses.[37,38] It is known that small heat shock proteins, such as HspB8, can bind denatured proteins as molecular chaperones to prevent irreversible protein aggregation during stress.[39] Given that HspB8 has been found to exhibit chaperone-like qualities both in tissue [40] and during in vitro cell culture,[41] it is likely that sufficient HspB8 is necessary for stem cells to adapt to these stresses caused by in vitro culture for maintaining their stemness properties. We reasoned when the primary stem cells are isolated from tissues and placed in cell culture condition, they experience a dramatic change in the environment from in vivo to in vitro conditions, and such change may create stresses to the cells, and induce the cells to express HspB8. After certain passages, the cells decrease HspB8 expression because of desensitization to the stress. Subsequently, the DPSCs lose their differentiation potential. It would be significant in future studies to elucidate the regulatory mechanism of HspB8 in maintaining differentiation potential of DPSCs, as well as the downstream factors that are affected by HspB8.

Although knock-down of Gipc2 appeared to increase BSP and DCN expression in DPSCs, neither expression of majority of other

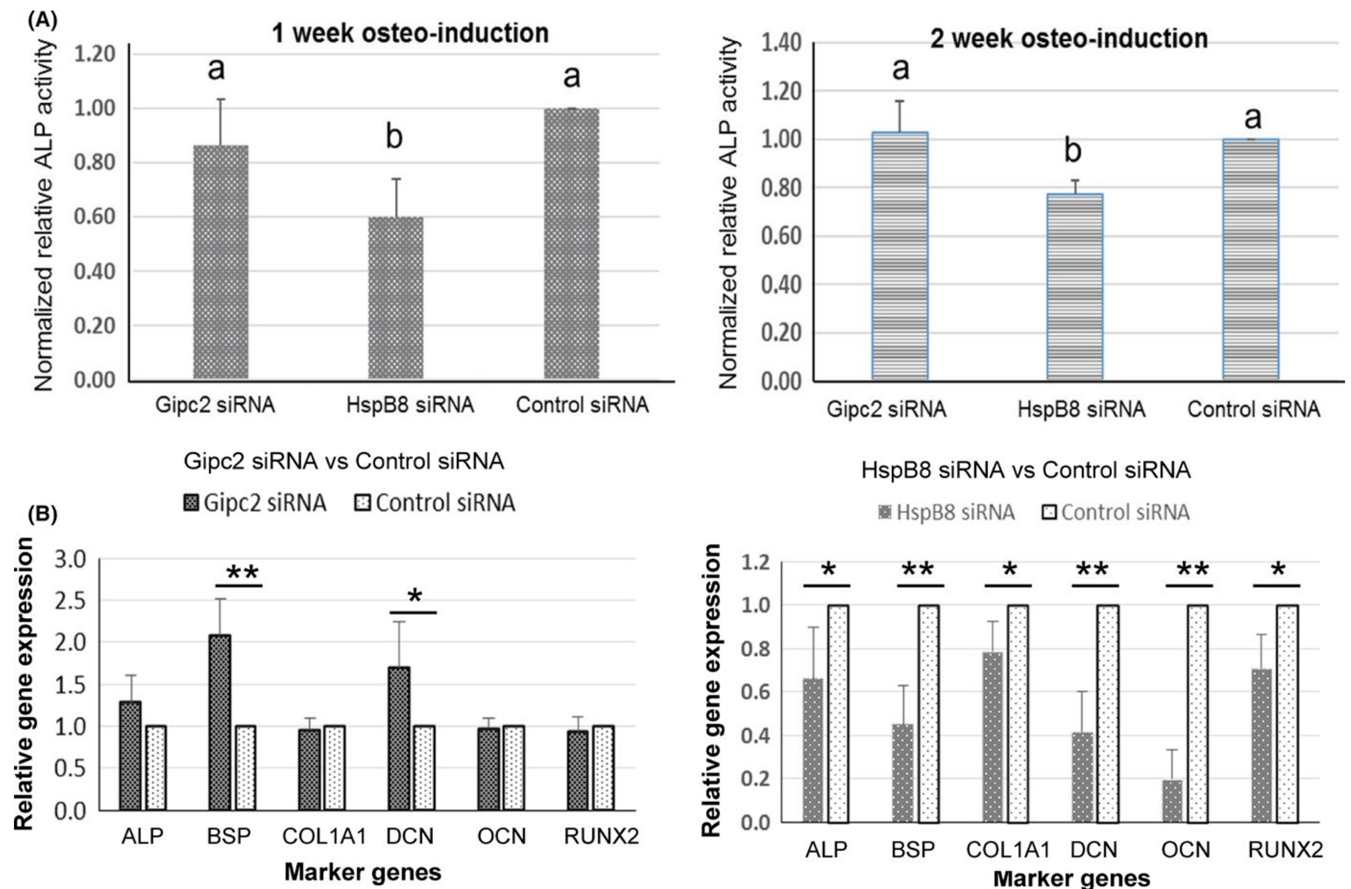

FIGURE 6 Evaluation of selected osteogenic markers after osteogenic induction of siRNA-transfected DPSCs. (A) relative ALP activity normalized to the siRNA control after 1 and 2 weeks of osteogenic induction. (B) Expression of osteogenic marker genes in the siRNA-transfected DPSCs after one week of osteogenic induction. Note that significant reduction in ALP activity and expression of ALP, BSP, COL1A1, DCN and RUNX2 in HspB8 siRNA transfection as compared to the control. Asterisks, * and ** indicate statistical significance between the two means at $P \le .05$ and $P \le .01$, respectively

osteogenic genes including the key transcription factor, RUNX2, nor calcium deposition were increased after osteogenic induction. Thus, Gipc2 appeared to have no significant effect on osteogenesis of DPSCs. Gipc2 was first reported highly expressed in ascending colon, kidney and pancreas and can potentially bind to transforming growth factor beta (TGF-β) type III receptor or some class of WNT receptor.[42] Although it is well known that TGF-β signalling pathway is involved in many cellular processes including cell growth and osteogenic differentiation,[43] TGF-β type III receptor does not have a recognizable signalling domain and ligand binding to it does not directly activate TGF-β signalling transduction.[44] This may explain why downregulating Gipc2 has no effect on osteogenic differentiation of DPSCs.

Dental pulp stem cells are capable of differentiating into various cell lineages.[45] This study demonstrates that HspB8 is critical for maintaining osteogenic differentiation potential of DPSCs. Further study would be needed to determine whether HspB8 plays roles in differentiation of DPSCs to other cell lineages. Although Gipc2 appeared having no effect on osteogenic differentiation, its effect on DPSC differentiation to other cell lineages needs to be determined. For example, Gipc2 was found to increase expression in patients with obesity and diabetes.[46] However, to our knowledge, the role of Gipc2 in adipogenesis remains to be determined. This study discovered that Gipc2 is expressed in DPSCs and its expression is greatly reduced during in vitro expansion. Studies should be carried out to determine whether Gipc2 functions for adipogenesis of DPSCs.

In conclusion, the compositions of DPSC cultures remain relatively stable for CD90+ and CD271+ cells while osteogenic differentiation capability is gradually reduced during in vitro proliferation (or expansion). Expression of HspB8 was significantly reduced during the in vitro expansion of DPSCs when the cells lose differentiation potential. Knock-down of HspB8 in the early-passage DPSCs resulted in decrease in osteogenic differentiation potential of DPSCs. Thus, reduction in HspB8 expression in the DPSCs seen during in vitro expansion likely contributes to loss of differentiation potential of the cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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