Intervertebral disc degeneration is a very common medical condition causing pain and disability, and it cannot be reversed by available treatment options. Here we report that targeting β-catenin, a pivotal factor associated with disc degeneration, ameliorates disc degeneration in a mouse model of disc injury. Degenerative changes in the disc in response to disc injury include decomposition of nucleus pulposus (NP), replacement of notochordal cells in the NP by chondrocyte-like cells, and disorganization of annulus fibrosus (AF). Importantly, downregulation of β-catenin through intradiscal injection of CRISPR-Cas9-expressing adeno-associated virus significantly mitigated all these pathological changes, by preserving notochordal cells and attenuating chondro-osteogenesis in the NP, as well as maintaining the AF structure. Moreover, β-catenin loss-of-function decelerated the rapid induction of catabolic reactions in disc matrix and attenuated pain-related neural events during disc degeneration. Thus, our data demonstrate that targeting β-catenin in disc cells through CRISPR-Cas9 has multifaceted therapeutic effects on disc degeneration, and we suggest that β-catenin plays a fundamental role in the remodeling and degenerative processes of the disc. In addition, this study proposes that CRISPR-Cas9 is a useful tool for identifying new drug targets and developing therapeutic strategies for disc degeneration.

INTRODUCTION

Low back pain is one of the leading causes of disability and a heavy socioeconomic burden all over the world. A major cause for low back pain is considered to be intervertebral disc degeneration (IVDD), which is characterized by the structure failure of the disc. Histologic studies of human lumbar intervertebral discs suggest that disc degeneration is age related and may occur as early as in the first decade of life. Disc degeneration is also a complicated process including alterations of structural matrix molecules, upregulations of cytokines, as well as increased proliferation and death of disc cells. Currently, several approaches have been tested by employing biologics for alleviation of disc degeneration, such as cell-based therapy, biomaterial-based disc repair, and gene therapy. Nevertheless, there are still very limited options other than surgery for effective treatment of disc degeneration.

For the treatment of disc degeneration, preclinical explorations have been performed by overexpressing growth factors or transcriptional factors in the disc of IVDD animal models. While these studies show promising results such as increased proteoglycan synthesis or restored disc height, the search for new therapeutic targets is still necessary to achieve more effective alleviation of disc degeneration. It has been demonstrated that β-catenin, a protein encoded by Ctnnb1 gene, is a major conductor of classical Wnt/β-catenin signaling pathway and plays important roles in regulating disc development and disc metabolism. Moreover, the expression level of β-catenin is significantly elevated in degenerative disc tissues from IVDD patients, suggesting that β-catenin is associated with disc degeneration. Genetically modified mice with conditional activation of β-catenin expression in disc tissues show severe disc degeneration phenotype. Therefore, reducing β-catenin expression by ablating the Ctnnb1 gene represents a promising strategy for treating disc degeneration.

Since the very beginning of the invention of clustered regulatory interspaced short palindromic repeat (CRISPR) gene editing technology, its application in treating human diseases has attracted intense attention. Over the past few years, this revolutionary genome-editing tool has experienced tremendous progress in the optimization for its uses in disease treatment. For example, SaCas9 (CRISPR associated protein 9 from Staphylococcus aureus), a Cas9 gene of relatively smaller size, can be packed into a single adeno-associated virus (AAV) vector together with a single guide RNA (sgRNA) module, making in vivo delivery of CRISPR-Cas9 system less challenging. Because of its high efficiency in generating non-homologous end-joining and thus producing gene-inactivating mutations in targeted genes, CRISPR-Cas9 is a useful tool for reducing the expression levels of disease-associated genes. Thus, application of CRISPR-mediated gene editing-based therapy would be a new, promising strategy for the treatment of IVDD.

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In this study, we generated a mouse model of disc degeneration and administered Ctnnb1-ablating AAV vectors to the injured discs to study how β-catenin loss-of-function intervenes in disc degeneration by modulating anabolic, catabolic, and neural events unfolded during those dynamic pathological changes. We found that the CRISPR-Cas9-mediated gene editing efficiently reduced the levels of β-catenin in disc cells, preserved notochordal cells, decelerated pathological remodeling of disc matrix, and attenuated neurite formation, which resulted in a comprehensive alleviation of disc degeneration in the mouse model. Thus, our study suggests that β-catenin is a promising drug target for treating disc degeneration and that CRISPR-mediated gene ablation is useful for development of new therapeutic strategies in preclinical models.

RESULTS

β-catenin is upregulated in a mouse model of disc degeneration

We generated a mouse model of disc degeneration in this study, through inserting a needle to cause a surgical injury to the coccygeal discs in the mice. Three months after the surgery, the discs were collected for histologic examination and immunohistochemistry analysis of β-catenin. In the sham group that did not receive the stab injury, the structure of the discs was intact: the NP contained an abundance of vacuolated notochordal cells and was surrounded by blue-stained proteoglycan matrix, and the AF consisted of highly-organized concentric lamellae. The boundary between AF and NP became distinctly due to the lesion. Evaluation of disc degeneration using the Thompson grading scheme confirmed that the stab injury caused severe degenerative changes in both NP and AF at 3 months after the injury (Figures S1B and S1C). Moreover, these degenerative changes in the disc aggravated with time, as revealed by the histological results of the discs collected 6 months after the injury (Figure S1D). In these NP tissues, notochordal cells have been completely depleted and proteoglycan matrix has been replaced by fibrocartilage that was largely calcified. The distinction between AF and NP became more profoundly obscure, and the vast majority of the lamellae in these NP tissues, notochordal cells have been completely depleted (Figures S1E and S1F). Together, our results suggested that the stab injury in the disc induces severe, comprehensive degenerative changes in the mice.

Next, we analyzed the protein level of β-catenin in disc tissues through fluorescent immunohistochemistry (IHC) to evaluate whether disc degeneration is associated with the expression level of β-catenin. In normal disc tissues from the sham group, β-catenin proteins were strikingly decreased due to the lesion. Evaluation of disc degeneration progression, as revealed by the IHC results at 6 months still showed evident β-catenin upregulation. In the sham group that did not receive the stab injury, it was speculated that β-catenin upregulation was mostly contributed by β-catenin-expressing chondrocyte-like cells, which migrated into the NP and proliferated locally to replace notochordal cells. Importantly, β-catenin upregulation was found throughout the progression of disc degeneration progression, as the IHC results at 6 months still showed evident β-catenin upregulation.
expression in degenerated disc tissues (Figures S2C and S2D). Together, our results demonstrated that disc injury can cause degenerative changes in the mice relevant to human disc degeneration, and that \( \beta \)-catenin upregulation is associated with and may be causative factor in severe, irreversible degenerative changes. Thus, antagonism of \( \beta \)-catenin could be a promising strategy to ameliorate disc degeneration.

**CRISPR-Cas9 efficiently ablates \( \beta \)-catenin expression in mesenchymal cells**

For in vivo \( \beta \)-catenin targeting, we chose the CRISPR-Cas9 system derived from *Staphylococcus aureus*, as SaCas9 is significantly smaller than the majority of Cas9 homologs and thus allows efficient AAV packaging. For the mouse *Ctnnb1* gene, we designed two single guide RNA (sgRNA) sequences and constructed two vectors accordingly. To test the *Ctnnb1* targeting efficiency of the sgRNAs, we stably transfected mouse CD45– bone marrow stromal cells with the AAV plasmids. Quantitative reverse-transcription PCR (qRT-PCR) of the transfected cells demonstrated that *Ctnnb1* mRNA was drastically reduced by either sgRNA or their combination (Figure 1A), confirming their effectiveness in ablating *Ctnnb1*, likely through generating genetic mutations that induce nonsense-mediated mRNA decay to degrade *Ctnnb1* mRNAs. Moreover, western blot results confirmed a tremendous loss of \( \beta \)-catenin protein (Figure 1B). We also genotyped the cells transfected with both sgRNAs through PCR and sequencing of the targeted regions, and found that the combination of both sgRNAs can generate a 679-nt deletion in the *Ctnnb1* gene (Figure 1C), which would lead to a frameshift mutation and introduce premature stop codons to prevent production of functional \( \beta \)-catenin proteins. Together, our results suggested that SaCas9 and the selected sgRNAs can achieve an effective antagonism of \( \beta \)-catenin in the mouse cells.

**AAV-delivered \( \beta \)-catenin targeting ameliorates disc degeneration**

Next, we prepared \( \beta \)-catenin-targeting AAV and tested whether its administration has any effects on the degenerative course in the injured disc. Three months after the injury and AAV treatment, disc tissues were collected for histological analysis. Compared with the control group that suffered stab injury and received a control AAV capsid, the mice administered with \( \beta \)-catenin-targeting AAV exhibited significantly reduced degenerative changes in the disc, as evidenced by better-organized parallel fibrocartilage lamellae in the AF as well as preservation of vacuolated notochordal cells in the NP (Figure 2A). The annular/nucleus border still remained clear, the NP retained...
much of its original volume, and the fissures in the AF left by the puncture were much less extensive than those in the control group. Histological evaluation using the Thompson grading scheme also confirmed mitigated degeneration in both AF and NP of the discs treated with β-catenin-targeting AAV (Figures 2B and 2C).

As disc degeneration evolves over time, we also looked at the long-term effects of AAV administration by harvesting disc tissues 6 months after the surgery and treatment. Compared with the sham group without stab injury, the control group showed comprehensive degenerative changes, including complete loss of notochordal cells, widening of cracks and fissures induced by the trauma, wide-ranging calcification in the disc including both AF and NP, and reduction of blue-stained cartilaginous matrix possibly due to calcification (Figure 2D). In particular, these degenerative changes are also more severe than those observed in the discs at 3 months after the injury, suggesting that disc degeneration is rapidly aggravated with time if without efficacious intervention. Significantly, the treatment of β-catenin targeting decelerated those pathogenic processes, as exemplified by better preserved cartilaginous matrix, reduced disc calcification, diminished fissures in the disc, and lower scores based on the Thompson grading scheme (Figures 2D–2F). We also examined the discs adjacent to those subjected to surgical operation and AAV administration to identify whether β-catenin targeting affected non-operated discs. Our histological analyses did not find significant effects of Ctnnb1 ablation on the structures of these discs (Figures S3A and S3B), suggesting that β-catenin targeting mainly modulates the discs with a direct exposure to AAV.

**Efficient ablation of β-catenin in both annulus and nucleus**

To examine the efficiency of β-catenin targeting in vivo, we performed fluorescent IHC of β-catenin in the disc tissues. The control group, in which the disc underwent the injury and received control injection, showed widespread expression of β-catenin in both AF and NP area, whereas administration of β-catenin-targeting AAV reduced the expression of β-catenin in the majority of both AF and NP cells (Figures 3A and 3C). It is notable that there are significant differences in terms of cellular composition between the control and the Ctnnb1-targeting group. Thus, the dramatic downregulation of β-catenin was in part due to fewer chondrocyte-like cells in the injured NP. Nevertheless, the chondrocyte-like cells in the treated discs still retained fewer β-catenin proteins, suggesting that our β-catenin sgRNA-expressing AAV efficiently transduced the disc cells and potently reduced β-catenin expression. At 6 months, β-catenin elevation induced by disc degeneration was still sustained in the control group but well contained by the CRISPR treatment (Figures 3B and 3D), suggesting long-lasting effects provided...
by AAV-delivered gene editing of Ctnnb1. In consideration of improved histological features in the group receiving β-catenin ablation, we concluded that β-catenin downregulation attenuates the progression of disc degeneration induced by traumatic injury.

**Reduction of β-catenin alleviates fibrocartilage replacement and eventual calcification of the nucleus**

It is notable that blue-stained proteoglycan matrix rapidly replaced the notochordal cells in the injured disc (Figure 2A). Thus, we reasoned that these morphological changes could be attributed to enhanced anabolic responses by those newly arriving and proliferated cells that replaced notochordal cells and mediated osteochondral differentiation in both annulus and nucleus. Specifically, our IHC data showed that the notochordal marker Brachyury was drastically reduced in the injured discs at 3 months after the injury, whereas this reduction of Brachyury could be considerably attenuated by β-catenin targeting (Figures 3E and 3G). With the progression of disc degeneration, the notochordal cells were almost completely depleted at 6 months after the injury, but they could be still kept at a detectable level when treated with Ctnnb1-targeting AAV (Figures 3F and 3H). Therefore, our data suggested that β-catenin loss-of-function decelerates the depletion of notochordal cells in disc degeneration.

Moreover, we found that type II collagen was abundantly expressed in the newly formed matrix that replaced the notochordal cells in the injured disc, while β-catenin targeting significantly limited the collagen II-positive area by preserving notochordal cells, when the disc were analyzed 3 months after the injury (Figure 4A). At this point, type X collagen was also evident in the tissues that were depleted of notochordal cells, which were also efficiently blocked by β-catenin loss-of-function (Figure 4B). We also examined type I collagen, the osteogenic marker, and our data demonstrated that its expression was largely concomitant with cartilage replacement of the nucleus in the injured disc but alleviated by β-catenin targeting (Figure 4C). Six months after the injury, the expression of collagen II started to fade away (Figure 4D) and collagen X picked up (Figure 4E), especially in the control group, suggesting that cartilage replacement of nucleus had been almost completed and calcification had been advancing in the injured disc. Nevertheless, β-catenin antagonism blocked these matrix remodeling events remarkably, as shown by the reduced levels of type X and type I collagens (Figures 4E and 4F). Notably, the difference of type II collagen expression became nonsignificant at 6 months, suggesting that notochordal replacement by chondrocyte-like cells has come to the end in the control group, while β-catenin targeting effectively delayed such a pathological change during disc degeneration. Collectively, our results suggested that
β-catenin plays a pivotal role in mediating matrix remodeling upon disc injury, and its downregulation may serve as an effective therapeutic strategy in decelerating pathological processes and thus ameliorating disc degeneration.

**Catabolic responses to disc injury are mitigated by β-catenin loss-of-function**

Since there were structural changes and matrix remodeling in the injured discs, we also investigated the catabolic responses in the discs by performing IHC to detect ADAMTS5 and MMP13, two prominent matrix proteases. Our data showed that both enzymes were significantly upregulated in the AF and NP of the discs at 3 months after the injury. Particularly, the most intensive immunopositivities were displayed in those newly formed tissues that replaced notochordal cells, suggesting a heightened activity of matrix catabolism (Figures 5A and 5B). At the later stage, i.e., 6 months after the injury, the protein levels of ADAMTS5 and MMP13 were still not subdued, indicative of sustained catabolic reactions (Figures 5C and 5D). Significantly, one-time injection of β-catenin-targeting AAV appeared to have substantial effects in combating matrix degradation at the time points of both 3 months and 6 months after injury, which may contribute to better-organized annulus and narrowed fissures in the CRISPR group (Figures 5A–5D). Thus, our data suggested that β-catenin targeting not only attenuated the anabolic pathways that stimulate cartilage replacement and calcification of nucleus, but it also ameliorated those catabolic reactions responsible for degradation of matrix components.

**Antagonism of the pain pathway by β-catenin downregulation in disc degeneration**

As pain is a major symptom associated with disc degeneration, we studied the expression changes of nerve growth factor (NGF) and βIII tubulin, two neuronal markers indicative of neurite growth in the pain genesis. Our IHC data demonstrated a dramatic increase of both NGF and βIII tubulin in the discs at 3 months after the injury, suggesting extensive nerve growth and induced pain sensation caused by the stab injury, while CRISPR-mediated Ctnnb1 ablation significantly reduced those pain-related molecular changes (Figures 6A and 6B). At 6 months, those neuronal activities were still extensive, as shown by abundant expression of NGF and βIII tubulin in the injured discs, while lower levels of β-catenin considerably contained these neural molecules (Figures 6C and 6D). Therefore, our results demonstrated that pain-related neural growth is associated with disc degeneration and can be restrained by downregulation of β-catenin. Further, our study suggest that β-catenin is a promising drug target not only for deterring structural deterioration in injured discs, but also for providing relief from pain associated with disc degeneration.

**DISCUSSION**

In this study, we applied the CRISPR-Cas9-based gene editing technology through AAV delivery to generate the loss-of-function of β-catenin in a disc degeneration animal model. Our results demonstrated that β-catenin deficiency significantly ameliorated injury-induced disc degeneration, suggesting a critical role of β-catenin in disc pathophysiology. Particularly, a prominent characteristic of disc degeneration in the mouse model is rapid depletion of notochordal cells in the NP, which is associated with significant upregulation of β-catenin (Figures S1 and S2). Thus, our results suggested that β-catenin negatively regulates the maintenance of notochordal cells in the disc. As notochordal cells have
been implicated to promote proteoglycan expression and inhibit inflammation and angiogenesis, their depletion in response to the disc injury leads to extensive replacement of proteoglycan matrix by fibrocartilage that undergoes subsequent calcification in the NP (Figure 2). It has been well established that β-catenin regulates chondrocyte hypertrophy and endochondral ossification, so β-catenin upregulation in disc degeneration is expected to promote the remodeling events such as fibrocartilage replacement and calcification. Moreover, it was reported that activation of WNT/β-catenin signaling induces cellular senescence and upregulates matrix metalloproteinases in NP cells, thereby promoting IVDD. Thus, the fundamental and far-reaching roles of β-catenin in both initiation and progression of disc degeneration warrant further investigation of the potential of β-catenin as a drug target for management of disc degeneration.

With regard to disc pathophysiology, mice and humans are remarkably different. In humans as young as 2 years of age, degenerative changes start to be seen in the NP, coincident with the rapid decrease of large vacuolated notochordal cell number following birth, which reflects the facts that mature mouse NP are populated by notochordal cells, but human NP are composed of chondrocyte-like cells. Thus, the findings from the mice should be extrapolated to human discs with caution. Nevertheless, the observations from both humans and animals unambiguously demonstrated that notochordal cells are important to disc health, and that studies of notochordal cells may provide new insights and propose new molecular targets for prophylactic or therapeutic care of disc degeneration. Moreover, our data also showed that β-catenin also controls the anabolic and catabolic responses in the disc after the notochordal cells are replaced with fibrocartilage, which bears a strong resemblance with human disc conditions. Therefore, our study using CRISPR-mediated gene editing of Ctnnb1 in the mouse model is a useful platform to study disc degeneration.

It is critical to identify an effective target gene for an in vivo study based on gene editing or gene therapy to treat disc degeneration. Thus, great efforts have been devoted to searching for a promising target. Adenoviral expression of SOX9, a chondrocyte-specific transcription factor, increased the production of type 2 collagen in human disc cells and preserved the NP structure in a rabbit disc degeneration model. As well, administration of adenovirus expressing human transforming growth factor β1 (TGFβ1) induced proteoglycan synthesis in the NP of a rabbit model. As disc degeneration is a complicated process involving multiple events such as upregulation of matrix-degrading enzymes and changes of cellular composition, search for a target that plays multipotent roles in disc degeneration would be preferable. As β-catenin has been demonstrated to play multifunctional roles in regulating NP cell proliferation, cellular senescence and apoptosis, and disc metabolism, modulation of β-catenin was thought to have significant effects on disc homeostasis. Moreover, β-catenin is significantly upregulated in degenerated disc. Thus, knocking down β-catenin could be a promising option to attenuate the progression of disc degeneration. In this study,
we demonstrated that β-catenin ablation by CRISPR-Cas9 preserved the NP structure, deterred the loss of vacuolated notochordal cells that are essential for disc health, and reduced the expression of matrix proteases such as MMP13 and ADAMTS5. Thus, our results have delineated an important molecular mechanism underlying disc degeneration and elucidated pivotal roles of β-catenin in the pathogenesis of disc degeneration through exploring a new therapeutic approach that utilizes the CRISPR-Cas9 system targeting β-catenin to treat disc degeneration in a mouse model. Furthermore, our data suggest that β-catenin is a promising drug target, and its targeting is an effective therapeutic strategy to attenuate the progress of disc degeneration.

MATERIALS AND METHODS

Animal studies
All animal procedures in the study were performed in accordance with the approved guidelines as well as the animal protocol approved by the Institutional Animal Care and Use Committee of Rush University Medical Center. The injury-induced disc degeneration mouse model was used in this study as previously described. Ten-week-old male wild-type C57BL/6 mice housed in the animal facility of Rush University Medical Center were used for generation of the disc degeneration model. X-ray was used through Faxitron (Faxitron Biopics, Tucson, Arizona) to locate the disc between fifth and sixth coccygeal vertebrae (C05/6). A 26G needle was inserted into the disc from the lateral side for about 1.5 mm to injure the tail disc. We injected 1 × 10^10 vector genomes of AAV in a volume of 10 μL into the disc before withdrawing the needle. The sham group did not receive disc puncture, and the control group received disc puncture and administration of control AAV. The mice were randomly selected for different treatments, and the injections were performed in a blind manner where the investigator was not aware of the study groups. Three or six months after AAV injection, the animals were euthanized and tail tissues were collected.

AAV vector preparation
We used px601-AAV-CMV-NLS-SaCas9-NLS-3xHA-bGHpA; U6:BsaI-sgRNA, a gift from Feng Zhang (Addgene plasmid # 61591). The px601 vector expresses the CRISPR-Cas9 system derived from Staphylococcus aureus. The AAV vectors were constructed by ligation of a specific 21–22 nt guide sequence into the sgRNA scaffold. For the gene of Ctnnb1 that encodes β-catenin, we designed two guide sequences and accordingly constructed two vectors. Recombinant AAV packaging was performed by SignaGen Laboratories (Rockville, MD, USA).

Histology, immunohistochemistry, and molecular studies
For histology and IHC, tissues were fixed in 10% formalin, decalcified, and embedded in paraffin. The sections were stained with Alcian blue/ hematoxylin & orange G (AB/H&OG) for histological analysis. Modified Thompson scoring was performed to evaluate disc degeneration essentially as previously described. Immunohistochemistry was performed essentially as described. The antibodies and their working concentrations are listed as follows: 1/50 β-catenin (610154, BD Biosciences), 5 μg/mL Brachyury (AF2085, R&D Systems), 1/2000 Type I collagen (AB745, Millipore), 1/200 Type II Collagen (Ab3092, abcam), 1/5000 Type X collagen (Ab58632, abcam), 1/200 MMP13 antibody (Ab39012, abcam), 1/500 ADAMTS5 antibody (Ab41037, abcam), 1/100 NGF antibody (ab6199, abcam), and 1/200 β-III Tubulin Antibody (801201, Biologend). Images of histology and IHC were captured using CellSens Imaging Software (Olympus) on an Olympus BX43 microscope. Positive signals of IHC were quantified using ImageJ that measures average integrated densities. For profiling gene expressions, total RNA was extracted, and qRT-PCR was performed, using the primer pairs for Ctnnb1 (5’-ATGGAGCGGACAGAAAGC-3’ and 5’-CTTGCCACTCAGGAAAGG-3’), and β-actin (5’-GGGTTGTTTCCCCTCCATCG-3’ and 5’-CCAGTTGGTACAAATGGCCATGT-3’). To test the efficiency of the Ctnnb1-targeting sgRNAs, we stably transfected CD45^+ bone marrow stromal cells, or a chondrogenic cell line ATDC5, with px601 plasmids, together with pMX-GFP that contains puromycin resistance to be used as a selection marker, using Lipofectamin 3000 (Invitrogen, Carlsbad, CA, USA). We extracted genomic DNAs from cells that had survived from 1-week puromycin selection and performed PCR to amplify the region targeted by the sgRNAs as previously described. Then we sequenced the PCR amplicons to confirm that they contain gene-abating mutations. Western blots were performed as described previously, using antibodies against β-catenin (1:500, 610,154, BD Biosciences, Franklin Lakes, NJ, USA) and β-actin (1:10,000, A5441, Sigma-Aldrich, St. Louis, MO, USA).

Experimental design and statistical analyses
The sample size for each experiment was determined based on our previous experiences. All the data were expressed as mean ± SD as indicated in the figure legends. Statistical analyses were completed with Prism GraphPad. Unpaired Student’s t test (for two groups) and one-way ANOVA (for multiple groups) were used followed by the Tukey-Kramer test. p < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
Y.F. and J.H. conceived the study. J.H., L.Z., and Y.F. designed the experiments. J.H., L.Z., and Y.F. performed the experiments. The authors declare no competing interests.

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