

Basic Science

A novel and efficient method for culturing mouse nucleus pulposus cells

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Received 7 February 2019; revised 4 April 2019; accepted 5 April 2019

Abstract

BACKGROUND CONTEXT: As degeneration of the nucleus pulposus (NP) is a major cause of intervertebral disc degeneration, research directed toward nucleus pulposus cells (NPCs) is drawing increased attention. However, caused by the difficulties associated with their harvest and culture, there are few reports describing cultivation methods for mouse NP cells (mNPCs).

PURPOSE: To establish efficient culture methods for mNPCs.

STUDY DESIGN: In vitro animal study.

METHODS: After primary 3-dimensional (3D) gel culture of mNPCs and analysis of gene expression, cells digested from the gel were cultured in various bio-coated dishes with and without basic fibroblast growth factor (bFGF), and their growth kinetics and changes in gene expression profiles were evaluated. Next, the mNPCs obtained after sequential 3D gel and 2D culture were subjected to micromass culture and the effects of adding transforming growth factor- β 3 (TGF- β 3) on their gene expression profile and extracellular matrix (ECM) synthesis were evaluated.

RESULTS: The cell morphology and gene expression pattern of mNPCs proliferated in primary 3D collagen gel culture resembled those of mNP. In contrast, mNPCs could not proliferate in conventional monolayer culture. Cell adhesion (colony number) and proliferation (colony size) were greater in fibronectin-coated dishes than in dishes with other bio-coatings. The addition of bFGF enhanced mNPCs proliferation, but the gene expression characteristics of mNPCs were lost as passage number increased. 2D culture with bFGF followed by micromass culture allowed for the recovery of the mNPC gene expression profile in primary 3D-gel culture, and TGF- β 3 supplementation during micromass culture enhanced ECM synthesis.

CONCLUSIONS: We established novel culture methods for mNPCs. These methods will benefit basic cell-based and molecular research involving these cells. © 2019 Elsevier Inc. All rights reserved.

Keywords:

Fibroblast growth factor; Fibronectin-coated dish; Micromass culture; Mouse nucleus pulposus cells; Transforming growth factor- β ; 3D collagen gel culture

FDA device/drug status: Not applicable.

Author disclosures: **JK:** Nothing to disclose; **TK:** Asahi Kasei Pharma (B), Nuvasive (B), B-Braun (B), Kyocera (B), Medacta (C), Medtronic (B), **Grant:** JSPS Grant-in-aid (C), JSPS bilateral Joint Research Project; **RC:** Nothing to disclose; **RO:** Nothing to disclose; **HI:** Nothing to disclose; **ZB:** Nothing to disclose; **JK:** Nothing to disclose; **ST:**

Nothing to disclose; **TM:** Nothing to disclose; **YS:** Nothing to disclose; **HY:** Nothing to disclose.

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Introduction

The intervertebral disc consists of superior and inferior cartilaginous endplates, outer annulus fibrosus (AF), and central gel-like nucleus pulposus (NP). Intervertebral disc degeneration (IDD) is a major cause of lower back pain, and the burdens caused by IDD-related morbidities extend to society and the economy as a whole [1–3]. Current therapies cannot cure IDD and are limited to conservative treatments (such as medication or physical therapy) or surgical interventions [4]. Regeneration therapy for IDD mainly targets the NP, the degeneration of which is considered a major cause of the condition [4,5]. Therefore, understanding the cell morphology and gene expression profile of NP cells (NPCs) is critical to the development of new therapeutic strategies for IDD.

The ability to culture NPCs while maintaining their gene expression profile is essential in experiments using these cells, as dysregulation of multiple intracellular signaling pathways may play a key role in IDD [6,7]. Several cell-based or cytokine therapies have been investigated using rat, rabbit, bovine, and human NPCs culture [5,8–10], but molecular details such as NPC intracellular signaling pathways are still not fully understood [1,4].

The mouse is one of the most studied species. A variety of specific gene transgenic mice can be used for basic research. However, to date, there have been few reports using mouse NPCs (mNPCs) caused by the difficulty associated with harvesting adequate quantities of these cells from extremely small mouse intervertebral discs. Furthermore, the gene expression profile of mNPCs is easily lost with conventional 2 dimensional (2D) monolayer culture, which does not replicate the 3 dimensional (3D) hydrophilic aggrecan-rich gelatinous extracellular matrix (ECM) with unique avascular, hypoxic, nutrient-deficient, high osmotic pressure characteristics in which NPCs are naturally supported. Therefore, a novel culture method is required which allows for the number of mNPCs to be expanded while maintaining their gene expression profile. In this study, we aimed to establish novel, efficient primary culture, and subsequent expansion culture methods for mNPCs.

Materials and Methods

Animals

Male C57BL6J mice aged from 10 to 12 weeks were purchased from Oriental Yeast Co., LTD. (Tokyo, Japan) and maintained under standard animal housing conditions (12 hours light, 12 hours dark cycles, and free access to food and water). All procedures involving animals were conducted in accordance with the Regulations on Animal Experimentation guidelines at Osaka University Graduate School of Medicine.

Nucleus pulposus and annulus fibrosus cells isolation

NP and AF tissue was identified and separated from mouse tail intervertebral discs (IVDs) under a microscope

(Fig. 1A, B). Tissues were digested with 0.1% pronase (Roche, Indianapolis, IN, USA) at 37°C for 30 minutes and with 0.2% collagenase type 2 (Worthington, Lakewood, NJ, USA) at 37°C for 2 hours. Any remaining tissue debris was removed through a 100 µm pore-size nylon mesh (Corning Inc., Corning, NY, USA).

Conventional monolayer culture of mouse nucleus pulposus cells and mouse annulus fibrosus cells

The mNPCs and mouse AF cells (mAFCs) isolated from IVDs were plated on a 6-well culture dish (Corning Incorporated, Corning, NY, USA) and cultured as a 2D monolayer in Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque, Kyoto, Japan) with 10% Fetal Bovine Serum (FBS, Thermo Scientific HyClone, Logan, UT, USA) and 1% antibiotic/antimycotic solution (A/A, Sigma-Aldrich, St. Louis, MO, USA) under a 5% CO₂ and 5% oxygen atmosphere at 37°C.

Primary 3 dimensional culture of mouse nucleus pulposus cells in collagen gel

The mNPCs isolated from NP tissue were washed with PBS and centrifuged at 1500 rpm for 3 minutes. The cell pellet was mixed with 1 mL of Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan) and released into 12-well plates for 30 minutes at 37°C (Fig. 1A). After gelation, the gel was cultured in DMEM with 10% FBS and 1% A/A under a 5% CO₂ and 5% oxygen atmosphere at 37°C. The medium was replaced every 2 to 3 days.

Subsequent monolayer culture after primary 3 dimensional collagen gel culture

After 14 days of primary 3D collagen gel culture, the gel was digested in 0.1% collagenase type 2 at 37°C for 30 minutes. After washing, the mNPCs were plated on a culture dish to assess whether mNPCs number could be expanded in 2D monolayer culture.

Colony-forming assay of mouse nucleus pulposus cells using bio-coated dishes

The colony-forming assay was performed as previously reported [11]. In short, 100 mNPCs obtained from primary 3D collagen gel culture were plated on various bio-coated 6-well plates (fibronectin, type 1 collagen, type 4 collagen, poly-D-lysine [PDL], laminin, and an uncoated dish; BD Biosciences, Bedford, MA, USA) and cultured in DMEM with 10% FBS and 1% A/A for 14 days. The NPCs were subsequently fixed with 4% paraformaldehyde, stained with 0.5% crystal violet in water for 5 minutes, and washed with distilled water. The number of crystal violet-stained colonies larger than 2 mm in diameter was counted.

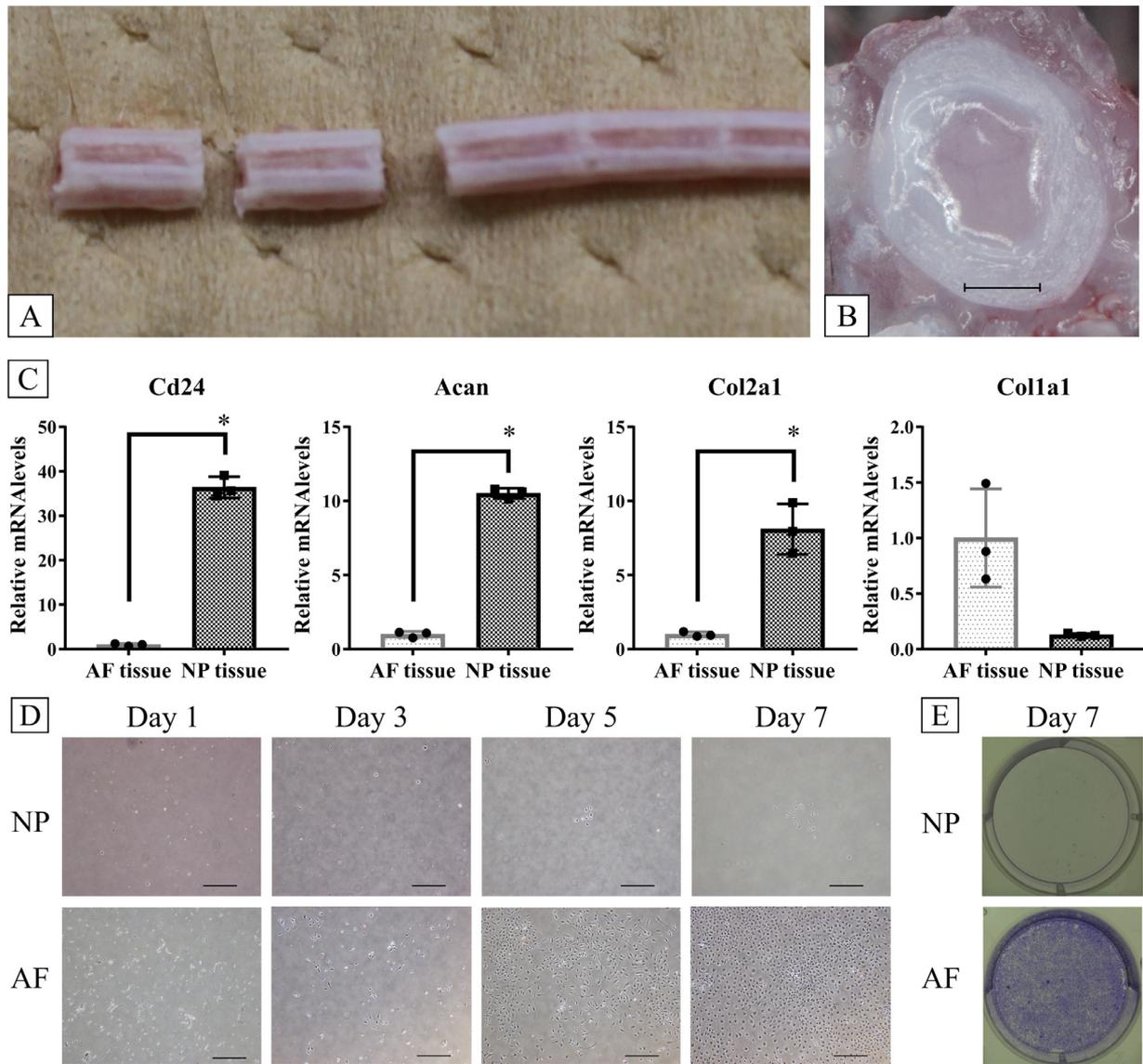


Fig. 1. Isolation of nucleus pulposus (NP) and annulus fibrosus (AF) tissue, and the conventional monolayer culture of NP and AF cells. A. Isolation of NP and AF. The mouse tail was cut off and its skin removed. Each vertebra was detached at the intervertebral disc level. B. Axial view of the intervertebral disc from the cranial side. The central gel-like tissue is the NP. The outer circle fibrous tissue is the AF (bar=500 μ m). C. Gene expression of NP tissue compared with AF tissue (N=3, each; *, $p < .05$ by Welch's t test). D. Conventional monolayer culture of NP and AF cells (bar=500 μ m). E. The cultured 6-well dishes were stained with 0.5% crystal violet at day 7 for both NP and AF cells. Cells in the dish in which mAFCs had been cultured were almost confluent. On the other hand, no cell colonies were seen in the dish in which mNPCs had been cultured.

Effect of basic fibroblast growth factor addition on 2 dimensional culture after 3 dimensional collagen gel culture

The mNPCs obtained from primary 3D collagen gel culture were plated on fibronectin-coated dishes at 2500 cells/cm² and cultured in DMEM with 10% FBS and 1% A/A, with or without 10 ng/mL of bFGF (recombinant human basic fibroblast growth factor, FUJIFILM Wako, Osaka, Japan) in 5% CO₂ and 5% oxygen incubator at 37° C for 7 days (passage 1). Thereafter, the mNPCs were passaged at 2500 cells/cm² every 7 days until their expansion potential was lost. The cells at passage 3 were

used for gene expression analysis and were considered representative.

Redifferentiation assay of mouse nucleus pulposus cells with or without transforming growth factor- β 3

The mNPCs obtained from primary 3D collagen gel culture were cultured with four different protocols, which included bFGF and TGF- β 3 (Transforming growth factor- β 3, Pepro Tech, Inc., Rocky Hill, NJ, USA) treatment to determine the ideal conditions for the expansion of mNPCs (Fig. 5A). At passage 0, the mNPCs in all groups were cultured in collagen gel. At passages 1 and 2, the mNPCs were

cultured in expansion medium (DMEM with 10% FBS and 1% A/A) with or without 10 ng/mL of bFGF. At passage 3, the mNPCs were cultured in micromass (5×10^4 cells/10 μ L) in chondrogenic medium (DMEM with 1% ITS [insulin, transferrin, selenium, Corning Inc., Corning, NY, USA], 50 μ g/mL ascorbic acid [Sigma-Aldrich], 40 μ g/mL L-proline [FUJIFILM Wako, Osaka, Japan], and 1% A/A) with or without 10 ng/mL of TGF- β 3. In each group, cells were cultured for 7 days in each passage. The mNPCs were also cryopreserved in Cellbanker 1 (Zenoaq, Fukushima, Japan) after passage 1. Then, the mNPCs were thawed and cultured in expansion medium with or without 10 ng/mL of bFGF from passage 2, and cultured in micromass in chondrogenic medium with or without 10 ng/mL of TGF- β 3 at passage 3 as mentioned above. At the end of passage 3, the mNPCs were fixed with 4% paraformaldehyde, stained with alcian blue (pH 1.0) for 3 hours, and washed with distilled water. The mNPCs from each group were used for real-time polymerase chain reaction (PCR). At passage 4, the mNPCs were reseeded in 3D collagen gel, and used for real-time PCR.

Real-time PCR assay

The IVD tissues (mNP and mAF), and cultured cells were homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted using Phasemaker Tubes (Invitrogen, Carlsbad, CA, USA) and a Direct-zol RNA kit (Zymo Research, Orange, CA, USA), and was converted to cDNA using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Gene expression was measured using real-time PCR with SYBR green master mix (Applied Biosystems, Foster City, CA, USA) using a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences used for real-time PCR are listed in [Supplementary Table 1](#). The results are expressed as mRNA levels, and were calculated using the relative quantitation standard curve method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in each sample.

Statistical analysis

All data are expressed as means \pm standard deviation (SD). Data were analyzed using the Statistical Package for the Social Sciences version 23.0 for Windows (IBM Corporation, Armonk, NY, USA). Differences in the measured variables between the two groups were analyzed with Welch's *t* test or a paired *t* test, as appropriate. Differences between multiple groups were analyzed with one-way ANOVA followed by Bonferroni test. Differences with a *p* value of $<.05$ were considered significant.

Results

Isolation of mouse nucleus pulposus

The mNP isolation was validated by comparing gene expression profiles of mAF isolated at the same time. As

reported previously, significantly higher expression of Cd24, Acan, and Col2a1, and lower expression of Col1a1, was found in mNP than in mAF tissue ([Fig. 1C](#)) [5].

Mouse nucleus pulposus cells did not proliferate in conventional 2D monolayer culture

Next, we tried the conventional 2D monolayer culture of mNP and mAF. In contrast to mAFs, which adhered to the culture dish and proliferated, the isolated mNPCs rarely adhered. Furthermore, those mNPCs which did adhere did not proliferate ([Fig. 1D](#)). These results suggested that conventional 2D monolayer culture is not suitable for primary culture of mNPCs.

Mouse nucleus pulposus cells proliferated and maintained gene expression profile in primary 3D collagen gel culture

3D collagen gel culture has been reported to resemble the *in vivo* environment of NPCs, and can therefore be used to maintain their original gene expression profile [12,13]. We therefore attempted primary 3D collagen gel culture of mNPCs ([Fig. 2A](#)).

After gelation, two different types of cells were observed in the 3D collagen gel. The first consisted of large vacuolated cells, which we considered “notochordal” cells ([Fig. 2B](#) Day 0- α). The second comprised small round cells, which we considered “chondrocyte-like” cells ([Fig. 2B](#) Day 0- β) [5]. On day 3, spindle-shaped protrusions were observed in the large vacuolated cells ([Fig. 2B](#)). After day 5, the large vacuolated cells were no longer observed. Instead, the cell population consisted of small cells which formed cell colonies ([Fig. 2B](#) Day 5-14). The gene expression profile of mNPCs cultured in 3D collagen gel on day 14 was similar to that of NP tissue ([Fig. 2C](#)). These results suggested that mNPCs cell numbers can be expanded without loss of phenotype by using 3D collagen gel culture.

Fibronectin coated dish was most appropriate for subsequent 2 dimensional culture after primary 3 dimensional gel culture

To further increase the number of mNPCs, we performed 2D expansion culture using various types of bio-coated dishes. Unlike mNPCs cultured using the primary conventional 2D culture method, those expanded by 3D collagen gel culture could adhere to all types of bio-coated dishes tested. Of these dishes, using those with fibronectin coatings resulted in the largest colony sizes and the greatest number of colonies stained with crystal violet ([Fig. 3A, B](#)). These results suggested that fibronectin-coated dishes are appropriate for adhesion and expansion of NPCs after 3D collagen gel culture.

Basic fibroblast growth factor supplementation increased mouse nucleus pulposus cells proliferation, but caused the loss of their gene expression profile

Next, we attempted the monolayer expansion culture of mNPCs using fibronectin-coated dishes. First, conventional

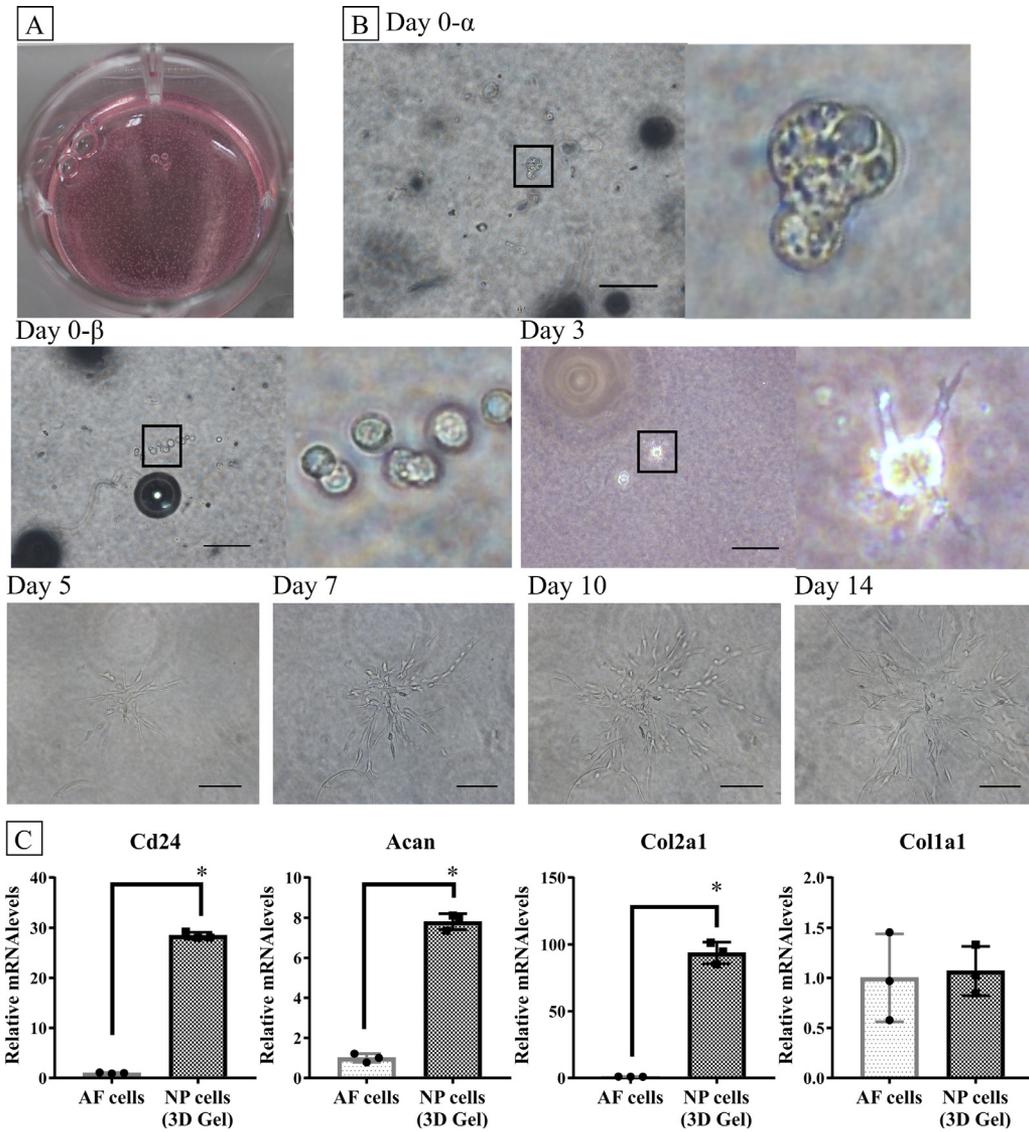


Fig. 2. The 3D culture of mNPCs in collagen gel. A. The isolated mNPCs pellet was mixed with 1 mL of Cellmatrix type I-A and released into 12-well plates. B. The appearance of mNPCs in 3D collagen gel (bar=100 μ m).

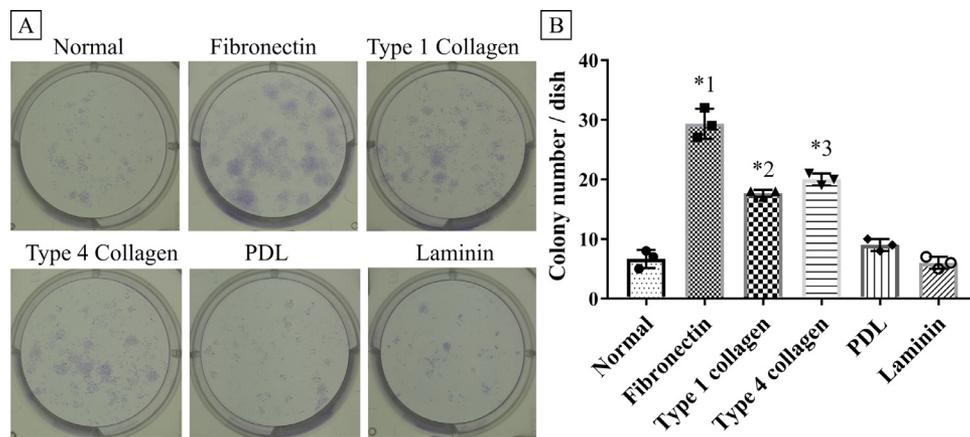


Fig. 3. The mNPCs colony-forming assay using bio-coated dishes in monolayer culture. A. Colony formation by NPCs in various bio-coated dishes after 14 days of culture. Representative culture dishes stained with crystal violet are shown. B. Colony number per dish in various coated dishes. Colony numbers were counted after 14 days of culture (N=3, each. *1; adjusted $p < .0001$ vs. Normal, Type 1 collagen, Type 4 collagen, PDL, Laminin *2; adjusted $p < .0001$ vs. Normal Laminin, adjusted $p = .0001$ vs. PDL *3; adjusted $p = .0001$ vs. Normal, PDL, Laminin by one-way ANOVA followed by Bonferroni test).

expansion culture medium (DMEM+10% FBS+1% A/A) was used for mNPCs culture. However, their growth was halted after 2 to 3 passages, with cells showing evidence of senescence by becoming hypertrophic and flattened. To maintain cell phenotype and proliferation capacity we examined the effects of bFGF, which has been reported to enhance the proliferation of IVD cells [14,15].

Once supplemented with bFGF, the mNPCs steadily proliferated up to passage 4 to 6 (Fig. 4A). At every point, the number of mNPCs cultured with bFGF was significantly larger than that of those cultured without bFGF. At passage 5, the number of mNPCs cultured with bFGF was three times larger than that of those cultured without bFGF (Fig. 4B). Next, gene expression profiles of mNPCs cultured with or without bFGF at passage 3 were evaluated.

Expression levels of Cd24, Acan, and Col2a1 of expanded mNPCs, both with or without bFGF, were significantly lower than in the primary mNPCs cultured in 3D gel (Fig. 4C). These results suggested that the addition of bFGF is advantageous for cell proliferation, but that mNPCs lose their original gene expression profile as cell numbers increase.

Micromass culture after 3 dimensional collagen gel and 2 dimensional monolayer culture can recover the gene expression profile of mouse nucleus pulposus cells

To explore means of recovering the gene expression profile of expanded mNPCs, the micromass culture was performed both with or without TGF- β 3. The micromass

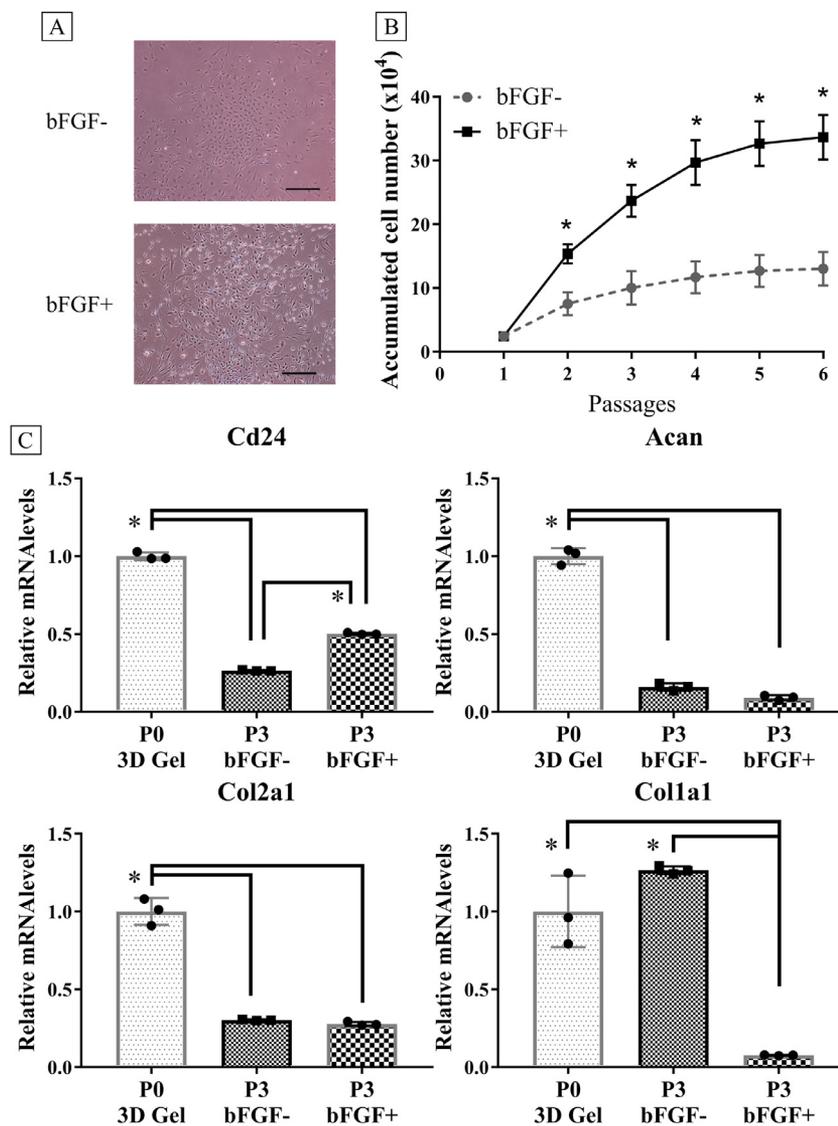


Fig. 4. Expansion culture of mNPCs after 3D gel digestion with or without bFGF. A. The appearance of mNPCs with or without bFGF (Passage 3, day 5, bar=500 μ m). B. Growth kinetics of mNPCs obtained from 3D gel culture and 2D expansion culture. Starting from Passage 1, each population of mNPCs was replated at 2500 cells/cm² every 7 days. Accumulated cell numbers are shown at each passage (N=3, each. *; p<.05 by paired *t* test). C. Gene expression in mNPCs cultured in primary 3D collagen gel, cultured with bFGF at passage 3, and cultured without bFGF at passage 3 (N=3, each. *1; adjusted p<.0001 by one-way ANOVA followed by Bonferroni test).

culture method, which cultures cells at high density, has been reported to promote chondrogenic differentiation, and to increase ECM synthesis [16]. The TGF- β is an anabolic growth factor which induces aggrecan expression and glycosaminoglycans (GAG) synthesis in NPCs [17,18]. The TGF- β 3 has been reported to be the most effective factor in the TGF- β family [18].

A schema of this assay is shown in Fig. 5A. The expansion of mNPCs with or without bFGF was accomplished using a 3D micromass culture, which was performed with or without TGF- β 3. Alcian blue staining was used to assess GAG synthesis (Fig. 5B). As expected, TGF- β 3 stimulated GAG synthesis by mNPCs in the micromass culture, with the strongest staining observed in mNPCs treated with bFGF for 2D expansion culture and TGF- β 3 for micromass culture (group 4).

The expression of Acan in groups 1 and 2 was significantly lower than that of the primary 3D collagen gel culture. On the other hand, the expression of Acan in groups 3 and 4 was significantly higher than that in the primary 3D collagen gel culture. The highest level of Acan expression was observed in group 4. Expression of Col2a1 in groups 1 and 2 was significantly lower than of the expression in the primary 3D collagen gel culture. Expression of Col2a1 in groups 3 and 4 was significantly higher than in the primary 3D collagen gel culture. The expression of Cd24 and Col1a1 in group 3 was similar to that in the primary 3D

collagen gel culture (Fig. 6A). The similar gene expression results were confirmed from the mNPCs cryopreserved during expansion culture (data not shown). The similar gene expression results were also confirmed from the mNPCs reseeded in 3D collagen gel after micromass culture (Fig. 6B). At passage 4 (the mNPCs reseeded in 3D collagen gel after micromass culture), groups 1 and 2 also showed significantly lower expression of Acan than the primary 3D collagen gel culture. On the other hand, groups 3 and 4 also showed significantly higher expression of Acan than the primary 3D collagen gel culture. The highest level of Acan expression was observed in group 4. Similarly, groups 1 and 2 also showed significantly lower expression of Col2a1 than the primary 3D collagen gel culture, and groups 3 and 4 showed significantly higher expression of Col2a1 than the primary 3D collagen gel culture. The expression of Cd24 and Col1a1 in group 3 was also similar to that in the primary 3D collagen gel culture. These findings suggested that the phenotype of mNPCs expanded by 3D collagen culture and 2D monolayer culture can be recovered mNPCs by subsequent micromass culture.

Discussion

In this study, we established novel efficient primary and subsequent expansion culture methods for mNPCs, using a series of primary 3D collagen gel culture, 2D monolayer

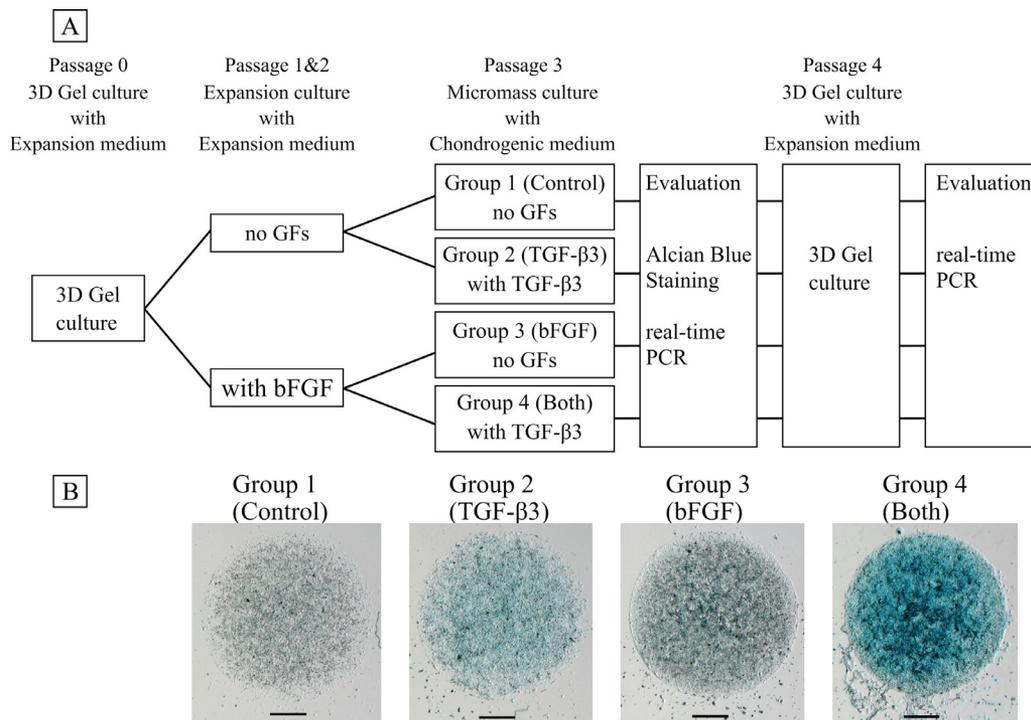
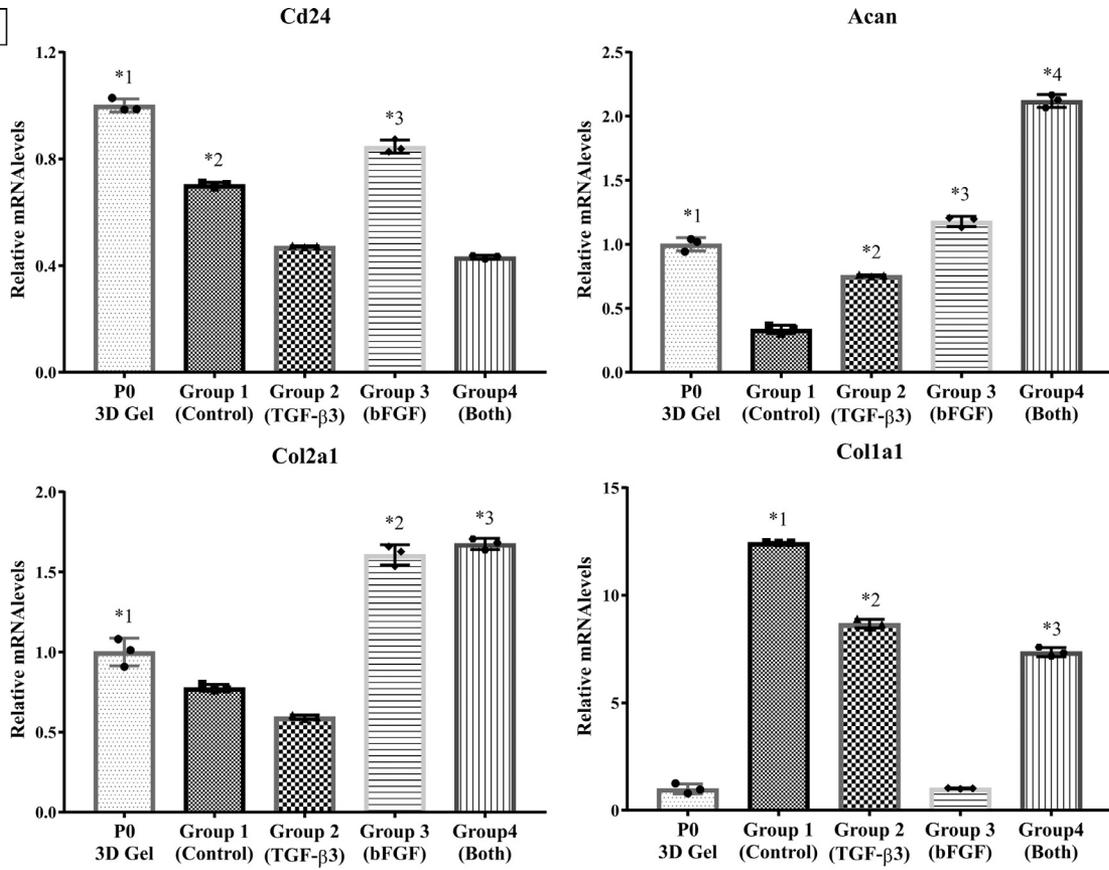
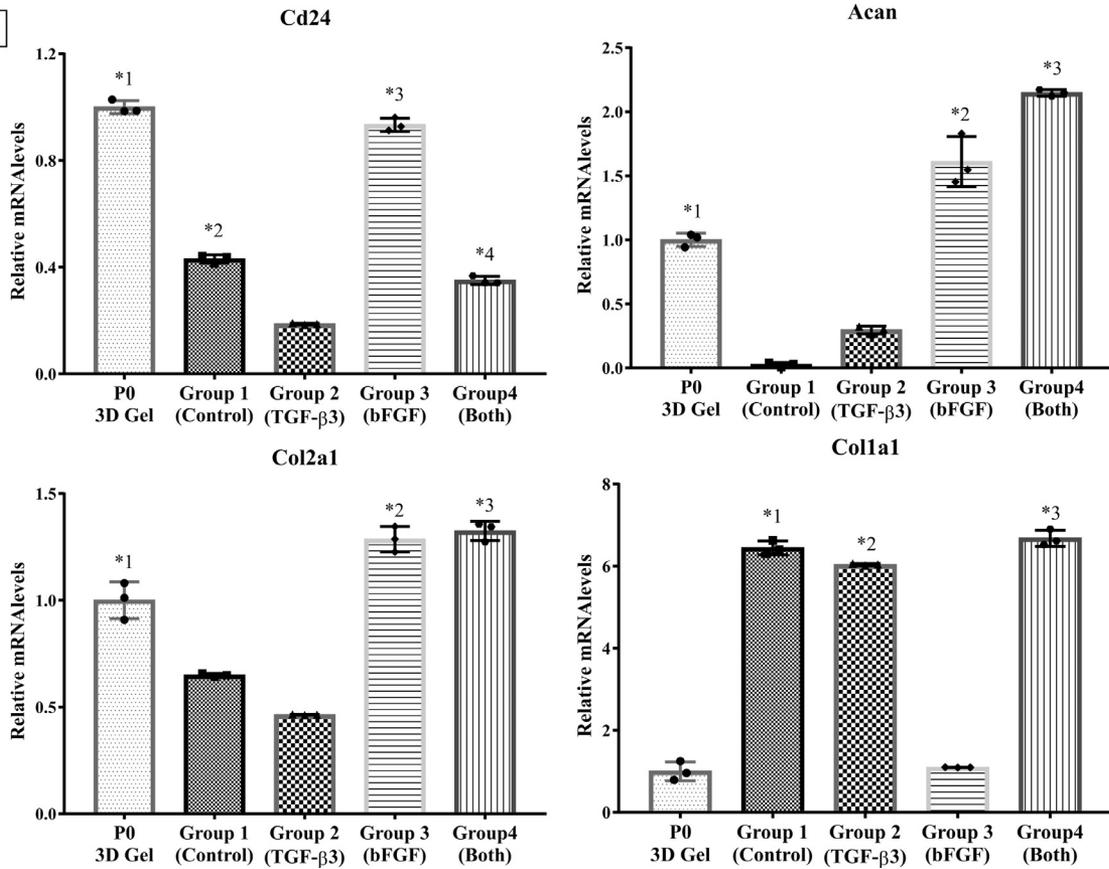


Fig. 5. A. A protocol used for each culture group. At passage 0, the mNPCs were cultured in collagen gel in all groups. In groups 1 and 2, the mNPCs were cultured in expansion medium until passage 2. In groups 3 and 4, the mNPCs were cultured in expansion medium with 10 ng/mL of bFGF until passage 2. At passage 3 the mNPCs in every group were cultured in micromass in chondrogenic medium. In groups 2 and 4, 10 ng/mL of TGF- β 3 was added to the chondrogenic medium. B. Alcian blue staining of micromass in each group (bar=1000 μ m). GFs; growth factors, bFGF; basic fibroblast growth factor, TGF- β 3; transforming growth factor β 3.

A



B



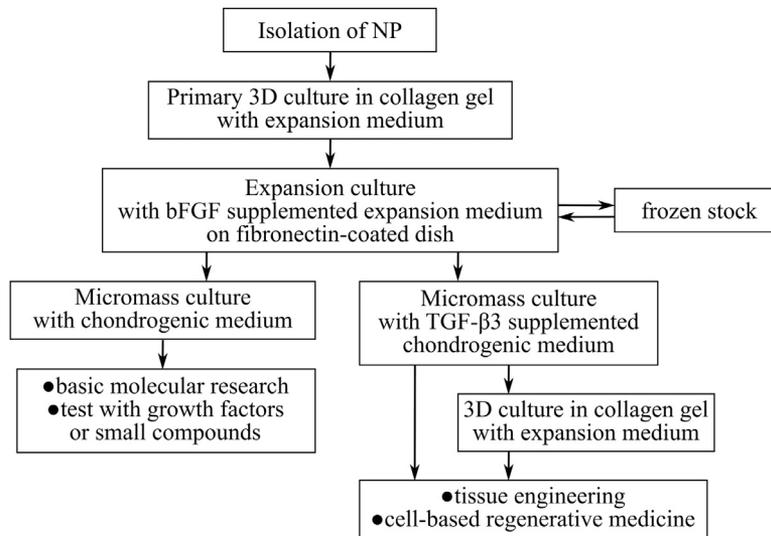


Fig. 7. Step-by-step instructions for culturing mNP cells.

culture on fibronectin-coated dishes with bFGF, and micromass culture with or without TGF-β3.

Where the 3D culture of mNPCs in collagen gel is concerned, culture attempts were successful in every trial. Despite the small number of mNPCs initially present in the 3D collagen gel, successful proliferation was achieved. This is possibly attributable to the fact that mNPCs are surrounded by ECM and connective tissues in vivo and may therefore favor environments in which they can be anchored to collagen gel. The 3D collagen gel culture used in this study also maintained the NP phenotype, as confirmed by the expression patterns of marker genes (Cd24, Acan, Col1a1, and Col2a1).

Regarding the 2D monolayer culture for cell expansion, the addition of bFGF accelerated the proliferation of mNPCs. This result is consistent with previous studies which have reported enhanced cell proliferation following FGF administration [7,14,15]. However, consistent with previous reports [19,20], 2D monolayer culture of mNPCs both with or without bFGF leads to phenotype loss. In this study, the addition of bFGF alone did not affect ECM synthesis, although the effect of FGF on ECM production remains controversial [14,21].

Micromass culture after 2D monolayer culture leads to the recovery of the mNPC phenotype. Culture in chondrogenic medium following culture with FGF has been

reported to increase chondrocyte-specific gene expression and ECM synthesis [22–24]. In this study, mNPCs showed the appearance of “chondrocyte-like” cells after primary 3D gel culture. Therefore, the use of the chondrogenic medium for micromass culture in this study might contribute to the recovery in the NPCs related gene expression (Fig. 5, Group 3).

The addition of TGF-β3 to the micromass culture also enhanced ECM production and increased gene expression related to ECM synthesis in mNPCs cultured in expansion medium both with and without bFGF supplementation (Fig. 5 Groups 2 and 4). The combined use of expansion culture with bFGF and micromass culture with TGF-β3 further enhanced ECM production, and increased gene expression associated with ECM synthesis.

This study has several limitations. First, we did not distinguish between “notochordal cells” and “chondrocyte-like cells” among NPCs [5,25]. However, morphological differences between “notochordal” cells and “chondrocyte-like” cells have recently been put forward as differences in the stages of cellular differentiation [5,26–31]. In this study, both large vacuolated cells and small round cells were observed during the early stages of 3D collagen gel culture; however, the small cells dominated the cell population as cell proliferation increased. Accordingly, NP “chondrocyte-like” cells were mainly used in this study.

Fig. 6. A. Gene expression in mNPCs from each group at passage 3 (N=3, each. Cd24 *1; adjusted p<.0001 vs. Groups 1, 2, 3, 4. *2; adjusted p<.0001 vs. Groups 2, 4. *3; adjusted p<.0001 vs. Groups 1, 2, 4. Acan *1; adjusted p<.0001 vs. Group 1, adjusted p=.0002 vs. Group 2. *2; adjusted p<.0001 vs. Group 1. *3; adjusted p=.0026 vs. P0, adjusted p<.0001 vs. Groups 1, 2. *4; adjusted p<.0001 vs. P0, Groups 1, 2, 3. Col2a1 *1; adjusted p=.0034 vs. Group 1, adjusted p<.0001 vs. Group 2. *2; adjusted p<.0001 vs. P0, Groups 1, 2. *3; adjusted p<.0001 vs. P0, Groups 1, 2. Col1a1 *1; adjusted p<.0001 vs. P0, Groups 2, 3, 4. *2; adjusted p<.0001 vs. P0, Groups 3, 4. *3; adjusted p<.0001 vs. P0, Group 3 by one-way ANOVA followed by Bonferroni test).

B. Gene expression in mNPCs from each group at passage 4 (N=3, each Cd24 *1; adjusted p<.0001 vs. Groups 1, 2, 4 *2; adjusted p<.0001 vs. Group 2 *3; adjusted p<.0001 vs. Groups 1, 2, 4 *4; adjusted p<.0001 vs. Group 2 Acan *1; adjusted p<.0001 vs. Groups 1, 2 *2; adjusted p=.0001 vs. P0, adjusted p<.0001 vs. Groups 1, 2. *3; adjusted p<.0001 vs. P0, Groups 1, 2, adjusted p=.0003 vs. Group 3 Col2a1 *1; adjusted p<.0001 vs. Groups 1, 2 *2; adjusted p=.0005 vs. P0, adjusted p<.0001 vs. Groups 1, 2. *3; adjusted p=.0002 vs. P0, adjusted p<.0001 vs. Groups 1, 2. Col1a1 *1; adjusted p<.0001 vs. P0, Group 3 *2; adjusted p<.0001 vs. P0, Group 3 *3; adjusted p<.0001 vs. P0, Group 3 by one-way ANOVA followed by Bonferroni test).

Further research is needed to establish appropriate culture methods for NP “notochordal” cells. Second, we evaluated only TGF- β supplemented medium during micromass culture. However, the other growth factors such as growth and differentiation factors, platelet-derived growth factors, insulin-like growth factors, or bone morphogenetic proteins have also been reported to activate NPC differentiation [32–35]. Further research is needed to optimize the combined use of growth factors required for the differentiation of NPC phenotype.

Our method has the potential to become a useful tool for basic research aimed at elucidating molecular mechanisms in NPCs, and in developing cell-based regenerative therapy for IDD using mNPCs, which cannot be obtained using a conventional monolayer culture method [36]. Furthermore, it will be useful for research related to tissue engineering or regenerative medicine in which ECM production plays an important role (Fig. 7).

Conclusions

We established a novel and efficient primary culture and expansion culture method for mNPCs consisting of sequential primary 3D collagen gel culture, 2D monolayer culture on fibronectin-coated dishes with bFGF, and micromass culture either with or without TGF- β 3. Our method will pave the way for basic research into the molecular mechanisms of NPCs, and for the development of cell-based regenerative therapies for IDD.

Acknowledgment

We thank Ms. Fumiko Hirayama for her great contribution to this work.

Supplementary materials

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.spinee.2019.04.005>.

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