

## PROTOCOLS, METHODS, AND RESOURCES

# In vitro nucleus pulposus tissue model with physicochemical stresses

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### Abstract

Intervertebral discs (IVDs) are exposed to changes in physicochemical stresses including hydrostatic and osmotic pressure via diurnal spinal motion. Homeostasis, degeneration, and regeneration in IVDs have been studied using in vitro, ex vivo, and animal models. However, incubation of nucleus pulposus (NP) cells in medium has limited capability to reproduce anabolic turnover and regeneration under physicochemical stresses. We developed a novel pressure/perfusion cell culture system and a semipermeable membrane pouch device for enclosing isolated NP cells for in vitro incubation under physicochemical stresses. We assessed the performance of this system to identify an appropriate stress loading regimen to promote gene expression and consistent accumulation of extracellular matrices by bovine caudal NP cells. Cyclic hydrostatic pressure (HP) for 4 days followed by constant HP for 3 days in high osmolality (HO; 450 mOsm/kg H<sub>2</sub>O) showed a trend towards upregulated aggrecan expression and dense accumulation of keratan sulfate without gaps by the NP cells. Furthermore, a repetitive regimen of cyclic HP for 2 days followed by constant HP for 1 day in HO (repeated twice) significantly upregulated gene expression of aggrecan ( $P < .05$ ) compared to no pressure and suppressed matrix metalloproteinase-13 expression ( $P < .05$ ) at 6 days. Our culture system and pouches will be useful to reproduce physicochemical stresses in NP cells for simulating anabolic, catabolic, and homeostatic turnover under diurnal spinal motion.

### KEYWORDS

cyclic and constant hydrostatic pressure, in vitro, nucleus pulposus tissue model, osmolality

## 1 | INTRODUCTION

Intervertebral disc (IVD) degeneration is associated with herniated discs, spinal stenosis, and spondylolisthesis.<sup>1</sup> Surgical interventions for disc herniation are predominantly the excision of pathologic discs and/or spinal fusion, resulting in a loss of biomechanical function.<sup>2</sup> However, no surgical interventions are directed towards the underlying pathomechanism of disc degeneration itself. Thus, we sought to develop

cell-based therapy for IVD degeneration, focused on the regenerative capability of nucleus pulposus (NP) cells. The NP is composed of negatively charged amorphous extracellular matrix (ECM) and abundant intratissue fluid.<sup>3,4</sup> Spinal motion in an upright position and in a prone/recumbent position create changes in physicochemical stresses in IVDs, both moment-to-moment and in daily cycles.<sup>5,6</sup> The combination of those stresses—hydrostatic pressure (HP), deviatoric stress, and osmotic pressure—affects metabolic turnover and behavior in NP cells.<sup>7-12</sup> Thus,

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we included these stresses in designing experimental models to study homeostasis, regeneration, and degeneration in IVD.

We developed an in vitro dynamic culture model to reproduce the effects of these changes in physicochemical stresses on NP cells/clusters, isolated enzymatically from bovine NP (bNP),<sup>13</sup> which we refer to as our NP tissue model. We enclosed the NP cells/clusters suspended freely within a semipermeable membrane pouch; the pouch was suspended in culture medium, which was compressed for application of HP using a pressure/perfusion culture system. Our latest study demonstrated that combined HP and high osmolality (HO; 450 mOsm/kg H<sub>2</sub>O) stimulated the gene expression of ECM molecules, but created gaps within the newly accumulated ECM. Conversely, no gaps were formed within the ECM accumulated under low osmolality (LO; 320 mOsm/kg H<sub>2</sub>O). We became interested in these contradictory results, because upregulation of the mRNA of ECM molecules did not form dense ECM without gaps in histology. We sought to understand this inconsistency result between gene expression and histology by reproducing various types of metabolic turnover in NP tissue in vitro. Another study using bNP explants demonstrated that cyclic HP and constant HP inhibited exudation of negatively charged matrix from freely swelling bNP explants. Thus, we began to believe that HP affects accumulation of dense ECM.<sup>13</sup>

We hypothesized that a regimen of cyclic HP followed by constant HP in HO results in dense ECM accumulation without gaps by NP cells/clusters. Although various modes of continuous HP application are possible, we focused on duration and repetition, using our model to suspend bNP cells/clusters in pouches under a regimen of cyclic HP followed by constant HP. We evaluated matrix accumulation and the gene expression of ECM molecules using quantitative-polymerase chain reaction (qPCR) and immunohistology.

## 2 | MATERIALS AND METHODS

### 2.1 | NP cell/cluster isolation and suspension culture prior to HP loading experiments

We purchased bovine tails (from 2- to 3-year-old cows) from a local USDA-certified slaughterhouse. We harvested caudal NP tissues from each segment of IVD of the bovine tails using a blade (#15 and #22, Bard-Parkers, Aspen Surgical, Caledonia, Michigan).<sup>14,15</sup> Subsequently we digested the NP tissues in 0.15% collagenase (CLS1, Worthington, Lakewood, New Jersey) dissolved in Ham's F12 medium (Gibco, ThermoFisher, Waltham, Massachusetts) sterilized with a 0.45 μm filter (Nalgene, ThermoFisher) at 37°C overnight on a rotator. Then, we collected NP cells and cell clusters by centrifugation, rinsed them with Dulbecco's phosphate-buffered saline (D-PBS, Gibco) twice, and seeded them onto 1.5% cell-culture-grade agarose-coated six-well plates (Sigma-Aldrich, St. Louis, Missouri). We combined all NP cells and clusters and incubated them in Dulbecco's Minimal Eagle Medium/Ham's F-12 (1:1) with 10% fetal bovine serum and antibiotics (Gibco) for 2 to 3 days. During incubation, the suspended NP cells grew to come with each other and

accumulated ECM. We designated these connected cells and cell aggregations seen in the digested tissue as "cell clusters."<sup>16</sup> The nondigested debris were removed piece by piece with a pipette under a dissection microscope.

### 2.2 | Preparation and performance of a semipermeable membrane pouch

We cut hollow fiber tubing made of polyvinylidene fluoride, 1 mm ID, 1.2 mm OD, and 500 kD molecular weight cut-off (Repligen, Rancho Dominguez, California) into pieces 35 mm in length.<sup>16,17</sup> These pieces of tubing were immersed in 100% ethyl alcohol for 30 minutes, rinsed in D-PBS, and autoclaved at 121°C for 15 minutes (Figure 1).

Prior to the experiments using NP cells, we evaluated the performance of a semipermeable membrane pouch at LO. Each piece of tubing was filled with D-PBS and sealed with stainless steel clips at both ends to create a pouch. We placed the pouches in a pressure-proof chamber filled with 1% bovine serum albumin to load cyclic HP at 0.2 to 0.7 MPa, 0.5 Hz with medium replenishment at 0.1 mL/min or to load constant HP at 0.3 MPa with medium replenishment at 0.1 mL/min and 5% CO<sub>2</sub> in air using a pressure/perfusion culture system (TEP-2, Purpose, Shizuoka, Japan; Figure 1). We confirmed that more than 95% of the D-PBS in the pouches was replaced by albumin within 24 hours.

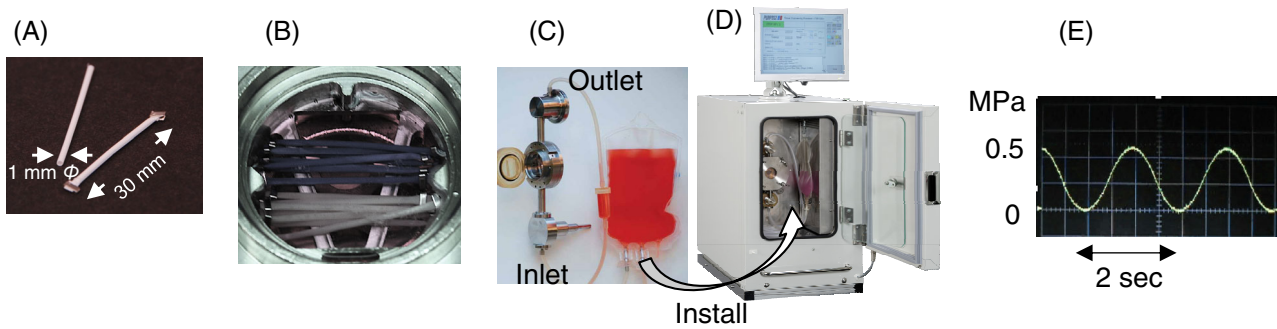
### 2.3 | Incubation of NP cells/clusters with various regimens of changes in HP and medium osmolality

We conducted two sets of experiments to elucidate: (A) the effects of constant HP and (B) the effects of repetitive regimens of cyclic HP followed by constant HP on metabolic turnover in bNP cells (Figure 2). We injected the DNA equivalent of  $1.0 \times 10^5$  NP cells/clusters into the pieces of tubing and closed both ends with stainless steel clips to form pouches. The culture medium at HO was supplemented with sodium chloride at 4.6 g/L.<sup>13</sup> The osmolality was confirmed with a freeze point osmometer (Osmet, Precision Systems, Inc., Natick, Massachusetts).

#### 2.3.1 | Experiment A: The effects of constant HP

Our recent study indicated that cyclic HP at 0 to 0.5 MPa and HO stimulated anabolic gene expression. In addition, there is always some degree of intradiscal pressure even when the body is in a recumbent position.<sup>4</sup> We thus hypothesized that minimal HP is needed to accumulate newly synthesized dense ECM without gaps. We sought to identify the optimal regimen of changes in HP and HO to prevent gap formation within newly accumulated ECM and consistently express upregulated levels of ECM genes.

The pouches were divided into four groups, each of which was cultured under different combinations of no HP or HP (cyclic HP at



**FIGURE 1** A semipermeable membrane pouch culture module and hydrostatic pressure (HP)/perfusion culture system. A, A semipermeable membrane pouch for enclosing nucleus pulposus (NP) cells/clusters. The pouch was made of polyvinylidene difluoride (1.0 mm in diameter, 1.2 mm outer diameter, 30 mm in length, 500 kD MWCO). B, Pouches placed in a culture chamber. C, A photograph of HP/perfusion culture chamber. D, HP/perfusion culture system. E, Profile of cyclic HP

0.2 to 0.7 MPa, 0.5 Hz followed by constant HP at 0.3 MPa) and LO or HO; in addition, the order of these combinations was changed on day 4 (Figure 2A). We compared metabolic turnover in NP cells/clusters among groups: (a) LO/no HP for 7 days (control), (b) LO/HP (LO and cyclic HP for 4 days followed by constant HP for 3 days), (c) HO/HP (HO and cyclic HP for 4 days followed by constant HP for 3 days), and (d) H-LO/HP (HO/cyclic HP for 4 days followed by LO/constant HP for 3 days). For no HP, we suspended the pouches within a stainless-steel mesh basket held in a 100-mL medium bottle with a stirrer at five spins/second to maintain sufficient mass transfer through the semipermeable membrane pouch. To identify the histologic effects of constant HP, we repeated the experiment three times.

### 2.3.2 | Experiment B: The effects of a repetitive regimen of cyclic HP followed by constant HP

Experiment A indicated that constant HP had the potential to accumulate dense ECM. To clarify effects of repetitive daily spinal movement, we compared metabolic turnover in NP cells/clusters between groups: (a) HO/no HP for 6 days and (b) cyclic HP at 0.2 to 0.7 MPa, 0.5 Hz for 2 days followed by constant HP at 0.3 MPa for 1 day, repeated twice over 6 days (Figure 2B). We repeated the experiment six times for obtaining statistical power.

### 2.4 | Evaluation of the cell viability and cell/ECM formation within semipermeable membrane pouches

Pouches were collected from each group, and NP cells/clusters from each pouch were collected and stained with live/dead cell viability assay dyes (Invitrogen). We harvested NP cells/clusters with their pouches and fixed them in a 2% paraformaldehyde/0.1 M cacodylate buffer (pH 7.4) at 4°C. We embedded them (again with their pouches) in plastic resin (Technovit, Electron Microscopy Sciences, Hatfield,

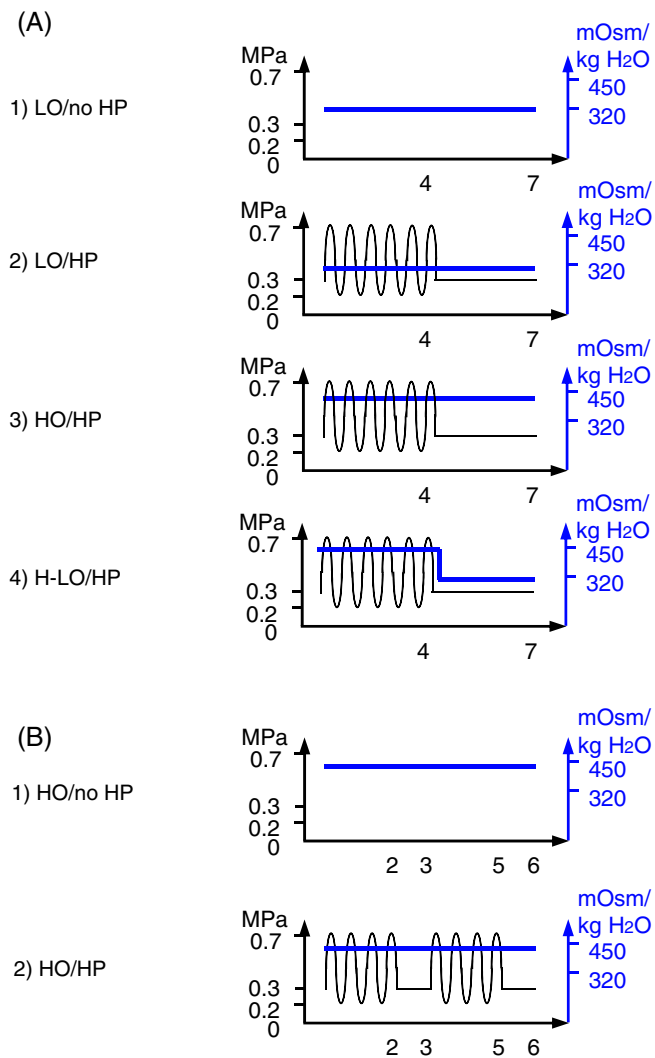
Pennsylvania), cut them into 7- $\mu$ m sections, and stained them with toluidine blue-O at pH 4.0 (Fisher Scientific).

### 2.5 | Evaluation of gene expression using qPCR

To analyze gene expression, we harvested the NP cells/clusters on defined days. The total RNA was extracted from the NP cells/clusters using an RNeasy kit (Qiagen, Germantown, Maryland). The samples were homogenized in the guanidine isothiocyanate-based proprietary component of the kit with 1%  $\beta$ -mercaptoethanol (RLT buffer) using a handheld homogenizer pestle (Fisher Scientific) in accordance with the manufacturer's protocol. The samples were amplified with a reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Thermo Fisher). A gene expression master mix and fluorescent-labeled specific primers (TaqMan, Thermo Fisher) were mixed, followed by qPCR (QuanStudio version 1.4, Applied Biosystems, Foster City, California). We measured the expression of the typical molecules aggrecan core protein (*Acan*) and collagen type-II (*Col2a1*) and the minor molecule collagen type-I (*Col1a1*) in the ECM of NP as well as a catabolic molecule in the NP matrix metalloproteinase-13 (*Mmp-13*). All expression levels were compared with an intrinsic control, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), in each experiment. The TaqMan primers were a *Col1a1*: Bt03225358\_g1; *Col2a1*: Bt03251837\_mH; *Acan*: Bt03212189\_m1; *Mmp-13*: Bt03214051\_m1; and *GAPDH*: Bt03210919\_g1 (Life Technology, Carlsbad, California). Expression Suite Software v1.0.4 was used to analyze the data.

### 2.6 | Data analysis of gene expression profiles using a qPCR assay

Relative quantities (RQs) of the expression of each gene were calculated according to the difference between the average of each condition and of the LO/no HP control, which was given a value of 1.0. RQ

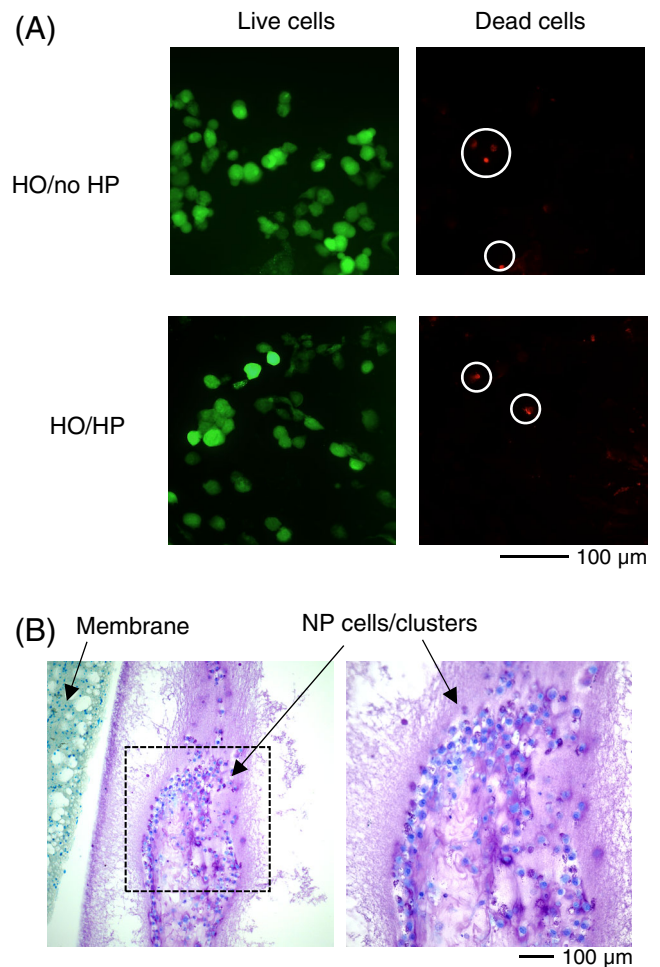


**FIGURE 2** Systematic culture conditions to compare interactions of changes in cyclic and constant hydrostatic pressure (HP). A, The effects of constant HP. B, The effects of a repetitive regimen of cyclic HP followed by constant HP. HP: cyclic hydrostatic pressure at 0.2 to 0.7 MPa, 0.5 Hz for 2 days followed by constant hydrostatic pressure at 0.3 MPa for 1 day. LO: low osmolality (320 mOsm/kg H<sub>2</sub>O). HO: high osmolality (450 mOsm/kg H<sub>2</sub>O). H-LO: HO for 2 days followed by LO for 1 day. no HP: atmospheric pressure

was analyzed using a one-way analysis of variance followed by a Bonferroni test to compare all conditions, with  $P < .05$  considered significant (SPSS software, IBM).

## 2.7 | Evaluation of the production of ECM utilizing immunohistology

We harvested cells/clusters by ejecting them from the pouches with D-PBS. We then fixed them in a 2% paraformaldehyde/0.1 M cacodylate buffer (pH 7.4) at 4°C, embedded them in paraffin, and cut them into 7- $\mu$ m sections for immunostaining. Dewaxed sections were stained with a primary antibody against keratan sulfate (KS, 1:500, Millipore Sigma, St. Louis, Missouri), collagen type II (Col-2, 1:50,



**FIGURE 3** A, Live and dead nucleus pulposus (NP) cells/clusters stained with fluorescent indicators: live cells in green and dead cells in red. B, NP cells/clusters within a semipermeable membrane pouch. A bar indicates 100  $\mu$ m

Chemicon, Temecula, California), and matrix metalloproteinase-13 (MMP-13, 1:50, Santa Cruz Biotechnology, Santa Cruz, California). The sections were then rinsed three times and incubated with a second biotinylated antibody according to the manufacturer's instructions (VECTASTAIN ABC kit, Vector Laboratory, Burlingame, California). Color was developed with 3,38-diaminobenzidine and nickel (DAB kit, Vector Laboratory). Counterstaining was performed with Harris's hematoxylin (Sigma-Aldrich) for Col-2, and KS; and Contrast Red (KPL, Laboratories, Gaithersburg, Maryland) was used for Col-1 and MMP-13.

## 3 | RESULTS

### 3.1 | Viability of NP cells

NP cells/clusters were easily ejected from pouches by flushing with D-PBS, RLT buffer, or fixative on day 6 or 7. With or without HP,



dead NP cells were negligible within the semipermeable membrane pouches. Viable cells with their characteristic sharp periphery were easily identified within aggregated clusters and individually in all experimental groups. Dead cells illuminated in red were rare, and small particles were often seen randomly within and around the aggregation (Figure 3A).

### 3.2 | Density of NP cell/clusters and performance of the semipermeable membrane pouch

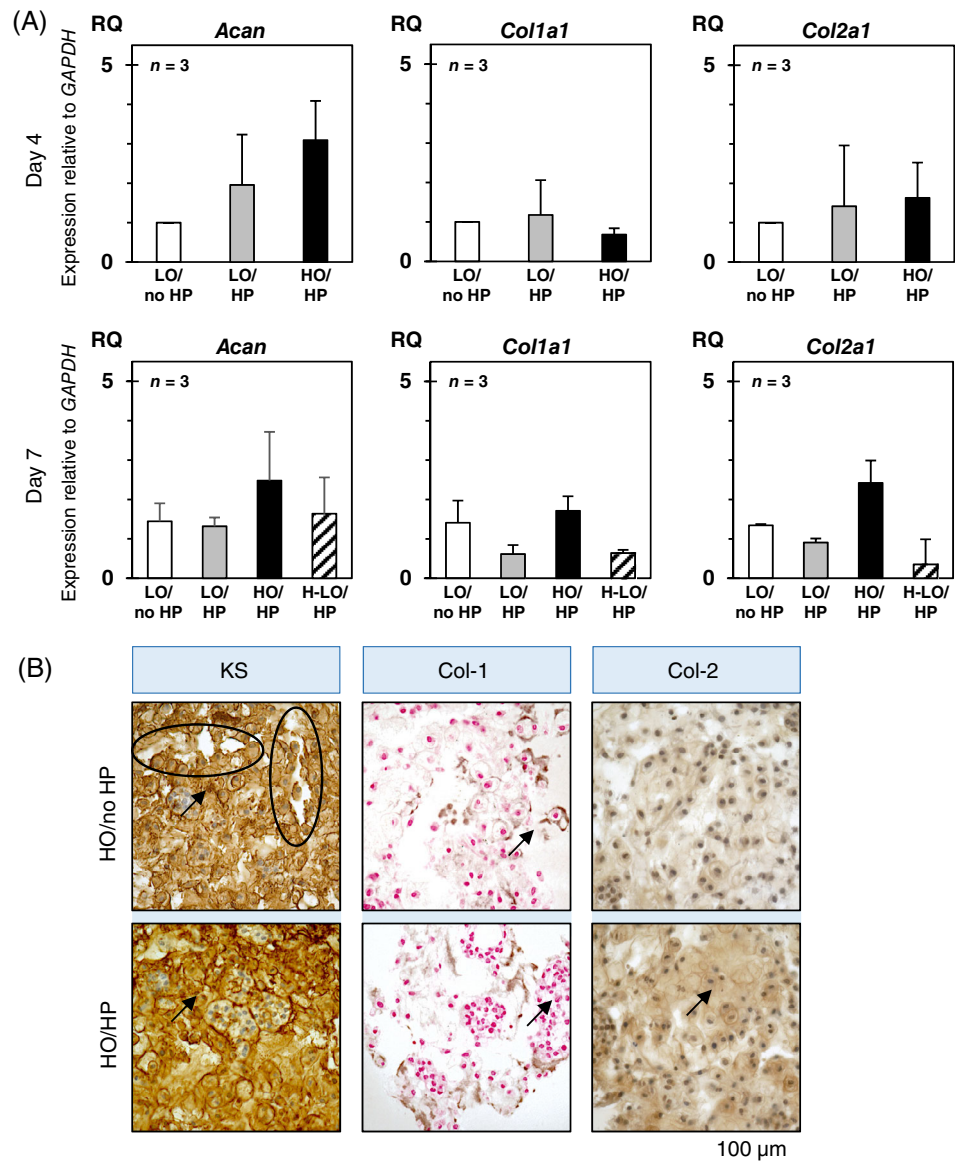
To evaluate the performance of the semipermeable membrane pouch, we fixed NP cells/clusters including their pouch. NP cells/clusters with newly synthesized metachromatic ECM and semipermeable membrane were seen in the same section (Figure 3B). NP cells and ECM were completely separate from the membrane. There was empty space within a pouch, which would be useful for

accumulation of newly synthesized ECM and growth of NP cells. With HO/HP, the NP cells/clusters were in contact with each other, while in LO and HO they were loosely connected or separate at early time points (days 3 and 4).

### 3.3 | The effects of constant HP in experiment A

We sought to identify a regimen of HP and the osmolality of culture medium that effectively upregulated gene expression of ECM molecules and achieved dense accumulation of the ECM consistently around the bNP cells. Based on our hypothesis, we particularly focused on the absence of empty gaps within accumulated newly synthesized ECM. We incubated the pouches with LO/no HP, LO/HP, and HO/HP for the first 4 days in experiment A (Figure 4A). With LO/HP *Acan* showed a trend towards upregulation compared to LO/no HP control at day 4. With HO/HP *Acan*

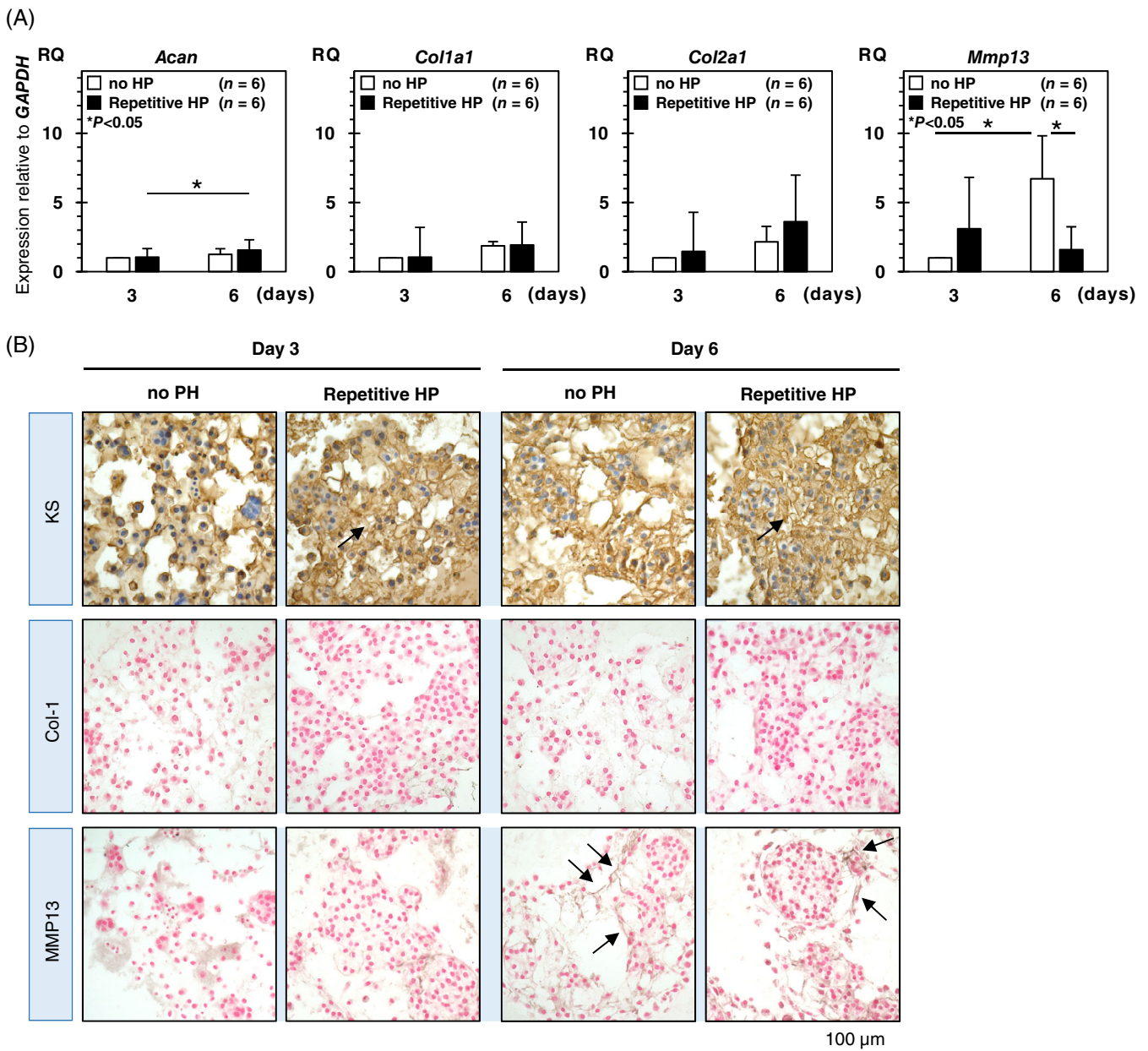
**FIGURE 4** Gene expression profiles and immunohistology of nucleus pulposus (NP) cells/clusters in response to changes in cyclic hydrostatic pressure (HP) followed by constant HP in high (450 mOsm/kg H<sub>2</sub>O) or low (320 mOsm/kg H<sub>2</sub>O) osmolality. A, The expression of aggrecan core protein (*Acan*), collagen type-I (*Col1a1*), and collagen type-II (*Col2a1*). RQ, relative quantity. Mean ± SD (*n* = 3). B, The accumulation of keratan sulfate (KS) in brown and counterstained with hematoxylin. Arrows indicate intense accumulation of KS and circles indicate empty spaces. C, The accumulation of collagen type-I (Col-1) in black and counterstained with Contrast Red. D, The accumulation of collagen type-II (Col-2) in brown and counterstained with hematoxylin. Arrows indicate intense accumulation of Col-2 and circles indicate empty spaces. Each section is 7 μm thick and a bar indicates 100 μm



showed threefold upregulation compared to LO/no HP control. With LO/HP and HO/HP *Col1a1* showed levels similar to the control at day 4 (Figure 4A). As would be seen in the typical phenotype for ECM of NP, *Col2a1* with LO/HP and HO/HP was expressed at similar level, 1.5- and 1.8-fold, compared to LO/no HP control at day 4, respectively (Figure 4A).

After 4-day loading with cyclic HP in LO or HO, we incubated the pouches with constant HP for three additional days (total 7 days) in the same osmolality, or we incubated pouches with constant HP for

three additional days in LO (having been switched from HO). At day 7, with LO/HP and H-LO/HP, *Acan* expression was similar to that in LO/no HP control (Figure 4B). With HO/HP, *Acan* showed twofold upregulation compared to LO/no HP (control) and LO/HP. Immunohistological staining with KS revealed that bNP cells/clusters accumulated denser KS as a specific molecule of aggrecan without gaps with HO/HP compared to HO/no HP at day 7 (Figure 4C). On the other hand, empty gaps were seen within accumulated KS with LO/no HP. *Col1a1* expression levels with HO/HP were similar to



**FIGURE 5** Gene expression profiles and immunohistology of nucleus pulposus (NP) cells/clusters in response to two rounds of repetitive changes in cyclic hydrostatic pressure (HP) applied for 2 days followed by constant HP for 1 day in high osmolality (450 mOsm/kg H<sub>2</sub>O). A, RQ of the expression of aggrecan core protein (*Acan*), collagen type-I (*Col1a1*), and collagen type-II (*Col2a1*) and matrix metalloproteinase-13 (*Mmp13*). RQ: relative quantity. Mean  $\pm$  SD ( $n = 6$ ). \*:  $P < .05$ . B, The accumulation of keratan sulfate (KS) in brown and counterstained with hematoxylin. Arrows indicate intense accumulation of KS and circles indicate empty spaces. The accumulation of collagen type-1 (Col-1) and metalloproteinase-13 (MMP-13) in black and counterstained with Contrast Red. Arrows indicate intense accumulation of KS and MMP-13. Each section is 7  $\mu$ m thick and a bar indicates 100  $\mu$ m

those in the LO/no HP control, but *Col1a1* with LO/HP and H-LO/HP had approximately one-half of the control levels. Col-1 accumulation was seen around cells, but no distinctive difference was seen between HO/HP and LO/no HP control (Figure 4C). *Col2a1* expression with HO/HP was approximately twofold greater than LO/no HP control, but with LO/HP and H-LO/HP downregulated. Col-2 accumulation was seen among cells and was denser with HO/HP than with LO/no HP control (Figure 4C).

### 3.4 | The effects of repetitive HP in experiment B

We tested repetition of a regimen of cyclic HP followed by constant HP, since we found in experiment A that the regimen had the potential to form dense ECM without gaps. We compared repetitive regimens of 2-day cyclic HP followed by 1-day constant HP (repetitive HP) and no HP at 3 days (once) and 6 days (twice). *Acan* expression was significantly upregulated under repetitive HO/HP at day 6 compared to day 3 ( $P < .05$ , Figure 5A). Immunohistological staining with KS was used to evaluate the consistency of *Acan* expression histologically, revealing denser ECM accumulation without empty gaps with repetitive HO/HP compared to no HP control at day 6 (Figure 5B). Expression of *Col1a1* with repetitive HO/HP twice showed a similar level of HO/no HP control. This similarity between the two regimens was confirmed immunohistologically with Col-1 staining (Figure 5B). *Col2a1* expression with repetitive HO/HP tended to be higher compared to HO/no HP control at day 6 (Figure 5A). *Mmp13* expression significantly increased under HO/no HP from days 3 to 6 ( $P < 0.01$ ), while no significant difference was found between under repetitive HO/HP and HO/no HP at day 3. At day 6, expression of *Mmp13* was significantly downregulated with repetitive HO/HP compared with HO/no HP control ( $P < .05$ ). Immunohistological staining for MMP-13 revealed slight localization around cells with repetitive HO/HP and in the HO/no HP control (Figure 5B).

## 4 | DISCUSSION

We have developed a novel cell culture method using a semipermeable membrane pouch device and a pressure/perfusion cell culture system for incubating NP cells/clusters. We evaluated the performance of these culture methods for simulating NP under diurnal spinal motion.<sup>4</sup> In addition, we assessed their consistency in producing major phenotypic gene expression markers of NP ECM and associated histological characteristics of newly accumulated ECM.

### 4.1 | Performance of the semipermeable membrane pouch

We used a semipermeable membrane pouch to enclose NP cells/clusters. Though we had previously successfully incubated

chondrocytes embedded in collagen gel within these pouches,<sup>16</sup> we here demonstrated the superior performance of the pouches using NP cells/clusters without extrinsic material (Figure 3B). The bNP cells/clusters showed excellent viability, and both nondigested native ECM and newly synthesized ECM were retained by NP cells within the pouch. We believe that the semipermeable membrane's cut-off size of 500 kD was useful in retaining ECM and NP cells/clusters suspended within the pouch and gradually accumulated newly synthesized ECM without disturbance by fluid motion. Since dynamic fluid flow was absent, the periphery of the NP cells/clusters took on a heterogeneous cellular and ECM surface. On the inner surface of the pouch, no NP cells adhered to the membrane (Figure 3B). Thus, all NP cells/clusters were self-reconstituting NP tissues model. The cut-off size (500 kD) of the semipermeable membrane used in this study was much higher than the one used previously for semiconfined explant culture (15 kD).<sup>18,19</sup> We believe that 500 kD was appropriate to retain the majority of ECM around the NP cells/clusters while allowing efficient nutrient supply and debris elimination.

### 4.2 | Cell viability

A number of small particles were stained red (denoting dead cells) with live/dead viability assay dyes (Figure 3A). These particles were probably DNA fragments that had been eliminated from dead cells. Though counting dead cells is likely to yield an overcount, live cells illuminated in green can be counted as true live cells, because a viable bNP cell needs to convert calcein-AM into green fluorescent calcein within a cell.<sup>20</sup> Maturing cells stained with KS antibody accumulated dense matrix, which may prevent infiltration of the dyes through the matrix and cell membrane. In extending culture times, we need to ensure the efficiency of penetration of such dyes, given the prevention of their movement by newly accumulated ECM.

### 4.3 | Cell density

Monolayer cultures of NP cells and 3D encapsulated cultures within biomaterial (eg, calcium-alginate) have often been used to create NP cell culture models.<sup>21,22</sup> However, the former requires multiple passages for maintaining live cells and removing debris including newly synthesized ECM, and the latter requires embedding of the cells within extrinsic matrix, increasing the risk of altering native characteristics. Additionally, there are concerns regarding the limitation of those types of systems in maintaining NP phenotypes for periods ranging from days to weeks. In our model, we seeded NP cells/clusters with native ECM (nondigested) and suspended them within a semipermeable membrane pouch. Throughout the culture process, the pouch allowed seeded NP cells/clusters to accumulate newly synthesized ECM around each cell and expand into the available space.

Cell density in native NP tissue is very low, and fluid and sulfated glycosaminoglycan are abundant. In practical terms, extraction

of total RNA from native NP tissue requires large amounts of tissue because of low RNA yield and fluid interference. Thus, the high cell density of the bNP tissue model provides an efficient means of gene expression analysis.

#### 4.4 | The effects of a regimen of constant HP

We successfully reduced gap formation within newly accumulated ECM with a regimen of cyclic followed by constant HP, which we believe caused entanglement of fibrous ECM, for example, collagen type-I. Recently, we demonstrated that cyclic HP and constant HP inhibited free swelling of NP explants (manuscript under review). Furthermore, in the human body, intradiscal pressure in a recumbent position has been measured as 0.1 to 0.3 MPa.<sup>6,23</sup> Thus, we shall incorporate changes in intradiscal pressure across time including minimal HP (>0.1 MPa) with any spinal motion and apply those physiological factors to our NP cells/clusters model *in vitro*.<sup>23-25</sup>

#### 4.5 | The effects of a repetitive regimen of cyclic HP followed by constant HP

Diurnal spinal motion encompasses constant and cyclic stresses as well as the absence of stress. Thus, studies of the effects of changes in physicochemical stresses should include a regimen of descending HP or post-HP loading.<sup>26</sup> We demonstrated that anabolic molecules in bNP cells were upregulated at day 3 even after 4-day HP loading (Figure 4A).<sup>13</sup> Since the transduction mechanisms of on- and off-loading remain unclear, we will explore those mechanical switching mechanisms by HP in future studies.

Another important aspect of diurnal spinal motion concerns repetitive HP loading.<sup>27</sup> We demonstrated that NP cells/clusters showed significantly upregulated anabolic turnover compared with the no-HP control at day 6 after a regimen of cyclic HP followed by constant HP, repeated twice. We believe that regeneration, degeneration, and homeostasis in NP tissue take days and require repetitive stresses. Such repetition may allow NP cells to adapt to changes in physicochemical stresses. In longer duration stress-loading experiments, newly synthesized ECM will accumulate and create new changes in conditions via negatively charged glycosaminoglycan, for example, changes in osmotic pressure.<sup>28</sup>

Experimental approaches exploring changes in physicochemical stresses should incorporate a conditioning period prior to loading stresses. This conditioning period becomes particularly important when new experimental apparatus is used as well as when a novel experiment is begun. The many steps involved in the NP cells/clusters model, with the greater mechanical stress of handling cells, increase the risk of interference with cellular behavior.<sup>29</sup> Thus, it is necessary to plan sufficient preincubation time or repetitive regimens in order to obtain reproducible results.

Related to the concept of repetitiveness, intermittent HP loading (loading followed by off-loading), for example, 1-hour loading followed

by 23-hour off-loading,<sup>30</sup> has often been used to simulate the effects of HP or compressive stresses on *in vitro* cell cultures or explants. Our study, however, suggests that continuous HP loading (repeating cyclic followed by constant HP) is required. This contradictory suggestion may be related to another important mechanical factor, confinement.<sup>23,31</sup> For example, during off-loading, the NP absorbs fluid, generating swelling pressure. In explant models, swelling pressure likely acts to counterbalance HP. Therefore, intermittent HP loading in a whole-IVD-segment model is technically similar to our model.<sup>23,26</sup>

#### 4.6 | Pure HP vs compressive loading

Most studies of compressive stresses, which include HP and deviatoric stress, have used whole vertebral segments (including the NP, annulus fibrosus, and endplates) and a precisely controlled culture system.<sup>32-36</sup> The vertebral segment has the advantage of reproducing dynamic compressive axial loading, but the disadvantage of undergoing nonrecoverable tissue deformation in a matter of days. Thus, this model has limitations in investigating anabolic and regenerative turnover which may take longer duration. In contrast, our present model can be used for investigating the effects of pure HP on anabolic turnover in NP tissue model. In future studies, we will add deviatoric stress to our pouch system and simultaneously apply pure HP.

#### 4.7 | Limitations

In this study, we focused on metabolic turnover in our NP tissue model under cyclic and constant HP, revealing the regenerative/degenerative capability of NP tissue exposed to intradiscal pressure typical of diurnal spinal movement. However, this model does not encompass all IVD tissue units and all physicochemical factors. Our approach will provide a highly useful platform technology in future investigations of the effects of specific molecules and stresses. NP cells/clusters accumulated newly synthesized ECM components within a semipermeable membrane pouch. Since this accumulated ECM was amorphous, we did not anticipate the effects of deviatoric stress on the cells/clusters. We now understand that the NP cells/clusters form denser NP cell matrix for the extended duration of incubation. Thus, the effects of deviatoric stress or strain on the NP cells and the accumulated ECM will be investigated in future studies.

## 5 | CONCLUSIONS

We evaluated metabolic turnover in isolated NP cells/clusters suspended in a semipermeable membrane pouch under cyclic and constant HP in high-osmolarity medium using a HP/perfusion culture system. Previously we demonstrated that bNP cells/clusters showed upregulation of gene expression of ECM molecules with cyclic HP in high-osmolality medium. However, newly accumulated ECM showed gaps within the matrix. To solve this inconsistency between gene



expression and histological features, we incorporated constant HP into a regimen of repeated cyclic HP instead of no HP between cyclic HP, mimicking physiological intradiscal pressure. With the repetitive regimens of cyclic HP followed by constant HP in high-osmolality medium, bNP cells/clusters upregulated gene expression of ECM molecules and accumulated dense ECM without gaps. Thus, this NP tissue model based on cells/clusters is capable of reproducing metabolic turnover in response to regimens with combined cyclic and constant HP generated by spinal movement.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

Yoshiki Takeoka, James D. Kang, and Shuichi Mizuno designed the experiments and analyzed the data including qPCR, statistical analysis, and immunohistology. All authors participated in preparation of the manuscript for submission. All authors contributed the writing of the manuscript and the designing studies reported. Yoshiki Takeoka and Shuichi Mizuno conducted studies and evaluated all results.

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## REFERENCES

1. Saal JA, Saal JS. Nonoperative treatment of herniated lumbar intervertebral disc with radiculopathy. An outcome study. *Spine*. 1989;14(4):431-437.
2. Ghiselli G, Wang JC, Bhatia NN, Hsu WK, Dawson EG. Adjacent segment degeneration in the lumbar spine. *J Bone Joint Surg Am*. 2004;86(7):1497-1503.
3. Urban JPG, Smith S, Fairbank JC. Nutrition of the intervertebral disc. *Spine*. 2004;29:2700-2709.
4. Ishihara H, Warensjo K, Roberts S, Urban JP. Proteoglycan synthesis in the intervertebral disk nucleus: the role of extracellular osmolality. *Am J Physiol*. 1997;272:C1499-C1506.
5. Urban JP, Maroudas A, Bayliss MT, Dillon J. Swelling pressures of proteoglycans at the concentrations found in cartilaginous tissues. *Biorheology*. 1979;16:447-464.
6. Wilke HJ, Neef P, Caimi M, Hoogland T, Claes LE. New in vivo measurements of pressures in the intervertebral disc in daily life. *Spine*. 1999;24:755-762.
7. Bezci SE, Nandy A, O'Connell GD. Effects of hydration on healthy intervertebral disk mechanical stiffness. *J Biomech Eng*. 2015;137:101007.
8. Bezci SE, O'Connell GD. Osmotic pressure alters time-dependent recovery behavior of the intervertebral disc. *Spine J*. 2018;43:E334-E340.
9. Schroeder Y, Sivan S, Wilson W, et al. Are disc pressure, stress, and osmolality affected by intra- and extracellular fluid exchange? *J Orthop Res*. 2007;25:1317-1324.
10. Ziv I, Moskowitz RW, Krause I, Adler JH, Maroudas A. Physicochemical properties of the aging and diabetic sand rat intervertebral disc. *J Orthop Res*. 1992;10:205-210.
11. Heneghan P, Riches PE. The strain-dependent osmotic pressure and stiffness of the bovine nucleus pulposus apportioned into ionic and non-ionic contributors. *J Biomech*. 2008;41:2411-2416.
12. O'Connell GD, Vresilovic EJ, Elliott DM. Human intervertebral disc internal strain in compression: the effect of disc region, loading position, and degeneration. *J Orthop Res*. 2011;29:547-555.
13. Mizuno S, Kashiwa K, Kang JD. Molecular and histological characteristics of bovine caudal nucleus pulposus clusters by combined changes in hydrostatic and osmotic pressures *in vitro*. *J Orthop Res*. 2019;37:466-476.
14. Maldonado BA, Oegema TR. Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. *J Orthop Res*. 1992;10:677-690.
15. Lee JT, Cheung KM, Leung VY. Systematic study of cell isolation from bovine nucleus pulposus: improving cell yield and experiment reliability. *J Orthop Res*. 2015;33:1743-1755.
16. Brown S, Matta A, Erwin M, et al. Cell clusters are indicative of stem cell activity in the degenerate intervertebral disc: can their properties be manipulated to improve intrinsic repair of the disc? *Stem Cells Dev*. 2018;27:147-165.
17. Mizuno S, Ogawa R. Using changes in hydrostatic and osmotic pressure to manipulate metabolic function in chondrocytes. *Am J Physiol Cell Physiol*. 2011;300:C1234-C1245.
18. van Dijk B, Potier E, Ito K. Culturing bovine nucleus pulposus explants by balancing medium osmolality. *Tissue Eng Part C Methods*. 2011;17:1089-1096.
19. van Dijk BGM, Potier E, Ito K. Long-term culture of bovine nucleus pulposus explants in a native environment. *Spine J*. 2013;13:454-463.
20. Papadopoulos NG, Dedoussis GV, Spanakos G, Gritzapis AD, Baxevanis CN, Papamichail M. An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytotoxicity. *J Immunol Methods*. 1994;177:101-111.
21. Rosenzweig DH, Tremblay Gravel J, Bisson D, Ouellet JA, Weber MH, Haglund L. Comparative analysis in continuous expansion of bovine and human primary nucleus pulposus cells for tissue repair applications. *Eur Cell Mater*. 2017;33:240-251.
22. Wuertz K, Urban JPG, Klasen J, et al. Influence of extracellular osmolality and mechanical stimulation on gene expression of intervertebral disc cells. *J Orthop Res*. 2007;25:1513-1522.
23. Walter BA, Korecki CL, Purmessur D, Roughley PJ, Michalek AJ, Iatridis JC. Complex loading affects intervertebral disc mechanics and biology. *Osteoarthritis Cartilage*. 2011;19:1011-1018.
24. Chan SCW, Walser J, Ferguson SJ, Gantenbein B. Duration-dependent influence of dynamic torsion on the intervertebral disc: an intact disc organ culture study. *Eur Spine J*. 2015;24:2402-2410.
25. Lee CR, Iatridis JC, Poveda L, Alini M. *In vitro* organ culture of the bovine intervertebral disc. *Spine*. 2006;31:515-522.
26. Schmidt H, Shirazi-Adl A, Schilling C, Dreischarf M. Preload substantially influences the intervertebral disc stiffness in loading-unloading cycles of compression. *J Biomech*. 2016;49:1926-1932.
27. Shah BS, Chahine NO. Dynamic hydrostatic pressure regulates nucleus pulposus phenotypic expression and metabolism in a cell density-dependent manner. *J Biomech Eng*. 2018;140:021003.
28. Holm S, Maroudas A, Urban JP, Selstam G, Nachemson A. Nutrition of the intervertebral disc: solute transport and metabolism. *Connect Tissue Res*. 1981;8:101-119.
29. Neidlinger-Wilke C, Mietsch A, Rinkler C, Wilke HJ, Ignatius A, Urban J. Interactions of environmental conditions and mechanical loads have influence on matrix turnover by nucleus pulposus cells. *J Orthop Res*. 2012;30:112-121.

30. Chan SC, Ferguson SJ, Wuertz K, Gantenbein-Ritter B. Biological response of the intervertebral disc to repetitive short-term cyclic torsion. *Spine*. 2011;36:2021-2030.
31. Arkesteijn IT, Mouser VH, Mwale F, van Dijk BG, Ito K. A well-controlled nucleus pulposus tissue culture system with injection port for evaluating regenerative therapies. *Ann Biomed Eng*. 2016;44:1798-17807.
32. Rosenzweig DH, Gwawri R, Moir J, et al. Dynamic loading, matrix maintenance and cell injection therapy of human intervertebral discs cultured in a bioreactor. *Eur Cell Mater*. 2016;31:26-39.
33. Gantenbein B, Grunhagen T, Lee CR, van Donkelaar CC, Alini M, Ito K. An in vitro organ culturing system for intervertebral disc explants with vertebral endplates: a feasibility study with ovine caudal discs. *Spine*. 2006;31:2665-2673.
34. Chan SCW, Walser J, Kappeli P, Shamsollahi MJ, Ferguson SJ, Gantenbein-Ritter B. Region specific response of intervertebral disc cells to complex dynamic loading: an organ culture study using a dynamic torsion-compression bioreactor. *PLoS One*. 2013;8:e72489.
35. Roberts S, Menage J, Sivan S, Urban JP. Bovine explant model of degeneration of the intervertebral disc. *BMC Musculoskelet Disord*. 2008;9:24.
36. Hartman RA, Bell KM, Debski RE, Kang JD, Sowa GA. Novel ex-vivo mechanobiological intervertebral disc culture system. *J Biochem*. 2012;45:382-385.

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