



# Identification and Characterization of a Novel Type of Cartilage Stem/ Progenitor Cells from Tibetan Mastiff

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## ABSTRACT

Based on the physiologically non-self-renewing property of articular cartilage, the treatment of articular cartilage lesions remains an important challenge. Articular cartilage, the target of osteoarthritis (OA), contains renewable cartilage stem/progenitor cells (CSPCs) to maintain the tissue homeostasis. Compared with other adult stem cells, the self-renewal capacity and preference for chondrogenesis of resident CSPCs have propelled their exciting therapeutic prospects for cartilage repair. Tibetan mastiff, the ancestor of large breed dogs worldwide, represents a valuable model for human diseases. To our knowledge, this is the first study to capture the emergence of Tibetan mastiff-derived CSPCs. Our study aimed to unveil the biological properties and differentiation multipotency of CSPCs. CSPCs were characterized by the clonogenicity, growth curves and karyotype analysis, respectively. The results showed that the CSPCs of Tibetan mastiff displayed self-renewal ability, proliferative potential, and hereditary stability. Moreover, the CSPCs of Tibetan mastiff also positively expressed recognized surface antigens of human CSPCs. Additionally, CSPCs exhibited differentiation multipotency, including osteogenic, adipogenic and chondrogenic potential *in vitro*. These findings may propell CSPCs application in a clinical study, suggesting potential therapeutic treatment for OA or related joint diseases.

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## Authors' Contribution

CM and HW conducted research. LL, GL and CW performed the statistical analysis. YG and SF reviewed the article. HW and CL designed the research and wrote the paper.

## Key words

Tibetan mastiff, Cartilage stem/progenitor cells, Biological properties, Differentiation multipotency, Cell therapies

## INTRODUCTION

By 2040, an estimated approximately 78.4 million adult population will have arthritis, characterized by joint pain and activity limitation, which will negatively affect their work productivity and life quality (Hootman *et al.*, 2016). In addition, the high medical costs of health care estimated up to \$81 billion a year in the United States (Yelin *et al.*, 2007). Osteoarthritis (OA), a prevailing form of arthritis, can lead to severe mobility loss (Jiang *et al.*, 2015; Felson, 2004). Cartilaginous tissues are comprised of hyaline cartilage, elastic cartilage and fibrocartilage and they can be distinguished on the basis of molecules

and matrix (Jiang and Tuan, 2015). Articular cartilage, a kind of hyaline cartilage, resides in the end of diarthrodial joints. Hypocellular articular cartilage is a vasculature-free structure without nerves, once destroyed, can give rise to the development of osteoarthritis (Jiang and Tuan, 2015).

When articular cartilage becomes damaged, chondrocytes and stem cells can be used to repair cartilage degeneration. However, chondrocyte-based therapies are thought to be a major challenge due to the uncertainty of expanded chondrocytes phenotype and their dedifferentiation properties, which have a negative effect on the generation of functional cells and hyaline cartilage (von der Mark *et al.*, 1977; Pappa *et al.*, 2014). At present, three groups of stem cells play significant roles in cartilage tissue engineering, including mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), and embryonic stem cells (ESCs). Whereas, the problems of tumorigenesis and ethical issues limit the applications of pluripotent iPSCs and ESCs (Nam *et al.*, 2018). Among multiple sources of adult MSCs, bone marrow MSCs (BM-MSCs), which can spontaneously ossify, commonly generate calcified cartilage bone instead of hyaline cartilage after treatment with chondrogenic differentiation

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medium (Huang *et al.*, 2017). Therefore, a more in-depth exploring of novel therapeutic approaches for cartilage repair was warranted.

Articular cartilage, the target of OA, contains core cartilage stem/progenitor cells (CSPCs) that maintain the tissue homeostasis (Jiang and Tuan, 2015). Moreover, the resident CSPCs exhibit great potential applications in cartilage regenerative medicine owing to their preference for chondrogenesis (Seol *et al.*, 2012; Pizzute *et al.*, 2015; Jessop *et al.*, 2020). Homing to the injury zones, CSPCs can respond to cartilage lesions and effectively promote joint cartilage repair attributed to self-renewal and multipotency (Seol *et al.*, 2012). Further study on the origin, function and regenerative mechanism of endogenous CSPCs will provide a potential therapeutic cell resource for the treatment of OA related degenerative joint diseases.

The Tibetan mastiff, ancestor of large breed dogs worldwide, has been used as a valuable animal model for human diseases to decipher the potential disease mechanisms and evaluate treatment strategies (Wang *et al.*, 2012; Li *et al.*, 2008; Kijas *et al.*, 2003). To date, there are numerous published reports on CSPCs, such as cartilage tissues of human, equine, sheep, chicken, rabbit and bovine, with the exception of Tibetan mastiff (Oda *et al.*, 2016).

To our knowledge, this is the first study on the successful isolation and culture of Tibetan mastiff derived CSPCs *in vitro*. The present study aimed to evaluate the biological characteristics of Tibetan mastiff CSPCs by virtue of expression of biomarkers, growth kinetics, clonogenicity. Furthermore, multilineage potency of CSPCs was additionally probed on the basis of adipogenic, osteogenic, and chondrogenic differentiation test *in vitro*, which laid an important theoretical basis and provided cell resource for cell therapy of some canine diseases.

## MATERIALS AND METHODS

### *Isolation and cultivation of Tibetan mastiff CSPCs*

The joint samples of newborn Tibetan mastiff (6 individuals, male, weight 475g) were randomly dissected from nonlesion articular cartilages. The use of animals and all experimental procedures were approved by Institutional Animal Care and Use Committee (IACUC) for Ethics of Bengbu Medical College. Then, CSPCs were collected by fibronectin adhesion assay as previously described (Li *et al.*, 2015a). Briefly, the cell pellets digested by 0.2% (wt/vol) collagenase II to single cell were inoculated in new 10 mg/ml fibrin-coated culture dishes at  $6 \times 10^3$  cells/cm<sup>2</sup>, and further cultured in sequential warm fresh chondro-medium (CM, DMEM/F12 supplemented with 10% foetal bovine serum and 2 mM L-glutamine) up to P24.

### *Population doublings (PD) of Tibetan mastiff CSPCs in vitro*

Under standard culture conditions, the growth of  $1 \times 10^4$  CSPCs from different passages CSPCs (P4/P12/P20) was detected in real time, and the cell density were measured every day. Likewise, PD time was monitored by direct cell counts using hemocytometer assay according to a previously reported protocol (Zhang *et al.*, 2018).  $PD = (t - t_0) \lg 2 / (\lg N_t - \lg N_0)$ , t: termination time; t<sub>0</sub>: starting time; N<sub>t</sub>: ultimate cell number; N<sub>0</sub>: initial cell number.

### *Colony-forming activity of Tibetan mastiff CSPCs*

The selfrenewal capacity of CSPCs cultures was characterized on the basis of the clonogenicity test using the Giemsa compound staining method (Jessop *et al.*, 2020). Following enzymatic digestion, fibronectin-adherent CSPCs was inoculated at the initial number of 200 cells and cultured in CM. After 8-12 days, colony-forming efficiency (CFE) was verified as a percentage of colony-forming unit (CFU) numbers from 200 cells/cm<sup>2</sup>.

### *Cell cycle analysis and karyotyping of Tibetan mastiff CSPCs*

The cell cycle distributions of the CSPCs stained with Propidium Iodide (PI) were carried out by flow cytometry (Cytomics FC 500, Beckman Coulter, USA). Chromosomes spreads of Tibetan mastiff CSPCs were prepared, fixed and stained following standard methods (Zhang *et al.*, 2018). After Giemsa staining, 200 well-spread metaphases were randomly selected and observed under an oil immersion objective.

### *Immunohistochemistry of Tibetan mastiff CSPCs*

CSPCs grown on coverslips were fixed with 4% paraformaldehyde, followed by permeabilization with 0.25% Triton X-100 (v/v) for 10 min and blocking with 1% bovine serum albumin (BSA, w/v) and 10% goat serum for 30 min at RT. Cells were then incubated with the following primary antibodies at 4°C overnight: CD90, CD105, CD166, Vimentin, and FGFR3 (1:200; BIOSS). Following which, cells were incubated with FITC-labeled secondary antibody (1:500; BIOSS) for 1 h at RT. The cell nucleus was counterstained with 10 µg/ml DAPI for 10 min. The results of the immunofluorescence staining were observed under a confocal laser-scanning microscope (Nikon corporation, Tokyo, Japan).

### *Cell surface markers detection by FACS and RT-PCR*

The CSPCs in the logarithmic phase were fixed with 70% precooled ethyl alcohol prior to treatment with primary antibodies. The expression of cell surface markers of CSPCs were detected following by colabelling with primary antibodies of CD90, CD105, CD166, Vimentin

and FGFR3 using flow cytometry, respectively. For cell markers expression levels, the data from CSPCs stained positively was acquired and processed. Moreover, the expression of cell surface markers (CD90, CD105, CD166, Vimentin) of CSPCs in mRNA level were detected by reverse transcription PCR (RT-PCR), and the primers and amplicon size are presented in [Table I](#).

#### *Multilineage potential of Tibetan mastiff CSPCs*

The CSPCs were induced to differentiate into adipocytes, osteoblasts, and chondrocytes under lineage-specific inducing conditions. For adipogenic differentiation, the cells in monolayer culture were

incubated in adipocytes-inducing differentiation (AID) medium, DMEM/F12 supplemented with 1.0 mmol/l dexamethasone, 0.5 mmol/l isobutyl-methylxanthine and 10 mg/l insulin for 12 days. Adipogenesis was evaluated by intracellular lipid accumulation by Oil Red O staining (Solarbio) and adipocytes specific genes detection by RT-PCR. For osteogenic differentiation, CSPCs were cultured in 6-well plates with a concentration of  $2 \times 10^5$  cells/well and induced in standard osteogenic induction medium (OID) for 14 days to make calcifying nodules formation ([Ma et al., 2017](#)). At day 14, the osteogenic differentiation potential of CSPCs were detected by alizarin red stain kit (Solarbio) and the expression levels of osteogenic specific genes

**Table I. Primer sequences used in RT-PCR assay.**

Gene name	Primer sequences	Product length (bp)	T <sub>m</sub> (°C)
<i>GADPH</i>	F:5'-GGTGATGCTGGTGCTGAGT-3' R:5'-GTCTTCTGGGTGGCAGTGAT-3'	299	56
<i>COL2A1</i>	F:5'-CTGTCCCATCTGCTCAACTG-3' R:5'-CCAGCCTTCTCATCAAATCC-3'	321	57
<i>Vimentin</i>	F:5'-CAGATGCGTGAAATGGAAGA-3' R:5'-TGTCAACCAGAGGGAGTGAA-3'	274	58
<i>SOX9</i>	F:5'-CTCAAGGGCTACGACTGGAC-3' R:5'-CGTTCTTCACCGACTTCCTC-3'	313	58
<i>ACAN</i>	F:5'-CTGAAGGGCAGGTGAGGAT-3' R:5'-CTGTTCCGGGTGTAGCAATGA-3'	293	60
<i>ITGB1</i>	F:5'-TTTCTGGATTGGACTGATTGG-3' R:5'-TCTGCTGTTCCCTTTGCTACG-3'	284	60
<i>ALCAM</i>	F:5'-CAGTTATCCAGACGGCAACA-3' R:5'-TTGATGGCAGTTTTTGATGG-3'	300	57
<i>COL1A2</i>	F:5'-CTCGCTCACCACCTTCTCTC-3' R:5'-CAGTTCTTGGCTGGGATGTT-3'	283	58
<i>FGFR3</i>	F:5'-CACAAGGTCTCCCGCTTTC-3' R:5'-CGTGGCATCATCTTTTAGCA-3'	312	56
<i>PPAR-γ</i>	F:5'-TGCTGTGGGGATGTCTCATA-3' R:5'-ACCTCTTTGCTCTGCTCCTG-3'	318	59
<i>LPL</i>	F:5'-TTTGGGATACAGCCTTGGAG-3' R:5'-CCTCTCTCTGCAATCACACG-3'	303	58
<i>SPP1</i>	F:5'-GATGATGGAGACGATGTGGA-3' R:5'-GGAAAGTAGGACGGCATTGA-3'	318	60
<i>CD90</i>	F:5'-TGGAGGGTTGGAGAAGGAGT-3' R:5'-GCACTGATGGGGGAGGTAAG-3'	265	58
<i>CD105</i>	F:5'-GAGCCCAGTGACTCTTTCCC-3' R:5'-AAACGTCACCTCACCCCTTG-3'	377	56
<i>CD166</i>	F:5'-GACCAAGCAGATTGGCGATG-3' R:5'-TCTCTGTTTTTCATTAGCAGAGACAT-3'	495	59

*COL1A2* and *SPPI* by RT-PCR. The chondrogenic capacity of CSPCs was explored following the previous study (Ma *et al.*, 2017). Briefly, cells were processed in 2D cultures and in 3D pellet cultures in specific chondrogenic differentiated medium (CID). For 2D cultures, induced cells were harvested after 21 days for RT-PCR analysis and alcian blue staining (Sigma). For 3D cultures, the pelleted cells ( $2.5 \times 10^5$ ) were placed into a 1.5 ml tube and treated with chondrogenic induction medium for 21 days, and intense stains of induced CSPCs were analyzed to investigate the glycosaminoglycan secretion *in vitro*.

#### Statistical analysis

All results were reported as the mean  $\pm$  standard deviation from at least three independent experiments. Statistical significance ( $P < 0.05$ ) was determined by Student's t-test. The software of GraphPad Prism 7.0 was used for statistical analysis and the generation of graphs.

## RESULTS

#### Morphological characteristics

The original isolated Tibetan mastiff CSPCs were plated in fibronectin-coated plates for 6-7 days under CM culture conditions, reaching confluence. Cytomorphology of CSPCs gradually adopted polygonal and spindle shapes in the growth phase for all passages (Fig. 1). Moreover, CSPCs could maintain stable morphology and undifferentiated states after subsequent subculture *in vitro* to at least 24<sup>th</sup> passages (Fig. 1). Afterwards, blebbing and karyopyknosis appeared sequentially potentially indicating senescence of CSPCs (Fig. 1).

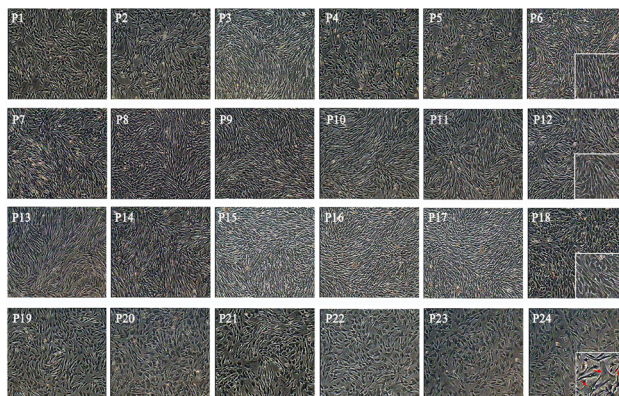


Fig. 1. Cell morphology of CSPCs at passage 1 to passage 24 cultured *in vitro*. After passage 20, CSPCs appeared senescent.

#### Colony formation, growth kinetics, and karyotyping analysis

The colony-forming units of CSPCs were detected

using Gimesa staining, and colony-forming efficiencies were determined as  $37.3 \pm 1.5\%$ ,  $29.1 \pm 0.8\%$  and  $25.6 \pm 1.2\%$  colonies/ 200 cells for P4, P12, P20 (Fig. 2a), indicated the clonogenic and self-renewal characteristics. Cell growth curve is a traditional assay to characterize proliferating adherent cells at different time points. Growth curves of CSPCs from P4, P12 and P20 were typical S-shaped, indicating that proliferative potential were similar (Fig. 2b). Based on the cell growth curves, the PD time of different passages was P4/34.09 h, P12/35.10 h, and P20/36.13 h, respectively. Karyotyping (Fig. 2c) confirmed the diploid chromosome frequencies of CSPCs of Tibetan mastiff from P4, P12, P20 with  $2n = 78$  were 93.6%, 92.8%, and 92.6%, which confirmed CSPCs was reproducibly diploid with no cross contamination. Likewise, no abnormalities were detected potentially indicating the stable character of CSPCs.

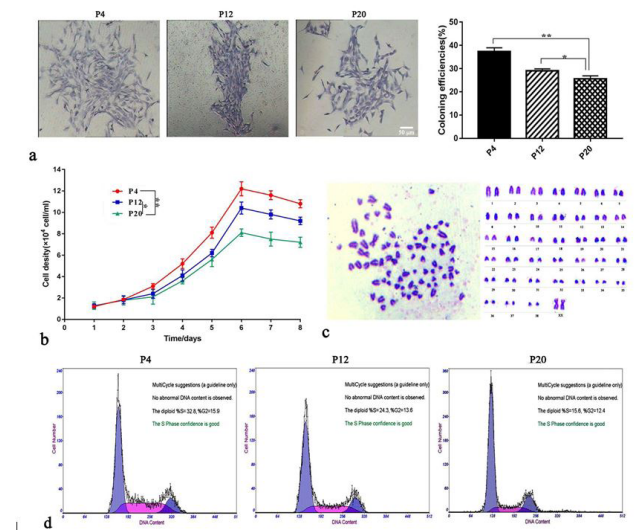


Fig. 2. Karyotype and cell cycle analysis of proliferating CSPCs. (a) Colonies and morphology of CSPCs by Gimesa staining at P4, P12 and P20 (bar, 50  $\mu$ m). Bar chart shows that colony forming efficiency of different passages; (b) Sigmoidal growth curves of CSPCs at different passages (P4/P12/P20); (c) Karyotype analysis of CSPCs ( $2n = 78$ ); (d) Cell cycle analysis of P4, P12 and P20.

#### Cell cycle analysis and cell-markers immunofluorescence characterizations in CSPCs

Cell cycle analysis showed that the rate of G0/G1 was about 51.3-72% (Fig. 2d), and only a small proportion was proliferating cells (in S/M phases). Moreover, there were no significant difference among the P4, P12 and P20. The results of immunofluorescence showed that CSPCs were positive for CD90, CD105, CD166, and Vimentin (Fig. 3a). And, RT-PCR demonstrated that the CSPCs



could express *CD90*, *CD105*, *CD166*, and *Vimentin* as well at mRNA level, which was consistent with the results of immunofluorescence (Fig. 3b). In addition, *FGFR3* was also validated in CSPCs by immunofluorescence analysis (Fig. 3a). Furthermore, FACS analysis further demonstrated that CSPCs of Tibetan mastiff highly expressed a subset of recognized markers (*CD90/105/166*, *Vimentin* and *FGFR3*) with over 95% viability (Fig. 3c), indicating similar fundamental characteristics of gene expression of human CSPCs.

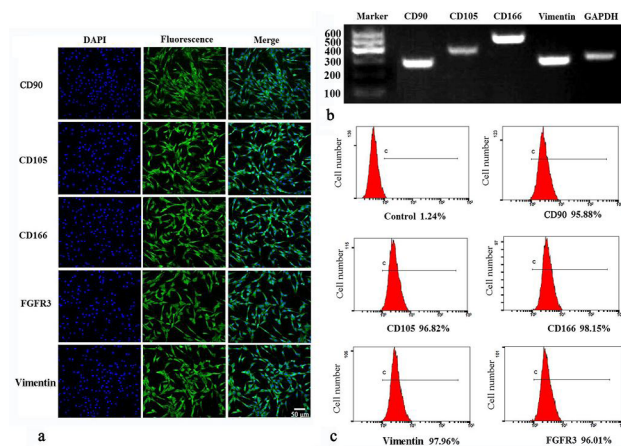


Fig. 3. Characterization of biomarkers on CSPCs. (a) The expression of biomarkers *CD90/105/166*, *Vimentin*, and *FGFR3* on CSPCs were analyzed by immunofluorescence (bar, 50  $\mu$ m); (b) RT-PCR was used to detect mRNA expression levels of cell surface antigens of CSPCs; (c) CSPCs were colabeled with biomarkers and analyzed by flow cytometry, and the positive rates of surface antigens of CSPCs were all above 95%.

#### Adipogenic differentiation

In the process of adipogenesis, the adipogenic potential of CSPCs was confirmed by Oil Red O staining and RT-PCR analysis. The morphology of CSPCs was gradually transformed from spindle shape into considerably flat shape, and followed by accumulation of lipid droplets under adipocyte-inducing conditions for 12 days (Fig. 4a). And, the lipid droplets were stained by Oil Red O staining (Fig. 4a). Likewise, adipogenesis was further confirmed by the expression of adipogenic marker genes of *PPAR- $\gamma$*  and *LPL* by RT-PCR (Fig. 4b).

#### Osteogenic differentiation

After incubation in osteoblast-inducing (OID) conditions for 14 days, the CSPCs showed osteogenic differentiation with calcium deposit nodules and increased expression of osteogenic marker genes. In the process of osteogenesis, fusiform or polygonal cell shape was

transformed into oval. Mineralization was visualized by Alizarin Red S staining (Fig. 4c). RT-PCR analysis showed that the increased expression of osteoblast differentiation marker genes, such as *COL1A2* and *SPPI* (Fig. 4d).

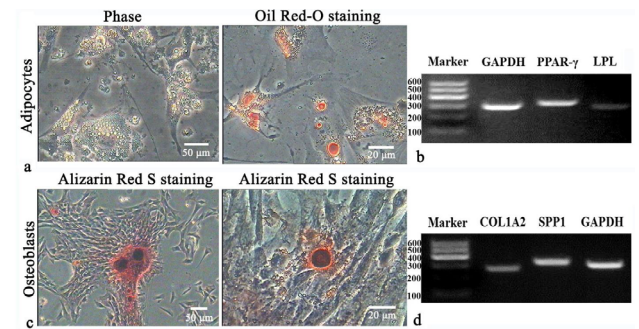


Fig. 4. Differentiation multipotency characteristics of cartilage-derived CSPCs. (a) Intracellular lipid droplets of adipocytes accumulated in cytoplasm (bar, 50  $\mu$ m) and were positive for Oil Red-O staining (bar, 20  $\mu$ m); (b) Adipogenic gene expression of *PPAR- $\gamma$*  and *LPL* was analyzed by RT-PCR. (c) Calcium deposits (bar, 50  $\mu$ m) within induced osteoblasts were positive for Alizarin Red S staining (bar, 20  $\mu$ m); (d) Osteogenic gene expression of *COL1A2* and *SPPI* was analyzed by RT-PCR.

#### Chondrogenic differentiation

After induction of chondrogenic differentiation for 21 days, the induced CSPCs formed numerous primmorphs or colonies, which were positive to Alcian Blue (Fig. 5a). The induced CSPCs in 3D monolayer cultures were smooth and iridescent, and stained positively for Alcian Blue and Toluidine Blue (Fig. 5c), suggesting the presence of glycosaminoglycans. In addition, the expression of chondrocyte-specific genes were further detected by RT-PCR, including *Vimentin*, *ALCAM*, *SOX9*, *ITGB1*, *ACAN*, *FGFR3*, and *COL2A1* (Fig. 5b). All these data clarify the chondrogenic differentiation potential of CSPCs.

## DISCUSSION

The poor self-repair nature of articular cartilage has partially posed a potential barrier to the development of tissue-engineered cartilage. On the other hand, compared with various tissues, cartilage tissues are theoretically thought to be easier to replicate attributable to their avascular and aneural characteristics (Jessop *et al.*, 2020). Nevertheless, the acquisition of original cartilage remains a challenge in the field of cartilage regenerative medicine (Abbott and Kaplan, 2015). Of interest, for chondrogenic potential, cartilage derived CSPCs that produce the stable cartilage with physiological relevance would be inherently advantageous over implants from unrelated cell

sources, such as mesenchymal stem cells (MSCs) from bone marrow, skeletal muscle and adipose tissue (Seol *et al.*, 2012; Pizzute *et al.*, 2015; Bauge and Boumediene, 2015). Previous study showed that a contributing signaling molecule NGF is tightly tied to the pathogenesis of OA, via involvement in OA joint pain and cartilage structural changes, with CSPCs as target cells (Jiang *et al.*, 2015). Further work will establish a suitable animal model to elucidate dual effects of the NGF signaling events by an *in vivo* study, suggesting a potential therapeutic treatment for OA or related joint diseases. To our knowledge, the present study is the first to describe stable biological characteristics and multilineage potential of Tibetan mastiff CSPCs.

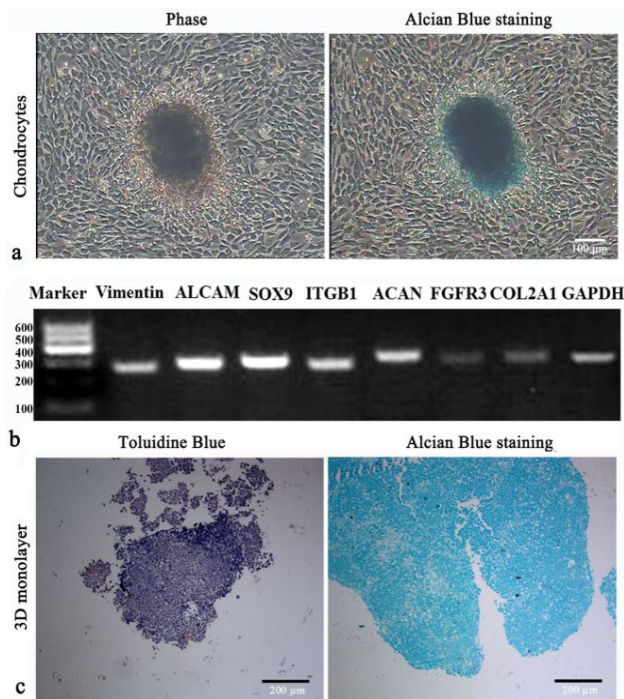


Fig. 5. Chondrogenesis derived from CSPCs by 2D and 3D culture. (a) Chondrocytes induced from CSPCs formed primmorphs, and were positive for alcian blue staining (bar, 100  $\mu$ m); (b) Chondrogenic gene expression of *Vimentin*, *ALCAM*, *SOX9*, *ITGB1*, *ACAN*, *FGFR3*, and *COL2A1* were analyzed by RT-PCR, respectively. (c) Chondrogenic differentiation of CSPCs in 3D monolayer culture was stained positive with toluidine blue and alcian blue staining, which depicted the presence of glycosaminoglycans (bar, 200  $\mu$ m).

The acquisition of desired cell types with a sufficient amount *in vitro* has always been a crucial scientific problem of stem cell and regenerative medicine. Herein, CSPCs positively responded to adipogenic, osteogenic, and chondrogenic induction, indicating their differentiation

multipotency. Adipogenic differentiation is initiated with preadipocyte phase, followed by further differentiation into mature adipocytes (Lee *et al.*, 2011). The interplay of adipogenic factors such as dexamethasone, insulin and isobutyl methylxanthine may facilitate preadipocytes terminal differentiation (Zhang *et al.*, 2018; Kim *et al.*, 2018). The transcription factor PPAR- $\gamma$ , a member of type II nuclear hormone receptor family, is known to exert a positive effect on phenotypic stability of mature adipocytes (Hallenborg *et al.*, 2014; Li *et al.*, 2015b). Moreover, lipogenic gene *PPAR- $\gamma$*  participates in the regulation of the storage and release of lipid during adipose differentiation through the interaction with FABP4. While family members PPAR- $\alpha$  and PPAR- $\beta$  have little effect on adipogenesis (Brun *et al.*, 1996; Lee *et al.*, 2019). The present study demonstrated the positive expression of adipogenic differentiation marker PPAR- $\gamma$  in Tibetan mastiff cartilage derived CSPCs.

Coordinated activity of osteogenic factors (L-ascorbic acid, dexamethasone, and  $\beta$ -glycerophosphate) may contribute to the alteration of spindle-shaped cells to cobble-stone-shaped osteoblasts (Zhang *et al.*, 2018). IGF and BMP signaling, as positive regulating factor of osteogenic differentiation, implicate possible molecular regulatory mechanism for treating bone loss at aging process (Chen *et al.*, 1998; Kang *et al.*, 2009; Wabitsch *et al.*, 1995; Yakar *et al.*, 2005). Differentiation related gene *SPPI* expressed in the late phase of osteogenesis and tightly connected to the mineralization of bone matrix (Jiao *et al.*, 2020). Our study herein presented the formation of mineralized bone nodules and *SPPI* expression.

Bioactive TGF- $\beta$ 3 could exert a positive effect on chondrocytes, and sequential coordination of heparin and chondrogenic factor TGF- $\beta$ 3 could maintain the differentiation stability of stem cells (Lei *et al.*, 2014). *SOX9*, the important marker of mature chondrocyte, plays a significant role in contributing to cartilage formation (Kim *et al.*, 2011; Park *et al.*, 2013). Herein, we detected the positive expression of *SOX9*, which suggested that CSPCs were successfully induced to differentiate into chondrocytes by chondrogenic medium supplemented with TGF- $\beta$ 3.

## CONCLUSION

In conclusion, we successfully isolated a novel type of cartilage stem/progenitor cells from Tibetan mastiff *in vitro*, and demonstrated its genetic characteristics and multilineage differentiation potential. The present study also illustrates the therapeutic potential of CSPCs and may serve as a fascinating candidate cell for regenerative therapies. However, more detailed studies are warranted

to decipher the precise molecular mechanisms of CSPCs differentiation and cartilage regeneration, as well as to probe into the difference of CSPCs between Tibetan mastiff and other species in future.

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## Statement of conflict of interest

The authors have declared no conflict of interest.

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