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RESEARCH ARTICLE

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Differential efficacy of two small molecule PHLPP inhibitors to promote nucleus Pulposus cell health

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Abstract

Background: Intervertebral disc (IVD) degeneration is associated with chronic back pain. We previously demonstrated that the phosphatase pleckstrin homology domain and leucine-rich repeat protein phosphatase (PHLPP) 1 was positively correlated with IVD degeneration and its deficiency decelerated IVD degeneration in both mouse IVDs and human nucleus pulposus (NP) cells. Small molecule PHLPP inhibitors may offer a translatable method to alleviate IVD degeneration. In this study, we tested the effectiveness of the two PHLPP inhibitors NSC117079 and NSC45586 in promoting a healthy NP phenotype.

Methods: Tail IVDs of 5-month-old wildtype mice were collected and treated with NSC117079 or NSC45586 under low serum conditions ex vivo. Hematoxylin & eosin staining was performed to examine IVD structure and NP cell morphology. The expression of KRT19 was analyzed through immunohistochemistry. Cell apoptosis was assessed by TUNEL assay. Human NP cells were obtained from patients with IVD degeneration. The gene expression of KRT19, ACAN, SOX9, and MMP13 was analyzed via real time qPCR, and AKT phosphorylation and the protein expression of FOXO1 was analyzed via immunoblot.

Results: In a mouse IVD organ culture model, NSC45586, but not NSC117079, preserved vacuolated notochordal cell morphology and KRT19 expression while suppressing cell apoptosis, counteracting the degenerative changes induced by serum deprivation, especially in males. Likewise, in degenerated human NP cells, NSC45586 increased cell viability and the expression of KRT19, ACAN, and SOX9 and reducing the expression of MMP13, while NSC117079 treatment only increased KRT19 expression. Mechanistically, NSC45586 treatment increased FOXO1 protein expression in NP cells, and inhibiting FOXO1 offset NSC45586-induced regenerative potential, especially in males.

Conclusions: Our study indicates that NSC45586 was effective in promoting NP cell health, especially in males, suggesting that PHLPP plays a key role in NP cell homeostasis and that NSC45586 might be a potential drug candidate in treating IVD degeneration.

KEYWORDS
FOXO1, intervertebral disc degeneration, NSC117079, NSC45586, nucleus pulposus, PHLPP, small molecule PHLPP inhibitor

1 | INTRODUCTION

Low back pain is the leading cause of physical disability worldwide, affecting 80% of the population at some point in their lives, and intervertebral disc (IVD) degeneration remains a major underlying factor for low back pain.[1] IVD degeneration is characterized by a cascade of degenerative changes in the extracellular matrix, chronic inflammation, and apoptosis, which increase with the severity of IVD degeneration.[2,3] Current treatment modalities for IVD degeneration rely on surgeries that can only temporarily ameliorate clinical symptoms. New treatment approaches are under investigation aiming to alleviate painful symptoms as well as to regenerate or heal degenerated and injured IVDs, such as tissue engineering,[4,5] gene therapy,[6,7] cell-based therapy,[8] non-coding RNA therapy,[9] and small molecule-based therapy.[10]

IVDs are comprised of a central gelatinous proteoglycan-rich nucleus pulposus (NP), a fibrocartilaginous annulus fibrosus that encases the hydrated NP at the periphery, and two cartilaginous endplates connected to the adjacent vertebral bodies superiorly and inferiorly.[11] The NP experiences the most extensive changes in the progression of IVD degeneration.[12] The juvenile human NP is comprised of large vacuolated notochordal-derived NP cells, which disappear after adolescence and are replaced by small chondrocyte-like mature NP cells. The rapid disappearance of notochordal cells during postnatal growth is considered to predispose the human NP to degenerative changes later in life.[13,14]

PH domain Leucine-rich-repeats protein phosphatase (PHLPP), originally identified as a negative regulator of protein kinase B (AKT) signaling, belongs to a relatively novel family of Ser/Thr protein phosphatases.[15] PHLPP's two isozymes, PHLPP1 and PHLPP2, promote apoptosis and inactivate AKT by directly dephosphorylating its hydrophobic motif (Ser473).[15] The isozymes differ in their selectivity for dephosphorylating specific AKT isoforms; PHLPP1 dephosphorylates AKT2 and AKT3, while PHLPP2 dephosphorylates AKT1 and AKT3.[16] PHLPP also regulates other pathways, such as mitogen-activated protein kinase (MAPK),[17] glycogen synthase kinase β (GSK3β),[16] and forkhead box O 1/3 (FOXO1/3)[16,18] through indirect dephosphorylation. These signaling pathways control a wide variety of fundamental cellular processes, such as cell survival, apoptosis, and matrix catabolism. Sierecki et al. identified two small molecule PHLPP inhibitors, NSC45586 and NSC117079, which can bind to the complex formed by PHLPP and its substrates and block its phosphatase activity.[18] One of the important downstream targets of AKT signaling is FOXO1, an isoform of the FOXO transcription factor family.[19] Phosphorylation of FOXO1 via AKT allows the translocation of FOXO1 from the nucleus to the cytoplasm, thus inhibiting its transcriptional functions.[19] FOXO1 participates in a wide range of biological processes, such as glucose metabolism, oxidative stress resistance, and cell cycle arrest, thereby promoting life span and delaying aging.[20–23] Recent studies demonstrated that FOXO knockout mice exhibited increased age-related damage in mouse IVDs.[24] We previously demonstrated that Phlpp1 knockout decelerated injury- and age-induced IVD degeneration by stimulating extracellular matrix (ECM) anabolism in mice.[25,26] We showed that in degenerated human NP cells, PHLPP1 knockdown increased FOXO1 expression and promoted NP cell regeneration.[26] In the present study, we tested the efficacy and underlying mechanism of the two small molecule PHLPP inhibitors NSC45586 and NSC117079 in promoting NP health in mice IVDs and human NP cells by utilizing mouse ex vivo and human in vitro cultures.

2 | MATERIALS AND METHODS

2.1 | Study design

Two PHLPP inhibitors were tested for their efficacy in counteracting IVD degeneration and promoting NP cell health in mouse IVD organ cultures and degenerated human NP cells (Figure 1A). Mouse IVD motion segments were cultured under low serum conditions which contained 1% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) to induce apoptosis, a hallmark for IVD degeneration. IVDs were divided into three groups: 1% FBS/DMEM, 1% FBS/DMEM + inhibitor NSC117079 (100 μm), 1% FBS/DMEM + inhibitor NSC45586 (100 μm). NP morphology, phenotype, and apoptosis were assessed to determine the protective effects of the inhibitors. Degenerated human NP cells from female and male donors were used to determine inhibitor cytotoxicity, evaluate the beneficial effects on NP cell phenotype, and identify the downstream targets of PHLPP (Figure 1B,C).

2.2 | Mouse IVD organ cultures

Sixteen C57BL/6J mice (strain #: 000664; 4 mice/sex/treatment) were obtained from JAX laboratory and used for this study. All experiments were performed in accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (U.S. Department of Health, Education, and Welfare, NIH 78-23, 1996). All animal protocols were approved by the Atlanta Veteran's Affairs Medical Center's Institutional Animal Care and Use Committee (Protocol V020-22). Mice were euthanized in a CO_2 chamber at 5 months of age, and the coccygeal vertebrae-IVD-vertebrae segments (CC5-11) were dissected using a Leica S9i stereomicroscope. Two IVD segments (positive controls) were fixed and stored for further tissue processing. Experimental IVDs were disinfected with 70% ethanol and washed with 1xPBS containing 3% penicillin–streptomycin (pen/strep) (15140122, Thermofisher Scientific) and 1.5% Amphotericin B (15290026, Thermofisher Scientific). IVDs

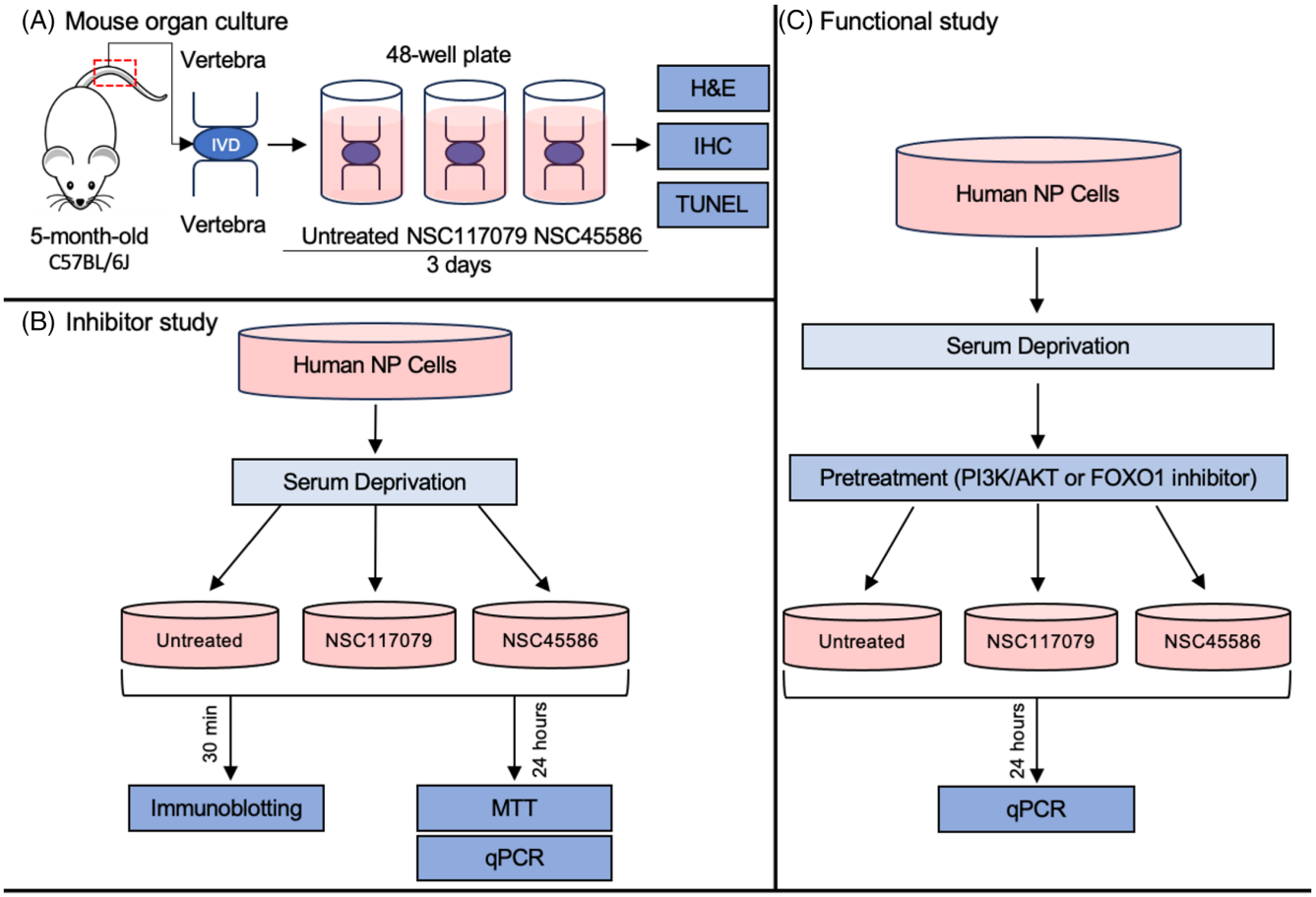

FIGURE 1 Study design. (A) Coccygeal vertebrae-IVD-vertebrae were collected and equilibrated in 10%FBS/DMEM for 24 h. IVD segments were then divided into three groups: 1% FBS/DMEM (untreated control), 1% FBS/DMEM with 100 μM NSC117079, and 1% FBS/DMEM with 100 μM NSC45586 and cultured for 3 days. (B) Degenerated human NP cells were serum deprived in 0.2%FBS/DMEM for 2 h, followed by treatment with different concentrations of NSC45586 or NSC117079. (C) Degenerated human NP cells were serum deprived in 0.2%FBS/DMEM for 2 h before pretreatment with an AKT/PI3K inhibitor Wortmannin or a small molecule FOXO1 inhibitor AS1842856. The cells were then treated with NSC45586 or NSC117079 for 24 h. IVD, intervertebral disc; NP, nucleus pulposus.

were then allowed to equilibrate in low glucose DMEM medium (Gibco, 11 885 084) containing 10% FBS (A4766801, Thermofisher Scientific) and 1% pen/strep for 24 h. IVDs were then randomly assigned into three groups (2 IVDs/mouse): 1% FBS/DMEM (untreated control), 1% FBS/DMEM with 100 μM NSC117079, and 1% FBS/DMEM with 100 μM NSC45586 and cultured for 3 days. To assess the potential of PHLPP inhibition in degenerating IVDs, we cultured mouse IVDs in 1% FBS/DMEM to induce IVD cell apoptosis, which was associated with IVD degeneration.[27] Low serum ex vivo culture conditions were chosen based on previous studies showing that IVD cells displayed degenerative changes in low serum culture conditions.[28,29] All IVDs were cultured at 37°C under 5% CO_2, 20% O_2, and 90% humidity.

2.3 | Histology and immunochemistry

IVDs were fixed in z-fix for 2 days and decalcified in 5% formic acid (Millipore Sigma, FX0440-6) for 3 days. Decalcified IVDs were embedded in paraffin, and 5 μm thick mid-sagittal sections were used for histology and immunochemistry. The IVD morphology was visualized using Hematoxylin and eosin staining (H&E, Thermofisher Scientific, 5026447 & 5031906). Changes in NP phenotype were visualized immunohistochemically with the expression of NP phenotypic marker KRT19 (Thermofisher Scientific, MA5-12663) as previously described.[26] IVDs were visualized using bright-field microscopy (DM6 B automated microscope with LAS X software, Leica, Germany), and one section per IVD was quantified for immunopositive cells by two independent observers who were blinded to the experimental groups.

2.4 | TUNEL assay

Click-iT Plus terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for in situ apoptosis detection (C10245, Thermofisher Scientific, Waltham, MA, USA) was used to detect apoptotic cells in mouse IVDs ex vivo.[26] In brief, deparaffinized IVD sections were washed with PBS, treated with TUNEL reagents as

TABLE 1 Patient information.

	Grade	Sex	Age
1	4/5	F	65
2	4	F	53
3	4/5	F	81
4	4	F	72
5	4/5	M	40
6	5	M	74
7	5	M	61
8	5	M	61

described by the manufacturer, and counterstained with ready-to-use 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, H-1200) to detect total cells in IVDs. One section per IVD was used for analysis. Images were captured using a Leica (DM6 B automated microscope with LAS X software, Leica, Germany) microscope at 20X magnification. The number of TUNEL-positive cells in the NP was counted using the ImageJ software. All sections were assessed by two researchers who were blinded to the experimental groups, and their counts were averaged for analysis.

2.5 | Human NP cell extraction and culture

In accordance with the institutional review board approval, after obtaining informed consent, human degenerated NP tissues from female and male donors (Grade IV or V on the Pfirrman grade[30]) were collected after discectomy surgeries for discogenic pain (Table 1). NP cells were harvested as previously described.[26] In brief, NP tissues were collected immediately after surgery and minced into small fragments (~1 mm^3). To release the cells from the tissues, tissues were digested in 0.2% protease (Sigma Aldrich, P5147-1G) for 1 h, followed by 0.025% collagenase P (Roche Diagnostics, 40 341 623) digestion for 4 h. Isolated NP cells were expanded in low glucose DMEM containing 10% FBS, 1% pen/strep, and 50 µg/mL L-Ascorbic acid 2-phosphate (Sigma, A8960) and maintained at 37°C under 5% CO_2, 20% O_2, and 90% humidity. Cells of passage 2–3 were used for experiments.

2.6 | Chemical inhibitor treatment

PHLPP inhibitors NSC45586 and NSC117079 (Glixx Laboratories Inc., MA, USA), FOXO1 inhibitor AS1842856 (Sigma, 344 355), and PI3K/AKT inhibitor Wortmannin (Sigma, W1628) were used in this study. The inhibitors were dissolved in dimethyl sulfoxide (DMSO). Degenerated human NP cells were cultured in 6-well plates and starved in medium containing 0.2% FBS for 2 h prior to stimulation. Cells were incubated in medium with either 0.08% DMSO (degenerated control) or different concentrations of NSC45586 or NSC117079 (25, 50, and 100 µM). To assess the effect on AKT phosphorylation and FOXO1 expression, cells were cultured for 30 min and harvested for western blot. We chose the 30-min timepoint to assess de/phosphorylation of AKT because AKT phosphorylation has been reported to be a rapid response to treatment and is commonly assessed within min after activation.[18] For gene expression analysis of KRT19, ACAN, MMP13, and SOX9, cells were incubated for 24 h, which is based on previous studies showing that gene expression changes can be detected after 24-h treatment.[31] The 0.08% DMSO group was used as a degenerative control. For FOXO1 inhibition or AKT inhibition, cells were pretreated with FOXO1 inhibitor AS1842856 (200 nM) or Wortmannin (100 nM) for 1 h before treating with NSC45586 or NSC117079 (100 µM) for 24 h.

2.7 | MTT assay

NP cells were seeded into 94-well plates at a density of 4×10^3 cells/well. After reaching 80% confluency, NP cells were starved in medium containing 0.2% FBS for 2 h prior to stimulation. Cells were incubated in medium with 0.08% DMSO or different concentrations of NSC45586 or NSC117079 (25, 50, and 100 µM) for 24 h. MTT assay (Thermofisher Scientific, V13154) was performed according to the manufacturer's protocol. Briefly, MTT was added into each well and incubated for 4 h at 37°C. After labeling the cells with MTT, all but 25 µL of supernatant was aspirated, and 50 µL DMSO was added into each well for 10 min at 37°C. Optical densities were measured at 540 nm using a microplate reader (GloMax, Discover). All experiments were performed three times, each in triplicate.

2.8 | Quantitative real-time PCR assays

Total RNA was isolated from NP cells collected in TRIzol reagent (Thermofisher Scientific, 15596018) and then cleaned using RNeasy Plus micro kit (Qiagen, 74 004) according to the manufacturer's protocol. RNA quality was measured using the NanoDrop-1000 spectrophotometer (Thermofisher Scientific, ND-2000), and 1 µg RNA was reverse transcribed using Superscript III Reverse Transcriptase (Thermofisher Scientific, 11754050). Quantification of mRNA expression was performed using the TaqMan Gene Expression Assays specific for KRT19 (Thermofisher Scientific, Assay ID: Hs01051611_gH), ACAN (Thermofisher Scientific, Assay ID: Hs00153936_m1), MMP13 (Thermofisher Scientific, Assay ID: Hs00942584_m1), and SOX9 (Thermofisher Scientific, Assay ID: Hs00165814_m1). Values were normalized to GAPDH (Thermofisher Scientific, 4331182, Assay ID: Hx02758991_g1). mRNA levels were presented as fold change compared to untreated cells according to the $2^{\Delta\Delta CT}$ method. All experiments were performed three times, each in triplicate.

2.9 | Immunoblotting

Whole-cell protein extracts were isolated from human degenerated NP cells, and equal amounts of protein (20 µg) were used to

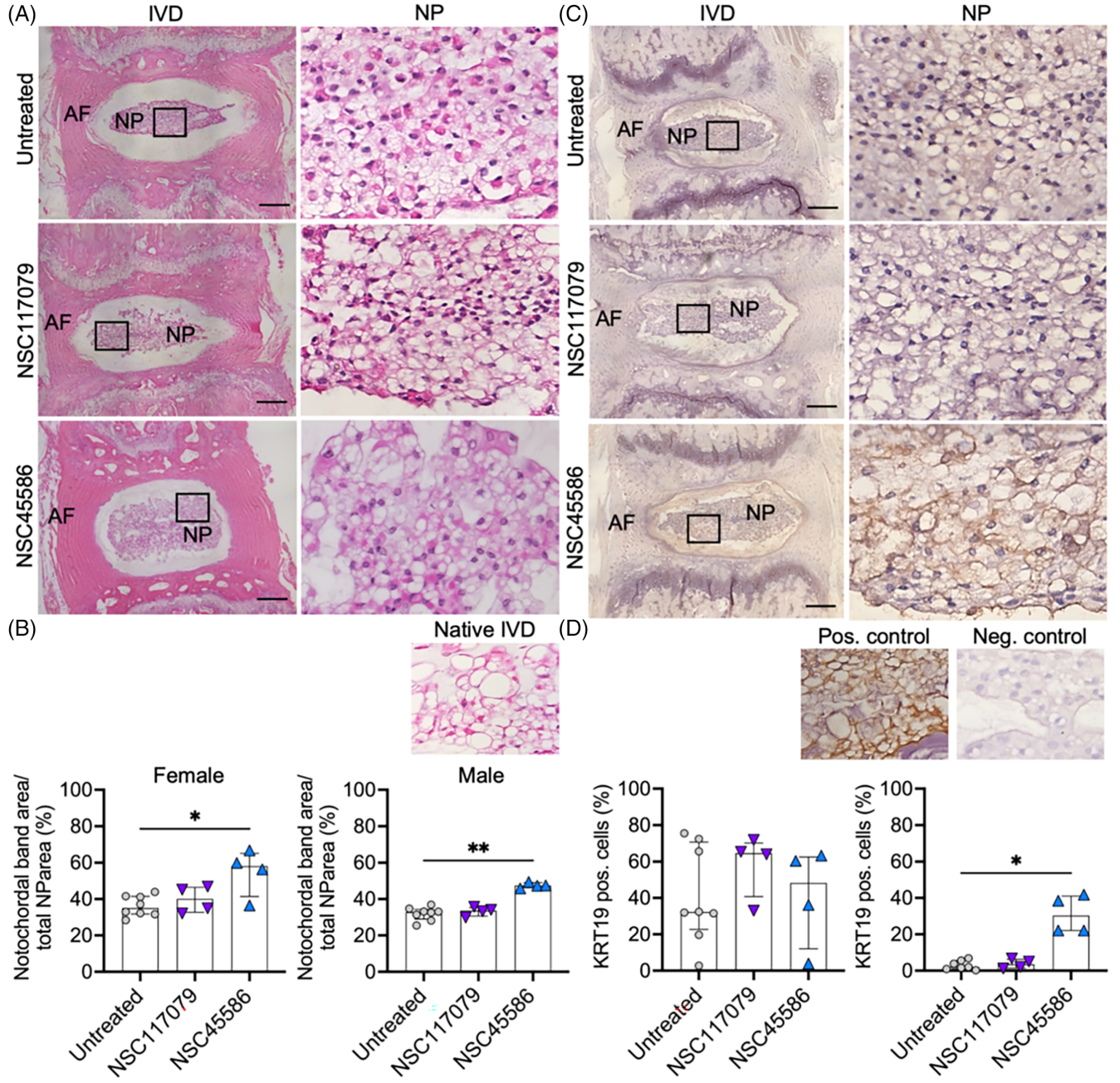

FIGURE 2 NSC45586 increased notochordal band area and KRT19 expression under low serum in an ex vivo mouse IVD organ culture model. A + C: representative images of IVDs 3 days after culture under low serum conditions (1%FBS) with and without PHLPP inhibitor treatments (male IVDs are shown). (A) H&E staining to visualize notochordal band morphology and size. Low serum (untreated IVDs) resulted in small NP cells, a known indicator for degenerative changes. NSC45586 (100 µm) treated cells maintained large notochordal band with large and highly vacuolated cells, as commonly observed in C57BL/6J mouse IVDs (see native IVD). NSC117079 (100 µm) treatment had no effect on notochordal NP cell morphology or notochordal band size. The native IVD image shows the NP morphology under physiological conditions. (B) Quantification of notochordal band area in female and male mice. (C) KRT19 immunostaining to evaluate the effect on the notochordal cell phenotype. Weak KRT19 expression was observed in the NP of untreated and NSC117079-treated IVDs. NSC45586 significantly increased KRT19 expression. The positive control shows the KRT19 immunostaining in the native IVD. The negative control shows the immunostaining without applying KRT19 antibody. (D) quantification of KRT19 positive cells in female and male mice. Neg.: negative. Pos.: positive. Scale bar = 100 µm; Kruskal–Wallis test with Dunn's post hoc testing was performed. The data are presented as median with interquartile range. *$p < 0.05$; **$p < 0.01$. Treatment group: $n = 4$. Untreated group: $n = 8$. IVD, intervertebral disc; NP, nucleus pulposus; PHLPP, PH domain Leucine-rich-repeats protein phosphatase.

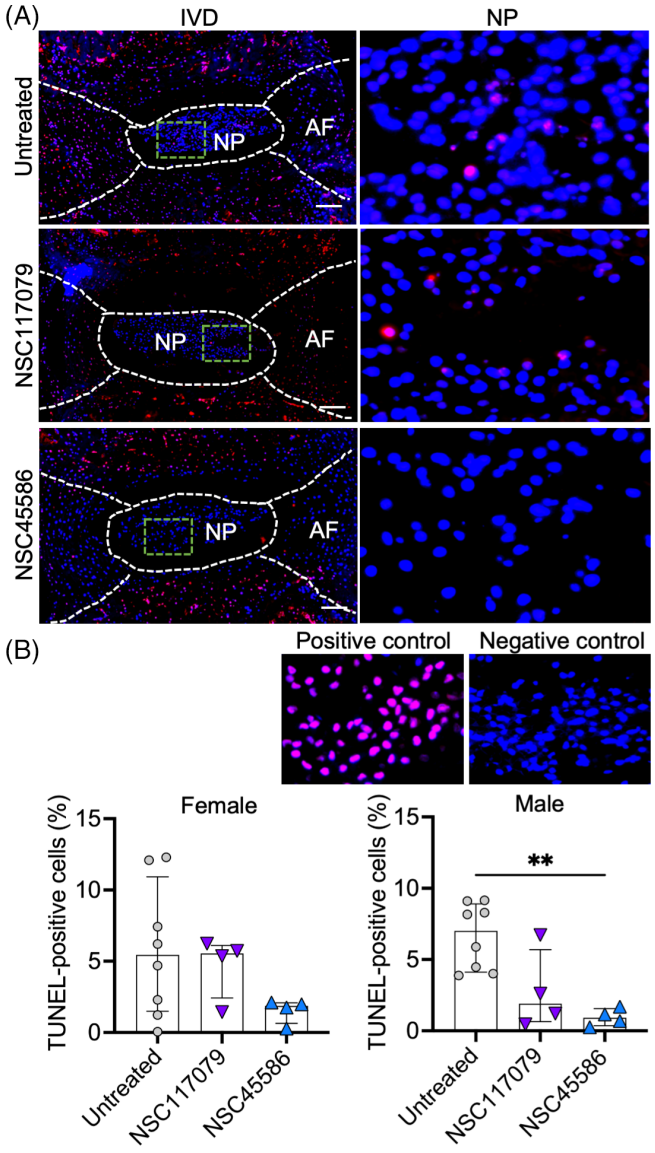

FIGURE 3 NSC45586 treatment was protective against apoptosis. (A) Representative images of IVDs 3 days after culture under low serum conditions (1%FBS) with and without PHLPP inhibitor treatments (male IVDs are shown). TUNEL-positive cells were present in the NP of untreated and NSC117079-(100 μm) treated IVDs. The NP of NSC45586-(100 μm) treated IVDs contained less TUNEL-positive cells, which was significant for males. The positive control shows the TUNEL staining of a DNase I treated sample. The negative control shows the TUNEL staining without applying the EdUTP nucleotide mixture. (B) Quantification of TUNEL-positive cells in female and male mice treated with NSC117079 or NSC45586. Scale bar = 100 μm; Kruskal–Wallis test with Dunn's post hoc testing was performed. The data are presented as median with interquartile range. $^{**}p < 0.01$. Treatment group: $n = 4$. Untreated group: $n = 8$. IVD, intervertebral disc; NP, nucleus pulposus.

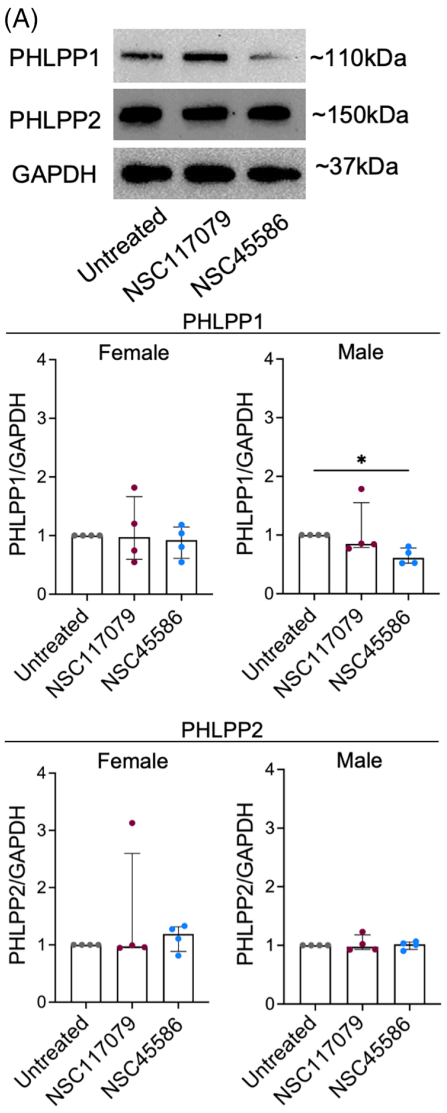

FIGURE 4 NSC45586 treatment reduced the protein level of PHLPP1 in degenerated human NP cells. Western blot analysis was performed to examine the protein level of PHLPP1 in NP cells treated with 100 μM NSC117079 or NSC45586. NSC117079 treatment did not affect the protein levels of PHLPP1 and PHLPP2. NSC45586 treatment reduced protein expression of PHLPP1, not PHLPP2, which was more pronounced in male than female groups. Kruskal–Wallis test with Dunn's post hoc testing was performed. The data are presented as median with interquartile range. $^{*}p < 0.05$. PHLPP, PH domain Leucine-rich-repeats protein phosphatase.

determine the protein expression of PHLPP1 (Sigma, 071341), PHLPP2 (Thermofisher Scientific, A300661A), AKT (Cell Signaling Technology, 4691S), and FOXO1 (Cell Signaling Technology, 2880S). Protein phosphorylation was determined using phospho-AKT (Ser473) (Cell Signaling Technology, 9271S). All proteins were normalized to GAPDH (Cell Signaling Technology, 884S), and band densities were calculated using imageJ as previously described.[26]

2.10 | Statistics

Kruskal–Wallis test followed by Dunn's post hoc test was performed to evaluate the effects of treatment for both organ culture and human

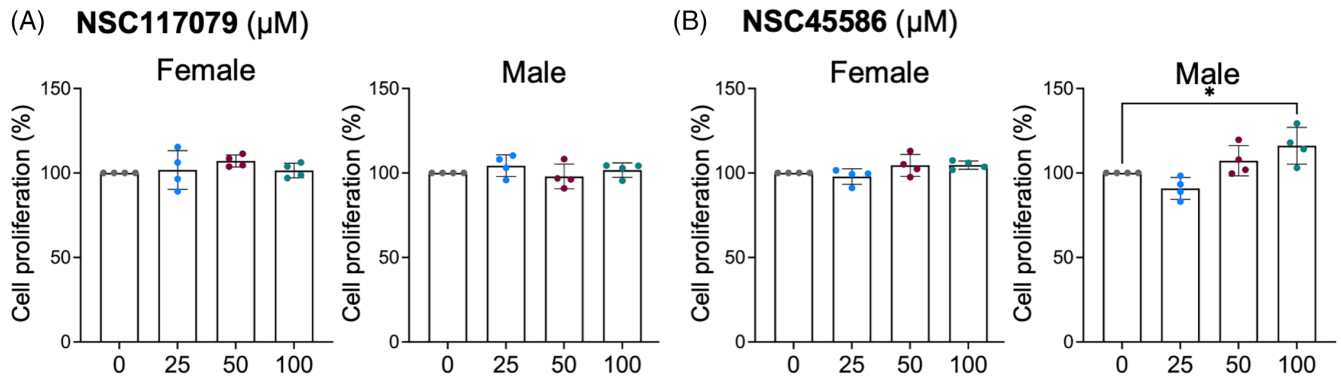

FIGURE 5 NSC45586 treatment increased cell proliferation in degenerated human NP cells. Cell proliferation was determined by MTT assay on human NP cells after being treated with different concentrations of NSC117079 or NSC45586 for 24 h. (A) NSC117079 treatment did not affect cell proliferation. (B) NP cells treated with NSC45586 had increased cell proliferation, which was significant for males; $n = 4$ per group. Kruskal–Wallis test with Dunn's post hoc testing was performed. The data are presented as median with interquartile range. *$p < 0.05$. NP, nucleus pulposus.

NP cell culture studies. The statistical analyses were performed using GraphPad Prism9 (GraphPad Software, Inc., La Jolla, CA). A *p*-value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | The small molecule PHLPP inhibitor NSC45586 maintained NP homeostasis in mouse IVD organ cultures

The notochordal bands of IVDs cultured under low serum and IVDs treated with NSC117079 were thin and contained mainly small, chondrocyte-like cells, which is a sign of IVD degenerative changes in mouse IVDs. In contrast, the notochordal band of NSC45586-treated IVDs was significantly larger and contained large notochordal cells (Figure 2A). No differences were observed in the total NP cell number (Figure S1), indicating that cell morphology alterations, not cell loss, contributed to the observed differences in cell band area. Accompanied by the morphological alterations in the notochordal band, only minor expression of the notochordal marker KRT19 was detected in IVDs cultured in low serum conditions and IVDs treated with NSC117079 (Figure 2C,D). In contrast, NSC44586 treatment increased KRT19 expression, which was significant for males. Collectively, only treatment with the PHLPP inhibitor NSC45586 maintained a healthy IVD homeostasis by preserving the NP morphology and phenotype.

3.2 | The small molecule PHLPP inhibitor NSC45586 protected against cell apoptosis in the NP in mouse IVD organ cultures

Low serum is a common method to induce apoptosis in IVD cells,[32] which is a hallmark of IVD degeneration. Compared to IVDs cultured in low serum conditions, NSC45586 treatment inhibited apoptosis, which was significant for males. However, NSC117079 treatment did not prevent apoptosis under low serum conditions (Figure 3).

3.3 | NSC45586 treatment reduced PHLPP1 protein expression in severely degenerated human NP cells

To examine whether PHLPP inhibitors affected the protein levels of the two PHLPP isoforms, we treated degenerated human NP cells (Pfirrman grade 4–5) with or without NSC117079 or NSC45586. The protein level of PHLPP1, but not PHLPP2, was significantly reduced in NSC45586 treated NP cells, which was more pronounced in males (Figure 4). NSC117079 did not impact the protein levels of PHLPP1 or PHLPP2.

3.4 | NSC45586 treatment promoted NP cell regeneration by increasing cell proliferation and the expression of NP phenotypic markers in severely degenerated human NP cells

To explore if therapeutical inhibition of PHLPP activity via its inhibitors would promote human NP cell regeneration, we cultured degenerated human NP cells (Pfirrman grade 4–5) with or without NSC45586 or NSC117079. NSC117079 had no effect on cell proliferation (Figure 5A). In contrast, NSC45586 had good cytocompatibility; cell viability was maintained during culture with a concentration of up to 100 μM. Moreover, treatment with NSC445586 significantly increased cell proliferation in males (Figure 5B). In addition, NSC45586 promoted KRT19, ACAN, and SOX9 expression while decreasing MMP13 expression, which was more pronounced in males, suggesting

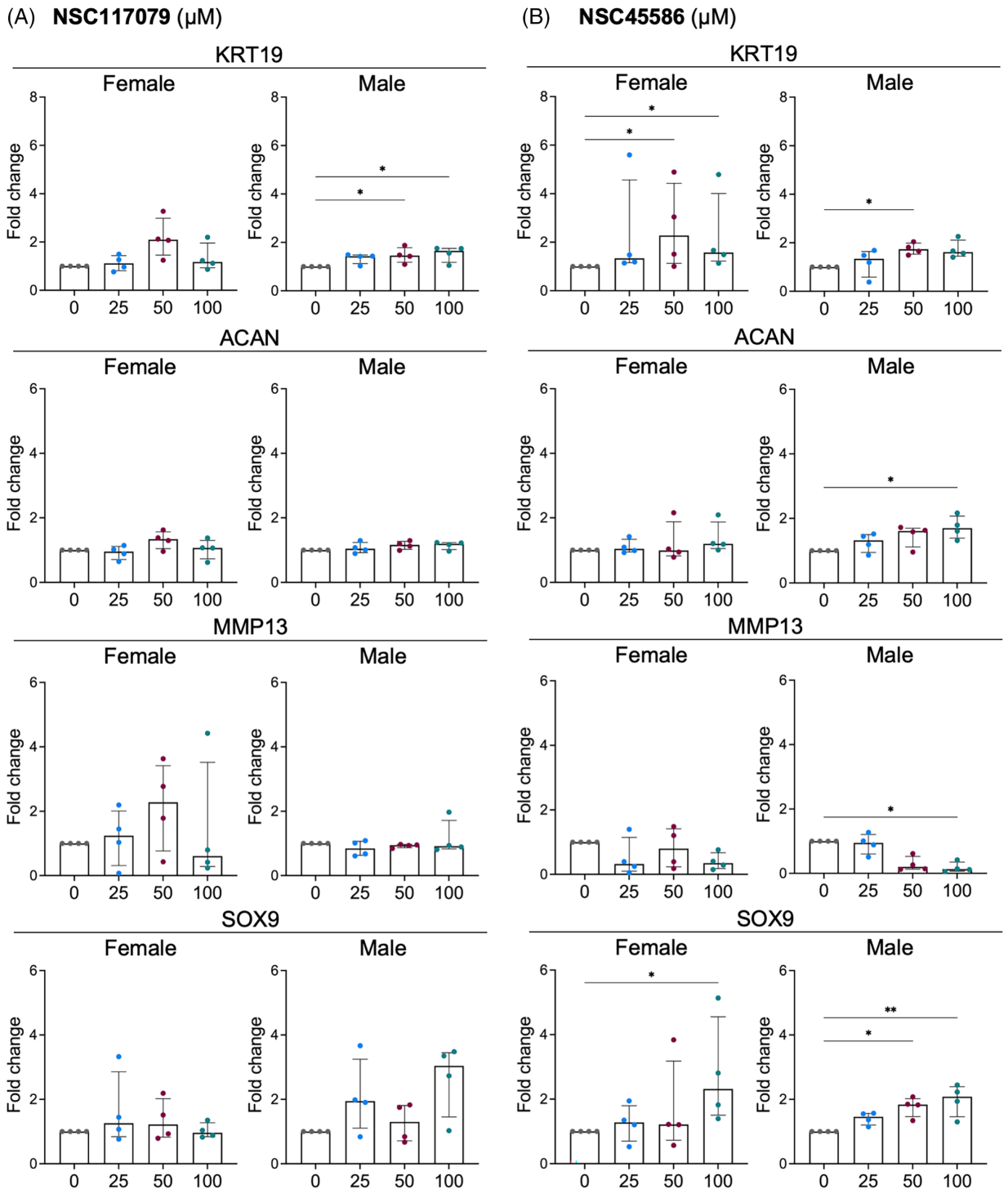

FIGURE 6 NSC45586 treatment promoted a healthy NP phenotype in degenerated human NP cells. Gene expression of the NP markers KRT19, ACAN, MMP13, and SOX9 were evaluated in NP cells treated with different concentrations of NSC117059 or NSC45586 for 24 h. (A): NSC117079 increased KRT19 gene expression in males but did not affect the gene expression of other genes examined. (B) NSC45586 promoted the gene expression of KRT19 and SOX9 in both females and males, and ACAN gene level was increased in mainly males. MMP13 gene expression was reduced in males after NSC45586 treatment. $n = 4$ per group. Kruskal–Wallis test with Dunn's post hoc testing was performed. The data are presented as median with interquartile range. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NP, nucleus pulposus.

the beneficial effects of NSC45586 in promoting NP phenotype (Figure 6B). In contrast, NSC117079 only increased KRT19 expression (Figure 6A). Taken together, these data indicated NSC45586 inhibited PHLPP activity, promoted cell proliferation, and had a regenerative effect in degenerated human NP cells.

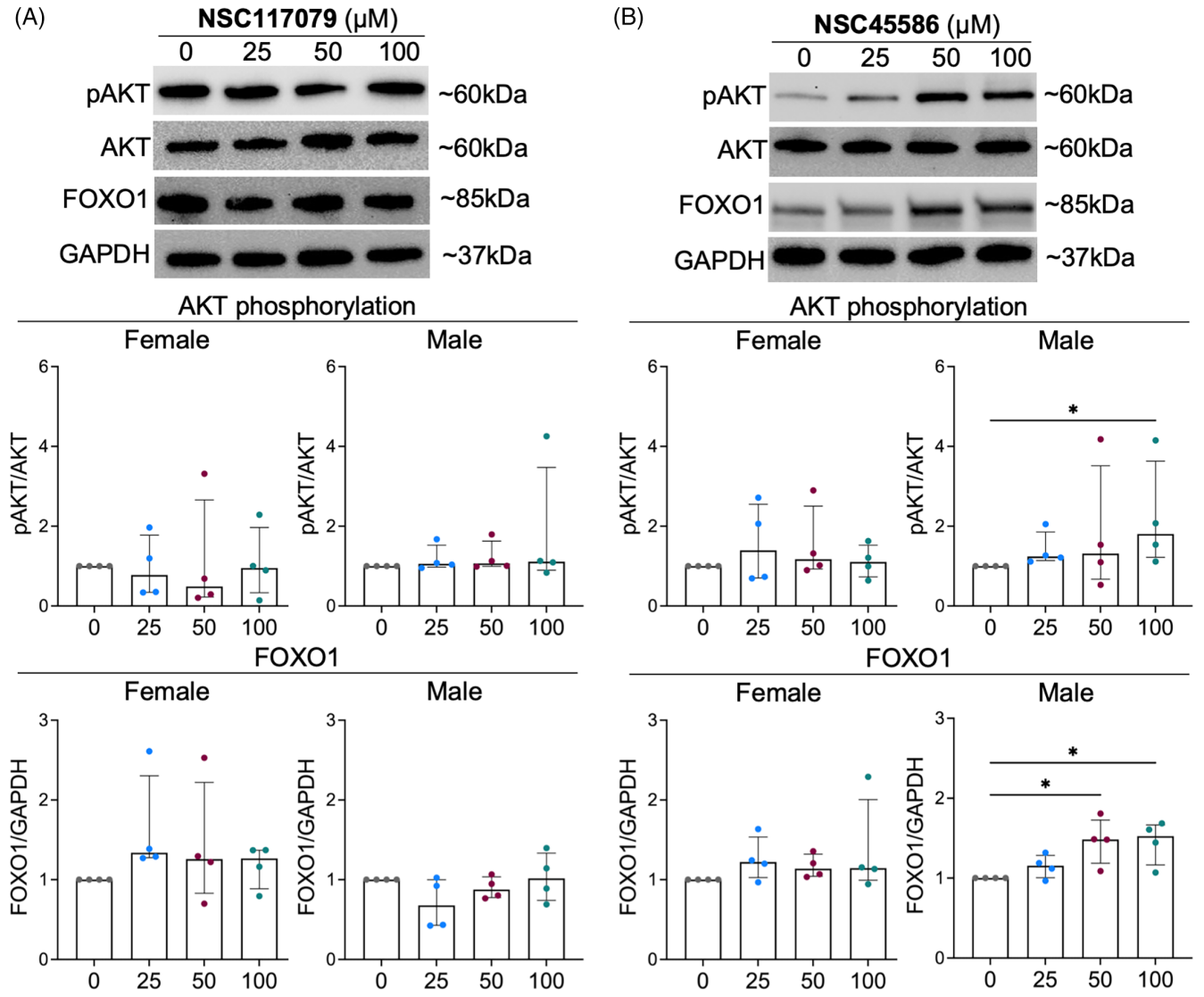

FIGURE 7 NSC45586 treatment increased FOXO1 expression in degenerated human NP cells. Western blot analysis was performed to evaluate the phosphorylation of AKT and the protein level of FOXO1 in human NP cells treated with different concentrations of NSC117079 or NSC45586 for 30 min. (A) AKT phosphorylation and FOXO1 expression was not altered in NSC117079 treated NP cells. (B) NSC45586 treatment increased AKT phosphorylation and FOXO1 expression in a dose-dependent manner, which was more pronounced in males. $n = 4$ per group. Protein levels were normalized against GAPDH. Kruskal–Wallis test with Dunn's post hoc testing was performed. The data are presented as median with interquartile range. $^*p < 0.05$. NP, nucleus pulposus.

3.5 | PHLPP inhibition increased FOXO1 expression in human degenerated NP cells

We first determined the NP-specific effects of PHLPP depletion on its substrate AKT. NSC117079 treatment did not affect AKT phosphorylation in either sex (Figure 7A). NSC45586 treatment increased AKT phosphorylation in NP cells of the male group (Figure 7B). The FOXO transcription factors are indispensable for maintaining IVD homeostasis, and our previous findings showed that PHLPP1 depletion promoted FOXO1 expression in the NP of both mouse and human IVDs.[25,26] Similar to PHLPP1 knockdown, culture with NSC45586 increased the protein abundance of FOXO1 in degenerated human NP cells from male donors (Figure 7B). The FOXO1 expression was not affected in NP cells treated with NSC117079 (Figure 7A).

3.6 | PHLPP inhibition alleviated degenerative changes via activating FOXO1 signaling in human degenerated NP cells

To determine the direct downstream effectors of PHLPP inhibitors in promoting NP cell phenotype, we pretreated human degenerated NP cells either with an AKT/PI3K inhibitor Wortmannin or a small molecule FOXO1 inhibitor AS1842856 that only binds to the active form of FOXO1. Pretreatment with an AKT/PI3K inhibitor or FOXO1

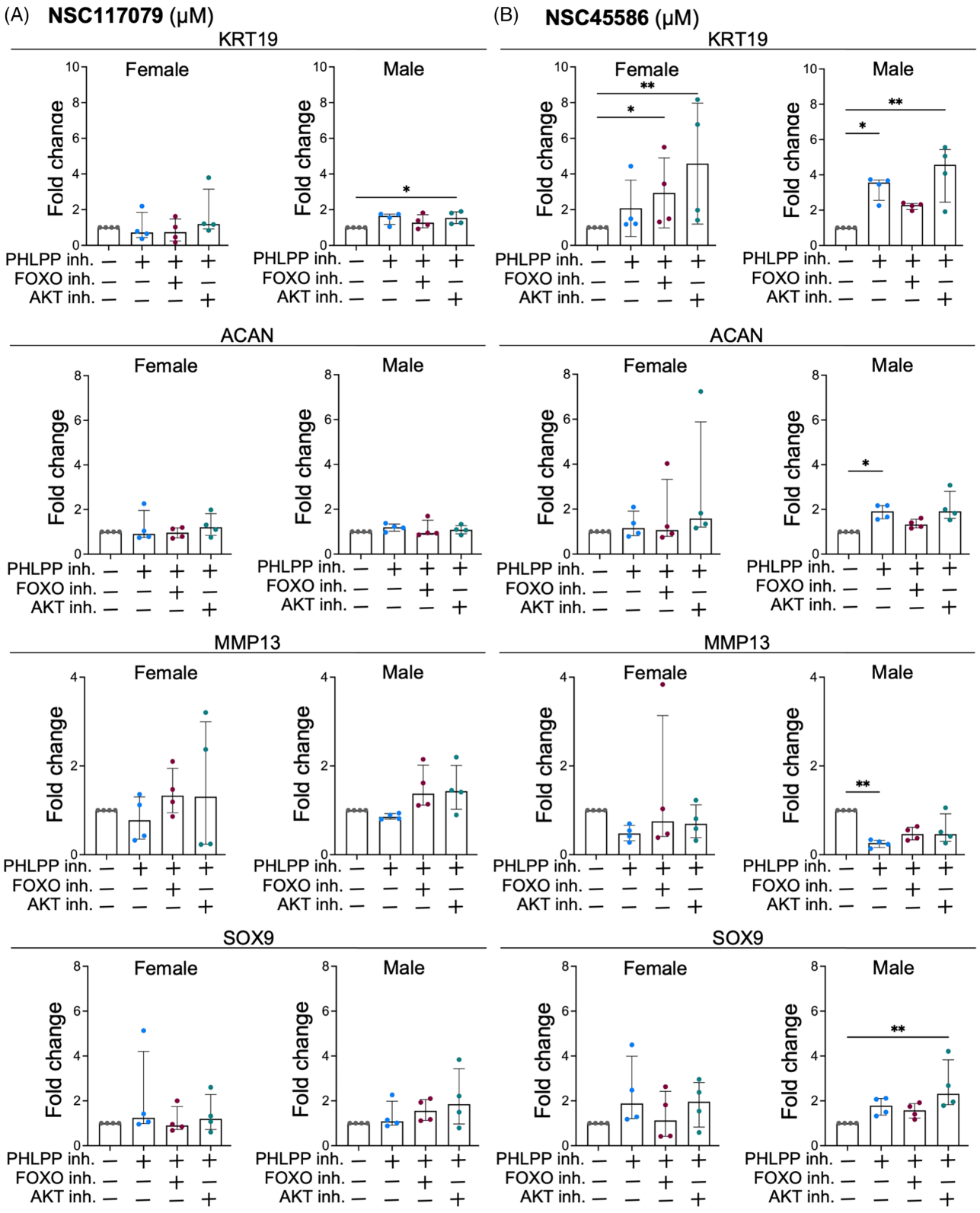

FIGURE 8 FOXO1 inhibition offset the effects of NSC45586 in degenerated human NP cells. Gene expression analysis of degenerated human NP cells. NP cells were pre-treated with either a FOXO1 inhibitor (200 nM) or a PI3K/AKT inhibitor (100 nM) for 1 h, followed by treatment with 100 μM NSC117079 or NSC45586 for 24 h. In (A) NSC117079 treated NP cells, FOXO1 or AKT inhibitor pretreatment did not affect the expression of all the genes examined. (B) NSC45586 increased the gene expression of KRT19 and ACAN, which was partially offset by the FOXO1 inhibitor in males. The gene expression of MMP13 and SOX9 was not affected by the FOXO1 inhibitor. No significant changes were detected in female NP cells treated with NSC45586. $n = 4$ per group. Kruskal–Wallis test with Dunn's post hoc testing was performed. The data are presented as median with interquartile range. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. NP, nucleus pulposus.

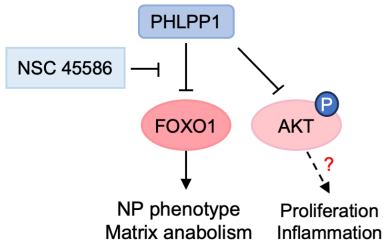

FIGURE 9 PHLPP inhibition promotes NP phenotype through FOXO1 activation: Graphic summary of the results. NSC45586 treatment promotes FOXO1 activation, which leads to increased expression of NP phenotypic marker KRT19 and decreased extracellular matrix catabolism. NP, nucleus pulposus; PHLPP, PH domain Leucine-rich-repeats protein phosphatase.

inhibitor did not affect the gene expression in NSC117079 treated NP cells (Figure 8A). When NP cells were pre-treated with the FOXO1 inhibitor, the beneficial effects of the PHLPP inhibitor NSC45586 were inhibited, and KRT19 and ACAN gene expression levels were decreased to levels similar to untreated degenerated NP cells (Figure 8B). Pretreatment with AKT/P13K inhibitor showed no significant effects on any of the genes examined in NSC45586-treated NP cells (Figure 8), suggesting that PHLPP inhibition with NSC45586 increased the regenerative potential of human degenerated NP cells via targeting FOXO1.

4 | DISCUSSION

PHLPP is involved in the progression of several degenerative diseases, such as osteoarthritis,[33] neurodegeneration,[34] and cardiac ischemia[35] by promoting pro-apoptotic pathways and suppressing tissue repair. Our previous studies demonstrated that the isozyme PHLPP1 increased with IVD degeneration and that its deficiency mitigated IVD degeneration in a model of spontaneous IVD degeneration in mice.[25,26] This study aimed to test if PHLPP inhibition has the potential to promote NP cell health by preventing degenerative changes and inducing a phenotypic shift toward a healthy NP cell phenotype.

The efficacy of NSC117079 and NSC45586 in promoting matrix metabolism and reducing catabolism has previously been demonstrated in articular cartilage.[36] Yet, the role of PHLPP and its inhibitors is known to be highly tissue-specific, and our data suggest that NSC45586, but not NSC117079, promoted NP cell survival and metabolism, maintained the notochordal morphology, enhanced the expression of NP phenotypic marker KRT19, and counteracted cell apoptosis in mouse organ cultures. Human degenerated NP cells, when treated with NSC45586, gained regenerative potential by enhancing cell proliferation and expressing higher levels of KRT19, ACAN, and SOX9 and lower levels of MMP13, which are indicators of a healthy NP phenotype. Combined, these data suggest that NSC45586 might be a potential therapeutic agent for treating IVD degeneration by promoting cell survival and matrix synthesis and suppressing matrix degradation.

Downstream effectors of PHLPP include FOXO transcription factors.[18] FOXO transcription factors are a class of highly conserved proteins that are involved in numerous physiological processes as well as aging and various diseases.[37–39] Its importance for tissue maintenance has been demonstrated in several mouse models where FOXO depletion accelerated age-induced axonal degeneration,[22] arthritis,[40] and IVD degeneration.[24] In cardiomyocytes, decreased PHLPP activity contributed to sustained activation of FOXOs, which in turn, increased AKT phosphorylation and kinase activity. In our study, we saw an increase in AKT phosphorylation in NP cells with PHLPP inhibition. However, blocking AKT pathway did not abolish the beneficial effects of NSC45586 on NP cell anabolism and NP marker expression. It is possible that AKT pathway might be involved in other cellular activities, such as cell proliferation, inflammatory responses, and senescence, as reported in previous studies.[41–43] Interestingly, pharmacological inhibition of PHLPP function increased FOXO1 expression and promoted NP cell anabolism and NP marker expression. Moreover, the regenerative potential of PHLPP inhibition was reversed when inhibiting FOXO1 activity, further suggesting FOXO1 as an important PHLPP target in IVDs.

Inhibition of PHLPP function exerted beneficial effects on IVD regeneration more robustly in males than in females, which was observed in ex vivo organ cultures and human in vitro cultures. It has been reported that Phlpp1 knockout in myeloid lineage cells led to an increase in bone mass in female, but not male, mice, and effects of the specific knockout of Phlpp1 in osteoclast cells on bone mass were lost following ovariectomy.[44,45] In addition, Farr et al. showed that short-term estrogen therapy slightly induced the expression of PHLPP1 in aged women when PHLPP1 expression was decreased in bone tissues.[45,46] These results indicate the existence of sexual differences in PHLPP1 function between females and males. In IVD organ cultures or NP cell cultures, inhibition of PHLPP function mainly had an effect in male groups suggesting intrinsic differences between the sexes in response to stimuli, independent of physiological hormonal influences.

It is possible that the lack of significance in females is due to the relatively small sample size and high variability in female groups. Yet, this variability within groups was observed in mouse and human studies, warranting further studies on exploring if sexual dimorphism exists in the effects of PHLPP inhibition on IVD health and the mechanisms of sexual dimorphism in response to PHLPP inhibition. It is noteworthy that low serum culture conditions for mouse IVDs cannot fully mimic the complicated degeneration process in humans, and other factors such as an inflammatory environment might additionally affect degenerative changes in ex vivo cultures. Future in vivo studies will build on our findings and provide information about the effects of NSC45586 on truly degenerating IVDs. Another limitation is the focus of KRT19 as sole NP marker. While the combination of KRT19, ACAN, SOX9, and MMP13 are indicators for NP phenotype, future in vitro and in vivo studies will include a panel of NP-related markers to verify the effect of NSC45586 on NP cell phenotype. While monolayer culture conditions could have caused the loss of NP-phenotypic markers, we chose these culture conditions to assess the immediate

response (30 min) on kinase phosphorylation. In addition, cultures under normoxia might have caused additional cell stress for the already degenerated human NP cells, further suggesting beneficial effects of NSC45586 on degenerated NP cells. Future studies will use pellet cultures under hypoxia conditions to further explore the effects of NSC45586 treatment on NP cell health.

In summary, NSC45586 was effective in promoting NP cell regeneration in degenerated human NP cells, which was most pronounced in males. The observed sex differences in the response to NSC45586 highlight the need for including sex as a biological variable. Our results suggest that PHLPP plays a key role in NP cell homeostasis and highlights the PHLPP-FOXO1 cascade as a potential therapeutic target for treating IVD degeneration (Figure 9).

AUTHOR CONTRIBUTIONS

Changli Zhang: study design, data collection and analysis, manuscript creation, and editing. **Madeleine D. Gordon**: IHC processing, image acquisition, data analysis, and manuscript editing. **Katherine M. Joseph**: IHC processing, image acquisition, data analysis, and manuscript editing. **Martha E. Diaz-Hernandez**: human specimen collection, data interpretation, critical suggestions, and manuscript review. **Hicham Drissi**: study design, data interpretation, critical suggestions, and manuscript review. **Svenja Illien-Jünger**: study design, data analysis, graphic designs, manuscript review and editing, and fund acquisition. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest associated with this work.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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