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Fate of Notochord Cells in Murine Models of Injury and Ageinduced Intervertebral Disc Degeneration

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FATE OF NOTOCHORD CELLS IN MURINE MODELS OF INJURY AND AGE-INDUCED INTERVERTEBRAL DISC DEGENERATION

Abstract

The first manifestations of low back pain are thought to be the result of degeneration of the intervertebral disc (IVD). There is surprisingly little known regarding the cellular composition and function of specific cell types within the nucleus pulposus (NP) of the IVD, particularly in models of degeneration. High-frequency, low-amplitude whole body vibration (WBV) is a form of mechanical stimuli that is currently used to treat a wide range of musculoskeletal disorders including osteoporosis, osteoarthritis and back pain. Recent data from the S-- Lab using a mouse model demonstrated that daily exposure to WBV results in degenerative changes to the intervertebral discs of the spine. Furthermore, the composition of the human NP changes with age; however, to date, no studies have been conducted to examine the origins of NP cells in older mice. To determine the fate of notochord cells in murine models of injury- and age-induced intervertebral disc degeneration, histological examination and molecular analysis were used to characterize the extent of degeneration and cell type. This study aims to better assess the changes in cellular composition of the IVD in response to injury and age, an important consideration in targeted therapeutic strategies to treat IVD degeneration and back pain.

Background

Low back pain is the second most common chronic condition in industrialized countries, with a lifetime prevalence of up to 84% (1). While the etiology of low back pain remains unknown, the first manifestations are thought to be a result of degeneration of the intervertebral disc (2). The physiological functions of the IVD include mechanical stabilization of the spine, load bearing during axial compression, and providing flexibility

to the spinal column. The IVD consists of the outer annulus fibrosus (AF), composed of bundles of type-I collagen fibers; the inner nucleus pulposus, composed of type-II collagen and proteoglycans; and the cartilage end-plates (CEP), which anchor the IVD to the adjacent vertebral bones. During degeneration of the IVD, there is a loss of proteoglycans which results in decreased osmotic pressure (3) and subsequent loss in disc height (4), which may lead to the pinching of nerves, especially resulting from changing mechanics of the facet joints in the three joint complex (5). Moreover, herniated discs may impinge on the sciatic nerve, exacerbating low back pain (6). Thus, IVD degeneration and changes in the extracellular matrix of the IVD account for a significant proportion of low back pain (7).

The formation of the NP begins with an embryonic structure called the node, which goes on to form the notochord (8). During development, the notochord plays an important role as a signaling center that patterns the embryo, after which it undergoes segmentation and forms the central NP (9). The AF and vertebral bodies form from somatic mesenchyme surrounding the notochord during embryonic development (10). The NP is thought to be the site where degenerative changes are initiated in the IVD (11), and therefore is our focus for understanding cellular changes associated with disc degeneration.

Although the NP has long been implicated in the initiation of disc degeneration, there is surprisingly little known regarding the cellular composition and function of specific cell types within the NP. The NP is composed of two cell types: smaller cartilage-like cells and larger notochord cells at birth. Notochord cells are thought to be important regulators of disc health providing signals that modulate the cartilage-like NP

cells. In the adult IVD, notochord cells protect NP cells through inhibition of caspase-9 and -3/7 activities, the up-regulation of anabolic extracellular matrix genes such as aggrecan and collagen type 2, and the down-regulation of matrix degrading enzymes such as MMP-3 (12). Furthermore, the loss of notochord cells precedes disc degeneration. The role of notochord cells within the IVD is demonstrated by breeds of chondrodystrophic dogs (i.e. Dachshund, Bulldog), where the NP is gradually replaced by cartilage-like cells beginning at 3 months of age, and the dogs are prone to early IVD degeneration. Conversely, non-chondrodystrophic dogs (i.e. Golden Retriever, Border Collie) which retain their notochord cells as the predominant cell type of the NP throughout their lives, are generally more resistant to disc degeneration (13).

There is evidence, however, that the morphological heterogeneity of cells within the NP reflects different cell stages, as opposed to different cell lineages (14). For example, notochordal cells are capable of proteoglycan production at a rate comparable to chondrocyte-like cells; other matrix protein expression analysis also further demonstrates a cartilage-like cell phenotype of notochordal cells, suggesting that they may act as progenitor cells (15). Furthermore, dynamic hydrostatic mechanical loading seems contribute to the transition from notochordal cells to cartilage-like cells in a porcine model (16). The origin of the morphological heterogeneity of cells within the NP, particularly the smaller cartilage-like cells, has not been definitively established.

There are currently two conflicting models that explain the origins of the cartilage-like NP cells: the niche-migration model and the notochord maturation model. The niche-migration model proposes that the smaller NP cells are of mesenchymal origin and migrate to the NP from the CEP. In this model, the notochord cells are proposed to

act as organizers directing the migration of mesenchymal cells and later undergo apoptosis (17). Cells from stem cell niches have been demonstrated to migrate to both the AF and NP in rabbit models (**Figure 1 A**) (18). Alternatively, the notochord maturation model hypothesizes that notochord cells are progenitors for all NP cells and undergo terminal differentiation to become cartilage-like cells (**Figure 1 B**) (19). Recent fate mapping experiments in mice using notochord-specific Cre recombinase expression driven by either *Shh* (20) or *Noto* (9) promoters support the notochord maturation model.

Research Project

Objective: Determine the fate of notochord cells in murine models of injury- and age-induced intervertebral disc degeneration.

Rationale

Previous work in the S-- lab has demonstrated that all cells in the healthy NP are derived from the notochord (9). However, the cellular composition of the unhealthy (degenerated) NP has not been established. The $Noto^{Cre}$ mouse model established by the S-- lab provides a unique opportunity to study the origins of NP cells in models of age or injury-induced degeneration by lineage tracing notochord-derived cells.

S-- lab has also recently developed a model of injury-induced IVD degeneration using repeated exposure to low amplitude, high frequency whole-body vibration. In this model, healthy 10-week old mice were subjected to 30 minutes of WBV at 45 Hz, 0.3 *g*, 0.72 mm vertical displacement for 5 days/week over 4 weeks. Histological evaluation revealed hallmark histological signs of disc degeneration, including increased

proteoglycan accumulation in the AF, fibrotic tissue in the NP, and the loss of demarcation between the AF and NP (21). Scoring of histological sections using the modified Thompson scale (22) demonstrated significant degenerative changes in the IVDs of mice exposed to WBV compared to non-vibrated sham controls (21).

Injury-induced degeneration is of particular interest in light of recent studies suggesting that mesenchymal-like stem cells home to sites of injury in a mouse model of IVD degeneration (23). Furthermore, greater homing of human bone marrow-derived mesenchymal stem cells (BMSCs) in bovine IVDs has been demonstrated under degenerative conditions compared to "healthy" physiological conditions *ex vivo* (24). Generally, BMSCs delivered systemically have been shown to migrate to sites of injury, such as wound healing (25), bone defects (26), myocardial infarction (27), and ischemic cerebral injury (28). In light of the potential for BMSCs to migrate to sites of injury, including potentially the IVD, we used a model of injury that has been previously characterized to induce disc degeneration in mice to investigate the origins of cells in the NP.

The second model of disc degeneration investigated in the current study was age-induced degeneration. Human notochordal cells are reported to disappear from within the IVD between 4-10 years of age (29). The loss of notochord cells precedes an increase in degenerative changes in human IVDs (30). Previous work in the S-- lab demonstrated that all cells in the NP of mice up to nine months of age were notochord-derived, supporting the notochord cell maturation model (9). To date, no studies have been conducted to examine the origins of NP cells in older mice. This study investigated the NPs of mice aged to 12 and 21 months, at which point histological examination revealed

hallmark features of disc degeneration. Investigating the persistence of notochord cells in mice will ultimately lead to a better understanding of the validity of the interspecies extrapolation of findings from mouse models of spine research to humans.

Hypothesis

All cells of the NP will be of notochord origin in mouse models of age- and mechanically-induced disc degeneration.

Experimental design

Mouse model:

The notochord-specific Cre mouse ($Noto^{Cre}$) was crossed with a conditional ROSA26 LacZ reporter mouse ($Rosa26^{LacZ}$) (9). The $Noto^{Cre/+}$; $Rosa26^{LacZ}$ mouse expresses LacZ only following Cre-mediated excision of a STOP sequence upstream of β-galactosidase. Thus, the $Noto^{Cre/+}$; $Rosa26^{LacZ}$ mouse expresses β-galactosidase in all notochord-derived cells and can be used to trace notochord-derived cells in the IVD (**Figure 2**).

Cohorts:

Lumbar spine segments were harvested from $Noto^{Cre/+}$; $Rosa26^{LacZ}$ mice at 12 months (n=4) and 21 months of age (n=3) or after exposure of 10 week-old mice to WBV, as described previously (45 Hz, 0.3 *g*, 30 min/day, 5 days/week , 4 weeks n=5, sham group n=4). Tail tissues were also harvested from $Noto^{Cre/+}$; $Rosa26^{LacZ}$ mice at 12 months of age.

Safranin O/fast green staining

In order to assess the presence/extent of disc degeneration in the IVD using histology, intact lumbar spinal segments (L1-L5) were stained with Safranin O/fast green to detect sulfated proteoglycans. Tissues were isolated and fixed in 4% paraformaldehyde. Spinal columns were decalcified for 4 days in Shandon's TBD-2 (Fisher Scientific) 5% EDTA in PBS (pH 7.0). Tissues were processed and embedded in paraffin. Spines were sectioned sagittally at a thickness of 5 μm using a microtome (Leica Microsystems). Using established protocols (31, 32), serial sections were stained with 1.5% Safranin O/0.02% fast green. Slides were imaged on a Leica DM1000 microscope, with Leica Application Suite (Leica Microsystems).

Whole mount staining

In order to assess the presence of β-galactosidase in whole-mount vertebral segments, β-galactosidase staining was carried out using standard protocols (9). Tissues were fixed for in 0.2% glutaraldehyde/1% formaldehyde/0.02% Igepal in Phosphate Buffered Saline (PBS) at room temperature. Following 3 washes in PBS/0.02% Igepal (5 min at room temperature), tissues were incubated in X-gal staining solution [5 mM K3Fe$(CN)_6$/5 mM $K_4Fe(CN)_6$/2 mM $MgCl_2$/1 mg/ml X-gal in PBS] overnight at room temperature on rocker. Tissues were then washed with PBS/0.02% Triton 3 x 5 min at room temperature and kept in PBS/0.02% Triton overnight to allow stain to develop.

Tissues were cryosectioned (Leica CM1850) in the transverse plane and slides were imaged on a Leica DM1000 microscope with Leica Application Suite.

Immunohistochemistry (IHC)

In order to visualize the cellular localization of β-galactosidase, lumbar tissues from all cohorts were stained using immunohistochemistry. Formalin-fixed tissues were decalcified for 5 days with gentle rocking, using Shandon TBD-2 decalcifier (Thermo Scientific) at a fluid-to-tissue ratio of 10:1. Following standard histological processing, samples were embedded in paraffin, and 5-micrometer thick serial sections were cut in the coronal plane. Sections were deparaffinized in xylene and rehydrated by successive immersion in descending concentrations of alcohol.

For IHC, antigen retrieval was performed with 10 mM sodium citrate (pH 6.0) for 12 minutes at 95°C. Slides were blocked by incubation for 1 hour with species-specific serum albumin (5%) in phosphate buffered saline plus 0.1% Triton X-100 (Sigma). The primary antibody directed against β-galactosidase (Abcam) was diluted 1:200 in PBST and incubated overnight at 4°C in a humidified chamber, followed by incubation with species-specific secondary antibody for 1 hour at room temperature (488–conjugated goal anti-rabbit IgG at a 1:200 dilution). Samples were mounted using Vectashield mounting medium with DAPI (Vector Laboratories). Species-specific isotype IgG controls were used to assess antibody specificity. Images (injury-induced degeneration: 2 discs per animal, 1 section per disc; age-induced degeneration: 3 discs per animal, 1 section per disc) were captured with a Zeiss Axio Imager.M1 fluorescence microscope using Northern Eclipse software.

The number of β-galactosidase positive cells per disc was counted for the above samples. The percentage β-galactosidase positive cells was averaged per mouse, then per cohort. An unpaired parametric t-test with Welch's correction was performed.

Results

Whole mount staining was used to detect the presence of β-galactosidase, and thus, notochord-derived cells, in the IVD. Blue staining detects X-gal, which stains cells that are positive for β-galactosidase, demonstrating the localization of notochord-derived cells in the NP of the IVD (**Figure 3**). The β-galactosidase staining was detected in five of six NPs collected throughout the tail of the 12-month mice and was not detected in any tissue other than the NP.

To assess degeneration within the IVDs, safranin O was used to detect sulfated proteoglycans and fast green was used as a counterstain. Hallmarks of IVD degeneration, such as proteoglycan accumulation in the AF, fibrotic tissue in the NP, and the loss of demarcation between the AF and NP, were detected more in mice following WBV than sham controls, and also detected in all 12- and 21-month old mice (**Figure 4 A** and **Figure 6 A**).

In order to quantify the contribution of notochord cells to the population of cells in the NP in the injury-induced model of degeneration, immunohistochemistry was performed to localize β-galactosidase positive cells (**Figure 4 B**). In the mechanical loading-induced model of degeneration, sham control mice demonstrated 99.5 ± 0.3 % NP cells that were positive for β-galactosidase while mice subjected to WBV demonstrated 99.6 ± 0.3 % NP cells that were positive for β-galactosidase. There was no

significant difference between the sham and vibrated cohorts in the injury-induced model of degeneration ($p = 0.7$) (**Figure 5**).

In order to assess the composition of the NP in our aging model of degeneration immunochemistry staining was likewise conducted on lumbar spine segments isolated from mice at 12 and 21 months of age (**Figure 6 B**). 12-month old mice had 91.2 ± 2.2 % NP cells that were positive for β-galactosidase; the 21-month old mice had 100.0 ± 0.0 % NP cells that were positive for β-galactosidase. There was a significant difference between the 12- and 21-month cohorts in the age-induced model of degeneration ($p = 0.03$) (**Figure 7**).

Discussion

Lineage tracing of the notochord cells in the IVD using the $Noto^{Cre}$ mouse demonstrated that both the notochordal cells and the cartilage-like cells of the healthy NP are notochord derived (9). To follow up on these studies we conducted Safranin O/fast green, whole mount, and IHC staining to determine whether all cells of the degenerated NP are still notochord-derived using 2 different models of disc degeneration. Our results from the whole mount staining validate the specificity of the $Noto^{Cre/+}$; $Rosa26^{LacZ}$ mouse model to trace notochord-derived cells in aged mice. The β-galactosidase stain was detected throughout five of six NPs, but nowhere else in the surrounding tissue, demonstrating the localization of notochord-derived cells to the nucleus pulposus of the IVD. At dissection, longitudal ligaments were left on the tail, over the IVD, to keep the tail tissue intact through the staining process (**Figure 3 A**); this may have interfered with

the whole-mount staining where the ligamentous tissue was thicker, resulting in one of six NPs not staining blue.

To confirm that the conditions of age and injury did indeed result in disc degeneration in our mice, tissue sections were stained with Safranin O/fast green or hematoxylin and eosin. Histological examination demonstrated that the IVDs of mice in the vibrated cohort had more degeneration than those of the non-vibrated sham cohort in the injury-induced model of degeneration. Similarly, IVDs of 12-month mice and 21-month mice also showed characteristics of disc degeneration. Thus, we validated that age and injury caused disc degeneration in our mice.

In our injury-induced model of degeneration, all NP cells were found to be notochord derived, thus supporting the notochord-maturation model. These results are not consistent with the studies in which mesenchyme-derived cells were reported to home to sites of injury. Needle-puncture was the mechanism of injury in studies whose results support the aforementioned homing effect (23, 24). Puncturing the disc with a needle causes acute trauma and creates an entry point for cells that otherwise would not be present in the IVD (33). The mesenchyme-derived cells that were reported to home to sites of injury may actually have migrated due to the unfilled space created by the puncture. The WBV model of injury-induced degeneration does not result in such an artificial entry point for cells, and thus, may be a better model for investigating whether mesenchyme-derived cells home to site of injury.

Furthermore, a study that transplanted bone marrow cells into mice with disc degeneration, induced through severe uneven loading by creating a loop in the tail, demonstrated that the migration of bone marrow cells was related to the severity of the

degeneration; even so, only a limited number of bone marrow cells migrated to the IVD, presumably because of its avascular nature (34). However, the needle puncture and the tail loop models of injury may not accurately recreate human disc degeneration, due to the artificial entry point and severity of uneven loading respectively. The WBV model is less drastic of an injury, and therefore, may be more appropriate for modeling the diseased state of disc degeneration.

In the age-induced model of degeneration, not all NP cells are notochord-derived at 12 months of age, but all NP cells are notochord-derived at 21 months. These finding may suggest the migration of BMSCs into the NP prior to 12 months. Degenerative conditions *ex vivo* have been shown to induce the release of factors promoting BMSC recruitment (24). Inflammatory cytokines are thought to trigger the chemotactic migration of BMSCs through extracellular matrix structures (35). However, the higher proportion of notochord-derived cells in the older 21-month mice compared to the 12-month mice is a counterintuitive observation if the source of the non-notochord-derived cells is from BMSC migration due to degenerative conditions.

The experimental design did not test for the presence of mesenchyme-derived cells. Therefore, one cannot conclude that the non-notochord-derived cells in the NP of the 12-month mice are the result of BMSC migration. Although the presence of non-notochord-derived cells does not necessarily support the notochord maturation model, the notochord-derived cells would have to stain positive for markers of mesenchymal cells to definitively support the niche-migration model. Furthermore, technical limitations, such as issues with the counterstain, mounting media, and nuclear imaging using DAPI, may have impeded the ability to quantify images. Investigating the origins of the non-

notochord-derived cells in the NP and optimizing protocols to overcome the aforementioned technical limitations would be the priorities of future directions with this project.

The results from the injury-induced model of degeneration compared to the age-induced model demonstrate that the origins of the NP cells may differ between the two different models of degeneration. The loss of notochord cells precedes an increase in degenerative changes in human IVDs, which suggests that notochord cells may serve a protective function against disc degeneration (30). The hypothesized protective function of notochord cells makes sense in the context of the data from the 12-month mice, which show both degenerative changes in the IVD and the presence of non-notochord-derived cells. However, although there were significant degenerative changes in the IVDs of mice exposed to WBV compared to non-vibrated sham controls (21), the vibrated mice have same proportion of notochord cells compared to the non-vibrated mice. Thus, the degenerative changes due to WBV do not seem to result from the loss of a protective effect of notochord cells. This finding suggests that the pathway of degeneration might be dependent on the mechanism. If the natural process of aging versus external physical forces causes differences in the pathway of degeneration, clinical implications might include different therapeutic targets for low back pain according to cause.

Alternatively, the fact that we did not control for the severity of degeneration between age and vibration groups may be responsible for the different results. Responsiveness to cytokines thought to mediate BMSC migration, such as transforming growth factor-β, interleukin-1β, and tumor necrosis factor-α, in the degenerative living human IVD can be different according to the degree of degeneration (36). This lack of

control between the two models of degeneration means that definitive conclusions cannot yet be drawn as to the significance of the different observations between the age-induced and injury-induced groups.

Future Directions

The surprising finding that there are non-notochord-derived cells at 12 months of age but not at 21 months is counterintuitive, and thus, bears revisiting using IHC co-localization. Future directions include the IHC co-localization of β-galactosidase with cytokeratin 8, a notochord cell marker. Previous research in the S-- lab demonstrated the embryonic NP consisted of only cytokeratin-8 positive cells at embryonic day 17.5, suggesting that all cells maintain a notochord phenotype at that age. However, only a subset of the NP cells maintained cytokeratin-8 expression at 9 months of age, suggesting that not all cells of the NP retain a notochord phenotype in older mice (9). Comparing the cytokeratin-8 expression at older time points, such as 12 and 21 months, will allow a better understanding of the morphology of the NP over time.

To investigate whether the non-notochord-derived cells are BMSC-derived, IHC co-localization staining of β-galactosidase and a marker of mesenchymal stem cells, such as platelet-derived growth factor receptor α (PDGFR-α) and stem cell antigen 1 (Sca-1) (37), can be performed. Performing IHC on serial sections would lead to a better understanding of composition of cells throughout the disc. For example, if BMSCs migrate into periphery of NP, but do not travel to middle of NP, then examining just one section will not yield a representative image of the whole disc.

An interesting observation to pursue would be the small pockets of β-galactosidase positive cells in the CEP of two of four of the non-vibrated sham mice used in the injury-induced model of degeneration, visualized under IHC staining. We hypothesize that these pockets may be notochordal remnant cells that fail to integrate into the NP, and in rare cases, can form the basis for chordomas (38). We can stain for brachyury, an NP cell phenotype marker, to further investigate.

Significance

In the long term, a better understanding of the changes in cellular composition of the IVD in response to injury and age may enable targeted therapeutic strategies to treat IVD degeneration and back pain. A recent publication demonstrated that native porcine NP tissue directed differentiation of human induced pluripotent stem cells to notochordal cells (39). This study was a proof of concept for eventual regenerative therapies for disc degeneration. With such research in notochordal cells as a promising cell source for cell-based therapy to treat IVD degeneration, there is an urgent need to understand of how notochordal cells act in degenerative disc.

References

1. Balague F, Mannion AF, Pellise F, Cedraschi C. Non-specific low back pain. Lancet. 2012;379(9814):482-91.

2. Lyons G, Eisenstein SM, Sweet MB. Biochemical changes in intervertebral disc degeneration. Biochimica et biophysica acta. 1981;673(4):443-53.

3. Urban JP, McMullin JF. Swelling pressure of the lumbar intervertebral discs: influence of age, spinal level, composition, and degeneration. Spine. 1988;13(2):179-87.

4. Frobin W, Brinckmann P, Kramer M, Hartwig E. Height of lumbar discs measured from radiographs compared with degeneration and height classified from MR images. European radiology. 2001;11(2):263-9.

5. Adams MA, Dolan P, Hutton WC, Porter RW. Diurnal changes in spinal mechanics and their clinical significance. The Journal of bone and joint surgery British volume. 1990;72(2):266-70.

6. Boos N, Rieder R, Schade V, Spratt KF, Semmer N, Aebi M. 1995 Volvo Award in clinical sciences. The diagnostic accuracy of magnetic resonance imaging, work perception, and psychosocial factors in identifying symptomatic disc herniations. Spine. 1995;20(24):2613-25.

7. de Schepper EI, Damen J, van Meurs JB, Ginai AZ, Popham M, Hofman A, et al. The association between lumbar disc degeneration and low back pain: the influence of age, gender, and individual radiographic features. Spine. 2010;35(5):531-6.

8. Yamanaka Y, Tamplin OJ, Beckers A, Gossler A, Rossant J. Live imaging and genetic analysis of mouse notochord formation reveals regional morphogenetic mechanisms. Developmental cell. 2007;13(6):884-96.

9. McCann MR, Tamplin OJ, Rossant J, Seguin CA. Tracing notochord-derived cells using a Noto-cre mouse: implications for intervertebral disc development. Disease models & mechanisms. 2012;5(1):73-82.

10. Dalgleish AE. A study of the development of thoracic vertebrae in the mouse assisted by autoradiography. Acta anatomica. 1985;122(2):91-8.

11. Taher F, Essig D, Lebl DR, Hughes AP, Sama AA, Cammisa FP, et al. Lumbar degenerative disc disease: current and future concepts of diagnosis and management. Advances in orthopedics. 2012;2012:970752.

12. Erwin WM, Islam D, Inman RD, Fehlings MG, Tsui FW. Notochordal cells protect nucleus pulposus cells from degradation and apoptosis: implications for the mechanisms of intervertebral disc degeneration. Arthritis research & therapy. 2011;13(6):R215.

13. Smolders LA, Bergknut N, Grinwis GC, Hagman R, Lagerstedt AS, Hazewinkel HA, et al. Intervertebral disc degeneration in the dog. Part 2: chondrodystrophic and non-chondrodystrophic breeds. Veterinary journal. 2013;195(3):292-9.

14. Risbud MV, Schoepflin ZR, Mwale F, Kandel RA, Grad S, Iatridis JC, et al. Defining the Phenotype of Young Healthy Nucleus Pulposus Cells: Recommendations of the Spine Research Interest Group at the 2014 Annual ORS Meeting. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2014.

15. Kim JH, Deasy BM, Seo HY, Studer RK, Vo NV, Georgescu HI, et al. Differentiation of intervertebral notochordal cells through live automated cell imaging system in vitro. Spine. 2009;34(23):2486-93.

16. Purmessur D, Guterl CC, Cho SK, Cornejo MC, Lam YW, Ballif BA, et al. Dynamic pressurization induces transition of notochordal cells to a mature phenotype while retaining production of important patterning ligands from development. Arthritis research & therapy. 2013;15(5):R122.

17. Kim KW, Lim TH, Kim JG, Jeong ST, Masuda K, An HS. The origin of chondrocytes in the nucleus pulposus and histologic findings associated with the

transition of a notochordal nucleus pulposus to a fibrocartilaginous nucleus pulposus in intact rabbit intervertebral discs. Spine. 2003;28(10):982-90.

18. Barreto Henriksson H, Lindahl A, Skioldebrand E, Junevik K, Tangemo C, Mattsson J, et al. Similar cellular migration patterns from niches in intervertebral disc and in knee-joint regions detected by in situ labeling: an experimental study in the New Zealand white rabbit. Stem cell research & therapy. 2013;4(5):104.

19. Risbud MV, Shapiro IM. Notochordal cells in the adult intervertebral disc: new perspective on an old question. Critical reviews in eukaryotic gene expression. 2011;21(1):29-41.

20. 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. Developmental dynamics : an official publication of the American Association of Anatomists. 2008;237(12):3953-8.

21. McCann M.R. PP, Pest M.A., Ratneswaran A., Kamphuis M.P., Esmail Z., Lee J., Barbalinardo M., Beaucage K.L, Holdsworth D.W., Beier F., Dixon S.J, Séguin C.A. Repeated exposure to high-frequency low-amplitude vibration induce degeneration of intervertebral disc and knee joint in a murine model. Arthritis & Rheumatology (In revision). 2015.

22. Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IK, Bishop PB. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine. 1990;15(5):411-5.

23. Tam V, Ng SY, Rogers I, Chan D, Cheung KMC. Investigation of the homing ability of mesenchymal-like stem cells to sites of injury using a mouse model of IVD degeneration. 2011.

24. Illien-Junger S, Pattappa G, Peroglio M, Benneker LM, Stoddart MJ, Sakai D, et al. Homing of mesenchymal stem cells in induced degenerative intervertebral discs in a whole organ culture system. Spine. 2012;37(22):1865-73.

25. Mackenzie TC, Flake AW. Human mesenchymal stem cells persist, demonstrate site-specific multipotential differentiation, and are present in sites of wound healing and tissue regeneration after transplantation into fetal sheep. Blood cells, molecules & diseases. 2001;27(3):601-4.

26. Kitaori T, Ito H, Schwarz EM, Tsutsumi R, Yoshitomi H, Oishi S, et al. Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. Arthritis and rheumatism. 2009;60(3):813-23.

27. Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, et al. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation. 2003;108(7):863-8.

28. Ji JF, He BP, Dheen ST, Tay SS. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem cells. 2004;22(3):415-27.

29. Chelberg MK, Banks GM, Geiger DF, Oegema TR, Jr. Identification of heterogeneous cell populations in normal human intervertebral disc. Journal of anatomy. 1995;186 (Pt 1):43-53.

30. Boos N, Weissbach S, Rohrbach H, Weiler C, Spratt KF, Nerlich AG. Classification of age-related changes in lumbar intervertebral discs: 2002 Volvo Award in basic science. Spine. 2002;27(23):2631-44.

31. McCann MR, Patel P, Beaucage KL, Xiao Y, Bacher C, Siqueira WL, et al. Acute vibration induces transient expression of anabolic genes in the murine intervertebral disc. Arthritis and rheumatism. 2013;65(7):1853-64.

32. Pest MA, Russell BA, Zhang YW, Jeong JW, Beier F. Disturbed cartilage and joint homeostasis resulting from a loss of mitogen-inducible gene 6 in a mouse model of joint dysfunction. Arthritis Rheumatol. 2014;66(10):2816-27.

33. Issy AC, Castania V, Castania M, Salmon CE, Nogueira-Barbosa MH, Bel ED, et al. Experimental model of intervertebral disc degeneration by needle puncture in Wistar rats. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al]. 2013;46(3):235-44.

34. Sakai D, Nishimura K, Tanaka M, Nakajima D, Grad S, Alini M, et al. Migration of bone marrow-derived cells for endogenous repair in a new tail-looping disc degeneration model in the mouse: a pilot study. The spine journal : official journal of the North American Spine Society. 2014.

35. Ries C, Egea V, Karow M, Kolb H, Jochum M, Neth P. MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines. Blood. 2007;109(9):4055-63.

36. Kim SH, Kuh SU, Kim KN, Park JY, Cho KH, Chin DK, et al. Biologic response of degenerative living human nucleus pulposus cells to treatment with cytokines. Yonsei medical journal. 2015;56(1):277-86.

37. Houlihan DD, Mabuchi Y, Morikawa S, Niibe K, Araki D, Suzuki S, et al. Isolation of mouse mesenchymal stem cells on the basis of expression of Sca-1 and PDGFR-alpha. Nat Protoc. 2012;7(12):2103-11.

38. Patel P, Brooks C, Seneviratne A, Hess DA, Seguin CA. Investigating microenvironmental regulation of human chordoma cell behaviour. PLoS One. 2014;9(12):e115909.

39. Liu Y, Fu S, Rahaman MN, Mao JJ, Bal BS. Native nucleus pulposus tissue matrix promotes notochordal differentiation of human induced pluripotent stem cells with potential for treating intervertebral disc degeneration. Journal of biomedical materials research Part A. 2015;103(3):1053-9.

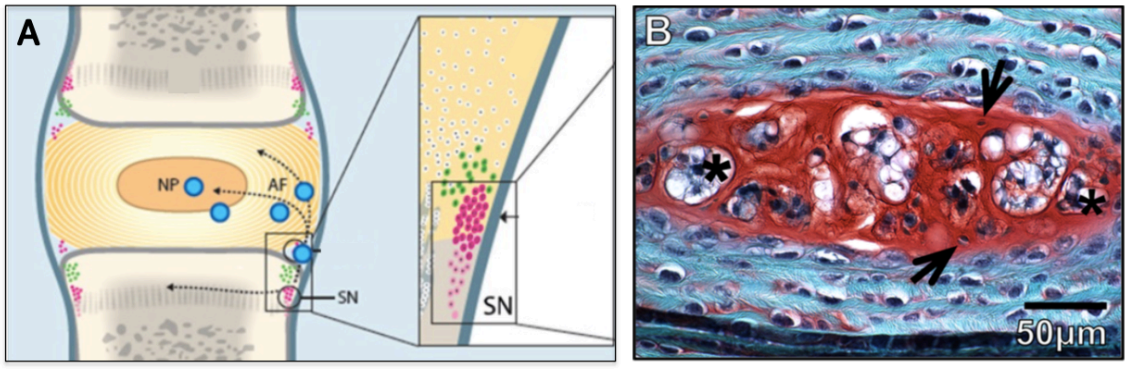

Figure 1: Two conflicting models of the origins of the cartilage-like cells in the nucleus pulposus: the niche-migration model and the notochord maturation model.

A. The niche-migration model proposes that the cartilage-like cells are of mesenchymal origin and migrate to the nucleus pulposus (NP) and the annulus fibrosus (AF) from stem cell niches (SN). Cells from stem cell niches have been demonstrated to migrate to both the AF and NP in rabbit models. Blue circles represent localization of detected migrating cells from the SNs. (Modified from Henriksson, et al. 2013). **B.** The notochord maturation model hypothesizes that notochord cells (*) are progenitors for all NP cells, and undergo terminal differentiation to become the smaller cartilage-like cells (arrows). (Modified from McCann, et al. 2011).

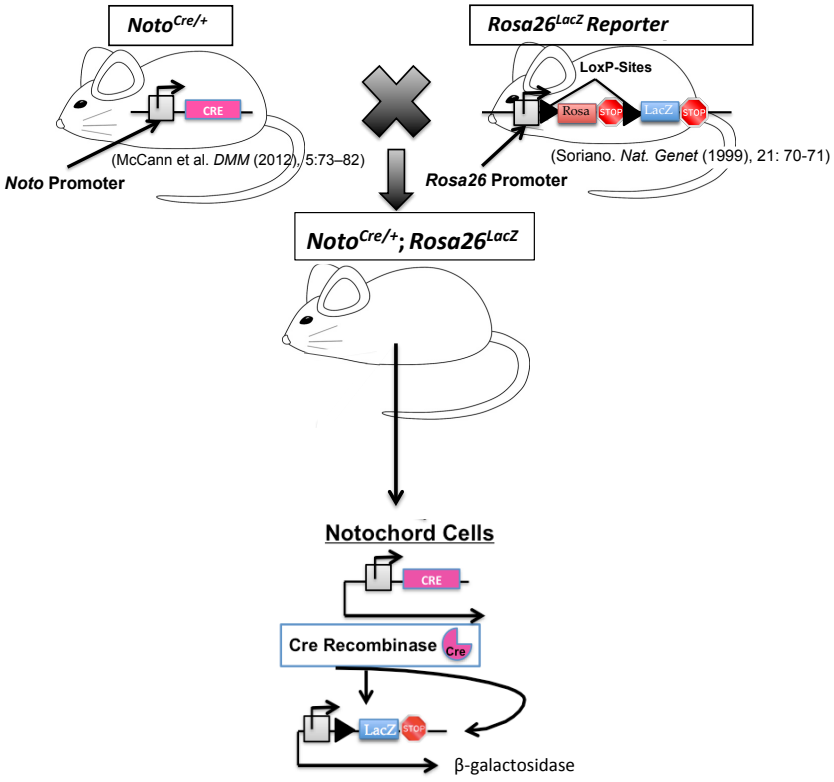

Figure 2: The *Noto*$^{Cre/+}$; *Rosa26*^{LacZ} mouse expresses LacZ exclusively following Cre-mediated excision of a STOP sequence upstream of β-galactosidase.

The notochord-specific Cre mouse (*Noto*^{Cre}) was crossed with a conditional ROSA26 LacZ reporter mouse (*Rosa26*^{LacZ}) (9). The *Noto*$^{Cre/+}$; *Rosa26*^{LacZ} mouse expresses LacZ only following Cre-mediated excision of a STOP sequence upstream of β-galactosidase. Thus, the *Noto*$^{Cre/+}$; *Rosa26*^{LacZ} mouse expresses β-galactosidase in all notochord-derived cells and can be used to trace notochord-derived cells in the IVD.

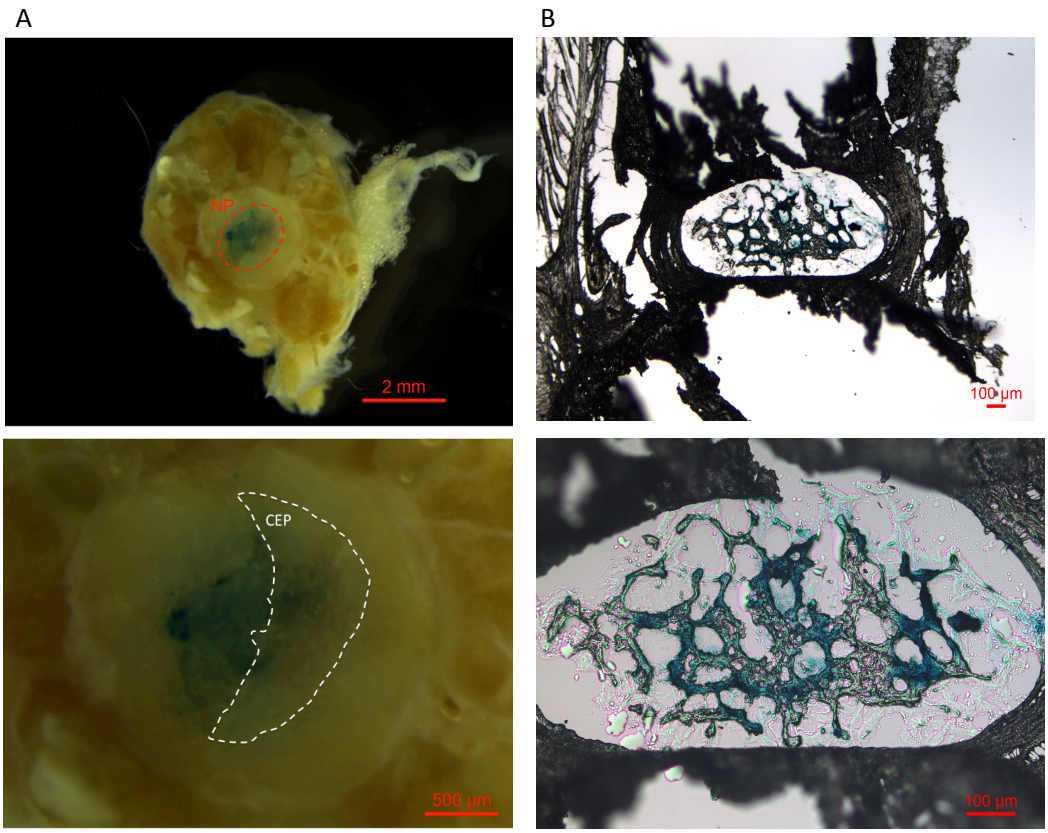

Figure 3: Whole mount staining of a tail IVD from a 12 month *Noto*^{Cre} ;*Rosa26*^{LacZ} mouse.

A. Representative image of transverse sections of caudal IVDs following whole mount β-galactosidase staining where the CEP was left partially covering the NP. **B.** Tail tissues were sectioned transversely at a thickness of 5 µm. Blue staining detects X-gal, which stains cells that are positive for β-galactosidase, demonstrating the localization of notochord-derived cells in the nucleus pulposus of the IVD. β-galactosidase staining was detected in five of six NPs throughout the tail and was not detected in any tissue other than the NP.

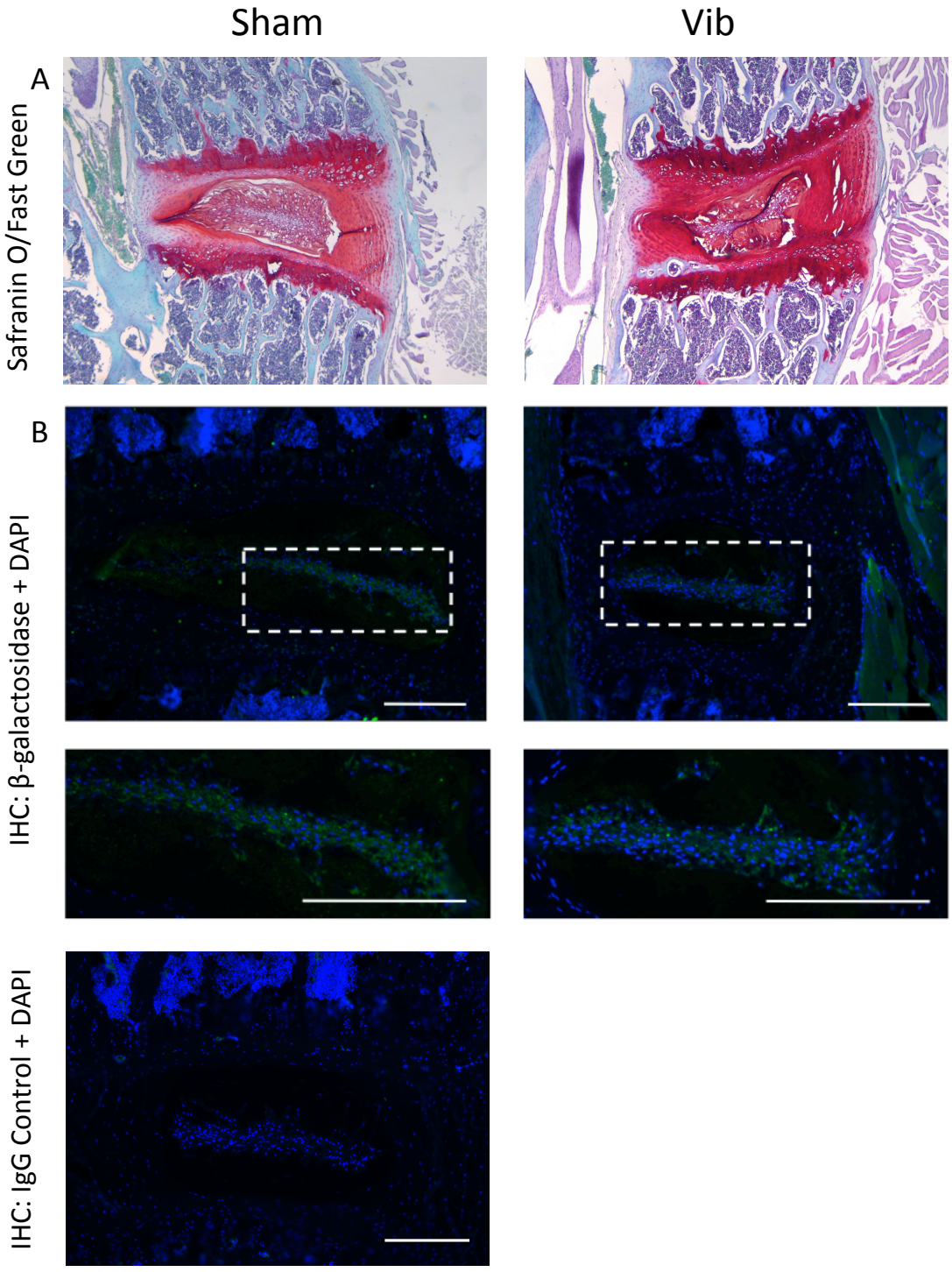
Sham
Vib
A
Safranin O/Fast Green
B
IHC: β-galactosidase + DAPI
IHC: IgG Control + DAPI

Figure 4: Safranin O/fast green stain and immunohistochemistry staining of β-galactosidase in lumbar tissue of mice following exposure to whole-body vibration as a model of disc degeneration.

A. Representative images of IVDs stained by Safranin O enabling visualization of proteoglycans and fast green as a counterstain demonstrate the induction of degeneration in mice exposed to WBV compared to non-vibrated sham control mice. **B.** Representative images of the immunolocalization of β-galactosidase (green) with DAPI (nuclear, blue) at 100x magnification (top) and 200x magnification (bottom) demonstrate that all cells of the NP in both sham and vibrated mice were β-galactosidase positive. Scale bar = 250μm. Box outlines the NP region. Species-specific isotype IgG controls were used to assess antibody specificity.

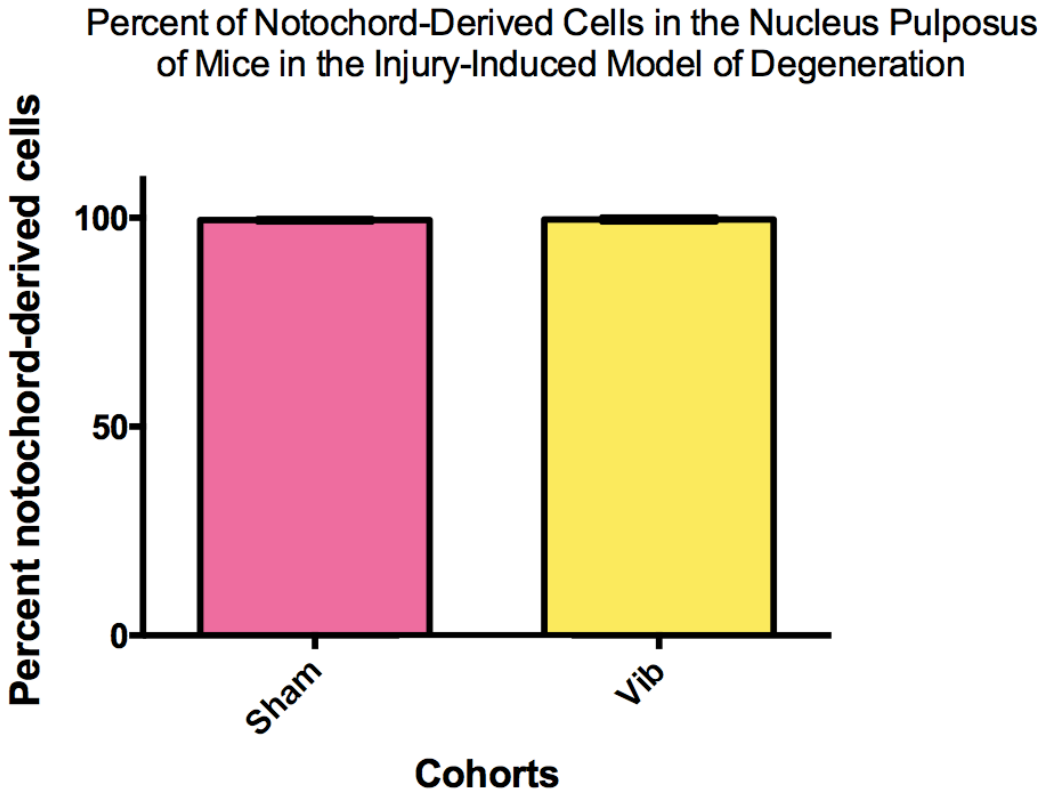

Figure 5: Percent of notochord-derived cells in the nucleus pulposus of mice in the injury-induced model of degeneration

An unpaired parametric t-test with Welch's correction was performed to compare the number of β-galactosidase positive cells per disc, averaged per mouse, then averaged per cohort (sham n=4, vib n=5). The sham cohort had 99.5 ± 0.3 % NP cells positive for β-galactosidase while the vibrated cohort had 99.6 ± 0.3 % NP cells positive for β-galactosidase. There was no significant difference between the sham and vibrated cohorts in the injury-induced model of degeneration ($p = 0.7$).

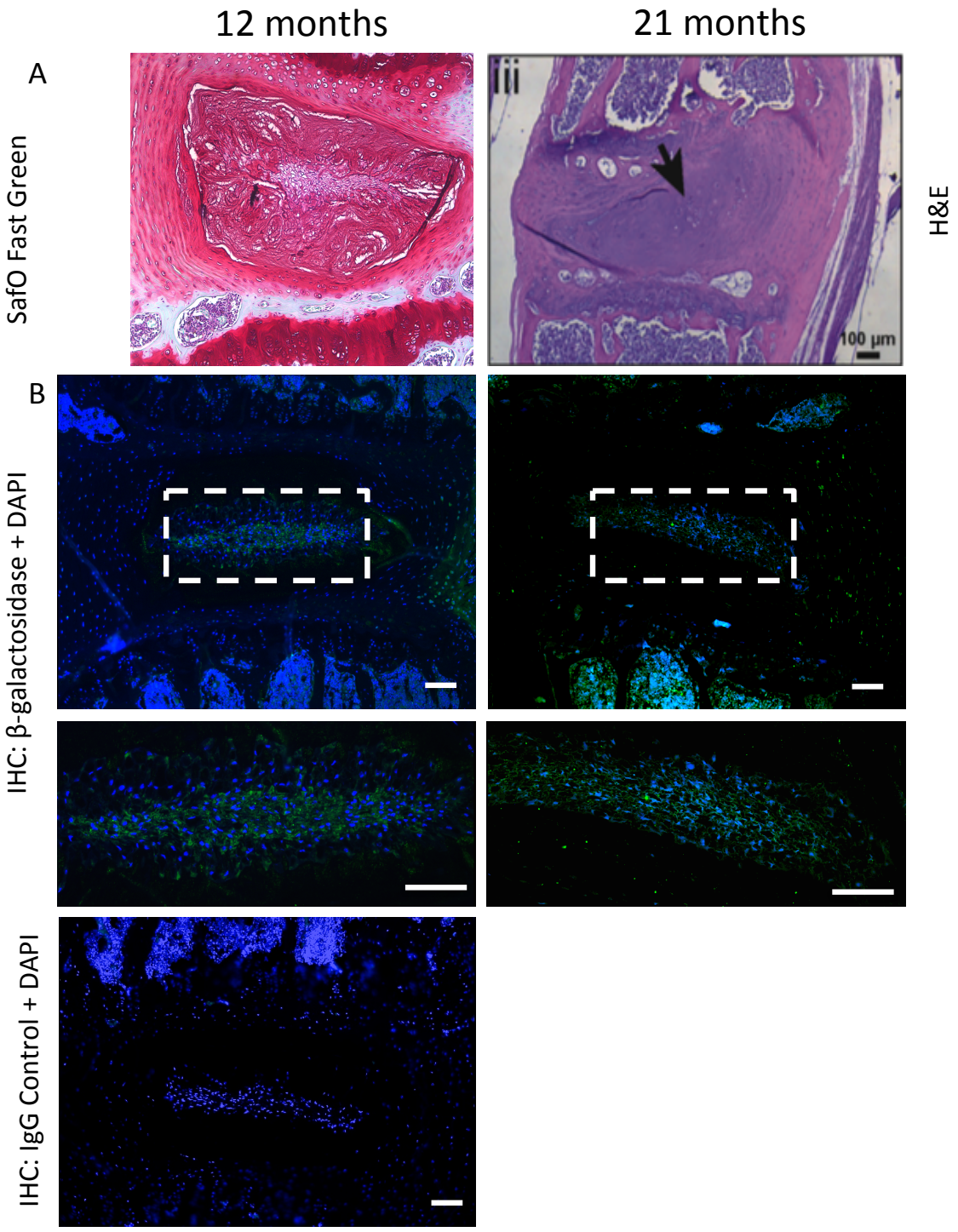
12 months
21 months
A
SafO Fast Green
iii
100 µm
H&E
B
IHC: β-galactosidase + DAPI
IHC: IgG Control + DAPI

Figure 6: Histological examination of lumbar IVDs of mice following exposure to whole-body vibration as a model of disc degeneration.

A. Representative images of IVD tissues stained by safranin O/fast green from 12-month old mice, or by hematoxylin and eosin from 21-month old mice show disc degeneration at both time points. (Modified from McCann, S-- lab.) **B.** Representative images showing the immunolocalization of β-galactosidase (green) with DAPI (nuclear, blue) demonstrate that not all cells of the NP in 12-month mice were β-galactosidase positive, but all cells of the NP in 21-month mice were β-galactosidase positive. Scale bars = 100μm. Box outlines the NP region. Species-specific isotype IgG controls were used to assess antibody specificity.

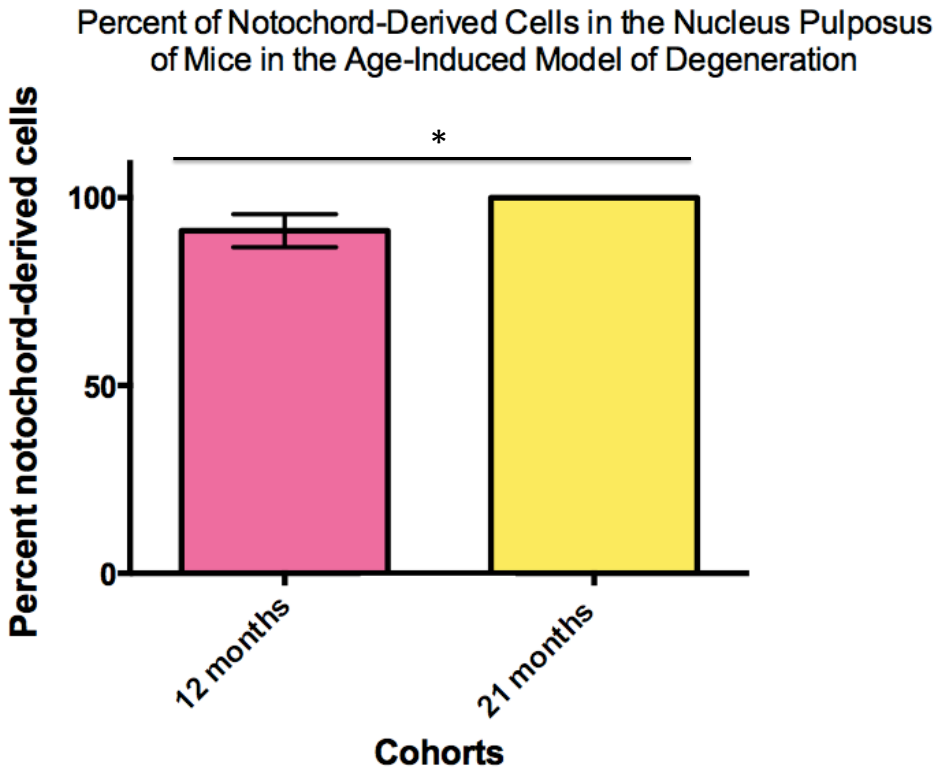

Figure 7: Percent of notochord-derived cells in the nucleus pulposus of mice in the age-induced model of degeneration

An unpaired parametric t-test with Welch's correction was performed to compare the number of β-galactosidase positive cells per disc, averaged per mouse, then averaged per cohort (12-months n=4, 21-months n=3). 12-month old mice had 91.2 ± 2.2 % NP cells positive for β-galactosidase; the 21-month old mice had 100.0 ± 0.0 % NP cells positive for β-galactosidase. There was a significant difference between the 12- and 21-month cohorts in the age-induced model of degeneration ($p = 0.03$).