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Microfluidic Disc-on-a-Chip Device for Mouse Intervertebral Disc —Pitching a Next-Generation Research Platform To Study Disc Degeneration

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Abstract

Low back pain is the most common cause of disability worldwide, and intervertebral disc degeneration is a major cause of low back pain. Unfortunately, discogenic low back pain is often treated with symptomatic relief interventions, as no disease-modifying medications are yet available. Both to-be-deciphered disc biology/pathology and inadequate in vitro research platform are major hurdles limiting drug discovery progress for disc degeneration. Here, we developed a microfluidic disc-on-a-chip device tailored for mouse disc organ as an in vitro research platform.

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Notes

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We hypothesize that continuous nutrients empowered by a microfluidic device would improve biological performance of cultured mouse discs compared to those in static condition. This device permitted continuous media flow to mimic *in vivo* disc microenvironment. Intriguingly, mouse discs cultured on the microfluidic device exhibited much higher cell viability, better preserved structure integrity and anabolic-catabolic metabolism in both nucleus pulposus and annulus fibrosus, for up to 21 days compared to those in static culture. This first “disc-on-a-chip” device lays groundwork for future preclinical studies in a relative long-term organ culture given the chronic nature of intervertebral disc degeneration. In addition, this platform is readily transformable into a streamlined *in vitro* research platform to recapitulate physiological and pathophysiological microenvironment to accelerate disc research.

Graphical Abstract

Keywords

low back pain; intervertebral disc degeneration; organ-on-a-chip; microfluidic; organ culture

INTRODUCTION

The most recent *Global Burden of Disease* identified back pain as the most common cause of disability worldwide.¹ Low back pain is the most common health problem in individuals between ages of 20 and 50 with a prevalence of 70–85% and an estimated annual cost of \$100 billion in the U.S.² Although etiology of low back pain has not been fully elucidated, intervertebral disc (IVD) degeneration plays a major role.³ Currently, discogenic low back pain is often treated with symptomatic interventions, such as epidural steroid injection, which do not adequately provide improved outcomes, as no disease-modifying medication are currently available.⁴

Our ability to effectively treat or retard disc degeneration is largely hampered by incomplete understanding of biological and pathological processes regulating disc development, function and disease. And thus, deciphering disc homeostasis and underlying mechanisms that drive the progression of disc disease appear as urgent tasks to tackle degenerative disc disease. However, it has been very challenging to investigate and interpret those processes within an *in vivo* animal model where mounting factors might be contributing to variation of experimental outcomes. Moreover, disc degeneration is a rather complex and multifaceted course, involving biochemical, mechanical, and genetic factors,^{4–8} as well as interplay of those factors. For this reason, currently reported animal models may not accurately recapitulate such a complex *in vivo* microenvironment.^{9–11} This may likely explain why

some preclinical studies fail in clinical trials. Therefore, researchers have developed various organ culture platforms, as powerful in vitro alternatives to define and manipulate variables using primary disc cells and various supporting structures.

In the past decade, several generations of bioreactors have been developed to culture discs from large animals and human. Unfortunately, most of these organ culture systems are static in media supply and suitable only for large discs with costly consumables and supplies.^{12–15} Furthermore, so far no in vitro organ culture model recapitulates in vivo continuous nutrient supply and all ranges of mechanical loading, not to mention genetic cues, which are critical factors for maintaining disc health. We therefore strive to build an in vitro mouse disc organ culture device with inherent and easy-to-implement capabilities as a new in vitro research platform to recapitulate various interweaving factors contributing to disc health.

Microfluidic devices have been proven to be advantageous over their conventional counterparts, such as large scale bioreactors, in a variety of aspects.^{16–23} The miniaturization, automation, and integration of fluidic control systems allow small amount reagent consumption, low cost, short turnaround time, and high throughput.^{23–28} In recent years, microfluidic organ-on-a-chip has emerged as a novel research platform to address a wide range of biomedical questions and for drug screening. For example, a well explored “liver-on-a-chip” platform is currently seeking its full potential from basic science research in drug screening to U.S. FDA testing of food safety.^{29,30} More importantly, miniaturized size of device and established fabrication process of microfluidic technology permit functionally driven optimization, further resonating with our conceptualization to develop a mouse disc culture device, referred as “disc-on-a-chip”. Furthermore, most developed microfluidic-chip assays were designed to test acute responses (typically a few hours or a couple of days) in 2D cells or 3D cell mixtures, exemplified as toxicity assays using the liver-on-a-chip platform, due to the nature of fast progression of pathological insult itself.^{31,32} In contrast, IVD degeneration is a chronic disease that progresses over months or even years in patients. To provide an in vitro research platform eventually unmasking disc biology/pathology and discovering new therapeutics in a long-term culture, we positioned our foremost strategy as to simulate in vivo microenvironment and to optimize disc organ culture in a time frame of several weeks. Here, we report a novel microfluidic disc-on-a-chip device ingrain continuous nutrient supply and refreshment as a unique approach to prolong in vitro health span of mouse disc at gene, protein, and structural levels, substantiating future investigations on disc metabolism.

MATERIALS AND METHODS

Fabrication of a Novel Microfluidic Device for Mouse Disc Organ Culture.

The disc-on-a-chip shown in Figure 1a, b was designed based on organ perfusion principle using AutoCAD (2D) and SolidWorks (3D) as previously described.^{33–36} Each device consists of four identical three-layer perfusion units. The top layer is a microchannel serving as flow inlet feeding fluid into perfusion chamber and an outlet for perfusates. The dimensions of the channel are 2 mm (width) × 500 μm (height). The middle layer is a circular perfusion chamber (7 mm in diameter and 3 mm in depth). The bottom layer consists of three tiny circular wells, 500 μm apart from each other, designed on the

dimensions of lumbar IVDs from 8–10 week old mice (about 2 mm in diameter and 1 mm in depth) factoring free swelling (Figure S1), which helps to gently immobilize IVDs without shielding them from the perfusion fluids while allowing adequate flow exposure of the IVD surface area. Mouse discs were loaded into perfusion chambers through the loading port from the top, which was then sealed to form a closed system.

Previous studies have demonstrated that shear stress is an important factor contributing to IVD degeneration.^{37–39} In microfluidic applications, where Reynolds numbers are relatively low, pressure difference P (kg/m s²) between two points along the fluid flow can be calculated from

$$\Delta P = QR \quad (1)$$

where Q (m³/s) is volumetric flow rate, and R (kg/m⁴ s) is a constant called fluid resistance. Previously, Fuerstman et al. found that the laminar flow of a liquid phase through rectangular channel approximately follows

$$\Delta P = \frac{a\mu QL}{WH^3} \quad (2)$$

and

$$a = 12 \left[1 - \frac{192H}{\pi^5 W} \tanh\left(\frac{\pi W}{2H}\right) \right]^{-1} \quad (3)$$

where μ is the viscosity of the liquid (kg/m s), L (m) is the length, W (m) is the width, and H (m) is the height of the channel.⁴⁰ Furthermore, using microfluidic channels with integrated micropillars, Gunda et al. measured the pressure drop of fluid flow through different alignments of microstructures and demonstrated that micropillars arranged in a squared pattern offered higher resistance to fluid flow when compared to their staggered counterparts.⁴¹ To reduce shear stress induced by flow in our device, a square-patterned micropillar array structure was designed near the inlet port to facilitate the pressure drop before flow enters into perfusion chamber. On the basis of established mathematic models, a computer simulation using COMSOL Multiphysics showed that the inserted micropillar array successfully reduced the pressure near perfusion chamber from 40 to 10% of original pressure applied near the inlet (Figure 1c).

It is imperative to have adequate flow exchange in the perfusion chamber, which ultimately depends on the dimension of perfusion chamber. Our computer simulation results indicated that, at current device dimensions, flow streams were evenly distributed in perfusion chamber and most of the solution flowed into the chamber before exiting the device (Figure 1d). Furthermore, glucose simulation demonstrated sufficient exchange of glucose molecules in the perfusion chamber (Video S1).

Mouse IVD Isolation and Organ Culture.

The animal protocol was approved by the Institutional Animal Care and Use Committee at University of Virginia. C57BL/6J mice (male and female, 8–10 weeks, Envigo, $n = 36$) were

ethanized in a carbon dioxide chamber. Lumbar spines were isolated aseptically under a dissection microscope following our published protocols.^{42,43} In brief, lumbar discs were dissected free from connective tissues. Bony end plate was meticulously excised from discs. And then discs were bathed in PBS and Dulbecco's modified Eagle's medium-F12 (DMEM-F12) containing 2% penicillin-strepto-mycin sequentially. Dissected discs were randomly divided into three groups: (1) freshly isolated native discs as control group, (2) on-chip culture group in which discs were cultured on microfluidic chip at a flow rate of 3.5 or 10 $\mu\text{L}/\text{min}$, and (3) static culture group in which discs was cultured in a 12-well plate. The complete medium for culture groups was DMEM-F12, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. For static culture, media was changed every 3 days. Both on-chip and static cultures were maintained in an incubator at 37 °C with 5% CO₂ for 10 and 21 days.

Alcian Blue and Picrosirius Red Staining.

Discs were fixed in 4% paraformaldehyde (PFA) for 12 h, embedded in paraffin, and sectioned in 5 μ m thickness. Tissue sections were stained with 1% alcian blue (in 3% acetic acid, pH2.5, Electron Microscopy Sciences, Hatfield, PA) and 0.1% picosirius red (in saturated picric acid, Electron Microscopy Sciences, Hatfield, PA) following our established procedure.¹¹ Images were taken with a Nikon Eclipse 600 Microscope (Nikon, Japan). Tissue sections for histological analysis were randomly selected.

Live/Dead Assay.

After culture, intact discs were stained with a Live/Dead Reduced Biohazard Viability/Cytotoxicity Kit (Thermo Fisher Scientific, Waltham, MA) for 2 h at room temperature. After fixed in 4% PFA for 30 min, samples were immersed sequentially in 15 and 30% sucrose for 1 day, embedded in O.C.T. Compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan), cryo-sectioned (5 μ m thickness), and mounted with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). Tissue sections for live/dead assay were randomly selected.

Cell viability was assessed with a Nikon Eclipse E600 microscope and a Nikon DS-Ri2 camera at a magnification of 200.^{44,45} Green and red fluorescent signals represented live and dead cells, respectively. Annulus fibrosus (AF) and nucleus pulposus (NP) regions were analyzed separately. Within AF region of each disc, 3–4 fixed regions of interests (ROI) were selected randomly to encompass both anterior and posterior of disc tissue. The entire NP region for each section was regarded as one ROI. Within each ROI, total numbers of red (dead) and green (live) cells were counted. For each disc sample, three adjacent sections were examined. Mean percentage of live cells over total cells was obtained per AF or NP regions per group.

DNA Assay.

Measurement and quantification of DNA content were performed as previously described.⁴⁴ In brief, disc samples were digested with papain (125 $\mu\text{g}/\text{mL}$ in $1\times$ PBE buffer, pH 6.5) and diluted in Tris-EDTA buffer. DNA content was measured with a Hoechst 33258 dye (Sigma-

Aldrich, St. Louis, MO). A calf thymus DNA was used as a standard. The numerical value was read with a DyNA Quant 200 Fluorometer (GE Healthcare, Chicago, IL).

Glycosaminoglycan (GAG) and Hydroxyproline (Hypro) Assays.

Since proteoglycan and collagen are major extracellular matrices in IVD, we measured sulfated glycosaminoglycan (GAG) to represent proteoglycan and hydroxyproline to determine collagen contents.⁶ In brief, the discs were digested with papain solution at 60 °C for 24 h. The amount of GAG was determined by a dimethylmethylen blue (DMMB) colorimetric assay using Chondroitin Sulfate as a standard. For hydroxyproline measurement, an aliquot of papain digestions was hydrolyzed in 6 N HCl for 16 h at 110 °C. Hydroxyproline was determined with a dimethylaminobenzaldehyde (DMBA)/chloramine T (Sigma-Aldrich, St. Louis, MO) colorimetric assay using hydroxyproline as a standard. Optical density (O.D.) was obtained with Spectramax ABS plus (Molecular Devices, San Jose, CA) at 560 nm.

Real-Time Reverse Transcription Polymerase Chain Reaction.

Total RNA was extracted from discs using TRIzol reagent (Invitrogen, Carlsbad, CA). One μg of RNA was reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), and real-time PCR was performed on a QuantStudio 3 (ABI Applied Biosystems, Foster City, CA). The primer sequences were listed in Table S1. The relative mRNA levels of target genes were calculated by the comparative CT method (also known as the 2^{-CT} method) using 18S as an internal control.

Detection of Denatured Collagen with a Collagen Hybridizing Peptide (CHP).

To specifically discern the degraded collagens during the disc degeneration process, we utilized a collagen hybridizing peptide (CHP) staining. CHPs, which are a class of peptides with a triple-helical structure comprised of a repeated Gly-X-Y amino acid sequence, have been shown to specifically bind to degraded collagens via triple helix hybridization.⁴⁶ The paraffin IVD sections (5 μm thickness) were stained with FITC-labeled CHP (F-CHP, 3Helix Inc., Salt Lake City, Utah). Five micrometer of F-CHP were freshly diluted in deionized water, heated at 85 °C for at least 5 min and then incubated on ice. Fresh F-CHP probes were applied on mouse IVD sections and incubated at 4 °C overnight in dark. After mounting with mounting media, fluorescent images were acquired using a Nikon Eclipse E-600 microscope.^{47,48} To study autofluorescence, disc sections were incubated with PBS buffer at 4 °C overnight and processed exactly the same as above. Tissue sections for CHP imaging were randomly selected.

To quantify the fluorescence intensity, we analyzed all images using the NIS Elements Software (Nikon, Japan). Images were captured at the same settings under 200 $\times$ magnification. A 100 μm square ROI was created in the NIS Elements software on a given image and mean fluorescence intensity was then recorded. At least three randomly selected areas in AF was analyzed per section, and at least six sections were used per disc to obtain a mean value.

Second Harmonic Generation (SHG) Imaging.

SHG microscopy has been extensively used to visualize fibrillar collagens and their organization with the resolution and detail of standard histology.^{49,50} The SHG imaging system consists of a Zeiss 780 confocal/multiphoton (MP) laser scanning system coupled to the Zeiss inverted epifluorescence microscope and controlled with the Zen software (Carl Zeiss Inc., Thornwood, NY). An ultrafast (150 fs) tunable 10W-pumped pulsed Chameleon laser, operating at 80 MHz and at 900 nm was used for SHG imaging (Coherent Inc., Santa Clara, CA). The laser was coupled to the Zeiss 780 unit to scan the specimen via an XY raster scanning mechanism using galvo mirror. The SHG image was collected at 450/10 nm with a 20 × apochromatic objective lens (NA = 0.8). Using Zen software, 4 × 4 tiling function was used to collect the SHG images of the paraffin processed IVD sections (5 μm thickness). Each element of the tiling SHG image was collected at 1024 × 1024 with 4 frames average. Tissue sections for SHG imaging were randomly selected.

Statistical Analysis.

Prism 6 software (Version 6.0; GraphPad Software Inc., CA, USA) was utilized for statistical analysis. For RT-PCR, DNA and biochemical assays, at least 6 mouse discs were used for each culture condition. For histology analysis, live/dead assay, CHP and SHG imaging, at least 6 tissue sections were used per mouse disc and at least 6 discs were used to represent each group. For all studies, three experimental replicates were performed. Data were reported as mean ± standard error (S.E.M). One-way ANOVA with multiple comparisons were used to analyze statistical differences among groups. For all tests, $p < 0.05$ was considered as statistical significant.

RESULTS

Disc-on-a-Chip Preserved Structural Integrity and Prevented Degeneration.

We first investigated whether our disc-on-a-chip device could sustain disc organ health over a culture period of 10 days. As revealed by alcian blue/picrosirius red staining (Figure 2), compared to well-aligned and tightly organized AF lamellae and high cellularity in the native control discs (top row), static cultured AF tissue showed loose and disorganized lamellae (bottom row, yellow arrows), a typical phenotype of AF degeneration, on day 10 and 21. Native NP showed abundant NP cells (green asterisk) embedded in extracellular matrix (ECM) rich in proteoglycan (blue), representing healthy jelly like NP tissue. Under static culture, NP exhibited a typical degenerative phenotype by exhibiting acellular fibrotic structure (black asterisk) and reduced quantity of NP cells and proteoglycan (black arrow). Similar to native discs, on-chip culture preserved structural integrity, cellularity, and abundant ECM contents in both AF and NP regions. Using histology, we also found disc structures of 21-day culture were similar to those of 10-day culture regarding cellularity, structural integrity and ECM (Figure S2). These histological analyses confirmed our hypothesis that continuous nutritional refreshment was critical to maintain a nurturing microenvironment, which potentially contribute to disc cell viability and cell phenotype.

To elucidate possible effects of flow rates, we tested 10 and 3.5 $\mu\text{L}/\text{min}$ for on-chip culture and demonstrated similar results in histology (Figure S3). For the sake of simplicity, we included flow rate of 10 $\mu\text{L}/\text{min}$ in this initial study.

Disc-on-a-Chip Sustained Disc Cell Viability in Both AF and NP.

Disc cell viability, an important criterion of disc health,^{42,51} was evaluated by a live/dead assay. Cultured discs were harvested on day 10, and live cells were stained with SYTO 10 as color of green and dead cells with red. As expected, on-chip culture significantly improved cell viability in both NP and AF regions, as compared to static culture for up to 10 days, both qualitatively and quantitatively (Figure 3a). Specifically, ~35% of NP and ~20% of AF cells were red in static culture (Figure 3b and 3c) whereas merely no reduction in cell viability (as of percentage of live-to-total cells) of AF (<10% decrease) and NP (no decrease) were detected in disc-on-a-chip culture, which was comparable to the freshly harvested native discs in both AF and NP (n.s. between on-chip and native discs). These promising observations were further corroborated by a DNA assay. As shown in Figure 3d, DNA content in on-chip group ($10.87 \pm 0.71 \mu\text{g}/\text{mL}$) remained at the same level as the native disc group ($10.12 \pm 0.28 \mu\text{g}/\text{mL}$, n.s.), whereas DNA content in the static group showed a significant reduction ($7.62 \pm 1.21 \mu\text{g}/\text{mL}$, $p < 0.05$). Cell viability results demonstrated that the newly developed disc-on-a-chip device held great promise in sustaining a viable disc organ culture by simply yet critically sufficing a continuously refreshment of nutrient in a dynamic micro-environment.

Disc-on-a-Chip Preserved Extracellular Matrix of Mouse Discs.

To evaluate ECM content, we further studied the impact of dynamic disc-on-a-chip culture condition on total content of GAG and collagen with biochemistry assays. Six discs with similar weight were digested in papain per group. As shown in Figure 4a, compared to static culture, GAG content of on-chip culture was comparable to the native control group. Collagen contents were maintained in a similar level among three groups (Figure 4b). In addition, ratio of GAG to Hypro had no apparent differences among three groups (Figure 4c).

Disc-on-a-Chip Decreased mRNA Expression of Catabolic and Inflammatory Genes.

We then studied the expression of catabolic genes to further elucidate impact of dynamic on-chip culture on disc health. As shown in Figure 4d, e, mRNA expressions of MMP13 and ADAMTs4 were significantly increased ($p < 0.05$) in static culture when compared to the native and on-chip cultured discs. After 10 days of culture, similar to freshly harvested native discs, on-chip cultured discs demonstrated basal expression level of both MMP13 and ADAMT4 genes. Because inflammatory markers are typically upregulated during disc degeneration, we further evaluated the expression of inflammatory cytokines. As seen in Figure 4f, g, TNF α and IL-6 was dramatically increased in static group compared to low levels in both native control and on-chip groups ($p < 0.01$). No significant difference was detected in mRNA expression of IL-1 among three groups (Figure 4h). Taken together, our data exemplified a distinct trait that on-chip culture maintained native disc homeostasis by preventing up-regulation of catabolic factors and inflammatory cytokines in mouse IVDs.

Disc-on-a-Chip Prevented Degradation of Collagen.

To detect and assess degenerative changes of collagen in molecular levels, we performed SHG and CHP imaging, because variation in collagen structure might not be accurately reflected by conventional methods. SHG imaging by a two-photon microscope depicted highly organized collagen fibrils of the AF region in both native and on-chip cultured discs, whereas static discs showed disorganized and interweaved fibrils in the AF region (Figure 5a). A similar trend was confirmed by fluorescence labeled F-CHP staining, which specifically detected degraded collagen at a molecular level.⁴⁶ Significantly higher CHP fluorescence intensity was observed in static culture compared to the native discs and on-chip culture after 10 days (Figure 5b, Figure S4). Autofluorescence of disc tissue was negligible (Figure S5). These data revealed strong molecular evidence to support that disc-on-a-chip system effectively protected collagen from degenerative changes under in vitro culture.

DISCUSSION

IVDs lie between adjacent vertebrae in the spinal column. It is connected to the adjacent vertebrae by a thin layer of hyaline cartilaginous end plate, which plays a critical role in maintaining spine stability and motion. Central gelatinous NP is surrounded by concentric collagen fibrils AF. Due to the avascular and aneural nature of IVD, nutrient and metabolite exchange of disc relies upon the cartilage end plate connecting to bone marrow. During aging and degenerative processes, the end plate may undergo calcification, blocking the porous structure and resulting in cell death, altered cell phenotype, and eventually mechanical dysfunction of disc unit.^{52–54} Despite the important functions of discs and significant socioeconomic impact of degenerative disc disease, understanding of disc biology, etiology, and pathology is still elusive. A limited research platform might contribute as a leading impact factor. Thus, in this very first study, we aim at developing an in vitro organ culture platform, specifically tailored for mouse IVD. The genuine significance disc-on-a-chip is to create a physiological simulating disc microenvironment for bridging in vitro IVD culture methodology with in vivo IVD biology/pathology, and ultimately to establish a streamlined drug discovery protocol for new therapeutic and regenerative strategies using an integrated microfluidic mouse IVD culture platform.

In our device, microscale structure such as microchannels and culture chambers allows fine manipulation of media supply with various flow rates and nutritional components through an automated media delivery pattern preprogrammed into the syringe pump. This enables long-term organ culture possible by preventing contamination, which accounts for a critical issue when performs in vitro culture up to weeks or even months. Three-dimensional organ culture retains its original geometry, matrix structure, and cell phenotypes. This has also been successfully demonstrated by Liu et al., who cultured the vertebrae-disc as a spine unit in vitro and applied the model for microCT imaging and finite element modeling.⁵⁵ However, inclusion of bony segments may confound results of nutrient exchange and function in disc and increase chances of contamination in a static culture condition. In terms of dynamic culture platform, several laboratories have created bioreactors for bovine and human discs with varied mechanical loading protocols, which showed promising results.^{56,57}

Nevertheless, these bioreactor systems are suitable only for large dimension of discs, as well as one disc in each unit, and require large amount of consumables with slow turnover. Our disc-on-a-chip culture possesses superior features including high throughput, low consumption, versatile customization and applications to the existing bioreactors. Importantly, the use of mouse IVD as a model system will provide unbeatable opportunities to study genetic factors in disc homeostasis using available resources of transgenic mouse strains.

Recently, Hwang et al. reported a “spine-on-a-chip” device to induce human AF cell degeneration with IL-1 for 72 h.⁵⁸ However, like most of reported microfluidic systems, AF cells were cultured in a monolayer for a short period of time, which does not replicate 3D environment of discs. Additionally, isolated human annulus cells may not maintain an annulus cell phenotype without proper ECM. Indeed, our disc-on-a-chip discs maintained its structural integrity and cellularity to a level comparable to freshly isolated mouse discs as revealed by histological and live/dead assays (Figures 2 and 3). Both native and on-chip discs possessed abundant live cells and proteoglycan in the NP region, and nicely aligned concentric collagen fibrils in the AF region. On the contrary, discs under static culture showed reduced cellularity in both NP and AF regions (Figures 2 and 3) and dramatically loosen collagen fibers in the AF regions (Figure 3). Such phenomena were already dramatic after 10 days of culture, and even worsened on day 21 (Figure S2). These results indicate that dynamic exchanging of culture media and metabolites plays a critical role in maintaining disc homeostasis, and our disc-on-a-chip system sustains disc health for up to 21 days, the longest time tested in this study. To our surprise, flow rate of medium does not affect disc health in this study, at least for two tested flow rates (10 $\mu\text{L}/\text{min}$ vs 3.5 $\mu\text{L}/\text{min}$, Figure S3). Detail investigation of optimal flow rate is undergoing.

Microfluidics device is a viable alternative to traditional in vitro techniques because of its physiological relevance: the laminar flow in microfluidic channels is a good way to mimic flows found in biological systems, like veins and small arteries, where the flow is usually unidirectional and laminar. However, there are still many challenges to design a microfluidics experiment. One of the challenges of microfluidic device is shear force, which is implicated as a potential contributor to degeneration of IVD.^{37–39} To address this problem, we designed a square arranged micropillar array next to the inlet, because shear stress is largely created by the fluid flow injection in microfluidic devices. Due to the high resistance of the micropillar array, flow rate, and hydrodynamic shear stress of single-phase flow through microchannels are significantly limited. In addition, another drawback to microfluidic systems for long-term cell culture is bubble generation and accumulation. In our design, a perfusion chamber on top of IVD culture pockets is used to overcome this issue. Over weeks of disc culture, bubbles form and stay at the top of the chamber because of their buoyancy. Thus, the accumulation of bubbles will not dramatically affect the fluid delivery to IVDs cultured at the bottom of the culture chamber.

Young healthy discs maintain a relatively small number of cells and a fine balance between anabolic and catabolic metabolism. Degeneration occurs as IVD suffers from aging, wear and tear, as well as other pathological triggers. It has been documented that degenerative changes of discs might be caused by deficiency in nutrient supply due to degenerative

changes in structure, cellularity, and cell phenotype.^{52,54} As a matter of fact, as suggested by our gene expression and biochemical analyses, on-chip culture prevented disc degeneration when compared to a static culture. Those discs in static culture expressed the highest level of catabolic genes MMP13 and ADAMTs4, and pro-inflammatory cytokine genes IL-6 and TNF- α . IL-1 is an important inflammatory cytokines involving pathogenesis of disc degeneration. In our study, we observed similar level of IL-1 mRNA among three groups that may be caused by a relatively long-term of 10-day static culture, whereas IL-1 modulation is an early event. Meanwhile, our system sustained high proteoglycan and collagen content to a similar level as freshly isolated discs. Our disc-on-a-chip restored anabolic-catabolic balance to a comparable level of freshly isolated native discs (Figure 4). A previous study suggested that high mechanical strain of primary IVD cells upregulated TLR2, TLR4, NGF and TNF α gene expression, along with elevated protein levels of IL-6, IL-8, IL-15, and TNF α in culture medium.⁵⁹ Upregulation of proinflammatory cytokines in turn elevated genes encoding matrix-degrading enzymes.⁶⁰ Consistent with literatures, our static condition induced catabolic gene expression (Figure 4). while on-chip culture with continuous nutrient refreshment (Figure 6) improved disc health and retarded disc degeneration (Figures 2–5). Therefore, culture condition might possess critical roles in regulating disc microenvironment and subsequently affecting disc health, e.g., cell viability, structure integrity, and matrix properties, which might be associated with expression of inflammatory cytokines by disc cells.

The fact we performed biochemical assays in whole discs may explain why we observed a similar level of collagen content among different groups. Separation of the NP and AF region may amplify the differences. Thus, we further evaluated collagen fibrils at the molecular level via two complementary methods: SHG and CHP staining. Collagen possesses a strong second-order nonlinear susceptibility that can be characterized by SHG with an intense laser beams. Using a combined two-photon excitation and SHG microscopy, we were able to image detailed structural changes in annulus tissue. It turned out that degenerative changes of discs were successfully visualized in static cultured discs, whereas both native and on-chip discs demonstrated typical highly organized collagen fibrils. These data were further corroborated by a fluorescence staining with CHP peptide, a novel and unique peptide that specifically binds to unfolded collagen chains (Figure 5 and Figure S4). The CHP peptide binds to the denatured collagen chains by hybridizing with the Gly-X-Y repeating sequence of native collagen and reforming the triple helical structure. All this evidence supports our hypothesis that dynamic nutrient supply sustained disc health. Our disc-on-a-chip platform not only supplied a continuous and controllable nutrient but also removed metabolic wastes accumulated in the perfusion chamber in a timely fashion, by which a desired fresh culture microenvironment was ensured to maintain disc homeostasis (Figure 6).

Genetic factors are considered to be the greatest contributor to degenerative disc disease.^{42,61–63} Mouse is the most-available animal species allowing genetic background study, with especially abundant readily available resources for genetically manipulated mice, which macroscale bioreactors tailored for human or large animal disc explants cannot offer. Our system can be equipped with the exclusive ability to customize the “chip” compartment to fine-tune “nutrient” microenvironment for diverse culture protocols. In vivo transgenic

mouse study, albeit of its wide application in biomedical research, is a very complex system with intercalated functions and communications between organs/tissues during development. In transgenic mouse models, genes might be knockout or knock-in in the entire body or in a tissue specific manner using a Cre/loxP system. However, as no cell-specific markers are identified for IVD cells so far, disc-specific knockout or knock-in in vivo can be very difficult.⁶⁴ On the contrary, our system could enable studies of interorgan communication in a well-defined manner, such as disc-immune cells, disc-nerve root, which cannot be easily realized using in vivo transgenic animal models. We can evaluate therapeutic effects of one particular protein in a gene deficient disc without confounding factors of in vivo setting. Additionally, future integration of molecular reporters and nanoscale sensors permits real-time and automated monitoring of disc health and function.

It is noteworthy that human discs are much different from experimental animal (quadrupedal) discs from both biomechanical and biochemical standpoints. For instance, sizes of mouse discs (in millimeters) are much smaller than those in humans (in centimeters). Although notochordal cells persist in the nucleus of the disc throughout adult life in the majority of animal species, they decline rapidly in humans after birth and completely disappear in the nucleus by the teenage years. These species-dependent differences shall be taken into consideration when designing future studies to explore mechanical properties and nutrient exchange of disc microenvironment.

CONCLUSIONS

In summary, we developed a microfluidic disc-on-a-chip device specifically for mouse disc organ culture. With continuous supply of nutrition, microfluidic on-chip culture successfully extended in vitro health span of mouse disc with improved cell viability, structure integrity, and cell phenotype. Our data also suggested that subtle changes of organ/cell microenvironment might lead to a cascade of events altering anabolic-catabolic balance and thus affect disc cell viability. For the first time, we demonstrated a microfluidic long-term disc organ culture platform as a new in vitro research platform for understanding disc biology/pathology and promoting therapeutic discoveries. This proof-of-concept microfluidic mouse disc-on-a-chip, once fully integrated and optimized, would not only fill in the technological blank of “disc-on-a-chip” but also shift the paradigm of conventional disc research via delivering a customizable research platform tailored for various downstream applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Microfluidic disc-on-a-chip device for in vitro mouse intervertebral disc. (a) Experimental setup of long-term mouse disc culture on a 4-unit disc-on-a-chip device. (b) Dimension of a single unit of disc-on-a-chip device. (c) Computer simulation of pressure changes without and with micropillar array inserted near the inlet. (d) Computer simulation of flow streams in the perfusion chamber.

Figure 2.

Disc-on-a-chip culture preserved disc structural integrity compared to conventional static culture for up to 10 days. Disc sections were stained with alcian blue/picrosirius red staining. Proteoglycans showed in blue and collagen fibers as red. Similar to native disc (top row), on-chip cultured discs (middle row) preserved NP cellularity (red asterisk) with abundant proteoglycan (black asterisk) while static cultured discs (bottom row) showed typical degenerative changes such as fibrotic network in NP cells (red asterisk) and loss of extracellular matrix (black asterisk). Similarly, on-chip cultured discs maintained AF structural integrity with dense AF fibers, whereas static cultured discs exhibited dramatically loose lamina layer (enlarged gap between AF layers, yellow arrowheads). Scale bar = 100 μm .

Figure 3.

Disc-on-a-chip culture enhanced mouse disc cell viability compared to conventional static culture for up to 10 days. (a) Representative fluorescence images of live–dead staining of mouse discs. On-chip continuous media supply significantly promoted cell viability (both in NP and AF) compared to static culture for up to 10 days. Note, green = live; red = dead. White arrows indicated dead cells. Quantitative analysis of percentage of live AF (b) and percentage of live NP (c) cells to total cells further confirmed superior on-chip condition to static culture. (d) DNA assay revealed similar DNA content between on-chip culture and freshly isolated discs, while discs in static culture showed least DNA content. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ and n.s. not statistically significant. Scale bar = 50 μm .

Figure 4.

Disc-on-a-chip culture maintained native disc homeostasis by preventing up-regulation of catabolic genes and inflammatory cytokines. Biochemical assays and real time PCR were conducted to measure extracellular matrix and catabolic genes, respectively. (a) GAG was significant decreased in static culture compared to native control and on-chip culture. (b) Collagen content was similar in three groups. (c) The ratio of GAG/Hypro was similar in three groups. In static culture, mRNA expression of catabolic genes (d) ADAMTs4 and (e) MMP13 were significantly increased compared to low basal levels in native and on-chip cultured discs. Similarly, in static culture, inflammatory cytokines (f) TNF α and (g) IL-6 were upregulated compared to low basal levels in native and on-chip cultured discs. (h) mRNA expression of IL-1 gene was of no significant difference among three groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and *n.s.* not statistically significant.

Figure 5.

Disc-on-a-chip effectively protected collagen from degeneration detected by second harmonic generation and collagen hybridizing peptide imaging. (a) Representative images exhibited collagen fibrils by alcian blue/picrosirius red (ABPR) staining and second harmonic generation taken with a two-photon microscope. Organized and distinct collagen fibrils were observed in native control and on-chip culture groups, while intercalated and cluttered collagen fibers was observed in AF of static culture. (b) Representative fluorescent CHP imaging suggested significant higher degree of collagen degradation (green) in static culture compared to native and on-chip discs after 10 days culture. Scale bar = 50 μ m. iAF, inner annulus fibrosus; oAF, outer annulus fibrosus.

Figure 6.

Hypothesis of advantageous dynamic nutrient refreshment in disc-on-a-chip culture.

Compared to static culture in a multiwell plate, disc-on-a-chip organ culture device provides dynamic nutrient supply and metabolite exchange in disc microenvironment, leading to enhanced disc cell viability and structural integrity in a long-term culture.