Minimal Sustainability of Dedifferentiation by ROCK Inhibitor on Rat Nucleus Pulposus Cells In Vitro

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Abstract:

Introduction: Intervertebral disc degeneration is strongly associated with low back pain. Cell transplantation has been extensively studied as a treatment option for intervertebral disc degeneration. It is often necessary to perform cell culture prior to cell transplantation; however, during cell expansion, the cells tend to dedifferentiate and lose their potency. Although the ability to suppress dedifferentiation by ROCK inhibitor (ROCKi) has recently been reported for chondrocytes, its effects on nucleus pulposus cells are still largely unknown.

Methods: Rat nucleus pulposus cells were cultured with or without the addition of ROCKi (Y-27632), and cell proliferation; CD24 positivity; expression of SOX9, COL2A1, Aggrecan, and COL1A1; and cell redifferentiation ability in pellet culture were evaluated.

Results: Although the addition of ROCKi tended to slightly increase the cell proliferative capacity, no significant differences were observed between treated and untreated conditions. The addition of ROCKi showed a trend of minimally increased COL2A1, ACAN, and SOX9 expression. Increases in COL1A1 expression was slightly suppressed by ROCKi. In pellet culture, strong increase in type II collagen deposition was observed by the addition of ROCKi. The addition of ROCKi did not significantly change the levels of CD24 positivity. The supplementation of ROCKi did not significantly enhance nucleus pulposus cell marker expression during monolayer expansion. However, ROCKi addition did result in an increased type II collagen deposition in 3D pellet culture.

Conclusions: Taken together, the results suggest a minimal effect by ROCKi on nucleus pulposus cell phenotype maintenance.

Keywords:

Intervertebral disc, Nucleus pulposus, ROCK inhibitor, Cell culture, Cell therapy, Dedifferentiation, Rat, *In vitro*

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Introduction

Intervertebral disc (IVD) degeneration is an irreversible pathology, where the cartilage-like IVD progressively changes to a fibrotic structure, which can lead to a variety of spinal diseases, *e.g.*, disc herniation, spinal stenosis, and radiculopathy[1)]. Moreover, IVD degeneration is strongly associated with low back pain, which has been identified as the primary global cause of disability[2,3)]. Although it has been reported that the IVDs possess some self-repairing ability, the potency remains insufficient to restore progressive IVD wear and tear[4)]. This might partly be ascribed to the avascular nature, limiting the attraction of active and stimulating cells and inadequate exchange of nutrients and waste products *in situ*[5)]. Currently, no therapeutic strategies are available that effectively treat degenerative IVDs, and interventions are limited to physiotherapy, analgesics, or surgical immobilization or excision of the affected discs. Therefore, various treatment commodities, such as cell therapy, cytokine injection, and gene therapy, are being explored as regenerative strategies for IVD repair[6-10)]. In particular, cell transplantation has gained significant momentum in the last decade[11)]. Nevertheless, for application and commercialization of cell products, it is often required to expand, cryopre-

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Table 1. Primer Sequence.

Target	Forward primer	Reverse primer
SOX9	GCACATCAAGACGGAGCAAC	AGGTTGAAGGGGCTGTAGGA
COL2A1	CCAGGTCCTGCTGGAAAA	CCTCTTTCTCCGGCCTTT
ACAN	GCAGGGATAACGGACTGAAG	CAAGAGTAAAGTGGTCATAGTTCAGC
COL1A1	CATGTTCAGCTTTGTGGACCT	GCAGCTGACTTCAGGGATGT
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA

serve, and enable cell recovery from their storage condition[12], which is hindered by rapid dedifferentiation of IVD-derived nucleus pulposus (NP) cells in monolayer culture, as indicated by a loss in Aggrecan, type II collagen (COL2A1), and SOX9 expression[13-15], forming an obstacle for the development of NP cell transplantation products[12].

Rho kinase (ROCK) is an intracellular serine/threonine-oxidizing kinase that was identified as a target of the Rho protein, a low-molecular-weight GTP-binding protein. The functions of ROCK include the regulation of smooth muscle contraction, cell morphology, and migration[14]. Y-27632, a specific ROCK inhibitor (ROCKi), is known to suppress cell death during cell dispersion and has been widely used for induced pluripotent stem cell and embryonic stem cell cultures[15]. Although it was reported that ROCKi suppresses dedifferentiation during monolayer culture of chondrocytes[16], the effect of ROCKi on NP cells is less established. As such, it might prove beneficial to examine ROCKi on NP cells to potentially maintain their phenotype for research and therapeutic purposes, in particular considering the need to expand NP cells for obtaining sufficient cell numbers for a therapeutic application[17], keeping in mind the limited cell yields from IVD specimen[13]. Therefore, we evaluated the impact of ROCKi Y-27632 as a culture medium supplement for maintaining cell phenotype of rat NP cells in monolayer culture for potential translation to prospective cell-based transplantation products.

Materials and Methods

The protocols used in this study were approved by Tokai University, School of Medicine committee for safe animal experimentation. (Application numbers 124007 and 131021)

Cell isolation and culture

NP cells were isolated from coccygeal discs of 11-week-old Sprague Dawley rats (n = 16). In short, rats were euthanized by excess pentobarbital sodium (Nembutal, Abbott. Laboratories, USA) injection. The coccygeal columns were removed aseptically, and IVDs were separated. Subsequently, NP tissue was digested in 0.125% trypsin solution for 15 min at 37°C, filtered, and spun down. The isolated cells were seeded at 200.000 cells/10-cm dishes maintained in 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin (ThermoFisher, USA) α-MEM, at 37°C, 21% O_2. 5% CO_2, with or without the addition of 10 μM Y-27632 (Wako, Japan), similar to the work of Matsumoto et al. applying Y-27632 on articular cartilage-derived chondrocytes[16]. Following Matsumoto et al., monolayer-cultured NP cells were passaged up to passages 1, 4, and 7 with medium (including ROCKi) being exchanged every 3 days[16].

Measurement of cell proliferation

Proliferation of NP cells was evaluated using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay[18]. NP cells were seeded on a 24-well plate at 1.5×10^4 cells per well and cultured for 72 hours. Thereafter, MTT (Dojindo, Japan) solution dissolved with serum-free phenol red-free DMEM at 0.5 mg/mL was added. Cells were cultured for 2 h at 37°C; medium was removed, and 78% isopropanol, 18% DMSO, and 4% Tween 20 solution was added. After thorough mixing, cell proliferation was measured by a microplate reader (SPECTRA MAX 250, Molecular Devices, Sunnyvale, CA) at 562 nm.

Real-time reverse transcription-polymerase chain reaction analysis

NP cells were cultured in 10-cm plates (5×10^5 cells/plate) with or without ROCKi for 72 h, and total RNA was extracted using the TRIzol RNA isolation protocol (Invitrogen, USA) at P1, P4, and P7. RNA was treated with RNase-free DNase I. Total RNA (100 ng) was used as a template for cDNA synthesis by the reverse transcription. Expression levels were determined by SYBR Green PCR Master Mix (Applied Biosystems, UK) to which gene-specific forward and reverse PCR primers (TaKaRa Bio Inc., Japan) for the genes SOX9, COL2A1, Aggrecan, and COL1A1 were added (Table 1). PCR reactions were performed by 7500 Fast system (Applied Biosystems, USA) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping control gene, and the arbitrary intensity threshold (Ct) of amplification was computed and further calculated as $2^{-\Delta\Delta CT}$ relative to levels of non-treated P1 cells.

Flow cytometric analysis and cell sorting

Using FACS analysis, ROCKi's ability to suppress the dedifferentiation and maintenance of marker expression was examined. Cells were suspended in blocking buffer (Dulbecco's phosphate-buffered saline, Sigma Aldrich, USA), 0.2% bovine serum albumin (BSA; Gibco), and 1 mM EDTA, followed by staining with antibodies against rat CD24 (clone HIS50, BD Pharmingen, USA). Cells were analyzed using a FACSVerse flow cytometer (BD Bi-

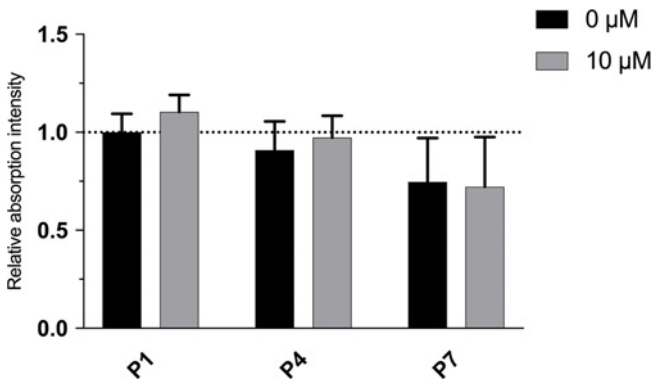

Figure 1. Measurement of cell proliferation.
Rat NP cell proliferation assessed by MTT assay at passage 1 (P1), 4 (P4), or 7 (P7) with or without 10 μM ROCK inhibitor, presented as absorbance intensity relative to the average value obtained from non-treated P1 NP cells. Graph demonstrates mean values (±SD).

osciences, USA). For cell sorting, cells from cultures after the first, fourth, and seventh passages were processed by a FACSAria I cell sorter (BD Biosciences).

Chondrogenic pellet culture

To evaluate whether dedifferentiation of ROCKi-treated cells was suppressed, immunohistochemical staining was performed to evaluate the regenerative ability within chondrogenic pellet culture. Monolayer-cultured cells up to P2 under treated and non-treated conditions were detached by trypsin, and pellet cultures were prepared by adding 2.5×10^5 cells in 1 ml medium to 15 ml conical polypropylene tubes. The cells were pelleted by centrifugation at 400×g for 6min and subsequently cultured at 37°C, 21% O_2, and 5% CO_2. Pellets were maintained in 5% FBS, 5 ng/mL TGF-β1 (PeproTech, Rocky Hill, NJ), 10 ng/mL R3 IGF-1 (Abcam, UK), 5 mg/mL recombinant human insulin, 5 mg/mL human transferrin, and 5 ng/mL selenous acid (ITS-Premix, BD Bioscience) Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM:F12, Wako) as previously described[19] and were cultured for 2 weeks[16].

Immunohistochemistry

After 2 weeks of culture, pellets were fixed with 10% formaldehyde (Sigma-Aldrich) and dehydrated with 10% sucrose (WAKO chemical, Japan) in PBS for an hour. Frozen sections were prepared, blocked by 3.0% BSA (Gibco) in PBS, followed by staining with anti-collagen type II (1:100, Daiichi Fine Chemical, Japan) at 4°C, overnight. Samples were washed with PBS and stained with Alexa 594-conjugated anti-mouse secondary antibody (ThermoFisher Scientific, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

To evaluate the effect of adding ROCKi on stress fiber formation, cultured NP cells in the ROCKi-treated and non-treated groups were evaluated by F-actin staining. Cells cultured up to P2 were seeded in a chamber slide (2 cm^2) at a concentration of 5.0×10^3 cells/ml. The next day, cells were fixed with 10% formalin, and then, actin filaments were visualized by Alexa Fluor 594 phalloidin (ThermoFisher Scientific, USA) staining.

Statistical analyses

Two-way ANOVA followed by Tukey testing was used to analyze the cell proliferation, RT-PCR, and FACS data. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., USA). Error bars represent 1 SD, and differences of $p < 0.05$ were considered significantly different.

Results

Measurement of cell proliferation

The MTT assay demonstrated a decline in cell proliferation potency with passage of NP cells (Fig. 1). No significant difference in the cell proliferation ability between the treated and non-treated groups was observed.

Real-time reverse transcription-polymerase chain reaction analysis

The gene expression of SOX9 displayed a decreasing trend with repeated passages for the non-treated group (Fig. 2A). With supplementation of ROCKi, the gene expression level of SOX9 remained elevated in P1 and P4 but declined at P7. The gene expression levels of COL2A1 also demonstrated a decreasing trend with repeated passages in both the treated and non-treated groups (Fig. 2B). Treatment of ROCKi showed a trend of improved COL2A1 expression compared with that in the non-treated group, with significantly higher levels at P1 and P4, compared with P7 for the ROCKi-treated cells. Expression of aggrecan showed a significant increase with progressive passaging (Fig. 2C); however, expression levels of aggrecan did not show a clear difference between culture conditions. The gene expression level of COL1A1 showed a slight increase with repeated passages in both the ROCKi-treated and non-treated groups (Fig. 2D). However, a clear trend of increased COL1A1/COL2A1 ratio could be observed for both groups, with a more intense increase for the untreated condition (Supplementary data 1).

Flow cytometric analysis

The percentage of CD24-positive cells in P1 was 73.9% in the non-treated group and 71.9% in the ROCKi-treated group, for P4 37.6% in the non-treated group and 34.4% in the ROCKi-treated group, and for P7 it was 7.6% in the non-treated group and 7.2% in the ROCKi-treated group (Fig. 3).

Immunohistochemistry

Pellet cultures were successfully established in both the ROCKi-treated and non-treated groups. In the non-treated

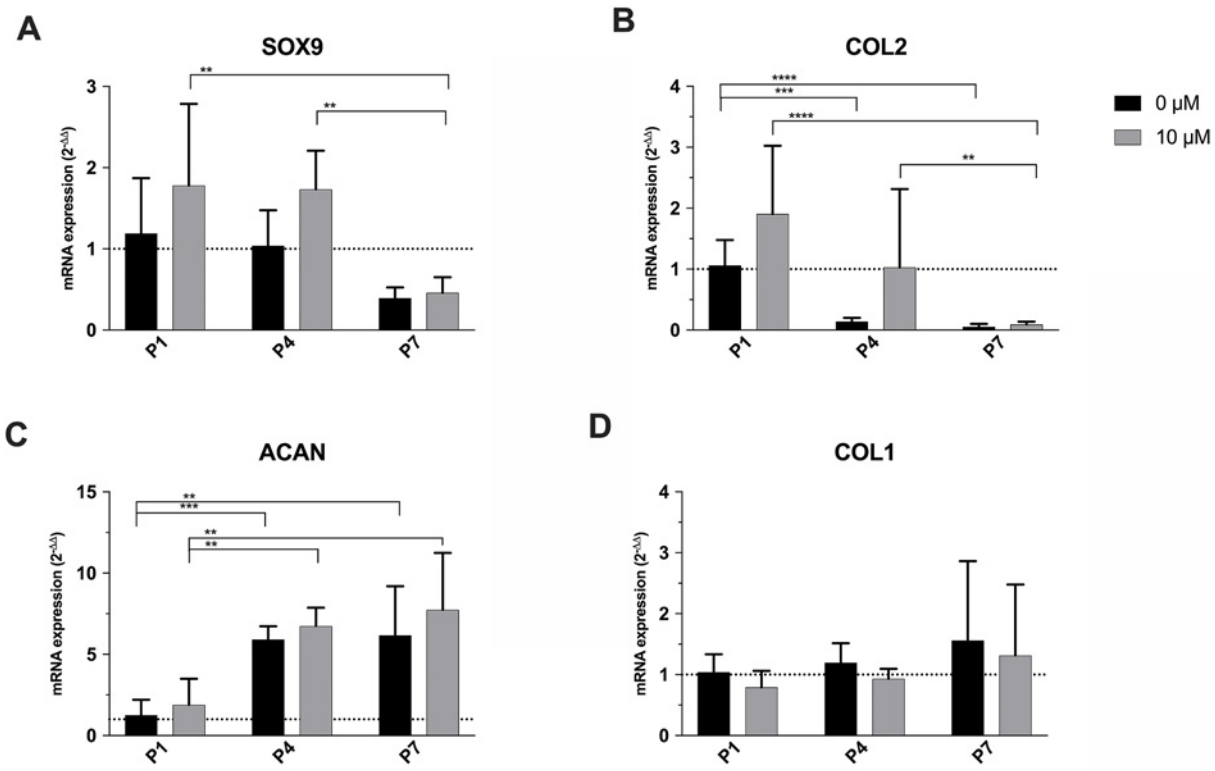

Figure 2. Real-time reverse transcription-polymerase chain reaction (RT-PCR) Analysis.
The expressions of (A) SOX9 and (B) COL2 (COL2A1) decreased with increasing number of passages in both the ROCKi-treated and non-treated groups. The expressions of SOX9 and COL2A1 were better maintained by treatment with ROCKi. (C) The gene expression of aggrecan showed enhanced expression in P1, P4, and P7. (D) The expression of COL1 (COL1A1) tended to increase its expression levels, with increasing number of passages in both the ROCKi-treated and non-treated groups. By treatment with ROCKi, the expression level of COL1A1 tended to be lower than that in the non-treated group. * ($p \leq 0.05$) ** ($p \leq 0.01$) *** ($p \leq 0.005$) **** ($p \leq 0.001$). Graph demonstrates mean values (±SD).

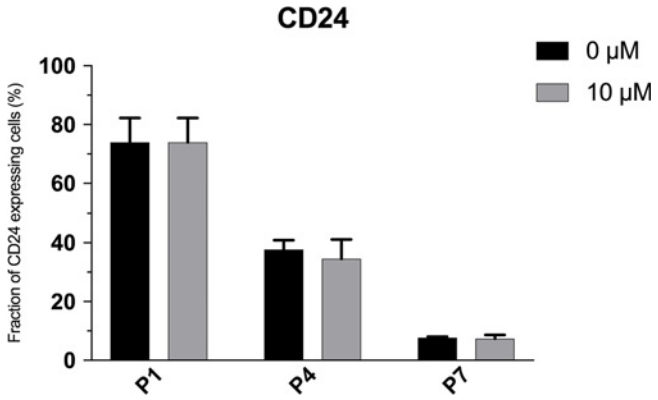

Figure 3. Flow cytometric analysis.
CD24 cell surface marker expression gradually decreased with every passage of monolayer culture as determined by flow cytometric analysis. No significant changes were observed between the ROCKi-treated and untreated group. Graph demonstrates mean values (±SD).

group, slight staining of type II collagen was observed, whereas in the cells cultured with ROCKi, increased intensity of type II collagen staining was observed (Fig. 4). Moreover, F-actin staining demonstrated a strong stress fiber formation in the non-treated group, but this was attenuated in the ROCKi-treated group (Fig. 5).

Discussion

SRY-related HMG-box proteins SOX9, SOX5, and SOX6 are essential for chondrocyte development, differentiation, and function[20-23]. In particular, SOX9 is expressed in all cartilage tissues *in vivo*, and genetically modified mice lacking SOX9 expression do not display endochondral ossification, and contrarily cartilage tissue can be induced by ectopic Sox 9 expression[21,22], indicating the pivotal role of SOX9 for chondrocyte development, differentiation, and function[21,22]. Matsumoto et al. reported that ROCK inhibition suppressed dedifferentiation of cultured chondrocytes along with SOX9 and SOX5/6 expression and regulated chondrogenic gene expression[16,24]. Work from Oh et al. demonstrated that short- or long-term passaged rat NP and annulus fibrosus cells cultured with ROCKi showed similar SOX9, COL2A1, and ACAN expression levels and concluded their cell populations were able to maintain their phenotype[25]. Nevertheless, it remained undetermined how these treated and immortalized cells compare with primary NP cells, or NP cells passaged without ROCKi[25]. The result of our study, applying primary and passaged NP cells, similarly demonstrate a trend of enhanced gene expression for SOX9 and COL2A1

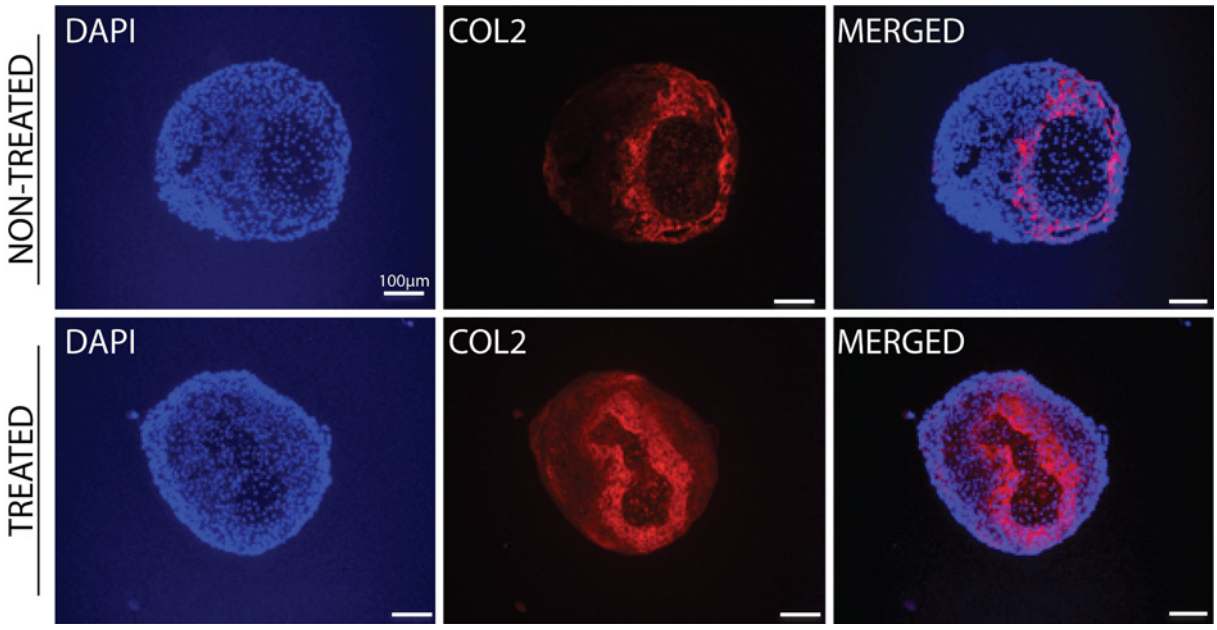

Figure 4. Immunohistochemistry.
Monolayer-cultured cells up to P2 in the ROCKi-treated and non-treated groups were detached with trypsin, and pellet culture was performed for 2 weeks. Type II collagen staining revealed strong type II collagen deposition in the ROCKi-treated group compared with the untreated group. Scale bar represents 100 μm.

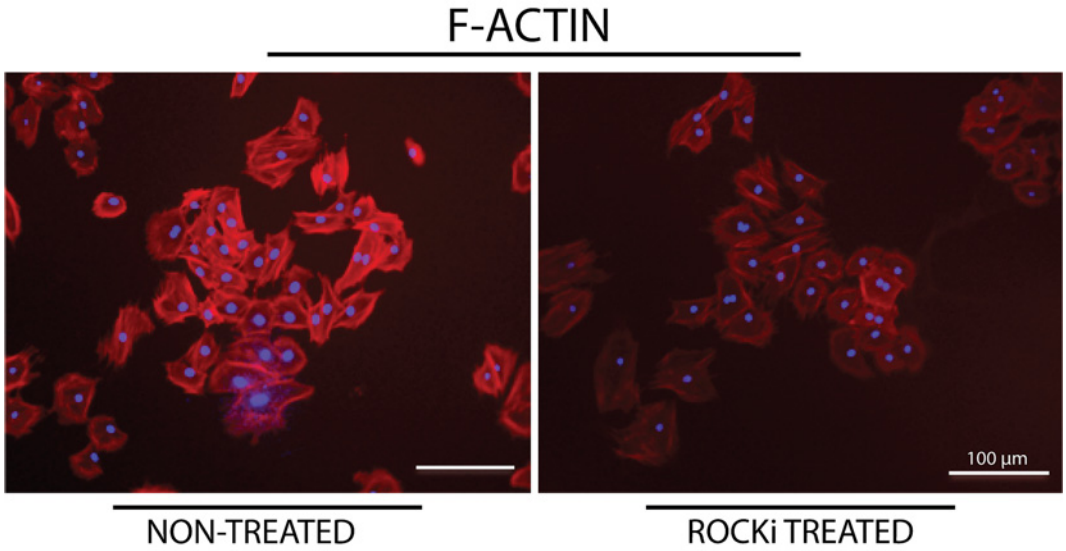

Figure 5. F-actin staining.
Nucleus pulposus cells treated with or without ROCK inhibitor. F-actin staining revealed intense staining in the non-treated group but attenuated stress fiber formation in the ROCKi-treated group. Scale bar represents 100 μm.

by treatment with ROCKi, although the differences were not significantly different. Data suggest ROCKi is able to maintain expression levels of NP markers up to passage 4 compared with passage 1 NP cells, but this effect is lost at passage 7. Moreover, COL1A1 expression revealed no increase in expression with passaging nor seemed to be affected by ROCKi. Previous studies have indicated that NP cells cultured in monolayer do not increase their COL1A1 expression[26,27], but instead increase their ratio of COL1A1/COL2A1[26,28]. COL1A1/COL2A1 ratio did show an increasing trend, concurring with previous reports (Supplementary data 1). Additionally, ROCKi treatment did present a strong increase in the deposition of type II collagen in 3D pellet culture. These data suggest that ROCKi did support maintenance of NP cell phenotypes and their extracellular matrix collagen production. Curiously, an increase in ACAN expression (Fig. 2C) for both treated and non-treated groups was observed with an increase in passage. Although unexpected, it is not uncommon to observe increased ACAN expression with advancing NP cell passage, as demonstrated

by the work of Ono et al.[29] Additionally, the notochordal cell type derived from rat NP tissue might have differentiated to a more chondrogenic phenotype *in vitro* monolayer culture conditions, resulting in enhanced ACAN expression[30,31]. As such, future studies might benefit from applying an animal model that lacks notochordal cells in their IVD[32].

It has been established that CD24 is a marker that is specifically expressed in rat NP cells[33] and has recently been reported as a marker for IVD stem/progenitor cells[34]. Sakai et al. reported that Tie2-positive cells from mouse, human, canine, and bovine NP can form spherical colonies and possess high multipotency and self-replicability[13,34,35]. Tie2-expressing cells, upon activation, differentiate to more mature CD24-positive cells, presenting high collagen type II and aggrecan expression. In our findings, cell passaging decreased CD24 positivity, and the resulting dedifferentiation was further confirmed by decreased gene expressions levels of SOX9 and COL2A1. ROCKi supplementation did not result in enhanced cell potency with passaging, although a small trend could be observed for ROCKi to maintain expression levels of SOX9 and COL2A1 up to passage 4. Noteworthy, however, is that the observed changes were evaluated on mRNA expression levels, and protein expression assessment, by, *e.g.,* western blotting, was not applied, and should be considered as a limitation when interpreting our findings.

ROCKi has also been reported to promote proliferation in corneal endothelial cells and chondrocytes[34], while other work on rat chondrosarcoma revealed decreased cell proliferation[36]. In this study, ROCKi did not demonstrate a proliferation-promoting effects on NP cells. The cause of these discrepancies remains undetermined. Generally, the NP-derived cells are considered chondrogenic-like, and we expected them to respond similarly to previously reports applying articular chondrocytes[16]. One explanation might be found in the more notochordal nature of the NP-derived cells, particularly for rat IVDs, resulting in a different response to ROCKi[30,37]. Or that a resembling chondrogenic phenotype of NP cells does directly relate to a similar response to ROCKi[38]. However, these hypotheses remain highly speculative. Therefore, further research is needed to elucidate the mechanism involved in ROCKi regulated proliferation.

ROCKi is being clinically applied as a cerebral vasospasm inhibitor after subarachnoid hemorrhage, as a therapeutic agent for pulmonary hypertension (Fasudil)[39] and recently as a treatment for glaucoma (Ripasil)[40]. In addition, Y-27632 is being clinically investigated as a corneal endothelial therapeutic agent. ROCKi is an inexpensive and relatively safe therapeutic agent that has been reported to enhance cell adhesion after transplantation[41]. Moreover, ROCKi poses a cheap and easily accessible supplementation that could potentially be applied to NP cells during expansion for cell transplantation product aimed at regenerating the IVD structure. Nevertheless, our results suggest no significant effects by ROCKi on maintaining rat-derived NP cell phenotype with passaging. However, the current work is limited by assessing the effect of ROCKi on rat-derived NP cells in a specific monolayer culture condition. The optimal condition of ROCKi treatment specific for rat NP cells has not yet been determined, and as such, additional research will be needed to determine the effect of ROCKi at different concentrations and within different culture setups. Moreover, future research will need to demonstrate the effects of Y-27632 on human NP cells and determine its potential as an additive to maintain the regenerative potency of NP cells and the resulting cell transplant products.

In conclusion, ROCKi Y-27632 showed a minimal trend of maintaining SOX9 and COL2A1 expression for rat NP cells expanded *in vitro*, up to passage 4. ROCKi supplementation did show a clear suppression of F-actin production and an increased type II collagen deposition in 3D pellet culture. Future research will need to determine the potential of ROCKi as a medium additive for regenerative NP cell products.

Conflicts of Interest: The authors declare that there are no relevant conflicts of interest.

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