Introduction

Low back pain (LBP) is common and a significant burden to the health care system. Population-based study shows that 40% under 30 years of age and 80% by age 50 have lumbar disc degeneration [1]. Although the precise etiology of LBP has yet to be determined, it is associated with degeneration of the intervertebral disc (IVD) [1]. While IVD degeneration is complex and considered a multifactorial process, age, genetics, trauma, overweight/obesity, and occupation have been suggested to be risk factors [2]. Biological approaches that deliver growth factors or so called mesenchymal stromal cells derived from a variety of tissue origins, including those from bone marrow (BM), adipose tissues or synovium, have been reported to arrest IVD degeneration or even partially restore its function though in most cases, the regeneration properties of such factors or cells were not clearly characterized [3-5], although our recent study of a bone marrow stromal cell-induced disc regeneration model suggests that it involves an activation of a native but yet uncharacterized stem cell population [6].

Recent pilot studies suggest that cells derived from human degenerated nucleus pulposus (NP) or annulus fibrosus (AF) have multi-differentiation potential and possess *in vitro* characteristics of mesenchymal stromal cell [7-9]. These observations implicate that intervertebral disc (IVD) may contain stem-like cells which possibly responds to stimuli and becomes activated in the degeneration or induced-repair processes. However, whether the populations of cells are derived from degeneration-induced non-IVD cells or are in fact specialized IVD-specific progenitor cells remains elusive. Moreover, it is not known whether their homeostasis is under control of defined niche components similar to other systems.

IVD degeneration is linked to a reduction of proteoglycan content and a deposition of

fibrocartilaginous matrix, which results in an overall structural and functional failure owing to loss of compressive strength and shock-absorbing property [10]. Biglycan or decorin belongs to the small leucine-rich proteoglycans (SLRP) which are reported to be key molecules in modulating the physiological and pathological process in many tissues by binding to collagens, growth factors, and other matrix components [11-14]. Mutation of SLRPs may predispose to degenerative disorders such as Ehlers-Danlos syndrome, corneal diseases, osteoarthritis, and muscular dystrophy [11-14]. Interestingly, the level of biglycan and decorin expression showed a unique pattern with age and IVD degeneration, in which their expression decreased progressively with age and with severity of degeneration [15]. Notably, biglycan-knockout mice developed an early episode of disc degeneration [16]. Thus, it is worth to investigate whether biglycan or decorin is responsible for the IVD tissue specific progenitor cell-supportive activities.

Here, we report the isolation of a subpopulation of IVD cells from Rhesus monkey that possesses progenitor characteristics, including clonogenicity, multipotency and retaining differentiation potential after extended expansion *in vitro* and *in vivo*. Our results also suggest that under low oxygen tension, the survival of these cells, termed disc progenitor cells (DPCs), depend on SLRP, which have been previously demonstrated to be essential extracellular matrix components in IVD and are associated to IVD degeneration [16]. Altogether, this study provides support to the existence of progenitor cells native to and normally quiescent in IVD, and a ~~novel~~ function of proteoglycans as a niche component in promoting their survival under the intrinsic microenvironmental stress.

Materials and Methods

Cell Isolation and Culture

The protocol for this study was approved by the Animal Experimentation Ethics Committee of Sichuan University. The health of the animals was monitored at all times. The L6-L7 and L7-S1 IVDs of healthy adolescent male *Rhesus Macaque* (Pingan Animals Breeding & Research Base, Chengdu, China), which are 3–5 years old and weighed 3–5kg, were harvested as previously described [7-9]. Briefly, samples were cleared of extraneous ligaments and the NP was separated from the AF. The tissues were digested by 100U/ml collagenase type II (Sigma-Aldrich, St Louis, MO, USA) for 2.5 hours in spinner flasks at 37°C. The primary cells were then cultured in standard culture dish in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 25μg/ml penicillin/streptomycin (Gibco, Carlsbad, CA, USA) under a humid atmosphere containing 5% CO_2 at 37°C until colonies were formed. Proliferative cells from NP tissue are described as NPC^P, and proliferative cells from AF tissue as AFC^P. Colonies (n=5) from NP cells were randomly isolated by trypsinization, which were in similar morphology, and expanded as clonal NP cells (clonal NPC) (17). BM was isolated from the ilium of the same healthy adolescent male *Rhesus Macaque* using heparin as anticoagulant. The cells were suspended in DMEM supplemented with 10% FBS and 25μg/ml penicillin/streptomycin (Gibco, Carlsbad, CA, USA) and centrifuged by Percoll density gradient centrifugation (500 x *g*, 20 mins). The cells in the middle layer were harvested and washed twice with DMEM medium. After 24 hours culture, non-adherent cells were removed and the remaining adherent cells were cultured for further use.

Multipotency Assay

The *in vitro* multi-differentiation potential of cells toward osteogenesis, adipogenesis and chondrogenesis was assessed using the StemPro differentiation kit (Invitrogen, Carlsbad, CA, USA). Alizarin red S (Sigma-Aldrich, St Louis, MO, USA) staining was used for detection of calcium accumulation. Oil red O (Sigma-Aldrich, St Louis, MO, USA) staining was used to reveal lipid droplets. Toluidine blue (Sigma-Aldrich, St Louis, MO, USA) staining was used to detect sulfated polysaccharides in cell pellets.

Quantitative Gene Expression Analysis

Total RNA was extracted using the Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA was obtained using the Super Script Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) as described in manufacturer's manual. Gene expression levels were examined by real-time polymerase chain reaction (qPCR) with IQ^{Tm} SYBR$^{®}$ Green Super mix Sample Kit (Bio-Rad, USA) and expressed as delta-delta CT in natural logarithm (Log 10). Non-induced cells were used as control and *Gapdh* as housekeeping gene for the normalization of qPCR results. See Supporting Information Table S1 for details of amplification primers.

Flow Cytometry

To study cell surface marker expression, cells were suspended at 1–5×10^7/ml in ice cold PBS, 10% FBS, and 1% sodium azide, and then immunolabeled with monoclonal mouse anti-human antibodies (1:200 dilution) against CD166, CD106, CD271, CD9, CD146, CD44, CD90, CD34 and CD45 (Abcam, Cambridge, MA, USA), or isotype-matched mouse immunoglobulin controls for 30 min at room temperature, followed by incubation with FITC-conjugated anti-mouse IgG (1:200 dilution) (Abcam,

Cambridge, MA, USA) for 30 min at room temperature. Labeled cells were analyzed by flow cytometer cytomics FC 500 with the CXP software (Beckman coulter, USA).

Tagging Cells for *In Vivo* Test

Cells for transplantation into mice were tagged with green fluorescent protein (GFP). Transduction was performed using lenti-EF1α-GFP lentivirus (Applied Biological Materials, China) in 6-well plates at 2ml/well (0.51-0.91 MOI [pfu/cell]) in the presence of 8ug/ml Polybrene (Sigma-Aldrich, St Louis, MO, USA). The viral supernatant was removed after 6 hours and replaced with complete medium (10% FBS in DMEM). Transduced cells were incubated for 72 hours at 37°C and then selected by 0.6 μg/ml puromycin (Sigma-Aldrich, St Louis, MO, USA) for 10-15 days to obtain GFP positive clones for further expansion and subsequently used in the *in vivo* differentiation tests.

***In Vivo* Differentiation Test**

The procedures were performed in accordance with specifications of a small-animal protocol approved by the institutional board of the Sichuan University. Cells were at first seeded on hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic (HA: TCP=3:2, a gift from Engineering Research Center in Biomaterials, Sichuan University, China) at 10^5 cells per particle (sized at 1-2 mm diameter). The composite (2~3 per mouse) was then transplanted subcutaneously into the dorsal surface of 8-12 weeks old male immunocompromised athymic nude mice (n=5) (BALB/c-Foxn1nu, Beijing HFK Bio-Technology Co., China) as described previously [18]. For re-derivation of transplanted cells, the subcutaneous implant was aseptically harvested 12 weeks after transplantation, minced and suspended in 100U/ml collagenase type II (Sigma-Aldrich, St Louis, MO, USA) and digested for 30 mins

at 37°C. The suspension was filtered and washed before plating in DMEM with 10% FBS under a humid atmosphere containing 5% CO_2 at 37°C.

Histological and Immunohistochemical Analysis

Freshly dissected tissues were fixed in 10% formalin for 24-48 hours and decalcified in ethylenediaminetetraacetic acid (EDTA) for 45 days at room temperature. The samples were embedded in paraffin and sectioned at 7 μm thickness using a microtome. The paraffin-embedded sections were stained with H&E and Masson trichrome. Cells cultured at passage 3 were treated with hypoxia and then immunolabeled with monoclonal mouse anti-human HIF-1α or HIF-2α (1:200), rabbit anti-human HIF-3α, or matched control antibodies (Abcam, Cambridge, UK) at 37°C for 1 hour, followed by incubation with polyclonal goat anti-mouse or anti-rabbit Texas Red-conjugated antibody (1:200) for 0.5 hour (Abcam, Cambridge, MA, USA). Cells were then counterstained with DAPI (4′,6-diamino-2-phenyndole) for 5 mins (1μg/ml). Signals were observed and captured under inverted fluorescence microscope (OLYMPUS IX71).

Cell Growth and Apoptosis Assay

Cell proliferation was examined by the BrdU Cell Proliferation ELISA (K-ASSAY@, US). Briefly, 100 μl of cell suspension at $2x10^5$/ml was mixed with 20 μl of diluted BrdU label solution, incubated in 37°C for 2 hours before incubation with BrdU antibody (100μl/well) for 1 hour at RT. The diluted anti-BrdU peroxidase-conjugated antibody (1:2000) was added and visualized by TMB Substrate (100μl/well). The absorbance at a wavelength of 450 nm was measured with microplate reader uQuant (BioTek, USA). The actual cell number was estimated by measuring the absorbance and then conversion with the standard

curve. Using the FITC-Annexin V/PI kit (Invitrogen, Carlsbad, CA, USA), apoptosis was performed according to Lee and Fujita et al [19, 20] which detects the presence of phosphatidylserine on the outer leaflet of plasma membrane. Cells that are annexin V+/PI− and annexin V+/PI+ were regarded as early apoptotic and complete apoptotic state respectively and distinguished by inverted fluorescence microscope (OLYMPUS IX71) and flow cytometer cytomics FC 500 (Beckman Coulter, USA).

Colony Formation Assay

The freshly isolated cells were plated at a range of 1-10 cells/cm^2 and cultured in complete DMEM medium with 10% FBS for 3 weeks. The cells were fixed in 70% ethanol for 5 mins and then stained with hematoxylin (Sigma-Aldrich, St Louis, MO, USA). Clusters that have more than 50 cells were qualified as colony [21].

Western blot analysis

Cells were harvested with trypsin-EDTA and pelleted by centrifugation (500 x *g*, 20 min). The supernatant was removed and the cell pellet was dried as much as possible. The nucleoprotein was extracted by NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo scientific, Waltham, MA, USA). Nucleoproteins were fractionated by SDS-PAGE (9%) and transferred onto polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membranes were incubated overnight at 4°C with a blocking solution (5% nonfat dry milk with PBS) and further incubated with monoclonal mouse anti-human HIF-1α or HIF-2α, or rabbit anti-human HIF-3α (Abcam, Cambridge, UK) at room temperature for 1 hour. Mouse anti-human GAPDH (Abcam, Cambridge, UK) was used as internal reference for equal protein loading. The membranes were incubated with anti-mouse or anti-rabbit

horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected using Pierce ECL Plus Western Blotting Substrate (Thermo, US) and captured by Bio-Rad Gel Doc™ XR system.

Statistical Analysis

Qualitative assays were derived from at least three independent experiments. The gene expression level among different genes and groups was assessed using the two-way ANOVA and Least Significant Difference (LSD) test, comparing experimental to control groups. The cell number counting among different groups was examined by SNK test. All the statistical tests were performed using the SPSS (version 11.0) program package. A *P* value <0.05 was determined as statistical significance.

Results

~~**IVD-derived cells possess clonogenic capability**~~**Clonogenicity of IVD derived cells**

We adopted a Rhesus macaque model as human and monkey share highly similar spine anatomy, mechanics, and physiology [22-25]. To test whether IVD-derived cells are clonogenic, we generated single-cell suspensions from NP and AF tissue isolated from adolescent monkey IVDs (Fig. 1 A). It is found that both NP and AF cells attached onto the plate and remained quiescent for 6-10 day before they started dividing. After 14-20 d, 5.2±0.6% of NP and 3.8±0.8% of AF cells formed colonies of heterogeneous size and cell density (Fig. 1B), reflecting different rates of proliferation. By three weeks of plating, it is demonstrated that the clonogenicity of these proliferative NP cells (NPC^P) was stronger than proliferative AF cells (AFC^P) (Fig. 1C).

~~**Proliferative NP and AF cells have stem characteristics**~~**Stem characteristics of proliferative NP and**

AF cells

To test if NPC^P and AFC^P are multi-potent, they were subjected to culturing in defined medium to induce adipogenesis, chondrogenesis and osteogenesis, followed by assessment of the cell phenotype. Cytochemical staining was performed to visualize the lipid droplets generated in adipogenic condition, sulfated polysaccharides generated in chondrogenic condition, and calcium accumulated in osteogenic condition (SI Fig. 1A). Real-time PCR analysis showed that the expression of the adipogenic markers peroxisome proliferator-activated receptor-gamma (*Ppar-gamma*) and adiponectin (*AdipoQ*), the chondrogenic markers sex determining region Y-box 9 (*Sox9*) and collagen II (*Col2a1*), and the markers osteopontin (*Opn*), also known as secreted phosphoprotein 1 that encodes an extracellular structural component of bone, and collagen I (*Col1a1*) in NPC^P and AFC^P was markedly upregulated after culturing in defined induction media (Fig. 2A). NPC^P and the clonal NPC^P have comparable differentiation efficiency to the BMSCs, whereas the BMSCs have higher differentiation efficiency than the AFC^P (Fig. 2A).

To determine the osteogenic differentiation potential *in vivo*, cells were pre-exposed to osteogenic induction medium for 1 week and transplanted subcutaneously with hydroxyapatite/tricalcium phosphate as carrier (SI Fig. 1B) into immunocompromised mice. After 12 weeks, bone tissue formation was observed on the surface of HA/TCP in all groups (Fig. 2B), suggesting the transplanted cells have potential of osteogenic differentiation *in vivo*. Interestingly, adjacent to the ectopic bone tissue in the NPC^P and clonal NPC^P group, we observed structures that contain large vacuolated cells intercalated by collagenous fibrous septa (visualized by Masson's trichrome staining), resembling nonmyxoid incipient chordoma (benign notochordal cell tumors) [26] (Fig. 2B).

We further investigated whether NPC^P and AFC^P retain differentiation potential after extended

expansion *in vitro* and *in vivo* (Fig. 2C). The NPC^{P} and AFC^{P} were tagged with GFP by lentiviral transduction and transplanted into the immunocompromised mice (SI Fig. 1C). By 12-14 weeks of transplantation, the transplant-derived cells retained their ability to proliferate and form colonies *in vitro* (Fig. 2D). The number of GFP^{+} colonies that were generated from the cells released from the transplant is about 2% by calculation of GFP^{+} colonies (more than 50 cells) under immunofluorescene microscopy, as well as ability to differentiate into osteoblasts, adipocytes and chondrocytes in *in vitro* differentiation assays (SI Fig. 1D). Re-transplanting the expanded cells into mice resulted in the formation of vascularized hyperplastic nodules that resemble chordoma-like structure without bone formation. These data suggested that after extended expansion *in vitro* and *in vivo*, the cells still retained clonogenic, and multipotent differentiation potential, and that the expansion and re-transplantation process may have facilitated a selection of disc progenitor-like cells. Altogether, our findings demonstrated that NPC^{P} and AFC^{P} shared the characteristics of progenitor cells. We named them as disc progenitor cells (DPCs).

CD146 expressioned ionas a marker for DPC^{NP}

To investigate the differences between the phenotype of DPCs and BMSCs, we first profiled and compared their expression of surface marker genes at the mRNA level. Data showed that both the NP-derived DPCs (DPC^{NP}) and AF-derived DPCs (DPC^{AF}) expressed the common BMSC markers, including CD44, CD166, CD90, CD146 and HLA-DR (Fig.3A and B). These cells also expressed Notch homology 1 (*Notch1*), which has been suggested as a marker for chondrogenic progenitor cells [27, 28]. DPC^{NP} were negative for CD90 and CD271, whereas DPC^{AF} were negative for CD106, CD29 and CD271. In comparison with BMSCs, CD44 and CD146 expression was significantly higher in a subset of DPC^{NP} and CD90 expression was higher in a subset of DPC^{AF}. However, at the protein level, flow

cytometry analysis showed that the expression of CD90, CD146 and CD166 were all significantly higher in DPC^{NP} compared to BMSCs (Fig. 3C). Both DPC^{NP} and DPC^{AF} showed lower expression in CD44 compared to BMSCs. DPC^{NP} appeared heterogeneous in phenotype, while DPC^{AF} and BMSCs showed more homogeneous expression of markers. Altogether, these data suggest that while DPCs express many BMSC markers, they exhibited some unique characteristics. Notably, the expression of CD146 may differentiate DPC^{NP} from BMSCs or DPC^{AF}.

Activities of DPCs ~~are incompetent to adapt to~~under oxygen tension

Owing to the avascular feature of the IVD, IVD cells live under hypoxic condition and are able to deal with low oxygen tension for survival and function [29]. Theoretically, this should also apply to any stem or progenitor cells that resided in IVD. We hypothesized that DPCs are able to maintain vitality under oxygen tension and therefore tested their proliferative activities under hypoxia. We found that DPCs, as well as BMSCs, showed significantly higher proliferation rate at an oxygen tension of 3.5%, which mimics the oxygen level within the IVD (30) (Fig. 4A). However, DPCs showed an abrupt deceleration of growth from day 5 onwards in this hypoxia condition. By day 10 of hypoxia, DPC^{NP} appeared apoptotic in morphology (Fig. 4B). Flow cytometry analysis indicated that the DPC^{NP}, and to a lesser extent DPC^{AF}, increased in Annexin V positivity under low oxygen tension (26.69% DPC^{NP} and 9.04% DPC^{AF}), suggesting hypoxia indeed induced apoptosis (Fig. 4C). Propidium iodide (PI) staining confirmed the presence of apoptotic cells. Consistent with previous report that BMSCs can resist hypoxia [31], BMSCs did not show sign of apoptosis under low oxygen tension (0.13%). Hypoxia-inducible factors (HIFs) are essential regulatory transcription factors for cells to adapt to low-oxygen tension [32, 33]. We tested whether the differential response of DPC^{NP} to hypoxia is related to the activation of

HIF-1α, the major HIF molecule that mediates hypoxic response. Data showed that HIF-1α expression level was significantly increased in the DPC^{AF} and the BMSCs under hypoxia (Fig. 4D). In contrast, while basal expression of HIF-1α is detected in the DPC^{NP}, its level remained constant under hypoxia (Fig. 4D). These findings suggest that the incompetency of DPC^{NP} to adapt to oxygen tension may be in part due to inability to upregulate HIF expression.

Effect of SLRPs ~~support survival of~~on DPCs under hypoxia

We postulated that the ECM in IVD forms a unique niche that regulates survival of DPCs. IVD composes largely of collagen and proteoglycan in particular collagen I and II, and small leucine-rich proteoglycans (SLRPs) decorin and biglycan [16]. We tested the growth of DPCs cultured on these ECM substrates for 7 days under hypoxia. We found that DPC^{NP} showed significantly higher proliferation rate on biglycan (Fig. 5A). DPC^{AF} showed slightly higher rates on collagen II, decorin, and biglycan (Fig. 5A). These suggest that SLRPs may possibly function to promote the maintenance of DPCs, especially DPC^{NP}. In the specialized microenvironment of IVD tissue, disc cells express HIFs for survival and tissue homeostasis [32, 33]. Since DPCs underwent apoptosis under extended hypoxic culture, we tested whether culturing DPCs on the SLRPs can modulate apoptosis induced by low oxygen tension [34]. Under hypoxia, the number of the apoptotic DPC^{NP} significantly decreased when cultured on biglycan or decorin (from 26.69±0.45% to 10.97±0.11% and 12.58±0.10%, respectively) (Fig. 5B). A similar inhibition on DPC^{AF} apoptosis was also observed (from 9.04±0.09% to 5.99±0.05% on biglycan and to 5.86±0.05% on decorin) (Fig. 5B). We further tested if the suppression of apoptosis is related to HIF activation/stabilization. By day 6 of culture, DPC^{NP} cultured on control surface (no substrate) displayed constant expression of HIF-1α, HIF-2α □□and HIF-3α □□under normoxia and hypoxia (Fig. 6A and

B). However when cultured on biglycan or decorin, DPC^{NP} showed an increase in expression for HIF-1α and HIF-2α □in response to hypoxia. DPC^{AF} were found positive for HIF-1α□□ and HIF-3α□□ but not HIF-2α□□ in normoxia, and the expression of HIF-1α was upregulated in the presence of biglycan or decorin in hypoxia (Fig. 6C and D). Expression of HIF-1α, but not HIF-2α□□ or HIF-3α, was induced in BMSCs cultured under hypoxia and the induction was not influenced by the presence of biglycan or decorin (Fig. 6E and F). These data therefore suggest that SLRP has a critical role in promoting survival of DPCs, in particular DPC^{NP}, under low oxygen tension by unblocking HIF induction/stabilization.

Discussion

In this study, we describe the isolation and culture of IVD cells from the Rhesus macaque, which is one of the bipedal non-human primate models that closely resemble to human in genetics, upright biomechanics, and the prevalence of age-related disc degeneration [22-25]. We have isolated a group of cells from normal IVD possesses characteristics of progenitor cells including clonogenicity as well as a tendency to differentiate into multiple cell lineage and retain differentiation potential after extended expansion *in vitro* and *in vivo*. We show that, similar to BMSCs, both the NP- and AF-derived DPCs possess multiple cell lineage differentiation capability *in vitro* and ability to differentiate in a subcutaneous transplantation model. While the DPCs generally express BMSCs surface markers, they are in higher expression of CD9, CD44, and CD146, and not expressing CD90 and CD271 in DPC^{NP} and CD29, CD106, and CD271 in DPC^{AF}. We postulate that the inconsistency between the protein and RNA expression of surface markers might be related to suboptimal specificity or cross-immunoreactivity of the primary human antibodies to monkey antigens. Nevertheless, the finding for CD146 is consistent in

the profiling assays, suggesting that it is a potential marker for DPC^{NP}. CD146 has been highlighted as a pericyte marker associated to stem cell characteristics [35]. It has been associated with a variety of cells with stem or progenitor properties, for example pericytes, endometrial cells, endothelial cells, and dental pulp cells [36-39]. The expression of CD146 therefore further supports a stem-like function of the DPC^{NP}.

Microenvironment, or niche, can modulate differentiation, self-renewal and a balance between quiescence and proliferative state of stem cells [40, 41]. While it is reported that supportive cells participate in the modulation and form part of the stem cell niche [42, 43], ECM proteins also have a major contribution to the niche in many tissues [11, 44, 45]. Our study suggests that the SLRPs may act as a unique niche component to regulate the activities of stem cells through HIF. HIFs are heterodimeric transcription factors composed of an α and $\alpha\beta$ unit and are crucial for a range of cellular functions, including response to stressors, angiogenesis and development [46]. HIFs also have strong anti-apoptotic effects and are involved in the regulation of stem cells in response to oxygen stress, such as embryonic stem cells, neural stem cells and glioblastoma stem cells [46-50]. Cells within IVD are under hypoxia and therefore presumably require HIF-related mechanisms to overcome the oxygen tension in order to survive and function. Our findings imply that regulation of HIFs activation or stabilization may be a key element that governs the homeostasis of DPCs, especially DPC^{NP}, under hypoxic stress. This is consistent with recent finding that notochordal NP cells, which are thought to be progenitor-like, demand more energy and are less resistant to nutritional stress, such as low oxygen tension, than chondrocyte-like mature NP cells [51]. Whether DPC^{NP} are in fact derivatives of the notochordal cells warrants further investigation. Interestingly, in the DPC^{NP} transplantation study, we observed chordoma-like structure

consisting of layers of vacuolated eosinophilic cells with eccentrically located nuclei and lack of myxoid matrix. Chordoma has been suggested to be arisen from notochordal cells [26]. In clinical cases, incipient chordomas may coexist with benign notochordal cell tumors [52]. We propose that chordomas may possibly be derived from descendents of notochord cells in form of DPCs which have migrated to ectopic sites during development and are activated under a favorable microenvironment.

Our findings imply an important role of SLRPs in regulating homeostasis of tissue-specific stem cells within hypoxic microenvironment. ~~To our knowledge, this is the first early report on the regulation of hypoxic survival of skeletal cells by SLRPs.~~ Previous studies have shown that biglycan or decorin can bind and modulate the transforming growth factor β (TGF- β)/bone morphogenetic protein 2 (BMP-2) to activate SMAD or the MAPK pathways [53, 54]. TGF-β can enhance HIF-1α translation by TGF-β receptor (ALK5) kinase activity in kidney cells and BMP-2 can increase the HIF-1α protein levels in periosteal cells via the MEK/ERK pathway [55]. We propose the biglycan or decorin might possibly enhance HIF translation or stabilization in DPCs in response to oxygen stress via interaction with TGF-beta or BMP signaling.

Our results suggest that an endogenous progenitor population exists in the healthy IVDs with similar characteristics to the MSC-like populations reported in degenerated IVDs. It has been previously reported that cell cultures derived from degenerated human IVD express many common MSC surface markers and are potent in differentiating into chondrogenic, osteogenic, and to some extent adipogenetic lineages [7, 8]. Furthermore, similar multipotency of AF cells have been isolated from scoliotic human IVD [9]. These reports relied on the common cell surface markers recommended by the International

Society for Cell Therapy (ISCT) that are expressed in virtually all fibroblastic cells, many of which are not defined as stem cells. In addition, *in vitro* differentiation assays employed in these reports may be prone to artifact and not faithfully indicate the differentiation capacity of cells. Moreover, no clonal analysis or other rigorous assays have been performed to test self-renewal *in vitro* and *in vivo*. Consistent with these reports, our findings have supported that DPCs possess differentiation potential after expansion *in vivo* and *in vitro*. A recent study also reported that human NP contains progenitor cells which exhibit self-renewal capacities *in vitro* and *in vivo* and express both tyrosine kinase receptor (Tie2) and disialoganglioside 2 (GD2) ($Tie^{+}GD2^{+}$) [56]. A cognate Tie2 ligand can activate these NP progenitor cells. It is worth to test the expression of Tie2 and/or GD2 in the DPCs, especially the DPC^{NP}, to further investigate if they are heterogeneous in phenotype and composed of subpopulations derived from different differentiation stages in a similar lineage as proposed for the $Tie^{+}GD2^{+}$ population of cells [56].

Conclusions

First, the findings of endogenous pool of cells with progenitor characteristics within normal IVD may facilitate the understanding of IVD homeostasis, especially to confirm whether IVD has the population of tissue-specific progenitor cells or to comprehend how the endogenous cells can be harnessed by critical niche components. Second, combined with tissue engineering or biomaterials, the understanding of these disc niche components may promote the management of IVD diseases. Whether the DPC niche components can become ~~a novel~~ biomaterials in disc regeneration deserve further investigation.

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Disclosures

All authors state that they have no conflicts of interest.