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Original Article

Leptin receptor–expressing cells represent a distinct subpopulation of notochord-derived cells and are essential for disc homoeostasis

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ABSTRACT

Background/objective: Intervertebral disc degeneration (IDD) remains to be an intractable clinical challenge. Although IDD is characterised by loss of notochordal cells (NCs) and dysfunction of nucleus pulposus (NP) cells, little is known about the origin, heterogeneity, fate and maintenance of NCs and NP cells, which further stunts the therapeutic development. Thus, effective tools to spatially and temporally trace specific cell lineage and clarify cell functions in intervertebral disc (IVD) development and homoeostasis are urgently required.

Methods: In this study, NP specimens were obtained from 20 patients with degenerative disc disease or scoliosis. LepR-Cre mice was crossed with R26R-Tdtomato mice to generate LepR-Cre; R26R-Tdtomato mice, which enabled fate-mapping of NPs from embryo stage to late adult. LMNA G609G/G609G mice was used to determine the effect of premature-aging induced IDD on LepR NPs. X-ray imaging was used to measure lumber disc height of mice.

Results: Here, we provide the first evidence that the leptin receptor (LepR) is preferentially expressed in NCs at embryonic stages and notochord-derived cells in the postnatal IVD. By using R26R-Tdtomato fluorescent reporter mice, we systematically analysed the specificity of activity and targeting efficiency of leptin receptor-Cre (LepR-Cre) in IVD tissues from the embryonic stage E15.5 to 6-month-old LepR-Cre; Rosa26-Tdtomato (R26R-Tdtomato) mice. Specifically, LepR-Cre targets a distinct subpopulation of notochord-derived cells closely associated with disc homoeostasis. The percentage of LepR-expressing NP cells markedly decreases in the postnatal mouse IVD and, more importantly, in the human IVD with the progression of IDD. Moreover, both spine instability–induced and premature ageing–induced IDD mouse models display the phenotype of IDD with decreased percentage of LepR-expressing NP cells. These findings uncover a potential role of LepR-expressing notochord-derived cells in disc homoeostasis and open the gate for therapeutically targeting the NP cell subpopulation.

Conclusion: In conclusion, our data prove LepR-Cre mice useful for mapping the fate of specific subpopulations of IVD cells and uncovering the underlying mechanisms of IDD.

The translational potential of this article: The translation potential of article is that we first identified LepR as a candidate marker of subpopulation of nucleus pulposus (NP) cells and provided LepR as a potential target for the treatment of intervertebral disc degeneration (IDD), which have certain profound significance.

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Introduction

Low back pain is a common clinical problem that imposes a tremendous socioeconomic burden on the society. Most people older than 65 years in industrialised countries suffer from back pain, which is thought to originate mainly from degeneration of the intervertebral disc (IVD), specifically the inner nucleus pulposus (NP) or the innervation from either the IVD or annulus fibrosus [1–4]. IVD degeneration (IDD) is characterised by increased breakdown of the extracellular matrix, abnormal matrix synthesis, inflammation, and ingrowth of nociceptive nerves and blood vessels into the typically aneural and avascular tissue [5]. Particularly, the cellular mechanism behind the onset and progression of IDD is the loss of notochord cells (NCs) and dysfunction of NP cells [6,7]. However, little is known about the origin, heterogeneity, and cell fate of NCs and NP cells, and the tools for targeting specific cell populations in IVDs are poorly developed, which hinders the understanding of their role in maintaining the physiological function of the IVD and in causing the pathological onset of IDD.

With the genetically modified mice models widely used in recent decades, an increasing number of studies have been focussing on *in vivo* lineage tracing of NCs at embryonic stages and NP cells under pathological conditions. Sonic hedgehog-Cre (Shh-Cre) and Sonic hedgehog-Cre^{ERT2} (Shh-Cre^{ERT2}) were first used to map the fate of Shh-expressing cells, including those residing in the notochord. Choi and Harfe et al. [8,9] first indicated that all NP cells in postnatal life were descendants from the embryonic notochord. Later, Mccann et al. [10] used a notochord-specific Cre mouse line by targeting the homeobox gene Noto to trace the fate of NCs within the IVD, and they also found that both NCs and NP cells were derived from the embryonic notochord. In addition, Chen et al. [11] and Henry et al. [12] used Col2a1-Cre^{ERT2} and Aggrecan-Cre^{ERT2} knockin mouse lines, respectively, to investigate the cellular component of IVD cells. Recently, Zheng et al. [13] have systematically analysed Cre recombinase mouse lines targeting postnatal IVD cells by using Aggrecan-Cre^{ERT2}, Col2a1-Cre, Col2a1-Cre^{ERT2}, Shh-Cre, Shh-Cre^{ERT2}, and Serine protease 7-Cre (Sp7-Cre), which provides a good guidance of using different mouse lines as valuable tools to investigate functions of a specific cell type in IVD development and homoeostasis. However, we have limited knowledge so far on whether all NP cells derived from the notochord are homogenous and contain different subpopulations because the specific marker for the NP cell subpopulation is not well defined.

The leptin receptor (LepR) gene, a member of the obesity gene family, encodes the protein to identify and transport leptin [14,15]. Recently, LepR has been fully discovered as a potential marker of bone marrow mesenchymal stromal cells and periosteum-derived stem/progenitor cells [16,17]. Studies used LepR-Cre knockin mice crossed with Rosa26-Tdtomato mice to map the fate of LepR-expressing cells in the adult bone marrow and found that these cells were abundant during adulthood, although rare during puberty. In addition, LepR-expressing cells were reported to form osteoblasts, chondrocytes (under fracture), adipocytes (under irradiation), and fibroblasts [16,18–21], which indicates that LepR-expressing cells might emerge at a very early differential stage and possess characteristics of stem cells. We previously demonstrated the LepR-Cre–labelled subpopulation of periosteum-derived stem/progenitor cells, which predominantly modulated cortical bone formation during adulthood [17]. We also showed that LepR-expressing mesenchymal stromal/progenitor cells could be the therapeutic target for skeletal ageing [22]. However, it is unknown whether LepR-expressing cells exist in the IVD during puberty or at even early embryonic stages and serve as a candidate marker for notochord-derived cells.

In this study, we discovered that the LepR could be a new potential marker for notochord-derived cells. In addition, by using LepR-Cre; R26R; Tdtomato reporter mice, we found that the embryonic notochord directly gave rise to NP cells of the IVD. Importantly, we identified that NP cells were heterogenous, and LepR-expressing cells represented a distinct subpopulation of notochord-derived cells essential for disc homoeostasis and could be a therapeutic target for IDD.

Table 1
Demographic data of patients.

Patient no.	Age	Gender	Level	Pfirrmann grading
Grade II/III group				
1	6	M	L3/4	II
2	16	F	L3/4	II
3	13	F	L2/3	II
4	12	F	L3/4	II
5	25	M	L5/S1	II
6	45	F	L4/5	III
7	61	F	L4/5	III
8	53	M	L4/5	III
9	28	M	L4/5	III
10	32	F	L4/5	III
Grade IV/V group				
11	52	M	L4/5	IV
12	36	F	L4/5	IV
13	31	M	L4/5	IV
14	26	M	L4/5	IV
15	71	F	L4/5	V
16	33	M	L5/S1	V
17	34	M	L5/S1	V
18	54	F	L5/S1	V
19	42	F	L4/5	V
20	58	M	L4/5	V

F = female; M = male.

Materials and methods

Patient samples

NP specimens were obtained from 20 patients (10 men and 10 women; mean age = 36.4 ± 17.4 years) with degenerative disc disease or scoliosis. The degree of IDD was assessed according to the modified Pfirrmann grading system by magnetic resonance imaging [23]. All the discs of patients with different degeneration grades who had received posterior discectomy (microendoscopic discectomy) surgeries were harvested. The annulus fibrosus (AF) tissue was cut using sharp-pointed knives during fenestration discectomy. NP tissue was taken from the inner part of the IVD, which was gelatinous. Different disc regions were cleanly separated from each other and clearly distinguished for unambiguous dissection. All the information of Grade II (n = 5), Grade III (n = 5), Grade IV (n = 4), and Grade V (n = 6) samples are showed in Table 1. Ethics approval was obtained from the Institutional Review Board of Xijing Hospital of Fourth Military Medical University, and informed consent was obtained from each donor.

Animals and treatment

We purchased the *LepR-Cre* mice (stock no. 008320), *R26R-TdTomato* mice (stock no. 007909) strain from the Jackson Laboratory (Bar Harbor, ME, USA). Lamin A (LMNA) G609G/G609G mice, generated as described previously [24], were kindly provided by Professor Baohua Liu (Shenzhen University, China). All animals were maintained under pathogen-free conditions at the Experimental Animal Centre of Fourth Military Medical University. We did not observe any abnormality in wild-type mice that were treated with the vehicle alone. The body weight of the mice was monitored weekly. The genotypes of the mice were determined by polymerase chain reaction analyses of genomic DNA extracted from mouse tail snips using the following primers: LepR-Cre mice: forward, 5′-CTT GGG TGG AGA GGC TAT TC-3′ and reverse, 5′-AGG TGA GAT GAC AGG AGA TC-3′; LMNA G609G/G609G mice: forward, 5′-CTATTGCATGCTTCTCCTCAG-3′ and reverse, 5′-TGAGCGCAGGTTGTACTCAG-3′, R26R-Tdtomato mice: forward, 5′-CCA GTT AGT CCA CTT ATG TTG-3′ and reverse, 5′-TAC CAG GAA GGC TTG GGA AG-3′; Stem cell factor-GFP (Scf-GFP) mice: forward,

5′-CTACGGCAAGCTGACCCTGAAG-3′ and reverse, 5′-CGTCGTCCTTGAAGAAGATGGTG-3′.

Immunocytochemistry and immunofluorescence analysis

Human NP tissues were fixed in 4% paraformaldehyde for 48 h, dehydrated, and embedded in optimal cutting temperature compound. The spine specimens of the mice were fixed in 4% paraformaldehyde for 48 h, decalcified in 10% ethylenediaminetetraacetic acid (pH 7.4) for 14 days, dehydrated, and embedded in optimal cutting temperature compound. Immunofluorescence analysis of the IVD sections was performed as described previously [22]. We processed 8-μm-thick longitudinally oriented sections (anterior to posterior) of the IVD for immunofluorescence analysis. We chose the medial sections as the selected plane, which enabled us to investigate the complete IVD, end plate, and AF. The slides were numbered to indicate the depth of the sections, and slides with close numbers from different groups were chosen to ensure similar anatomical levels were compared. We incubated the sections with primary antibodies to mouse LepR (1:200, BAF497; R&D Systems, Inc., Minneapolis, MN, USA), human LepR (1:100, ab104403; Abcam, for human samples), cytokeratin 8 (CK8, 1:200, ab53280; Abcam, Cambridge, MA, USA), Matrix metallopeptidase 13 (MMP13) (1:100, ab39012; Abcam), aggrecan (1:100, AB1031; Millipore, Burlington, MA,USA), Ki67 (1:100, NB500-170; Novus Biologicals, Centennial, CO, USA), and Col2 (1:100, ab185430; Abcam), followed by incubation with goat anti-rabbit, goat anti-mouse, and donkey anti-goat (1:100; Abcam) secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA). We counted the numbers of positively stained cells in the whole NP of the L4–L5 IVD in 5 sequential sections per mouse in each group. Images were acquired using a confocal laser microscope (Nikon C2; Nikon, Tokyo, Japan) under identical imaging conditions using identical acquisition parameters, and fluorescence signals were quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Study approval

The experimental protocols were reviewed and approved by the Fourth Military Medical University Animal Use and Care Committee.

Disc height measurement

X-ray imaging of lumbar discs of LMNA G609G/G609G and wild-type male mice was performed using cabinet X-ray imaging and irradiation systems (Faxitron Bioptics, LLC, Wheeling, IL, USA), as described previously [25]. Percentage of disc height was calculated as the average of three measurements per disc. The percentage of disc height of each group was calculated as the average height of three discs (L2–L5) from 5 mice.

Statistical analysis

Data are presented as mean ± standard deviation. Unpaired, 2-tailed Student t tests were used for comparisons between the 2 groups. For multiple comparisons, one-way analysis of variance with the Bonferroni post hoc test was used. All data were normally distributed and had similar variation between groups. Statistical analysis was performed using SAS, version 9.3, software (SAS Institute, Inc., Cary, NC, USA). A p value < 0.05 was deemed significant.

Results

LepR-Cre mediates recombination in NP cells of IVD tissues at postnatal stages

LepR-Cre is a knockin mouse line in which a GFP:Cre fusion gene is targeted into the LepR locus so that expression of Cre recombinase is turned on in cells that normally express LepR [26,27]. Previous

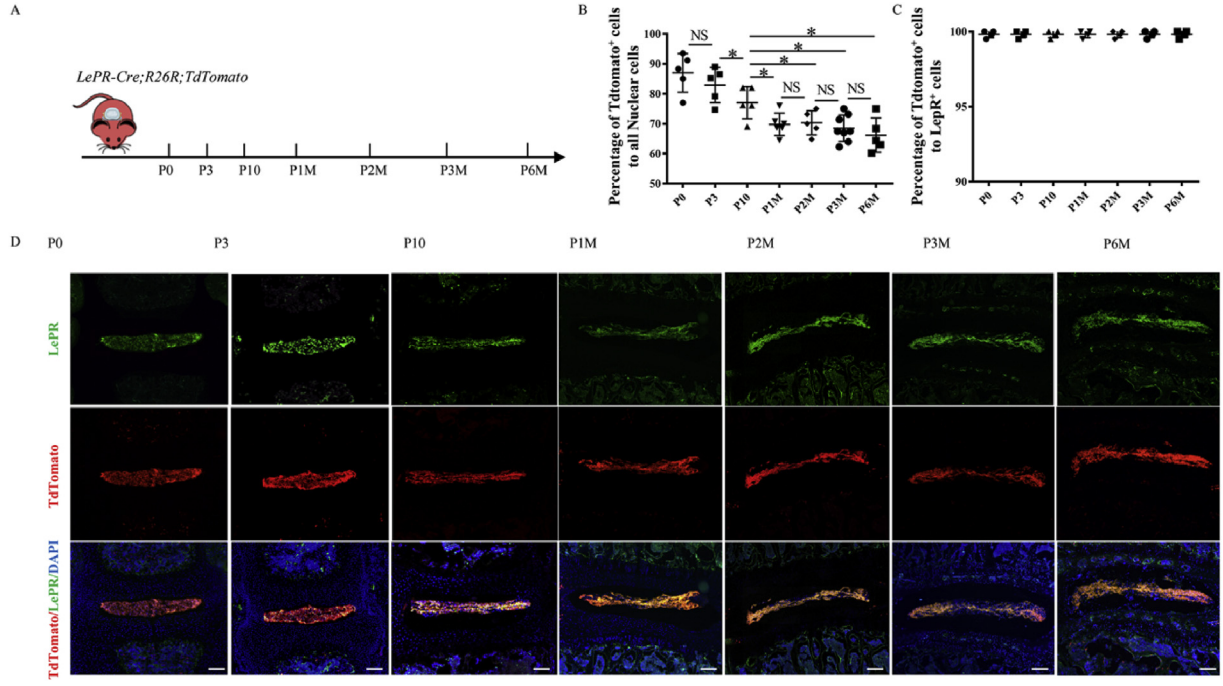

Figure 1. LepR-Cre mediates recombination in NP cells of IVD tissues at postnatal stages. (A) Diagram of the timeline analysing the specificity of LepR-Cre in NP cells of the IVD of LepR-Cre; R26R; TdTomato mice.(B) Quantification of the percentage of Tdtomato$^+$ cells to all NP cells from time points P0, P3, P10, P1M, P2M, P3M, and P6M. (C) Quantification of the percentage of Tdtomato$^+$ cells to all LepR$^+$ cells in the aforementioned time points. (D) Representative immunofluorescence staining images of the L4–L5 IVD using antibodies against LepR. P = postnatal. Scale bars, 100 μm ($n = 5$ mice per group). Data are presented as mean ± SD. * indicates $p < 0.05$, and NS indicates not significant, as determined by ANOVA with Bonferroni post hoc analysis. ANOVA = analysis of variance; IVD = intervertebral disc; LepR = leptin receptor; NP = nucleus pulposus; SD = standard deviation.

lineage-tracing studies discovered that LepR-expressing cells marked potential waves of bone marrow mesenchymal stromal/progenitor cells especially in the long bone of adult mice and gave rise to multiple lineage cells under certain circumstances [16,20]. To determine the cell-type specificity of LepR-Cre in IVD tissues, we crossed LepR-Cre mice with R26R-Tdtomato mice to generate LepR-Cre; R26R-Tdtomato mice. Whole-mount fluorescence analysis of the spine using the dissecting microscope showed that LepR-Cre specifically labelled all IVD tissues of mice (P0–P12M) from cervical to caudal vertebrae (Supplemental Fig. 1). To further characterise the specificity of LepR-Cre, the results of immunostaining of longitudinal lumbar 4–5 disc (L4–5) sections at different ages from early postnatal to late adulthood were thoroughly investigated in LepR-Cre; R26R-Tdtomato mice. Here, we first identify that LepR-Cre targeted postnatal NP cells (Figure 1A). In detail, LepR-Cre labelled 88.7 ± 5.1% of notochord-derived cells at the postnatal stage P0, and the percentage began to decrease since the postnatal stage P3 and reached a plateau at the age of one month, which consisted of nearly 75% of all NP cells and did not further decrease since the age of one month (Figure 1B–D). The gradually decreased percentage of Tdtomato$^+$ NP cells since the postnatal stage P3 revealed the potential role of LepR-expressing NP cells in disc homoeostasis because the loss of NCs in the postnatal stage is believed to be the sign of the onset of IDD. Moreover, we performed immunostaining of the LepR in the L4–5 vertebrae of LepR-Cre; R26R-Tdtomato mice and found that the signals of the LepR completely overlapped with tdTomato in IVDs, which indicated the high specificity and targeting efficiency of LepR-Cre in IVDs (Figure 1C and D). Of note, LepR-Cre started to label AF cells primarily in the outer AF (OAF) since the postnatal stage P14 and persist into adulthood (Supplemental Fig. 2), suggesting that Tdtomato$^+$ cells residing in the OAF after the postnatal stage P14 were not likely to migrate from NP cells (no Tdtomato$^+$ cells found in the inner AF) and possibly from the previously reported IVD niche (between the OAF and perichondrium) or circulation, which might promote AF calcification during disc degeneration [28].

LepR-Cre specifically targets notochord-derived cells at perinatal stages

The high percentage of Tdtomato$^+$ cells labelled in NP cells at postnatal stages impedes us to analyse whether Tdtomato$^+$ NP cells are derived from the notochord (Figure 2A). Of note, we found that LepR-Cre labelled nearly 95% of notochord-derived cells since the embryonic stage E15.5, and the percentage significantly decreased to 88.7 ± 5.1% after birth (Figure 2B and C). To determine whether LepR-Cre–labelled cells in IVD tissues during perinatal stages were actual notochord-derived cells, we chose the marker CK8, which was previously reported to have high specificity in all NCs and NP cells (Figure 3A and B) [29,30]. Immunostaining of CK8 was performed for longitudinal L4–5 sections of LepR-Cre; R26R-Tdtomato mice from the perinatal stage to late adulthood (Figure 3A–C). We found that Tdtomato$^+$ cells were all CK8 positive (Figure 3A), which indicated the high specificity of LepR-Cre targeting in notochord-derived cells. To further excluded whether the decreased percentage of LepR-expressing NP cells was due to apoptosis, we analysed cell apoptosis using the TUNNEL Kit (Beyotime Biotech, Shanghai, China) and found no TUNNEL$^+$ NP cells at postnatal stages P0 and P3 (data not shown). Given that the expression of Cre recombinase is turned on not only in cells that normally express LepR but also in the descendants of those cells, we could anticipate that the decreased percentage of Tdtomato$^+$ cells in the postnatal stage might result from the increased subpopulation of NP cells derived from other LepR$^-$ cells, which first indicated that NP cells might consist of different subpopulations during disc development.

NP cells can be divided into subpopulations which fulfil different functions in disc homoeostasis

To further validate whether NP cells can be divided into subpopulations, we performed immunostaining of CK8 and found that CK8 labelled nearly all notochord-derived cells. Importantly, LepR-Tdtomato was expressed in nearly 95% of CK8$^+$ NCs at E15.5, and the percentage decreased after birth and continued to drop to nearly 70% of all CK8$^+$ NP cells in adulthood (Figure 3A and C). Based on the immunostaining results and current understanding, we were the first to thoroughly investigate NP cell subpopulations via the fate-mapping mice model and further quantified the relative percentage of CK8$^+$Tdtomato$^+$ NP cells and CK8$^+$Tdtomato$^-$ NP cells. In details, CK8$^+$Tdtomato$^-$ NP cells were few at embryonic stages, and the percentage significantly increased to nearly 12% after birth. After the age of one month, the percentage of CK8$^+$Tdtomato$^-$ NP cells reached a plateau (nearly 25%) (Figure 3C). Given the fact that NP cells are heterogeneous, we could anticipate the

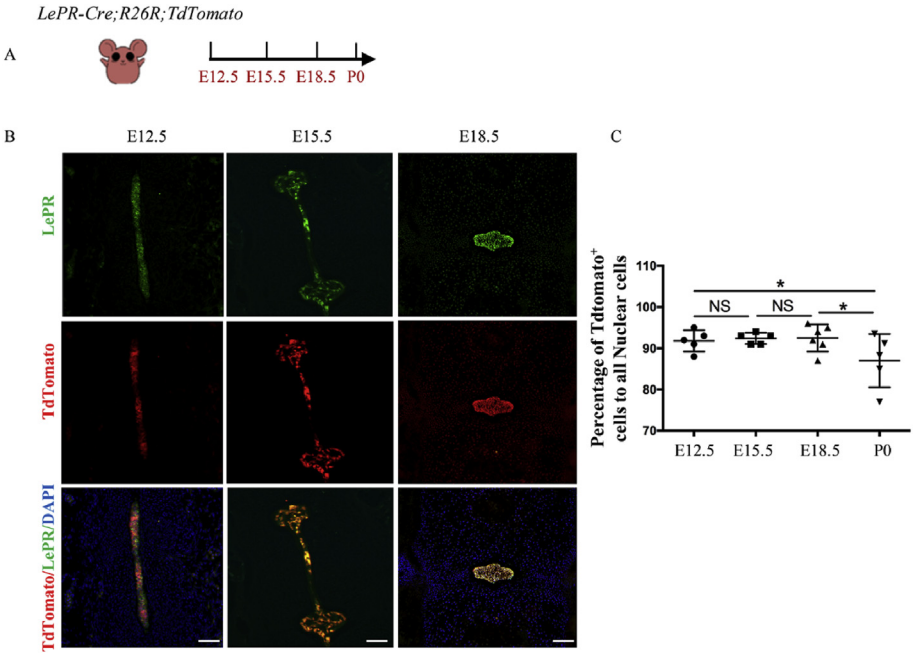

Figure 2. LepR-Cre specifically targets notochord-derived cells since perinatal stages. (A) Diagram of the timeline analysing the specificity of LepR-Cre in notochord-derived cells of the IVD of LepR-Cre; R26R; TdTomato mice. (B) Representative immunofluorescence staining images of the L4–L5 IVD from the embryonic stages E12.5, E15.5, and E18.5 using antibodies against LepR. (C) Quantification of the percentage of Tdtomato$^+$ cells to all notochordal cells in different time points. E = embryonic. Scale bars, 100 μm ($n \geq 5$ mice per group). Data are presented as mean ± SD. * indicates $p < 0.05$, and NS indicates not significant, as determined by ANOVA with Bonferroni post hoc analysis. ANOVA = analysis of variance; IVD = intervertebral disc; LepR = leptin receptor; SD = standard deviation.

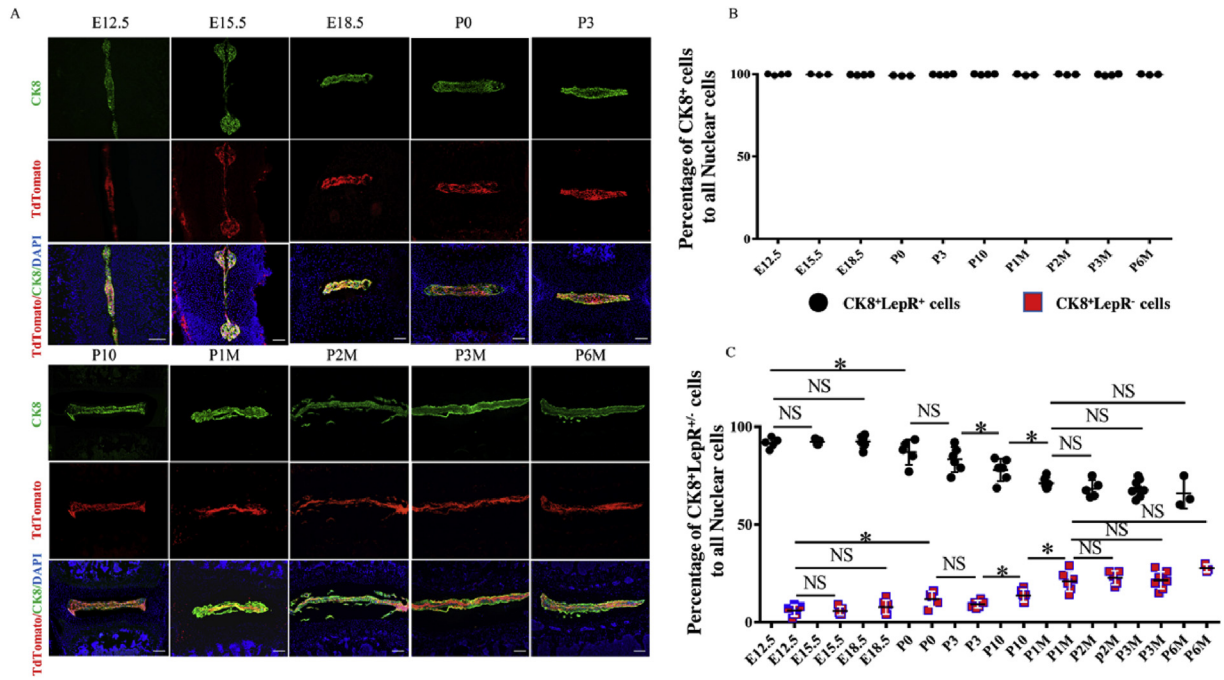

Figure 3. NP cells can be divided into two subpopulations from embryonic stages to adulthood. (A) Representative immunofluorescence staining images of the L4–L5 IVD from time points E12.5, E15.5, E18.5, P0, P3, P10, P1M, P2M, P3M, and P6M using antibodies against LepR and CK8. (B) Quantification of the percentage of $CK8^+$ cells to all notochord-derived cells in different time points. (C) Quantification of the percentage of $CK8^+LepR^+$ cells to all notochord-derived cells in different time points. The black dot indicates $CK8^+LepR^+$ NP cells, and the red square indicates $CK8^+LepR^-$ NP cells. P = postnatal; E = embryonic. Scale bars, 100 μm ($n \geq 3$ mice per group). Data are presented as mean ± SD. * indicates $p < 0.05$, and NS indicates not significant, as determined by ANOVA with Bonferroni post hoc analysis. ANOVA = analysis of variance; CK8 = cytokeratin 8; IVD = intervertebral disc; LepR = leptin receptor; NP = nucleus pulposus; SD = standard deviation.

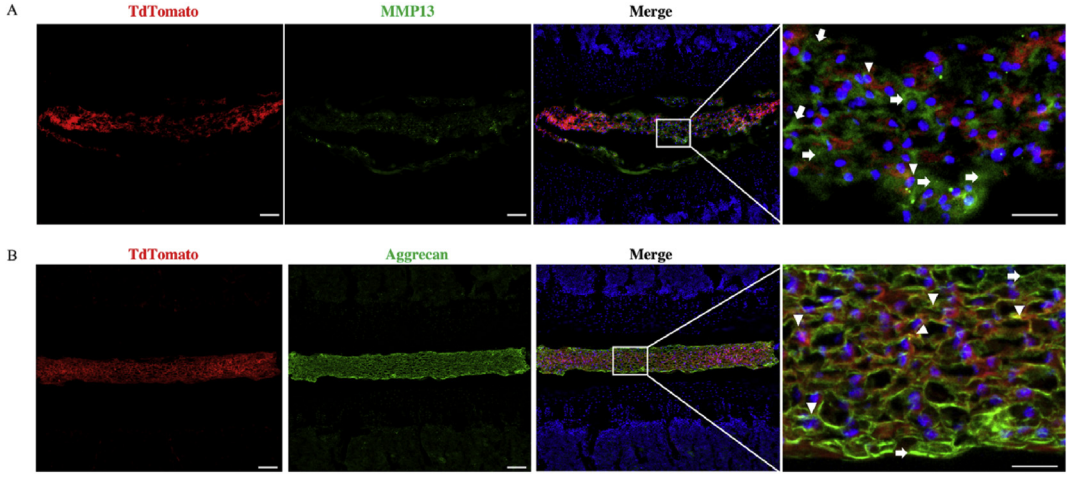

Figure 4. LepR-expressing NP cells are closely associated with the turnover of matrix proteins in the IVD. (A) Representative immunofluorescence staining images of the L4–L5 IVD from P3M LepR-Cre; R26R; Tdtomato mice using antibodies against MMP13. (B) Representative immunofluorescence staining images of the L4–L5 IVD from P3M LepR; R26R; Tdtomato mice using antibodies against aggrecan. Scale bars, 100 μm. Images on the right are magnification of certain areas in the merged images. Scale bars, 20 μm. Arrowheads indicate matrix protein closely associated with LepR-expressing NP cells; arrows indicate matrix protein distant to LepR-expressing NP cells (A and B) ($n = 5$ mice per genotype from 3 independent experiments). IVD = intervertebral disc; LepR = leptin receptor; NP = nucleus pulposus.

different functions and lineage fate between $CK8^+Tdtomato^+$ NP cells and $CK8^+Tdtomato^-$ NP cells.

To determine whether these two subpopulations have similar roles or phenotypes in disc homoeostasis, we performed immunostaining of Tdtomato with disc matrix proteins—aggrecan and MMP13 (Figure 4A and B). Surprisingly, most aggrecan protein (anabolic matrix protein) abundantly deposited in the pericellular matrix of $Tdtomato^+$ NP cells, whereas only few were adjacent to $Tdtomato^-$ NP cells (Figure 4A and B). On the contrary, the majority of MMP13 (catabolic matrix protein) were closely adjacent to $Tdtomato^-$ NP cells instead of $Tdtomato^+$ NP cells, which indicated that $Tdtomato^+$ NP cells together with $Tdtomato^-$ NP cells primarily contributed to the secretion and modulation of matrix proteins to maintain disc homoeostasis. To further determine whether $LepR^+$ NP cells share the similar characteristic with those of $LepR^+$ cells in the one marrow, Scf (stem cell factor, the ligand of c-kit)-GFP transgenic mice were used to determine whether $LepR^+$ NP cells were the niche cell because the previous study showed that SCF secreted by $LepR^+$ cells promoted the regeneration of stem cells and haematopoiesis [31]. However, we investigated no $Scf\text{-}GFP^+$ cells in disc tissues, including the NP and annulus fibrosus (Supplementary Fig. 3). These findings

demonstrate that LepR-expressing NP cells may play a pivotal role in maintenance of disc homoeostasis and the detailed mechanism should be further clarified.

The percentage of human LepR$^+$ NP cells significantly decreases during disc degeneration

After exploring the potential role of LepR-Tdtomato$^+$ and LepR-Tdtomato$^-$ NP cells in the IVD, we set out to elucidate the specificity of LepR$^+$ cells in human NP cells and investigate their correlation with IDD. We performed microendoscopic discectomy of the NP with different degeneration grades and carefully collected the pure NP cells for immunostaining. Notably, we found the percentage of LepR$^+$ NP cells negatively correlated with the progression of human IDD. In detail, LepR$^+$ NP cells consisted of nearly 75% of NP cells of Grade II, and the percentage significantly decreased to approximately 44% and 32% for Grade IV and V, respectively, although there was no significant difference in the percentage between Grades II and III (Figure 5A and B). These findings further indicated that LepR$^+$ NP cells might play potential roles in maintaining physiological disc homoeostasis and modulating the progression of human IDD.

The percentage of LepR-expressing NP cells significantly decreases under premature ageing–induced IDD

We previously demonstrated that premature ageing mice (LMNA G609/G609) displayed the early onset of IDD compared with the control littermates [32]. In this study, we used this model to further verify the relationship between LepR-expressing NP cells and IDD. Immunostaining of the LepR in longitudinal L4–5 sections was performed in premature ageing mice and indicated that the percentage of LepR$^+$ cells significantly decreased in G609/G609 mice with disc degeneration compared with the control littermates (Figure 6A and B). This finding further confirmed that LepR$^+$ NP cells was correlated with the progression of IDD and might become the potential target for therapeutic development. X-ray showed that the disc height of G609G/G609G mice was significantly lower than that of the control littermates (Figure 6C). Moreover, the expression of aggrecan and collagen II significantly decreased in the NP of G609G/G609G mice compared with that of the control littermates (Figure 6D and E).

Discussion

Low back pain is a common clinical problem that accounts for more than half of all musculoskeletal disabilities and is thought to originate from degeneration of the IVD, specifically the inner NP [9,33,34]. The limited understanding of disc development, maintenance, and degeneration entails the lack of effective treatment for this widespread problem. Particularly, the loss of NCs and dysfunction of NP cells are believed to be the signal of the onset and progression of IDD, respectively [6,7]. Therefore, great efforts have been made to understand the origin, subpopulation, lineage fate, and the function of NCs and NP cells using genetically modified mouse models. In this study, we first demonstrate that the LepR could be the potential marker for notochord-derived cells since the embryonic stage until late adulthood. In addition, we find that LepR-expressing cells represent a distinct subpopulation of notochord-derived cells that are closely associated with IDD and essential for disc homoeostasis.

The poorly defined notochord-specific markers and lack of effective lineage-tracing animal models complicate the identification of the subpopulation of notochord-derived cells. Shh-Cre transgenic mice crossed with ROSA26 reporter mice was first used to investigate the fate and origin of NCs, and all cells derived from the notochord were labelled, suggesting that all NP cells in postnatal life are descendants of NCs [9]. A following study used a novel notochord-specific Cre mouse by targeting the homeobox gene Noto in cross with LacZ to trace the NCs within the IVD, and fate-mapping studies demonstrated that NCs were embryonic precursors of all cells found within the NP of the mature IVD [10]. These findings suggest that NP cells in postnatal life are homogeneous and all are derived from NCs. However, a recent study showed Col2a1--Cre–mediated recombination in the majority of cells in IVD tissues at postnatal stages, and the fate-mapping study of Col2a1-Cre–labelled cells further demonstrated that NP cells were not homogenous and could be divided into two subpopulations. Another study identified two novel NP progenitor cell markers, disialoganglioside 2 (GD2) and tyrosine kinase receptor (Tie2), and found that Tie2$^+$GD2$^+$ cells possessed stem cell properties with self-renewal potentials and Tie2$^-$GD2$^+$ cells as potential NP cell progenitors, which also indicated the heterogeneity of postnatal NP cells [35]. Actually, the fate of the NCs within the NP has been debated for years. It has been proposed that small chondrocyte-like NP

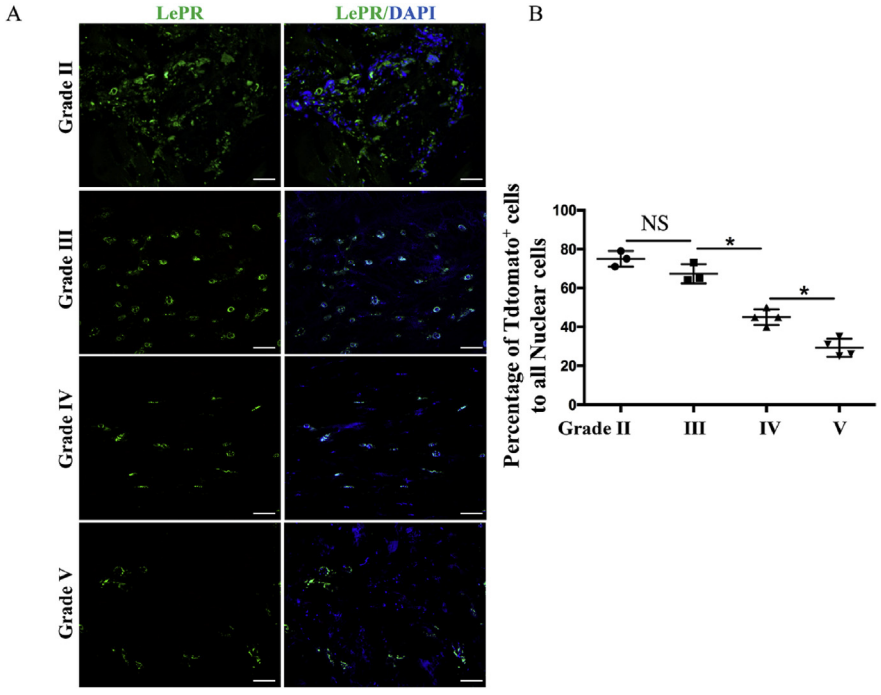

Figure 5. The percentage of human LepR$^+$ NP cells significantly decreases during disc degeneration. (A) Representative immunofluorescence staining images of NP cells from human IVD samples of Grades II to V using antibodies against LepR. (B) Quantification of the percentage of LepR$^+$ cells to all notochord-derived cells in different time points. Scale bars, 20 μm ($n \geq 3$ per group). Data are presented as mean ± SD. * indicates $p < 0.05$, and NS indicates not significant, as determined by ANOVA with Bonferroni post hoc analysis. ANOVA = analysis of variance; DAPI = 4,6-diamino-2-phenylindole; IVD = intervertebral disc; LepR = leptin receptor; NP = nucleus pulposus; SD = standard deviation.

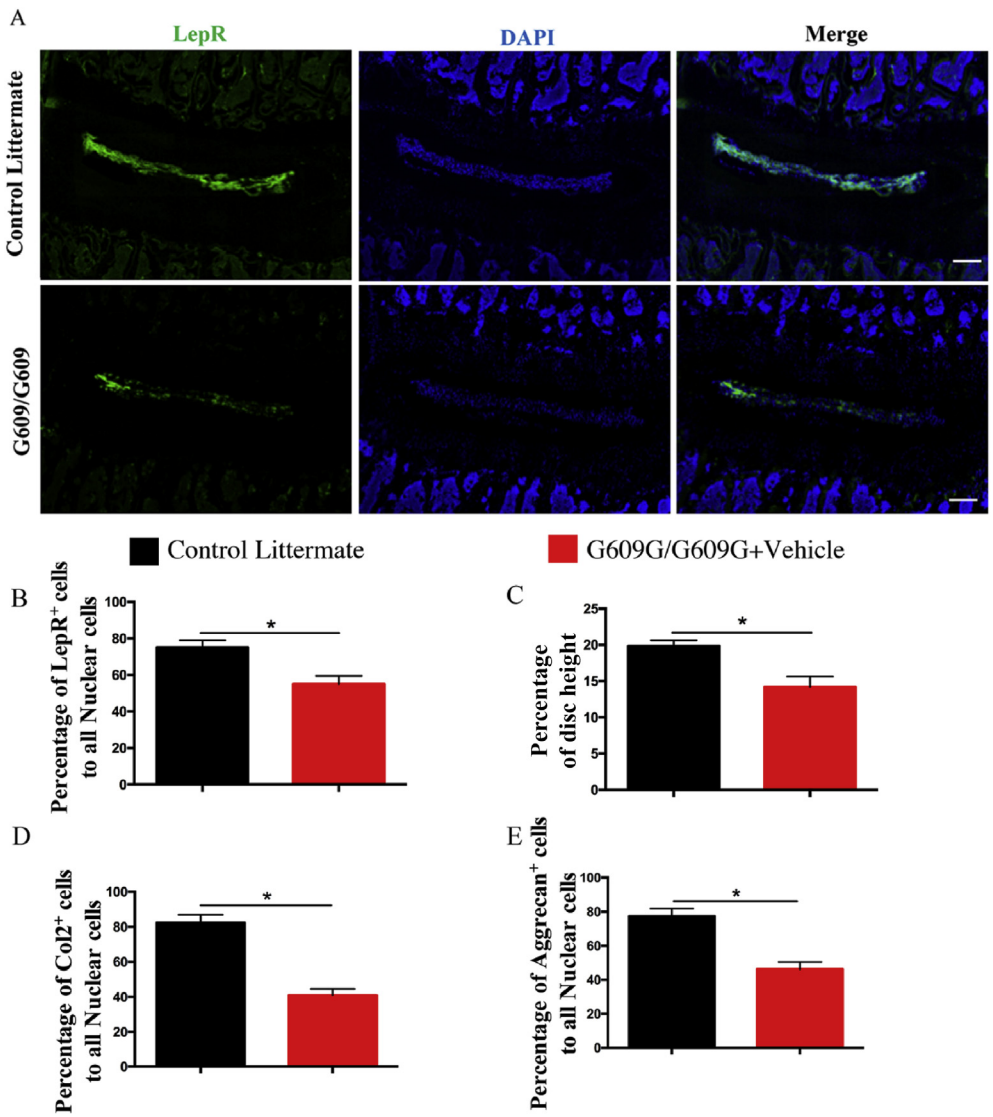

Figure 6. The percentage of LepR-expressing NP cells significantly decreases under premature ageing-induced IDD. (A) Representative immunofluorescence staining images of the L4–L5 IVD from P3M LMNA G609/G609 mice with their control littermates using antibodies against LepR. (B) Quantification of the percentage of $LepR^+$ cells to all NP cells of the IVDs. (C) Representative disc height indices (DHI) of the IVDs of LMNA G609G/G609G mice or control littermates using X-ray. (D) Quantification of the percentage of $Col2^+$ cells to all NP cells of the IVDs of LMNA G609G/G609G mice or control littermates. (E) Quantification of the percentage of $aggrecan^+$ cells to all NP cells of the IVDs of LMNA G609G/G609G mice or control littermates. Scale bars, 100 μm ($n > 3$ mice per group). Data are presented as mean ± SD. * indicates $p < 0.05$, and NS indicates not significant, as determined using two-tailed Student t tests. DAPI = 4,6-diamino-2-phenylindole; IDD = intervertebral disc degeneration; LepR = leptin receptor; NP = nucleus pulposus; SD = standard deviation.

cells were derived from mesenchymal cells, populating the NP after migration from the surrounding cartilage end plate or originating from transient amplifying cells in the perichondrium at the periphery of the disc [36,37]. In this study, we found that LepR-expressing cells accounted for approximately 95% of NCs at embryonic stages, and the percentage decreased to 70% when reaching adulthood. Owing to the characteristic that LepR-Cre can label not only the LepR-expressing cells but also their descendants, this finding provided solid evidence that NP cells in postnatal life were heterogenous, which warrants further clarification of the origin of postnatal NP cells. We also found that compared with $Tdtomato^-$ NP cells, majority of aggrecan-expressing cells were LepR-positive NP cells, whereas most of the MMP13-expressing cells were LepR-negative, indicating that the different subpopulation of NP cells might play disparate roles in IVD homoeostasis. However, the detailed function of different subpopulations of NP cells and whether a certain subpopulation dominates in triggering the onset and progression of IDD need to be further elucidated.

To further demonstrate the high specificity of $LepR^+$ cells in human NP cells and investigate their correlation with disc degeneration, we showed that the percentage of human $LepR^+$ NP cells significantly decreased during disc degeneration. These findings indicate that $LepR^+$ NP cells may play potential roles in maintaining disc homoeostasis and modulate the progression of IDD. One study reported that disc cells expressed leptin and its functional receptor so that leptin could stimulate proliferation of disc cells *in vitro*, which could be the onset signal of IDD [38,39]. Other related studies also showed that leptin induced angiogenesis *in vivo* and modulated the cytoskeletal elements in NP cells [40,41]. Of note, we should realise that LepR-expressing NP cells and the function of LepR in NP cells are not the same topic. Although an increasing number of studies have already clarified the function and lineage fate of LepR-expressing cells in the bone marrow and periosteum, only LepR-Cre; $Lepr^{f/f}$ mice can help us elucidate the function of LepR in LepR-expressing cells in the NP during IDD. Future studies should make efforts to determine the detailed function of LepR in $LepR^+$ NP cells from disc development to the onset and progression of IDD via using the valuable tool of the LepR-Cre; R26R; Tdtomato mice line. Interestingly, IVD cells express the LepR at embryonic stages, and people with obesity are reported to have higher levels of serum leptin [14,15,42,43]. It is likely that adipokines, such as leptin, may play pivotal roles in disc development modulated by the hypothalamus, thus linking obesity, IDD, and low back pain.

Until now, therapies for IDD mainly focused on alleviating the symptoms rather than eliminating the underlying causes or restoring the structure and biomechanical function of the discs. Of note, we have not found any published studies targeting the subpopulation of NP cells to treat IDD. The present study and our previous studies showed that

premature ageing mice (LMNA G609/G609) displayed early onset of IDD and the IDD phenotype compared with the control littermates [25]. In this study, we further demonstrated that the percentage of LepR-expressing cells significantly decreased in premature ageing mice with IDD, which indicated that LepR-expressing cells might be closely related to the onset and progression of IDD and could be the potential target to prevent and treat the widely spreading disease. Future study on IDD treatment should further discuss the possibility of targeting on a specific subpopulation of NP cells.

In this study, we identified the LepR as one of the novel markers for notochord-derived cells and demonstrated LepR-expressing cells as a distinct subpopulation essential for disc homoeostasis. Of note, we noticed that subsets of $LepR^+$ NP cells expressed Ki67 (data not shown), while Scf-GFP was not expressed in NP and AF cells. However, these phenotypes can only rule out the possibility that discs do not possess stem/progenitor cells essential for haematopoiesis, for Scf is secreted by $LepR^+$ cells in the bone marrow to maintain the hematopoietic stem cell (HSC) niche [31]. Whether subsets of $LepR^+$ NP cells represent stem/-progenitor cells either at the beginning of notochordal development or during IDD still needs further investigation. We just began to understand the heterogenous pattern of NP cells and tried to elucidate the role and function of LepR-expressing NP cells under physiological and pathological conditions. It would be important in future to clarify the detailed role of different subpopulations of NP cells including stem/progenitor cells and identify the IDD-related pathogenic subpopulation via single-cell transcriptome sequencing. Understanding the mechanism of LepR-expressing NP cells will undoubtedly benefit early prevention and treatment of IDD and provide translational insights into use of certain subpopulations for disc regenerative applications.

Conflict of Interest

The authors have no conflicts of interest to disclose in relation to this article.

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Author contributions

L.Y. and B.G. designed the experiments; B.G., J.Y., and X.X. carried out most of the experiments; J.F., W.L., J.S., D.W., C.Z., P.C., and L.L. helped to collect the samples; D.W. and C.Z. proofread the manuscript; L.Y. and Z.L. supervised the experiments, analysed the results, and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2019.11.005.

References

[1] Kepler CK, Markova DZ, Dibra F, Yadla S, Vaccaro AR, Risbud MV, et al. Expression and relationship of proinflammatory chemokine RANTES/CCL5 and cytokine IL-1beta in painful human intervertebral discs. Spine 2013;38:873–80 (Phila Pa 1976).
[2] Katz JN. Lumbar disc disorders and low-back pain: socioeconomic factors and consequences. J Bone Joint Surg Am 2006;88(Suppl 2):21–4.
[3] Freemont AJ, Peacock TE, Goupille P, Hoyland JA, O'Brien J, Jayson MI. Nerve ingrowth into diseased intervertebral disc in chronic back pain. LANCET 1997;350:178–81.
[4] Hayashi S, Taira A, Inoue G, Koshi T, Ito T, Yamashita M, et al. TNF-alpha in nucleus pulposus induces sensory nerve growth: a study of the mechanism of discogenic low back pain using TNF-alpha-deficient mice. Spine 2008;33:1542–6 (Phila Pa 1976).
[5] Kerr GJ, Veras MA, Kim MK, Seguin CA. Decoding the intervertebral disc: unravelling the complexities of cell phenotypes and pathways associated with degeneration and mechanotransduction. Semin Cell Dev Biol 2017;62:94–103.
[6] Wang SZ, Rui YF, Lu J, Wang C. Cell and molecular biology of intervertebral disc degeneration: current understanding and implications for potential therapeutic strategies. Cell Prolif 2014;47:381–90.
[7] Matta A, Karim MZ, Isenman DE, Erwin WM. Molecular therapy for degenerative disc disease: clues from secretome analysis of the notochordal cell-rich nucleus pulposus. Sci Rep 2017;7:45623.
[8] Choi KS, Harfe BD. Hedgehog signaling is required for formation of the notochord sheath and patterning of nuclei pulposi within the intervertebral discs. Proc Natl Acad Sci U S A 2011;108:9484–9.
[9] Choi KS, Cohn MJ, Harfe BD. Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: implications for disk degeneration and chordoma formation. Dev Dynam 2008;237:3953–8.
[10] Mccann MR, Tamplin OJ, Rossant J, Seguin CA. Tracing notochord-derived cells using a Noto-cre mouse: implications for intervertebral disc development. Dis Model Mech 2012;5:73–82.
[11] Chen M, Lichtler AC, Sheu TJ, Xie C, Zhang X, O'Keefe RJ, et al. Generation of a transgenic mouse model with chondrocyte-specific and tamoxifen-inducible expression of Cre recombinase. Genesis 2007;45:44–50.
[12] Henry SP, Jang CW, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. Generation of aggrecan-CreERT2 knockin mice for inducible Cre activity in adult cartilage. Genesis 2009;47:805–14.
[13] Zheng Y, Fu X, Liu Q, Guan S, Liu C, Xiu C, et al. Characterization of Cre recombinase mouse lines enabling cell type-specific targeting of postnatal intervertebral discs. J Cell Physiol September 2019;234(9):14422–31.
[14] Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. Nature 1998;395:763–70.
[15] Friedman JM. Leptin, leptin receptors, and the control of body weight. Nutr Rev 1998;56:s38–46. s54-75.
[16] Zhou BO, Yue R, Murphy MM, Peyer JG, Morrison SJ. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. Cell stem cell 2014;15:154–68.
[17] Gao B, Deng R, Chai Y, Chen H, Hu B, Wang X, et al. Macrophage-lineage TRAP+ cells recruit periosteum-derived cells for periosteal osteogenesis and regeneration. J Clin Investig 2019;129:2578–94.
[18] Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 2012;481:457–62.
[19] Sena I, Borges IT, Lousado L, Azevedo PO, Andreotti JP, Almeida VM, et al. LepR+ cells dispute hegemony with Gli1+ cells in bone marrow fibrosis. Cell Cycle 2017;16:2018–22.
[20] Yue R, Zhou BO, Shimada IS, Zhao Z, Morrison SJ. Leptin receptor promotes adipogenesis and reduces osteogenesis by regulating mesenchymal stromal cells in adult bone marrow. Cell stem cell 2016;18:782–96.
[21] Decker M, Martinez-Morentin L, Wang G, Lee Y, Liu Q, Leslie J, et al. Leptin-receptor-expressing bone marrow stromal cells are myofibroblasts in primary myelofibrosis. Nat Cell Biol 2017;19:677–88.
[22] Gao B, Lin X, Jing H, Fan J, Ji C, Jie Q, et al. Local delivery of tetramethylpyrazine eliminates the senescent phenotype of bone marrow mesenchymal stromal cells and creates an anti-inflammatory and angiogenic environment in aging mice. Aging Cell 2018;17:e12741.
[23] Pfirrmann CW, Metzdorf A, Zanetti M, Hodler J, Boos N. Magnetic resonance classification of lumbar intervertebral disc degeneration. Spine 2001;26:1873–8 (Phila Pa 1976).
[24] Osorio FG, Navarro CL, Cadinanos J, Lopez-Mejia IC, Quiros PM, Bartoli C, et al. Splicing-directed therapy in a new mouse model of human accelerated aging. Sci Transl Med 2011;3:106r–7r.
[25] Xu X, Wang D, Zheng C, Gao B, Fan J, Cheng P, et al. Progerin accumulation in nucleus pulposus cells impairs mitochondrial function and induces intervertebral disc degeneration and therapeutic effects of sulforaphane. Theranostics 2019;9:2252–67.
[26] Scott MM, Lachey JL, Sternson SM, Lee CE, Elias CF, Friedman JM, et al. Leptin targets in the mouse brain. J Comp Neurol 2009;514:518–32.
[27] Defalco J, Tomishima M, Liu H, Zhao C, Cai X, Marth JD, et al. Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. Science 2001;291:2608–13.
[28] Hu B, He R, Ma K, Wang Z, Cui M, Hu H, et al. Intervertebral disc-derived stem/progenitor cells as a promising cell source for intervertebral disc regeneration. Stem Cell Int 2018;2018:7412304.
[29] Gilson A, Dreger M, Urban JP. Differential expression level of cytokeratin 8 in cells of the bovine nucleus pulposus complicates the search for specific intervertebral disc cell markers. Arthritis Res Ther 2010;12:R24.
[30] Sun Z, Wang HQ, Liu ZH, Chang L, Chen YF, Zhang YZ, et al. Down-regulated CK8 expression in human intervertebral disc degeneration. Int J Med Sci 2013;10:948–56.
[31] Zhou BO, Yu H, Yue R, Zhao Z, Rios JJ, Naveiras O, et al. Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. Nat Cell Biol 2017;19:891–903.
[32] Xu X, Wang D, Zheng C, Gao B, Fan J, Cheng P, et al. Progerin accumulation in nucleus pulposus cells impairs mitochondrial function and induces intervertebral disc degeneration and therapeutic effects of sulforaphane. Theranostics 2019;9:2252–67.

[33] Bedore J, Sha W, Mccann MR, Liu S, Leask A, Seguin CA. Impaired intervertebral disc development and premature disc degeneration in mice with notochord-specific deletion of CCN2. Arthritis Rheum 2013;65:2634–44.
[34] Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. Nat Rev Rheumatol 2014;10:44–56.
[35] Sakai D, Nakamura Y, Nakai T, Mishima T, Kato S, Grad S, et al. Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. Nat Commun 2012;3:1264.
[36] Vujovic S, Henderson S, Presneau N, Odell E, Jacques TS, Tirabosco R, et al. Brachyury, a crucial regulator of notochordal development, is a novel biomarker for chordomas. J Pathol 2006;209:157–65.
[37] Henriksson H, Thornemo M, Karlsson C, Hagg O, Junevik K, Lindahl A, et al. Identification of cell proliferation zones, progenitor cells and a potential stem cell niche in the intervertebral disc region: a study in four species. Spine 2009;34:2278–87 (Phila Pa 1976).
[38] Li H, Zou X, Baatrup A, Lind M, Bunger C. Cytokine profiles in conditioned media from cultured human intervertebral disc tissue. Implications of their effect on bone marrow stem cell metabolism. Acta Orthop 2005;76:115–21.
[39] Zhao CQ, Liu D, Li H, Jiang LS, Dai LY. Expression of leptin and its functional receptor on disc cells: contribution to cell proliferation. Spine 2008;33:E858–64 (Phila Pa 1976).
[40] Anagnostoulis S, Karayiannakis AJ, Lambropoulou M, Efthimiadou A, Polychronidis A, Simopoulos C. Human leptin induces angiogenesis in vivo. Cytokine 2008;42:353–7.
[41] Li Z, Shen J, Wu WK, Yu X, Liang J, Qiu G, et al. The role of leptin on the organization and expression of cytoskeleton elements in nucleus pulposus cells. J Orthop Res 2013;31:847–57.
[42] Segar AH, Fairbank J, Urban J. Leptin and the intervertebral disc: a biochemical link exists between obesity, intervertebral disc degeneration and low back pain-an in vitro study in a bovine model. Eur Spine J 2019;28:214–23.
[43] Hart RA. The Spine Patient Outcomes Research Trial (SPORT): a continuing return on investment: commentary on an article by Jeffrey A. Rihn, MD, et al.: "The influence of obesity on the outcome of treatment of lumbar disc herniation. analysis of the Spine Patient Outcomes Research Trial (SPORT)". J Bone Joint Surg Am 2013;95:e5.