

Osteogenic potentials of osteophytes in the cervical spine compared with patient matched bone marrow stromal cells

Pei Zhao¹, Weidong Ni¹, Dianming Jiang¹, Wei Xiong², Feng Li², Wei Luo^{1,2}

ABSTRACT

Background: Osteophytes that form adjacent to degenerated disc have osteogenic potential. Studies suggest that their formation is associated with mesenchymal precursors arising from the chondrosynovial junction. This study is aimed to determine the cellular aging and osteogenic differentiation potential of osteophyte-derived mesenchymal cells (oMSCs) when compared to patient-matched bone marrow stromal cells (bMSCs).

Materials and Methods: oMSCs and bMSCs were isolated from tissue samples during anterior cervical discectomy and fusion surgery. Extensive expansion of cell cultures was performed and early and late passage cells (P₄ and P₉, respectively) were used to study cell senescence and telomerase activity. Furthermore, osteogenic differentiation was applied to detect their osteogenic capacity.

Results: The proliferation capacity of oMSCs in culture was superior to that of bMSCs and these cells readily underwent osteogenic differentiation. Our results showed that oMSCs had higher telomerase activity in late passages compared with bMSCs, although there was no significant difference in the telomerase activity in the early passages in either cell types. The telomerase activity was detectable only in early passage oMSCs and not in bMSCs.

Conclusions: Our results indicate that oMSCs retain a level of telomerase activity *in vitro*, which may account for the relatively greater longevity of these cells, compared to bMSCs. Furthermore, when compared to bMSCs, oMSCs maintained a higher proliferative capacity and the same osteogenic capacity, which may offer new insights of tissue formation.

Key words: Cervical spine, mesenchymal stromal cells, osteogenesis, osteophyte, telomerase activity

INTRODUCTION

Osteophytes are bony spur-like formations and are a distinctive feature of degenerative disc diseases. The function of osteophyte is not clear although it is regarded as the body's attempt to re-balance the load bearing of spine in order to recuperate the degenerated disc. Formation of

osteophytes adjacent to a degenerated disc is often implicated to cause compression of the spinal cord and/or nerves.^{1,2}

Recently developed cervical interbody cages and plate-screw devices offer the advantages of decreasing segmental flexibility and promoting a load-sharing environment in cervical spine reconstruction.^{3,4} Advances in bone graft technology have led to investigation of various osteoinductive materials for their ability to regulate and enhance skeletal bone formation and repair.^{5,6} However, higher resorption rates and lower osteogenic potential frequently compromise their ability to promote successful spinal fusion.^{7,8}

As we know, osteophytes which cause compression are excised during spine surgery. If the osteophyte had the qualified osteogenic potential, then it could be used as bone formation material in cervical interbody fusion surgery. Studies involving the ultrastructure of osteophytes suggest that their formation is associated with mesenchymal precursors arising from the chondrosynovial junction⁹ and it is regulated by a similar molecular mechanism of normal bone embryogenesis. Osteophytes have been found to

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consist of three different mesenchymal tissue regions, including endochondral bone formation within cartilage residues, intramembranous bone formation within fibrous tissue and bone formation within bone marrow spaces. The presence of bone and cartilage derived morphogenetic proteins in osteophytes indicates active bone formation.¹⁰ All of these features provide evidence of involvement of mesenchymal cells in osteophyte formation; nevertheless, this involvement remains to be characterized.

We aim to determine the cellular aging and osteogenic differentiation potential of osteophyte-derived mesenchymal cells (oMSCs). As the mesenchymal cells that form bone marrow (bMSCs) have a central role in the regenerative medicine, we compare it with oMSCs from the same patient. Till date no studies have comprehensively evaluated the osteogenic properties of osteophytes in the cervical spine.

MATERIALS AND METHODS

Sample collection

Tissue samples were collected from six patients following informed consent. The patients were between 55 and 67 years of age group undergoing anterior cervical discectomy and fusion surgery (four males and two females). Osteophyte tissues were obtained around the uncovertebral articulations and the spur of the vertebral body (four for C5 and two for C4). Bone marrow was also collected from the same patients by bone marrow aspirate from the vertebral body. The sample collection was done in accordance with the terms of the Human Ethics Committees of our institution.

Cell isolation and *in vitro* expansion

The osteophyte tissues were placed aseptically in sterile containers with 10 ml of Hanks' buffer (GIBCO, Invitrogen Corporation, USA). Small pieces (2 mm thick and $2 \times 4 \text{ mm}^2$) were dissected from the tissue for explant culture.^{11,12} Briefly, the tissue fragments were placed in six well plates with 2 ml of culture medium, low glucose Dulbecco modified Eagle medium (DMEM-LG; GIBCO, Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA) and 1% penicillin and streptomycin (P/S) (GIBCO, Invitrogen Corporation, USA). The expanded cells were trypsinized and cultured in a T-75 flask for future characterization. The cell surface marker (mesenchymal/stromal stem cells: CD90, CD105 and CD73) were used to detect its mesenchymal and stromal origin with fluorescence-activated cell sorter (FACS). For each assay, 5×10^5 cells were collected using 0.25% trypsin, fixed with 70% ice-cold ethanol and treated with 0.02 mg/ml RNase and EDTA. The DNA was stained with 0.1 mg/ml propidium iodide (PI). Cells were incubated in dark for

30 min and then filtered using 70 mm cell strainers. Samples were analyzed on an FACS (Becton Dickinson, San Jose, USA) and using the standard procedure of the Cell Quest software and the ModFitLT software version 3 (Becton Dickinson).

Isolation and culture of bone marrow-derived MSCs (bMSCs) were performed according to the previously published method in our lab.¹³ Briefly, the suspension of gelatinous bone marrow was filtered through a 74 mm nylon mesh and then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (FBS) on 25 cm² plastic flasks in 37°C with 5% CO₂. After 24 hrs, the nonadherent cells were removed by refreshing the medium and thereafter the medium was refreshed every 3-4 days. After 14 days, cells reached confluence.

Cell proliferation

To compare the capacity of cell proliferation between oMSCs and bMSCs, cell proliferation was conducted at the P₄ and P₉ using 3H leucine incorporation assay.¹³ All experiments were performed in triplicate.

Cell senescence assay

Cytochemical staining for senescence associated b-galactosidase assay was performed using a b-galactosidase staining kit. Cells from P₄ and P₉ were seeded at a density of 2×10^4 /well into four-well plates and allowed to attach overnight, then washed with PBS, fixed and incubated overnight at 37.8°C with X-gal chromogenic substrate at pH 6.0 according to manufacturer's protocol. The color development was observed under light microscope and the image was captured at 100× magnification. The percentage of stained cells as calculated from the averages from five MSC cultures from both early and late passages.

Telomerase activity assay

Telomerase activity in P₄ and P₉ oMSC and bMSC was assayed by a PCR-based assay designated telomere repeat amplification protocol (TRAP) (for telomeric repeated protocol) as telomerase assay kit (Dingguo, Inc., Beijing) described. In the TRAP assay, telomerase synthesized extension products then served as the templates for PCR amplification. The extended products were amplified for 35 cycles (94°C × 30 s, 55°C × 30 s, 72 °C × 1 min) after 3 min at 90°C. A typical assay gives several ladders with interval of 6 bp length DNA, reflecting the addition of one telomerase repeat unit. The size of ladders was representative of telomerase activity. Digital pictures were taken and analyzed using image analysis software (Genescan, ABI, USA). Relative gray value was used to reflect the DNA content of the ladder.

Osteogenic differentiation

Cells (oMSCs and bMSCs) in T-25 culture flask were cultured in growth medium (DMEM with 10% FSC, 1% Penicillin-Streptomycin, 1% Glutamine) at 37°C in a 5% CO₂ humidified incubator. When cells were approximately 80-90% confluent, dissociation with Trypsin-EDTA was performed. Cells were replated in growth medium at 3×10^3 cells/cm² in 6-well tissue culture plates with a medium volume of 2 mL per well. Cells were incubated at 37°C in a 5% CO₂ humidified incubator. We aspirated off the growth medium from each well and then added 2 mL osteogenic differentiation medium (DMEM with 10% FSC, 1% Penicillin-Streptomycin, 1% Glutamine, 50 µg/ml Ascorbate, 10 mmol/L β-Glycerophosphate, 10⁻⁸ mol/L Dexamethasone) after 24 hrs.

This medium was changed twice weekly for up to 3 weeks. Following 4% paraformaldehyde fixation, cell and matrix mineralization was detected by Alizarin red stain for calcium deposits.

Osteogenic assays

Cells were plated for osteogenic differentiation as above for up to 28 days, with medium changed three times per week. Samples were harvested in triplicate for the following assays. The alkaline phosphatase (ALP) activity in cell lysates was measured using SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec, San Jose, CA) following the manufacturer's instructions and normalized to total protein content through the Bradford assay (Bio-Rad, Hercules, CA).

Semiquantitative polymerase chain reaction

We undertook reverse transcription-polymerase chain reaction (RT-PCR) of the following osteogenic genes: ¹⁴ Osteonectin and ALP [Table 1]. PCRs were performed in triplicate. Thermal cycle conditions were 50°C for 2 min, 95°C for 10 min, then 50 cycles at 95°C for 15 s and 60°C for 1 min. Amplifications were monitored with the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Results were normalized against the

housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis

The experiment was repeated three times. All data are represented as the Mean ± SD and statistical analysis was carried out employing the SPSS software package (Version 12.0). Data was analyzed using the independent-samples *t* test. (*P* < 0.05 was considered statistically significant).

RESULTS

Cell culture and morphology

Within 3 days, cells started to migrate out from the osteophyte tissues and these cells showed a spear-like morphology and divided actively [Figure 1a-i]. The cells were confluent within 3 weeks, yielding approximately 3×10^5 cells from each piece of sample [Figure 1a-ii]. The osteophyte cells exhibited an attachment growth and acquired a fibroblast-like morphology when they were expanded after the first passage [Figure 1a-iii]. No obvious morphological difference could be detected between oMSCs [Figure 1a-iii] and bMSCs [Figure 1a-iv] after the first passage.

The proliferation capacity of osteophyte-derived mesenchymal cells

To assess the effect of extensive passaging on cell proliferation, the [³H] leucine incorporation assay was used to monitor cell proliferation. In an early passage (P₄), both oMSCs and bMSCs showed a similar proliferation rate with a similar increase in cell numbers over 7 days. At a later passage (P₉), oMSCs showed a significant increase in cell numbers compared with bMSCs (*P* < 0.05) from day 7 to day 21 [Figure 1b and Figure 1c].

β-Galactosidase expression in osteophyte-derived mesenchymal cells and bone marrow stromal cells

β-Galactosidase staining in both oMSCs and bMSCs cultures increased with the number of passages [Figure 2a]. Interestingly, bMSCs showed significantly higher cell numbers with the expression of β-galactosidase compared to oMSCs in P₉. The number of β-galactosidase-positive cells in early passage was low at 2-24% in oMSCs and 5-27% in bMSCs at P₄ culture; however, at P₉ β-galactosidase-positive cells increased to 12-37% in oMSCs and 58-90% in bMSCs [Figure 2a]. The percentage of β-galactosidase-positive cells in bMSCs was significantly higher than that in oMSCs. In bMSCs increase of β-galactosidase-positive cells in the late passage was correlated with the decrease of cell proliferation. There was a significant increase in cell proliferation in P₉ bMSCs compared with P₄ bMSCs and P₉ oMSCs.

Table 1: Design of primers and probes

Gene	Gene primers: 5'-3'
Osteonectin	F: AGATGATGGTGCAGAGGAA R: GGTGGTTCTGGCAGGGATT P: CCGAAGAGGAGGTGGTGGCGG
ALP	F: AGATGATGGTGCAGAGGAA R: GGACCTGGGCATTGGTGH P: CCTTCGTGGCCCTCTCCAAGACG
GAPDH	F: CCACCCATGGCAAATTCC R: GGATTCCATTGATGACAAGCTT P: TGGCACCGTCAAGGCTGAGAACC

ALP=Alkaline phosphatase, GAPDH=Glyceraldehyde-3-phosphate dehydrogenase, P=Probe, F=Forward, R=Reverse

Telomerase activity of marrow stromal cells

The telomerase activity of MSCs was determined by TRAP. The more and larger amplified products were, the higher telomerase activity. The extension ladders from bMSCs were less than that from oMSCs both in P₄ and in P₉. Image analysis of digital pictures showed

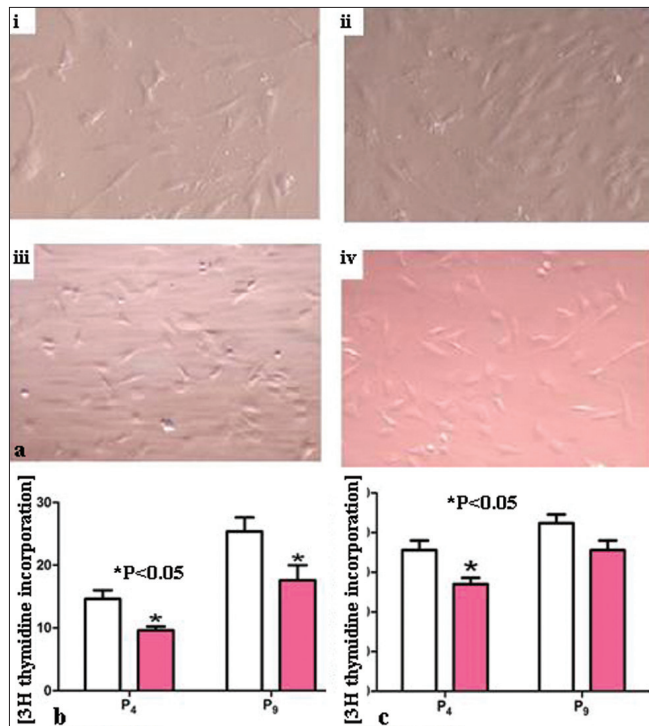


Figure 1: Characterization of oMSCs and bMSCs. (a) All MSC sources demonstrated a spindle-shaped morphology (100 \times). (i) oMSCs started to come off the tissue in 3 days; (ii) oMSCs were confluent within 3 weeks; (iii) oMSCs exhibited an attachment growth and acquired a fibroblast-like morphology when they were expanded after the first passage; (iv) bMSCs from the same patients after the first passage. (b) In the early passages, both oMSCs and bMSCs showed a similar proliferation rate with a similar increase in cell numbers over 7 days. (c) In the later passages, oMSCs showed a significant increase in cell numbers compared with bMSCs; * $P < 0.05$ vs control

the extension products' relative gray value from bMSCs was lower than that from oMSCs ($P < 0.05$) [Figure 2b]. This suggests that cell passing reduces the telomerase activity of MSCs.

Osteogenic differentiation assays by alizarin red stain

The results showed that the number of osteocytes, which were derived from oMSCs [Figure 3a-i] after 7d culture, were much higher than that from bMSCs [Figure 3a-ii].

Osteogenic differentiation assays by the alkaline phosphatase activity

After induction with osteogenic inducer, the ALP activity, as markers of osteoblasts, were lower in cells from bMSCs group than that from oMSCs group ($P < 0.05$) [Figure 3b]. This indicates that cell from osteophytes have a bigger osteogenic differentiation capacity.

Osteogenic differentiation assays by semiquantitative polymerase chain reaction

When exposed to osteogenic medium over 28 days, oMSCs underwent more robust osteogenic differentiation, generating more extracellular mineralization than the other MSC types, as shown by more intense alizarin red staining [Figures 3b and c]. Two osteogenic genes were upregulated earlier (ALP) or more robustly (osteonectin) in oMSCs than in bMSCs. oMSCs demonstrated earlier expression of osteonectin and higher expression of ALP than bMSCs [Figure 4].

DISCUSSION

Osteophyte is a frequent cause of cervical spondylotic myelopathy and radiculopathy in people over the age of 50 years.^{1,4} Variations in stress and strain in the spinal structure adjacent to a degenerated disc may affect spinal

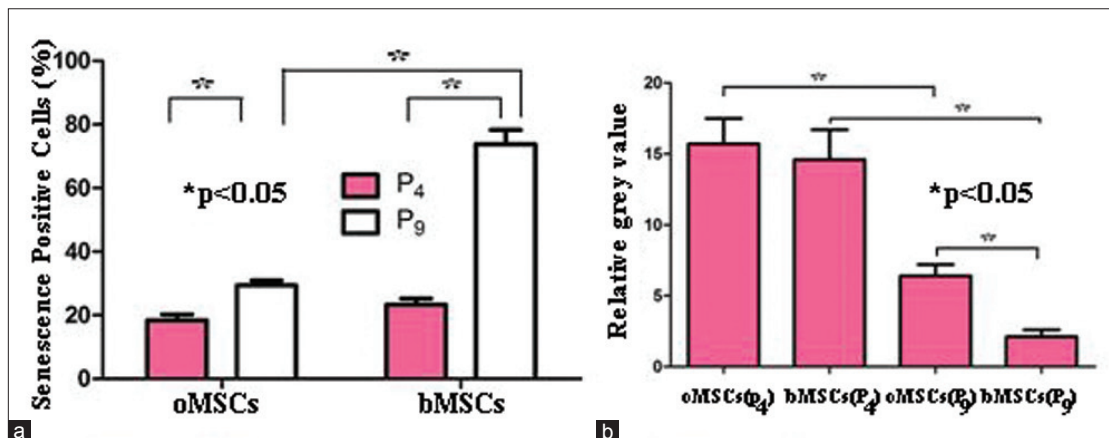


Figure 2: The aging of the MSCs. (a) β -Galactosidase expression in osteophyte-derived mesenchymal cells and bMSCs (P₄ and P₉). The percentage of β -galactosidase-positive cells in bone marrow stromal cells was significantly higher than that in oMSCs. (b) The telomerase activity of MSCs. The extension ladders from bMSCs were less than that from oMSCs both in P₄ and in P₉ * $P < 0.05$

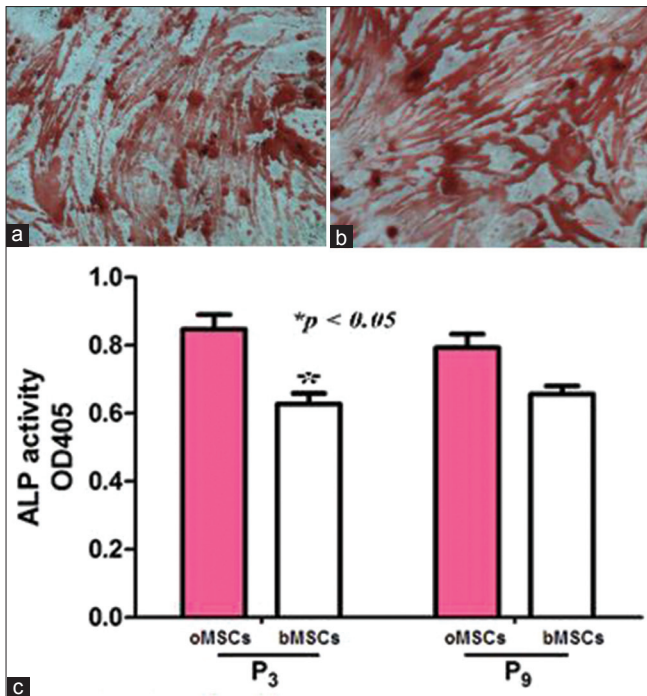


Figure 3: Markers for induced osteogenesis of osteophyte-derived mesenchymal cells. (a,b): Alizarin red stain for calcium deposits: the number of osteocytes, which were derived from oMSCs [Figure b] after 7d culture, was much higher than that from bMSCs [Figure a]. (c) The ALP activity by PNPP (P₄ P₉), the ALP activity were lower in cells from bMSCs group than that from the oMSCs group *P<0.05. Bar: 100 μm

stability, which lead to the formation of osteophytes.^{15,16} The osteophytes re-balance the load bearing surface of the cervical spine in order to recuperate the degenerated disc, with the aim to re-gain spinal stability. Unfortunately, osteophytes cause nerve compression (nerve roots and/or spinal cord) in the process of re-balancing. A discectomy followed by spinal fusion is considered the classic procedure in the treatment of symptomatic cervical spine pathology. Interbody cage with or without osteo-inductive materials (such as autogenous or allogenic bone, allogenic or ceramic materials and growth factors) is one of the choices for spinal fusion.^{5,6,17,18} High quality scaffolds with bMSCs are reasonable solutions for bone graft materials in recent basic research and have already been in clinical application.¹⁸ In this study we evaluated the osteogenic differentiation potential of oMSCs, with the aims to verify its ability as an osteo-inductive material. We found that oMSCs were capable of retaining proliferative capacities at higher passage numbers when compared with patient-matched bMSCs. We further found that the proliferative potential of MSCs from bone marrow and osteophytes appeared to be closely linked to telomere length, which is controlled by telomerase activity. However, there was no statistical significance in oMSC proliferation between P₉ and P₄, even though telomerase activity was significantly decreased in P₉ oMSCs. The significantly higher telomerase activity in the early passage oMSCs (P₄) may be responsible for the maintenance of

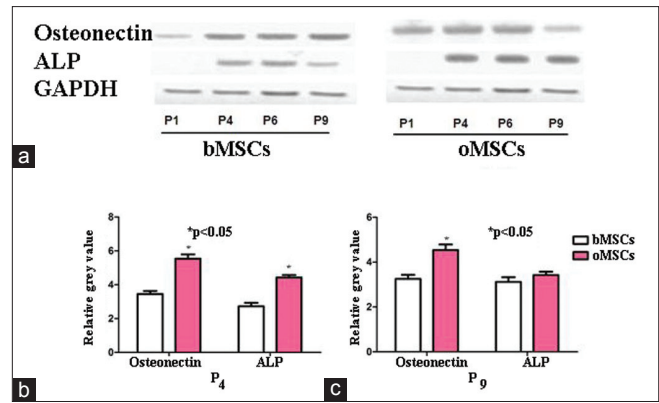


Figure 4: Osteogenic differentiation assays by reverse transcription-polymerase chain reaction. All two osteogenic genes were upregulated earlier (ALP) or more robustly (osteonectin) in oMSCs than in bMSCs. (a) mRNA expression of ALP and osteonectin. (b, c) image analysis of digital pictures of products relative gray value. *P<0.05

proliferation in P₉oMSCs. Previous studies have showed that stem cells in their niches have longer telomeres in comparison to their more differentiated counterparts.^{19,20} Our results, together with the previous studies,^{12,20} show the higher proliferating ability of OMSCs. This is good for quantity, but bad for quality in some way. Cell may be more prone to undergo malignant transformation in the proliferating process. Even though MSCs derived from healthy tissues do not appear to exhibit tumorigenic characteristics,²¹ it remains unclear whether MSCs derived from diseased tissues, such as osteophytes, show adverse cytogenetic variation in comparison to MSCs from bone marrow. Previous studies showed that osteophyte tissues contain pools of MSCs which exhibit normal traits, enter senescence after extended *in vitro* culture and have a normal karyotype compared to bMSCs.²⁰ Furthermore, the aim of our research was to verify the utility of vertebral body spur transplantation; we did not need a vitro cellular proliferation. Previous work has suggested that chondrogenesis and osteogenesis during the formation of osteophyte tissue may be similar to that seen during normal bone development;^{12,22} hence, we assessed oMSC differentiation into cells of the osteophytes lineages. In contrast to a previous study showing that nonbone marrow-derived MSCs have a reduced differential capacity,²³ our study showed oMSCs differentiate in a manner similar to bMSCs and were able to form a denser cellular matrix during osteogenesis. In terms of osteogenic differentiation, oMSCs demonstrated similar differentiation capacities in comparison to bMSCs. Interestingly, in one bMSC sample during osteogenic differentiation, an absence of calcium deposition was noted, but it was present in the comparative oMSCs. We further confirmed lack of osteopontin expression in the bMSCs and positive expression in the same patient's oMSCs. It has been revealed that there may be an age-related decline in osteogenic potential in bMSCs.²⁴⁻²⁶ Our results support the

possibility that the differentiation potential of MSCs is not affected by age.

The concept of 'age-dependent' expression in cell cultures was very distinct, not only between passages, but also between cell sources.^{27,28} When compared to our previously reported results for cell proliferation,^{12,20} oMSCs appeared to have either more primitive cells or be somehow more resistant to aging. Various studies have indicated that cellular aging may be, in part, due to critical size telomere length that leads to replicative senescence.²⁹⁻³² Evidence suggests that stem cells maintain longer telomeres relative to other cells within the tissue, such as in lingual mucosa, where *in situ* hybridization has revealed longer telomeres in the basal cells³³ and stem cell compartments of the skin, small intestine, cornea, testis and brain.¹⁹

In summary, we have isolated and characterized a cell population from osteophyte tissue in cervical spine. These osteophyte tissues contain pools of MSCs that exhibit normal traits. We noted that unlike bMSCs, these cells maintained their proliferative capacity at late passage and had a slightly higher osteogenic differential capacity, which indicates that these cells may offer an alternative to bMSCs in some way.

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