

Article

Optimization of Spheroid Colony Culture and Cryopreservation of Nucleus Pulposus Cells for the Development of Intervertebral Disc Regenerative Therapeutics

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Abstract: After the discovery of functionally superior Tie2-positive nucleus pulposus (NP) progenitor cells, new methods were needed to enable mass culture and cryopreservation to maintain these cells in an undifferentiated state with high cell yield. We used six types of EZSPHERE[®] dishes, which support spheroid-forming colony culture, and examined NP cell spheroid-formation ability, number, proliferation, and mRNA expression of *ACAN*, *COL1A2*, *COL2A1*, and *ANGPT1*. Six different types of cryopreservation solutions were examined for potential use in clinical cryopreservation by comparing the effects of exposure time during cryopreservation on cell viability, Tie2-positivity, and cell proliferation rates. The spheroid formation rate was 45.1% and the cell proliferation rate was 7.75 times using EZSPHERE[®] dishes. The mRNA levels for *COL2A1* and *ANGPT1* were also high. In cryopreservation, CryoStor10 (CS10) produced $\geq 90\%$ cell viability and a high proliferation rate after thawing. CS10 had a high Tie2-positive rate of 12.6% after culturing for 5 days after thawing. These results suggest that EZSPHERE enabled colony formation in cell culture without the use of hydrogel products and that CS10 is the best cryopreservation medium for retaining the NP progenitor cell phenotype and viability. Together, these data provide useful information of NP cell-based therapeutics to the clinic.

Keywords: intervertebral disc; nucleus pulposus; nucleus pulposus progenitor cells; cryopreservation; Tie2; spheroid colony; EZSPHERE; CryoStor 10



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1. Introduction

Low back pain is the primary cause of disability worldwide and often leads to poor quality of life for patients [1,2]. Low back pain is thought to relate to intervertebral disc (IVD) degeneration, although the mechanisms have not been clearly identified [3]. The IVD is composed of three distinct tissues: the nucleus pulposus (NP), annulus fibrosus (AF), and cartilage endplate. The NP comprises the gelatinous tissue located in the center of the IVD. The AF comprises layered collagen structures that surround the NP [4]. Previous reports have indicated that IVD homeostasis is determined by the NP [5,6] and that IVD degeneration is thought to be caused by dysregulation of extracellular matrix (ECM) homeostasis within the NP tissue [7,8].

Disc degeneration is associated with a decline in NP cell numbers and potency, and renewal by stem or progenitor sources appears to be limited [9]. Interestingly, in 2012, Sakai et al. discovered a specific NP progenitor cell population species [9]. NP progenitor cells were shown to have the ability for self-renewal and multipotency, including

adipogenic, osteogenic, and neurogenic differentiation potential [9–12]. NP progenitor cells were reported to differentiate into two types of colony-forming units upon culture in methylcellulose semisolid medium: spheroid colony-forming units (CFU-s) with high ECM production and fibroblastic colony-forming units (CFU-f) with low functionality [9,13]. The propensity of NP progenitor cells for CFU-s was linked to the expression of the cell surface marker angiopoietin-1 receptor (Tie2) [9]. Tie2-expressing NP progenitor cells have since been identified in human, canine, murine, and bovine IVD samples [12–14]. Interestingly, the presence of Tie2-expressing cells within the IVD declines sharply with aging [9] and is similarly linked with progression of disc degeneration [9]. Thus, Tie2 progenitor cells provide a promising therapeutic target in the treatment of IVD degeneration.

Cell therapy is a rapidly evolving field and is thought to be safe and effective for the treatment of low back pain associated with degenerating IVDs [15]. Cell therapy has been suggested to be effective through repopulation of the IVD cells or reactivation of endemic cells through paracrine signaling, thereby switching the disc environment from catabolic to anabolic [15]. Our group has conducted one of the first IVD cell therapy clinical trials, which involved the transplantation of autologous NP cells that were reactivated using coculture with mesenchymal stem cells (MSCs) [16]. The trial revealed that NP cells could be safely transplanted and that cell transplantation appears to be effective in preventing disc degeneration [16].

Although these results are promising, NP materials are often derived from aged and diseased patients, which can compromise their regenerative potential [17–19]. We believe that the potency of NP cell therapy could be increased by using a cell population with high Tie2-positive rates. However, Tie2-positive cells remain difficult to maintain in culture because they rapidly lose their Tie2 expression and consequently their proliferative capacity and potency [13]. In addition, mass production of these cells is limited by the high viscosity of the methylcellulose medium and unsuitability for industrialization.

A variety of studies have investigated ways to improve the culture conditions [10,14,20] or isolation methods [21] to increase the number of Tie2-expressing cells. For example, Tekari et al. reported beneficial effects of fibroblast growth factor 2 (FGF2) and hypoxic culture conditions [14]. Recent work by Guerrero et al. [10] showed the benefits of three-dimensional (3D) pellet culture environments for maintaining the multipotent capacity and retaining Tie2 expression in Tie2-positive NP progenitor cells. These findings provide evidence for the potential of culture augmentation to increase the retention of NP progenitor cells. However, an optimized culture method that would be scalable, marketable, and clinically applicable remains to be determined.

Another consideration that could hinder the translation of Tie2-positive NP progenitor cells to the market is their preservation. Tanaka et al. has shown that cell viability, differentiation ability, and ECM production do not change significantly from before to after NP cell cryopreservation [22]. This finding suggests that activated NP cells could be used in a cryopreserved state whenever needed according to the patient's condition, thus increasing their potential as a commercial, mass-produced, off-the-shelf (OTS) regenerative therapeutic product. In a canine study, an OTS discogenic NP cell product was shown to be as effective in regenerating induced disc degeneration compared with freshly cultured cells [23]. These products are currently undergoing clinical trial evaluations (NCT03347708, NCT03955315).

There is a large difference between the actual clinical application and the results in laboratory-based reports. In addition, the time required before and after freezing is rarely considered [24]. The cytotoxicity of animal serum and dimethyl sulfoxide (DMSO) contained in the cryopreservation solutions may also present safety and regulatory problems during clinical applications [13,25]. Furthermore, during the product management and transportation after mass culture, the process from cell isolation to storage becomes more complicated and time consuming.

We wanted to examine the potential for optimization of the practical aspects of NP cell production in terms of cell culture, storage, and logistic handling. Given the needs of

future commercialization, we aimed to identify optimal cryopreservation solution(s) that could limit the functional deterioration and maintain the high cell viability of Tie2-positive NP cells. We examined the potential for using specialized nonadherent EZSPHERE® dishes, which have been used with induced pluripotent stem cells and cancer cells but not with NP cells. This culture dish supports 3D cell cultures without the need for hydrogel encapsulation to enable mass production of spheroid-forming NP cells.

2. Materials and Methods

2.1. Human NP Cell Isolation

Human NP tissues were collected from 10 patients undergoing surgery for lumbar disc herniation at Tokai University Hospital and related facilities (Table 1). All patients provided their informed consent for the collection and use of surgical waste for research purposes. All research procedures described in this study were approved by the Institutional Review Board for Clinical Research, Tokai University (application number 16R-051: 15 July 2016).

Table 1. Details of clinical samples. Age, sex, total weight of IVD tissue collected at the time of surgery, and weight of NP tissue after selection are listed for each sample. LDH: lumbar disc hernia, IVD: intervertebral disc, NP: nucleus pulposus.

	Code	Age	Sex	Disease	IVD Tissue Amount(g)	NP Tissue Amount(g)
1	T19	19	M	LDH	8.05	7.59
2	A29	29	F	LDH	3.76	2.57
3	K30	30	F	LDH	4.26	2.5
4	E32	32	F	LDH	4.75	4.75
5	A16	16	F	LDH	3.58	1.9
6	K21	21	F	LDH	7.52	4.79
7	A24	24	M	LDH	8.04	4.69
8	T23	23	M	LDH	5.06	4.6
9	T18	18	M	LDH	3.38	3
10	A18	18	M	LDH	5.88	3.73

NP cells were isolated as described previously [13]. Briefly, the collected NP tissue was washed with excess saline and the tissue wet weight was measured. In a 100 mm dish, the tissue was cut into 3–5 mm-sized pieces with sterilized scissors and scalpels. The shredded NP tissue was carefully transferred to a 50 mm conical tube and centrifuged at 1200 rpm for 5 min at 4 °C. After centrifugation, the supernatant was removed, and the tissue was resuspended in 10 mL of 10% (*v/v*) fetal bovine serum (FBS), minimal essential medium α (MEM α , Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), and 10 mL of TrypLE Express (Thermo Fisher Scientific, Tokyo, Japan). The suspension was incubated with gentle swirling at 37 °C for 1 h. The tissue dissolution state was confirmed and the sample was centrifuged at 1800 rpm for 5 min. Next, the tissue was resuspended in 15 mL of 10% FBS–MEM α and 5 mL of 0.25 mg/mL collagenase P (Roche, Basel, Switzerland), and incubated for 2 h at 37 °C. After digestion, the tissue was again centrifuged and resuspended in 20 mL of 10% FBS–MEM α , the cell suspension was filtered through a 40 μ m cell strainer (Corning, Corning, NY, USA), and the cells were subjected to experimental cryopreservation or culture conditions as described below.

2.2. NP Cell Culture Conditions

Primary human NP cells ($n = 4$, Table 1: T19, A29, K30, E32) were suspended at 10,000 cells/mL in 10% FBS–MEM α . Cells were seeded in 2 mL onto different types of EZSPHERE® plates (spheroid-formation culture vessel: IWAKI, Tokyo, Japan) and cultured at 37 °C in 5% CO₂ and 5% O₂ for 14 days. EZSPHERE® plates have a uniform well structure on the surface of the dish that allow cells to form different shapes of germ layers or spheroids depending on the size and type of the well (Figure 1). Here we used the following EZSPHERE® dish types: 4000-900 (caliber: 500 μ m, depth: 100 μ m,

number of wells: 2300); 4000-901 (caliber: 200 μm , depth: 100 μm , number of wells: 9200); 4000-902 (diameter: 500 μm , depth: 200 μm , number of wells: 2300); 4000-903 (diameter: 800 μm , depth: 400 μm , number of wells: 600), and 4000-904 (diameter: 800 μm , depth: 300 μm), number of wells: 200); type 4000-905 (caliber: 1400 μm , depth: 600 μm , number of wells: 2300). After 2 weeks of culture, the established colonies were collected carefully by pipetting. For analysis of marker expression, standard monolayer culture was performed by seeding NP cells at a density of 4.5×10^5 cells/ cm^2 into 100 mm plates and culturing them under identical conditions for 7 days. These cells were used as the control.

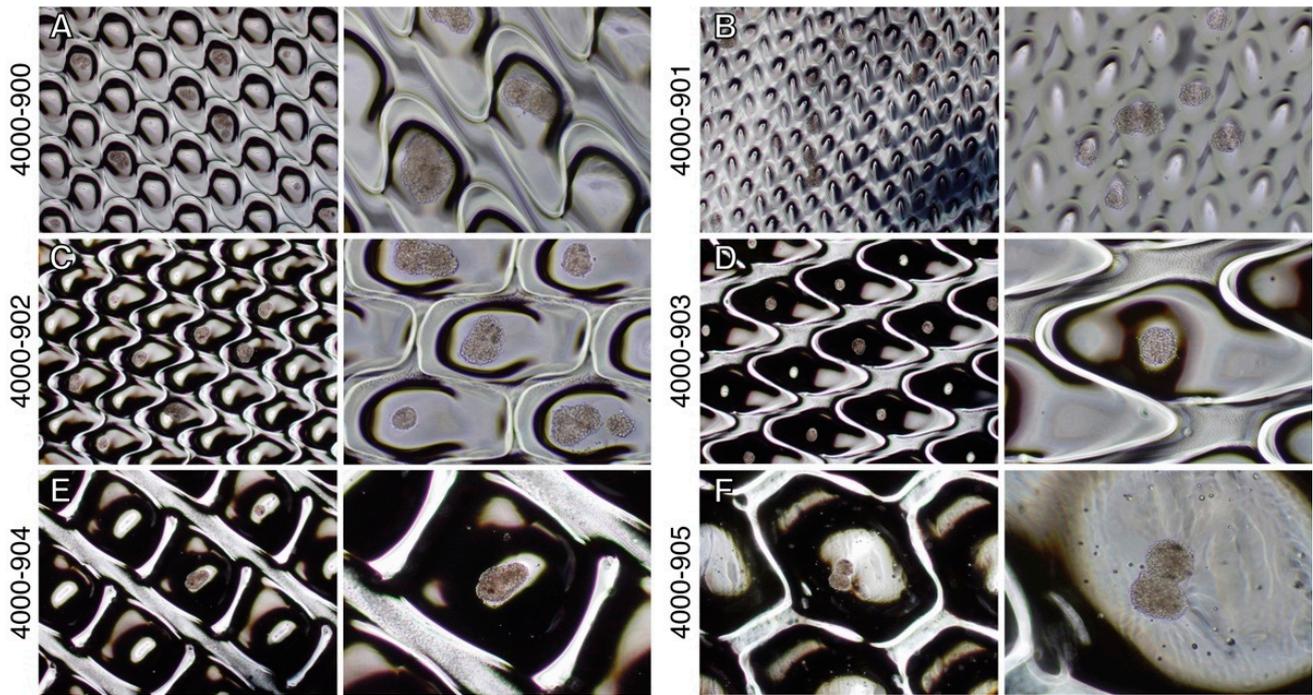


Figure 1. Cells cultured giving rise to spheroid forming units within EZSPHERE[®] dish (A) type 4000-900, (B) type 4000-901, (C) type 4000-902, (D) type 4000-903, (E) type 4000-904, and (F) type 4000-905 at 4 \times (left) and 10 \times (right) magnification. Images visualized by inverted phase contrast microscope. 20,000 human nucleus pulposus cells were seeded per dish and cultured for 14 days at 5% CO₂, 5% O₂, and 37 $^{\circ}\text{C}$.

NP cells ($n = 1$, donor E32) were also cultured in EZSPHERE[®] dish type 4000-903 with normal medium or medium supplemented with 100 ng/mL epidermal growth factor (EGF; R&D Systems, Minneapolis, MN, USA), 10 ng/mL FGF2 (PeproTech, Cranbury, NJ, USA), 100 ng/mL platelet-derived growth factor (PDGF; R&D Systems), or a combination of all three factors. The colonies obtained were similarly harvested and evaluated.

2.3. Cryopreservation of NP Cells

After confirming they were free of contamination and the state of the cells, primary NP cells were treated with 5 mL of TrypLE Express for 3 min and then collected into a 15 mL conical tube. Samples were centrifuged at $500 \times g$ for 5 min at 4 $^{\circ}\text{C}$, resuspended in 5 mL of buffered saline, and centrifuged again at $500 \times g$ for 5 min at 4 $^{\circ}\text{C}$. The cells were counted using the trypan blue exclusion method to determine their cell number and viability.

For the first experiment, the cell suspension (donor T19) was centrifuged once more to prepare a 1 mL suspension of CELLBANKER 1 (CB1), STEM-CELLBANKER GMP grade (SCB), or STEM-CELLBANKER DMSO Free GMP grade (SCBD-Free) (all from Zenoaq Resource, Fukushima, Japan) (Table 2), each containing 1×10^6 cells in their respective cryopreservation solution to be dispensed into a cryotube. The samples were kept at room temperature or on ice for 0.5, 1, 2, 3, 4, and 5 h, and cell viability was directly analyzed either or analyzed following cryostorage at -80°C for 1 day. Cryostorage was prepared with

BICELL containers (Nihon Freezer, Tokyo, Japan) in same way as previously reported [22] to control the cooling rate.

Table 2. Details of cryopreservation solutions. Whether include the animal serum, clears GMP grade, and concentration of DMSO are listed for each cryopreservation. CB-1: CELLBANKER 1, SCB: STEM-CELLBANKER GMP grade, SCBD-free: STEM-CELLBANKER DMSO Free GMP grade, CS10: CryoStor[®] CS10, and PF: Pro Freeze[™]-CDM.

Cryopreservation	Animal Serum	Concentration of DMSO	GMP Grade
CB1	Included	10%	Not Cleared
SCB	Not Included	10%	Cleared
SCBD-free	Not Included	0%	Cleared
CS10	Not Included	10%	Cleared
CP-1 [™]	Not Included	5%	Not Cleared
PF	Not Included	15%	Not Cleared

Next, the effects of cryopreservation solutions on NP cell potency and surface marker expression were examined. Based on the previous step, a 3 h incubation time on ice was selected. NP cells ($n = 3$, T19, A24, and T23) were aliquoted at 1×10^6 cells in 1 mL of CB1, SCB, or SCBD-Free. The cells were then frozen at -80°C for 1 day, thawed, and cultured in monolayer culture for 5 days. The cells were retrieved using TrypLE Express and analyzed by flow cytometry.

Tie2 expression and cell viability of NP cells ($n = 1$, donor, T19) were examined using six different cryopreservation compositions: CB1, SCB, SCBD-Free, CryoStor[®] CS10 (CS10) (Stemcell Technologies, Vancouver, BC, Canada), CP-1[™] (Kyokuto Pharmaceutical Industry, Tokyo, Japan), and Pro Freeze[™]-CDM (PF) (Lonza Bioscience, Basel, Switzerland). The final tubes were incubated on ice, frozen at -80°C , and cultured before being analyzed as previously described.

Finally, 1×10^6 NP cells ($n = 3$, T19, A18, and T18) were kept in 1 mL of CB1 or CS10 on ice or at room temperature for 0, 2, 3, or 5 h. Cells were either analyzed directly or cryopreserved at -80°C for 1 day.

2.4. Thawing Protocol of NP Cells

Cells cryopreserved in cryotubes were slowly warmed in a bath with 37°C water for about one minute. The tube was removed from the pre-heated water and kept on room temperature until complete thawing was observed. When thawed, the solution was carefully transferred to a falcon tube, and dropwise media containing 10% FBS was added to slowly resuspend the cells and dilute the cryo-storage media. The samples were then centrifuged at 1200 rpm for 5 min at 4°C to collect the cells and applied for the respective experiments.

2.5. Colony Formation and Cell Proliferation Rates

After the cells were cultured for 14 days in EZSPHERE[®] plates, an inverted phase-contrast microscope was used to count the number of colonies that had formed per 100 wells. To calculate the number of cells, the entire colonies were collected, centrifuged, and treated with 1% Liberase (Roche) for 30 min at 37°C . The cells were centrifuged again and resuspended, and the number of cells derived from the colonies was counted.

2.6. Cell Viability

Cell viability was measured using the standard trypan blue exclusion method and an inverted phase-contrast microscope; the percentages of living and dead cells were counted.

2.7. Real-Time Polymerase Chain Reaction (PCR) Analysis

Cultured human NP cells ($n = 3$, E32, A16 m, and K21) were homogenized using ISO-GEN II (Nippon Gene, Tokyo, Japan), and RNA was prepared using an RNAqueous Micro RNA isolation kit (Applied Biosystems, Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions. High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems 4387406) were used to reverse transcribe mRNA and to synthesize cDNA. Based on TaqMan gene expression assays (Applied Biosystems), gene expression levels were detected using TaqMan Universal PCR Master Mix and the following primers: TaqMan Gene Expression (human): ACAN (Hs00153936; Applied Biosystems), COL1A2 (Hs01028956; Applied Biosystems), COL2A1 (Hs00264051; Applied Biosystems), and ANGPT1 (Hs00919202; Applied Biosystems). PCR was performed using an amplification machine (7500; RealTime PCR Systems) with stage 1 (95 °C for 20 s \times 1 cycle) and stage 2 (95 °C for 3 s, 60 °C for 30 s \times 50 cycles). C_T values were calculated relative to the C_T value for the housekeeping gene GAPDH (4333764F; Applied Biosystems).

2.8. Flow Cytometry and Fluorescence-Activated Cell Sorting

NP cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Only living cells were targeted by using a living (propidium iodide-negative) gate. The cell sorting methods and monoclonal antibodies used for analysis were performed as described previously [13].

2.9. Statistical Analysis

All data were collected and processed using Microsoft Excel (Microsoft, Redmond, WA, USA). Statistical analysis was performed using IBM SPSS Statistics software (version 26; IBM Corp., Armonk, NY, USA). Significant differences were identified using the Mann–Whitney U test and Kruskal–Wallis test. All values are presented as mean (\pm standard deviation). p values of <0.05 were considered to be significant.

3. Results

3.1. Spheroid Colony-Formation and Cell Proliferation Rates in EZSPHERE Dishes

The ability of EZSPHERE dishes to support NP cell colony formation was evaluated by determining the colony formation and cell proliferation rates (Figure 1). Interestingly, the colonies formed in each plate were similar in size (Figure 1). The average colony formation rates were 9.1% ($\pm 3.0\%$) for type 4000-901, 15.6% ($\pm 9.6\%$) for type 4000-900, 24.0% ($\pm 1.0\%$) for type 4000-902, 45.1% ($\pm 12.1\%$) for type 4000-903, 17.5% ($\pm 7.0\%$) for type 4000-904, and 15.1% ($\pm 5.1\%$) for type 4000-905. Dish 4000-903 significantly outperformed almost all other dish types (Figure 2A).

Similarly, the cell proliferation rates were highest using the 400-903 plate, although the only significant difference was compared with 4000-905 ($p = 0.029$) (Figure 2B). Dish type 4000-903 seemed to be the most suitable type for NP cell CFU culture. Next, growth factor supplementation showed strongly increased proliferation rates by up to 45 times when stimulated with FGF2 or 62.5 times when stimulated with all three factors combined (Figure 2C).

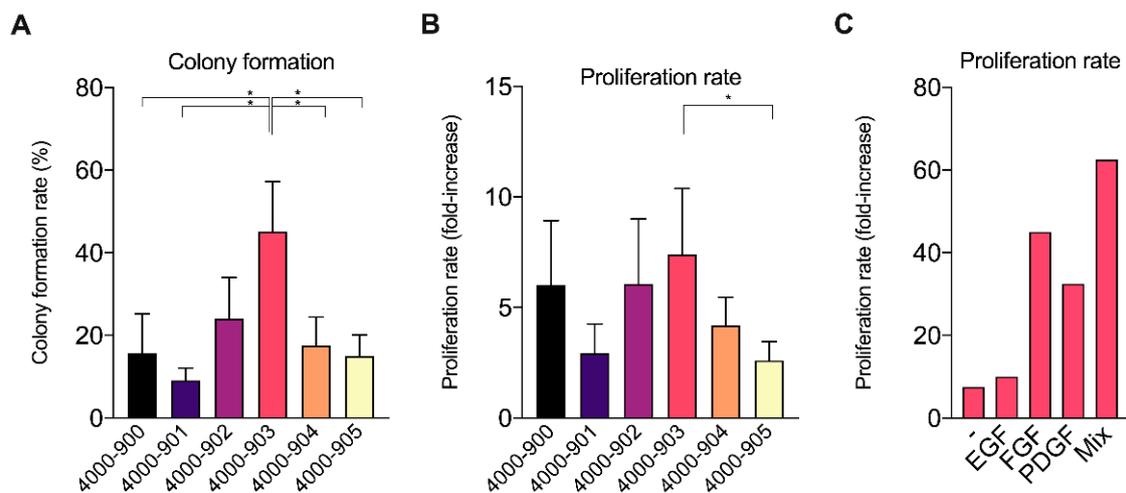


Figure 2. (A) The rate of spheroid colonies that formed and (B) fold increase of cells on different EZSPHERE[®] dish types derived from 20,000 human nucleus pulposus cells ($n = 4$) following 14 days of culture. (C) The fold-increase in cell numbers following culture of nucleus pulposus cells ($n = 1$) on EZSPHERE[®] dish type 4000-903, without (-) or with 100 ng/mL epidermal growth factor (EGF), 10 ng/mL fibroblast growth factor (FGF), 100 ng/mL platelet derived growth factor (PDGF), and a combination of all three growth factors (Mix). Data is presented as mean values with error bars presenting standard deviation. * $p < 0.05$,

3.2. NP Cell Marker Expression in EZSPHERE Spheroid Colonies

Real-time PCR was performed after culturing cells in each of the six types of EZSPHERE dishes. The expression levels of ANGPT1 and COL2A1 were higher in 3D compared with 2D culture (Figure 3A,D). The COL2A1 expression was particularly prominent, which supports previous reports of high ECM production performance in colony-forming cells [9]. Augmentation of EZSPHERE culture conditions by growth factor supplementation revealed strong reduction in the expression levels of ACAN, COL1A2, COL2A1, and ANGPT1. (Figure 3E–H). Notably, the standard deviation for type 4000-901 was observably high for COL2A1 and ANGPT1 outcomes, predominantly due to high expression found for the A29 donor. (Figure 3A,D) The expression levels for the FGF2- and PDGF-supplemented conditions were similar to those of the monolayer-cultured NP cells.

3.3. Cell Viability after Addition of Cryopreservation Solution

To examine the effects of different cryopreservation solutions on NP cells during mass cell batch production, we tested extended exposure of NP cells to three types of cryopreservation solution (CB1, SCB, and SCBD-Free). At room temperature, cells exposed to SCB and SCBD-Free showed a rapid decline in cell viability, with >50% cell loss within 1 h (Figure 4A). In the same process on ice, a high cell viability of $\geq 80\%$ was obtained in all solutions for up to 2 h, but the number of viable cells decreased with longer incubations in SCB and SCBD-Free (Figure 4C). Samples left at room temperature for ≥ 1 h followed by cryopreservation showed complete loss of viability (Figure 4B). Samples kept on ice before cryopreservation had better viability, although cell viability was 67% in SCB and 57.8% in SCBD-Free conditions after 2 h and decreased further with time (Figure 4D). Cells exposed to CB1 had a survival rate of $\geq 90\%$ even after being left for 5 h on ice, although the cell viability decreased with time at room temperature.

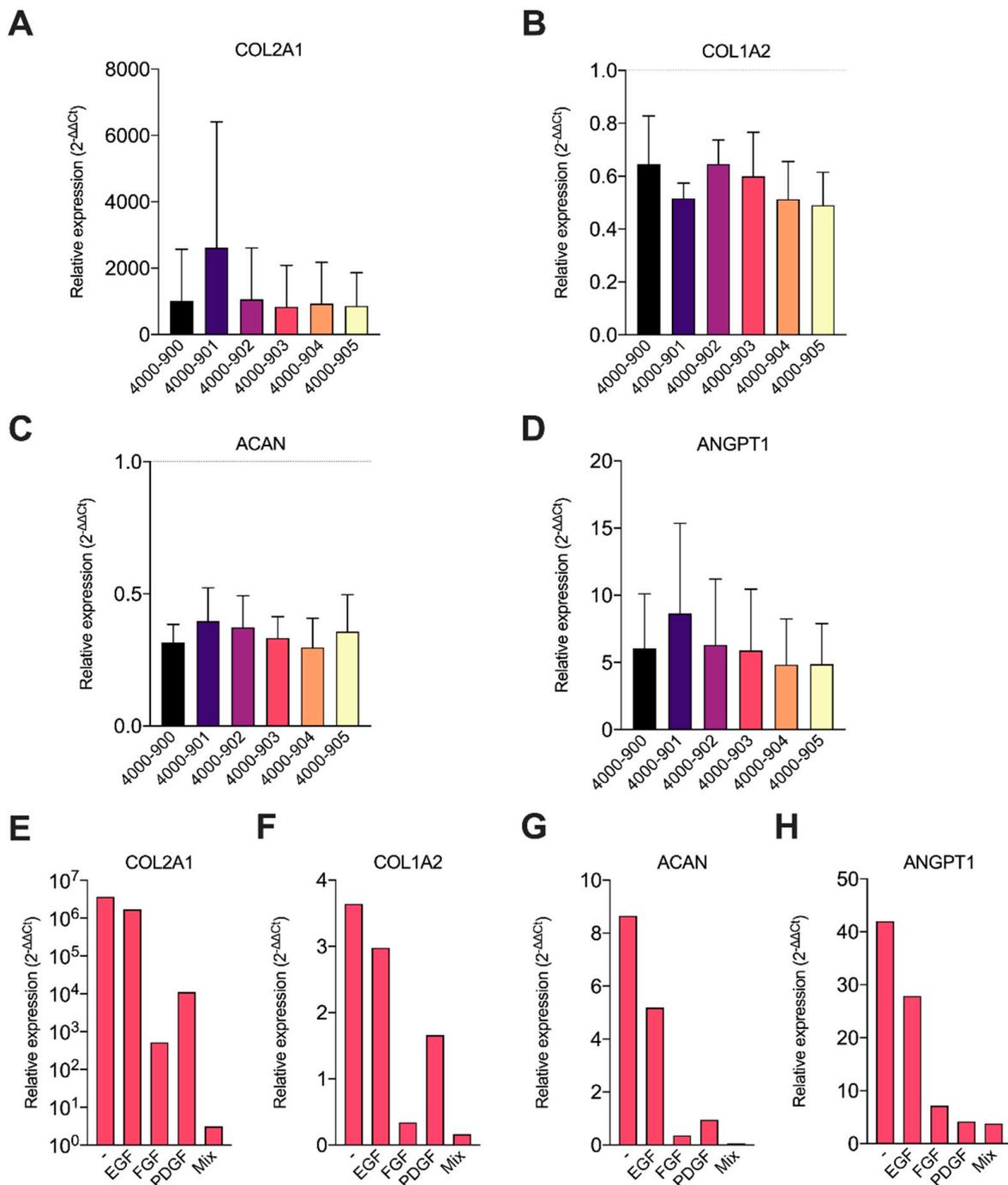


Figure 3. Relative gene expression levels of (A) COL2A1, (B) COL1A2, (C) ACAN, and (D) ANGPT1 for nucleus pulposus cell spheroid colonies cultured on different EZSPHERE® dish types. ($n = 4$) Relative gene expression of (E) COL2A1, (F) COL1A2, (G) ACAN, and (H) ANGPT1 of nucleus pulposus cell cultured on EZSPHERE® dish 4000-903 ($n = 1$) without (-) or with 100 ng/mL epidermal growth factor (EGF), 10 ng/mL fibroblast growth factor (FGF), 100 ng/mL platelet derived growth factor (PDGF), or a combination of all three growth factors (Mix). Data is calculated relative to monolayer cultured nucleus pulposus cells. Values derived using $2^{-\Delta\Delta Ct}$ method and presented as mean values with error bars presenting standard deviation.

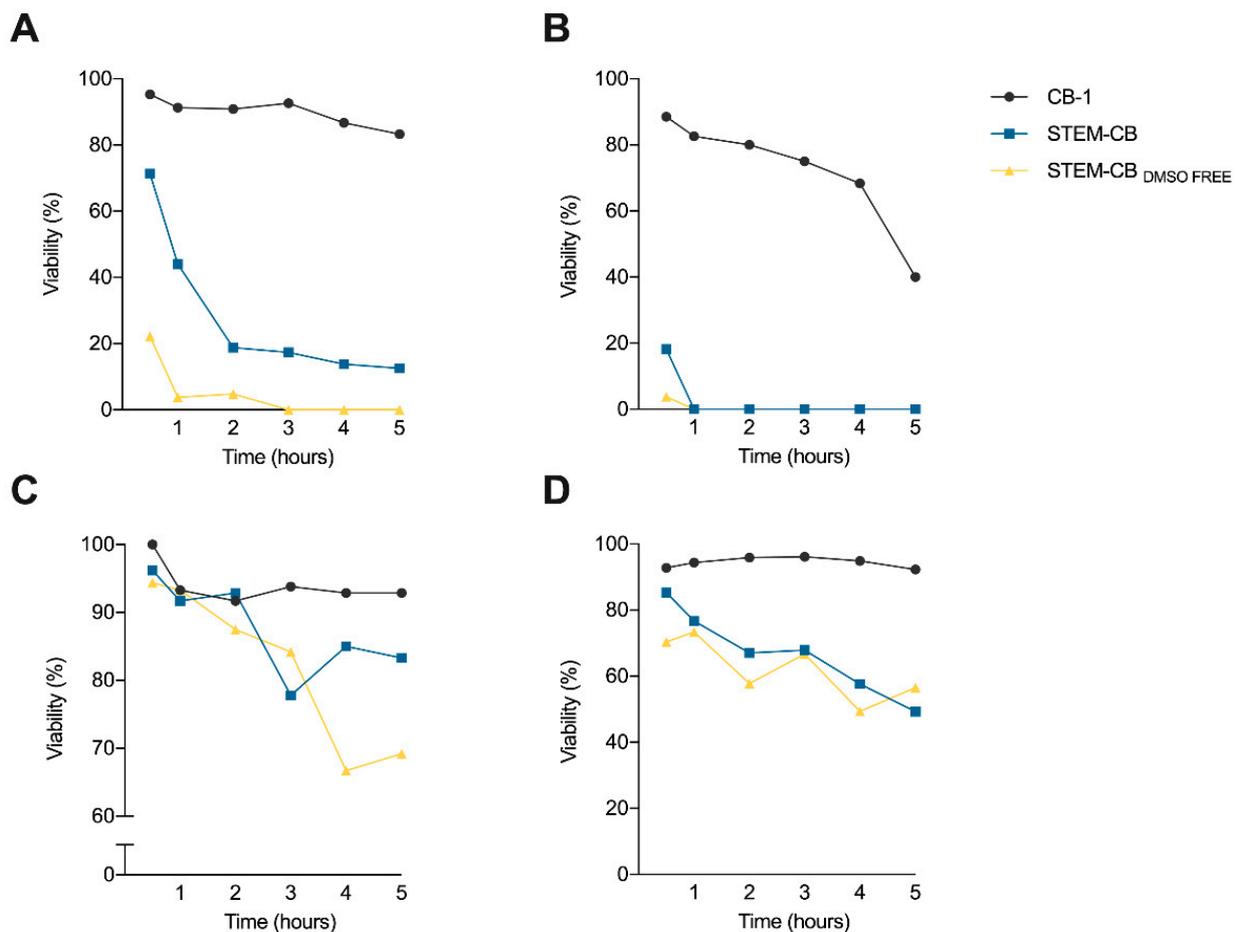


Figure 4. Cells ($n = 1$) were suspended in CellBanker-1 (CB-1), STEM-CELLBANKER (STEM-CB), or STEM-CELLBANKER without DMSO (STEM-CB_{DMSO FREE}) and kept for up to 5 h on (A,B) room temperature or kept (C,D) on ice. Cell viability tested at multiple time points either before or after 1 day of cryopreservation.

3.4. Cell Potential after Cryopreservation

Next, NP cells were exposed to cryostorage solutions (CB1, SCB, or SCBD-Free) for 3 h on ice before freezing. The cells were thawed and subsequently cultured. The cells frozen in CB1 had an average cell viability of 95.7% (Figure 4A), but the other two cryopreservation solutions resulted in considerable cell loss (Figure 5A). The average growth rate of cells frozen in CB1 was 5.9 times ($p = 0.53$), which was lower than the growth rate of cells frozen in SCB (8.93 times; Figure 5B). The Tie2- and CD24-positive rates in flow cytometry analysis showed a trend toward reduced Tie2- and CD24-expressing fractions for NP cells kept in CB 1 compared with SCBD-Free, although this difference was not significant (Figure 5C,D).

Additional investigation with a wider range of cryopreservation solutions (CB1, SCB, SCBD-Free, CS10, CP-1, and PF) revealed viability ranging from 63.6% (PC) to 84.6% (CS10) (Figure 6A). Interestingly, the Tie2-positivity rates of NP cells was 12.6% in cells in CS10, suggesting a higher Tie2 retention rate in cells in CS10 than in CB1 (Figure 6B).

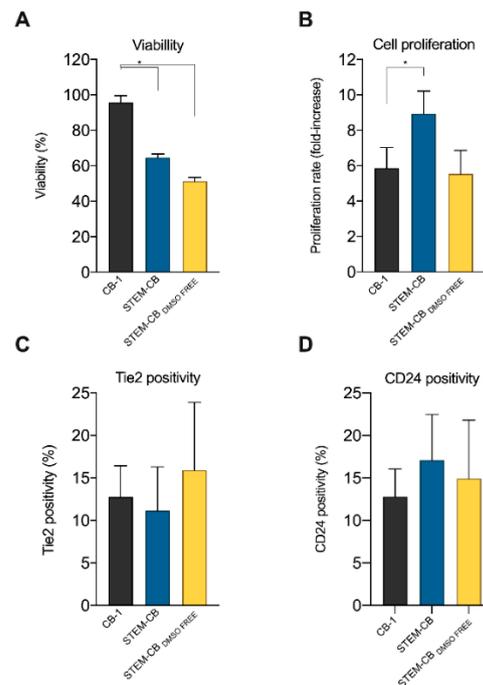


Figure 5. Cells ($n = 3$) were suspended in CellBanker-1 (CB-1), STEM-CELLBANKER (STEM-CB), or STEM-CELLBANKER without DMSO (STEM-CB_{DMSO FREE}) and kept for on ice for 3 h and frozen for 1 day. Subsequently (A) cell viability was assessed. Following cryostorage and thawing cells were seeded cultured for 5 days. The (B) fold-increase in cell numbers was analyzed, as well as the fraction of (C) Tie2 expressing and (D) CD24 expressing cells as analyzed by flow cytometry. Data is presented as mean values with error bars presenting standard deviation. * $p < 0.05$.

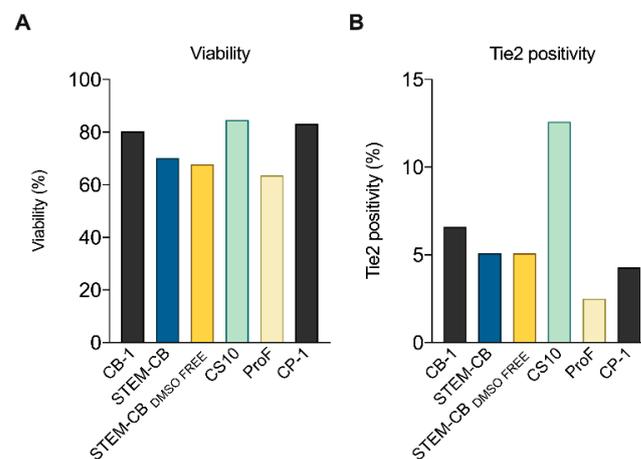


Figure 6. Comparison of 6 types of cryopreservation solutions, namely CELLBANKER 1 (CB-1), STEM-CELLBANKER GMP grade (STEM-CB), STEM-CELLBANKER DMSO Free GMP grade (STEM-CB_{DMSO FREE}), CryoStor[®] CS10 (CS10), and Pro Freeze[™]-CDM (ProF). Nucleus pulposus cells suspended in the cryopreservation solutions and kept on ice for 3 h before cryostoring them for 1 day. The cells were recovered and cultured for 5 days. Subsequently, the (A) viability rate and the (B) fraction of Tie2 positive cells were determined. Data is presented as mean values with error bars presenting standard deviation.

Finally, CS10 and CB1 were used to resuspended primary NP cells and the effects on cell viability in cells with time left at room temperature or on ice were examined. The average cell viability was 95.2% ($\pm 1.8\%$) for cells in CS10, and this value was maintained even after 5 h on ice (Figure 7A) and at 89.9% (6.5%) in cells stored at room temperature

(Figure 7B). CB1 produced similar results (Figure 6B). The average cell viability after cryopreservation and thawing also remained high: 88.4% ($\pm 4.6\%$) in cells on ice in CS10 or 87.7% ($\pm 6.0\%$) in CB1 (Figure 7C). However, when kept at room temperature, the survival rate after cryopreservation and thawing appeared to decrease to 67.5% ($\pm 29.9\%$) after 3 h in CS10 compared with 82.1% ($\pm 6.5\%$) in CB1 (Figure 7D).

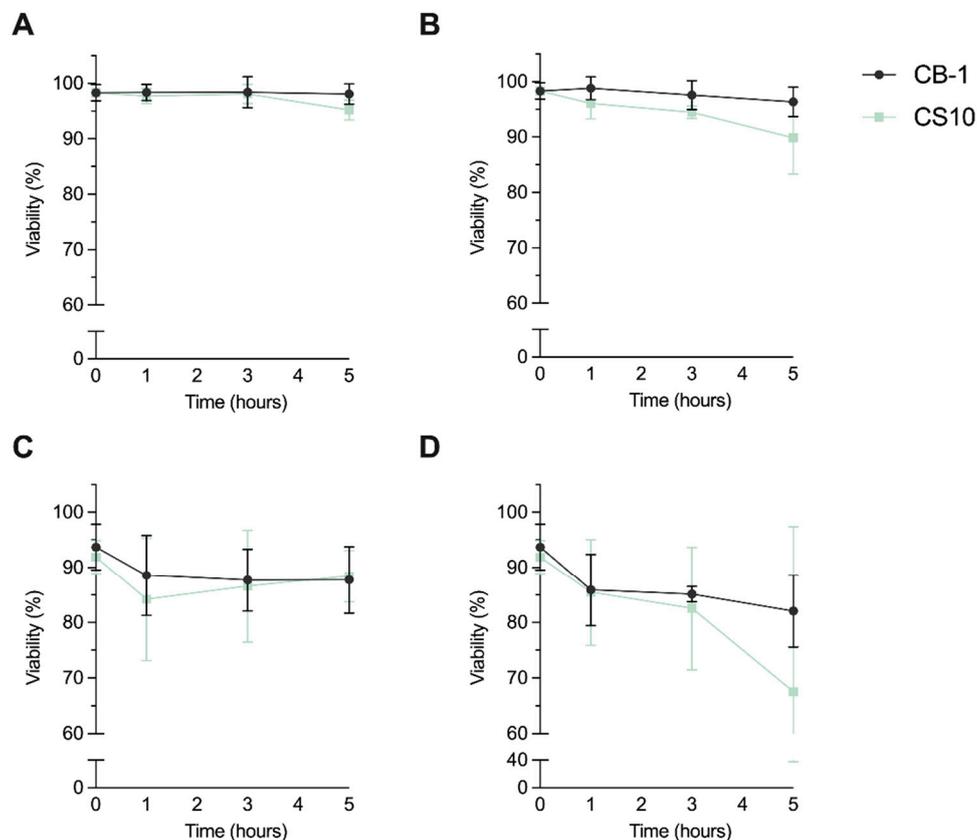


Figure 7. Cells ($n = 3$) were suspended in CellBanker-1 (CB-1) and CryoStor-10 (CS10) and kept for up to 5 h on (B,D) room temperature or (A,C) on ice. Cell viability tested at multiple time points either before or after 1 day of cryopreservation.

4. Discussion

In this study, we aimed to tackle some practical issues that potentially limit the market and clinical translation of NP progenitor cells as a regenerative cell transplantation product. First, we examined the ability of EZSPHERE cultures plates to allow large-scale colony-forming culture of NP progenitor cells. Second, we examined the effects of cryopreservation solution on NP cell viability and potency under real-world conditions.

Spheroid formation rates were highest using EZSPHERE plate type 4000-903, which has wells with a diameter of 800 μm and a depth of 400 μm . The proliferation rates were also the highest for this plate type. 3D cell cultures are used widely in research in various tissues, such as the vascular endothelium and cartilage, in an attempt to simulate the in vivo microenvironment. Better differentiation potential has been demonstrated in vitro compared with monolayer cultures of MSCs in spheroid cultures [26]. Previous work has shown that spheroid-forming NP cells have a greater ability to produce ECM [9,27] and are thus considered as promising cell types for regenerative and cellular therapeutics.

Our data support these previous findings, as EZSPHERE cultured cells exhibited a markedly higher *COL2A1* expression (on average 836-fold) compared with monolayer cultured NP cells. By contrast, *COL1A2* expression, as a negative NP marker [28], was markedly downregulated (on average 0.6-fold). The ECM produced by NP cells in human IVD tissue is classified mainly as type II collagen and proteoglycans, and IVD is not formed normally in type II collagen-deficient mice [29]. Therefore, type II collagen is

thought to play an important role in the formation of the IVD, which suggests that NP cells that can be collected by this culture method should have excellent functionality. This supports our previously stated belief that the ability for colonization is an indicator of NP functionality [7].

We were particularly interested in the potential application of EZSPHERE plates as an alternative to the current standard of methylcellulose-based CFU-s cultures [13]. Culturing in EZSPHERE plates has an additional potential advantage because the diffusion gradient of the cultured cells and solution can be controlled more closely, which should improve the reproducibility and quality control for commercialization [30]. Conversely, methylcellulose-based culture leads to the formation of spheroid colonies of different sizes and, because of the high viscosity of the culture medium, cell recovery can be complicated. By contrast, the size of colonies obtained using EZSPHERE culture relies on the well's dimension, which produces relatively uniform colony sizes (Figure 1A). Moreover, the colonies are not maintained in a hydrogel, and the recovery of large quantities of colonies is much easier.

The EZSPHERE culture systems offer a promising culture device that could be scalable for production of CFU-s NP cells. As a goal for commercialization, for example, cells from one sample must be extracted and available for at least 100 people. This is important because IVD cell therapy products generally involve the transplantation of a total of $1\text{--}100 \times 10^6$ cells per disc (although an optimal concentration has not been determined) (15). For example, in our previous clinical trial [16], we obtained an average of 1.24×10^6 primary cells/7.92 g tissue per patient donor material (Table 3). Assuming that a cell therapy product requires 1×10^6 NP cells, ~100 times the cell proliferation effect is required. However, with the EZSPHERE method alone, the cell proliferation rate was 7.75-fold in our study, which would mean that only nine patients on average could be treated with cells from a single-batch production following EZSPHERE culture methods. Our data in the present study show that growth factor supplementation can markedly increase the NP cell proliferation rate. These findings are consistent with previous work showing that FGF2 has a Tie2 expression-enhancing effect on NP cells [14]. In our study, FGF2 supplementation had positive effect by increasing the cell proliferation rate by up to 62.5%. This would make it possible to manufacture NP cell products for about 77 patients from a single tissue sample. However, cells cultured with other growth factors showed a marked decrease expression of *COL2A1* and *ACAN*, which may be a disadvantage compared with the original colony assay culture. Further analysis and in vivo experimentation are needed to elucidate these effects.

Table 3. Details of previous Mochida et al. clinical trial of autologous NP cell transplantation. In the clinical trial of Mochida et al. [16], surgically removed NP tissue and obtained cells were cultured and administered to prevent adjacent degenerative disc disease. The average time required to recover the transplanted cells was 122.9 min using a similar procedure in this study. IVD: intervertebral disc, NPC: nucleus pulposus cell.

	Age	Sex	IVD Tissue Amount (g)	Number of Collected NPC	Number of Cultured NPC	Cultured Cell Recovery Time (min)
1	21	M	8.2	1.25×10^6	7.2×10^6	115
2	28	M	4	0.93×10^6	5.85×10^6	113
3	28	M	10.9	3.42×10^6	10.80×10^6	105
4	27	M	8.9	1.20×10^6	5.8×10^6	174
5	29	M	3.6	0.71×10^6	3.36×10^6	100
6	28	M	7.7	1.0×10^6	3.02×10^6	120
7	21	M	11.9	1.05×10^6	2.88×10^6	120
8	29	M	6.6	0.5×10^6	2.48×10^6	125
9	21	F	9.1	0.93×10^6	2.63×10^6	115
10	25	M	8.3	1.43×10^6	2.80×10^6	142

The different types of cryopreservation solutions and exposure times used in the cryopreservation of cellular products are thought to affect the product quality [24]. Especially in undifferentiated cells such as MSCs, the maintenance and prevention of phenotype loss and changes in metabolism during storage are important. Reports have suggested inadequate therapeutic results can arise because of the effects of cryopreservation in clinical trials using MSCs [31,32]. Given our aim to deliver multipotent, undifferentiated NP progenitor cells with high Tie2 positivity, concerns were raised about the effects of cryopreservation on NP cells and their potency. In the trial reported by Mochida et al. [16], the collection of cultured cells before their administration in the operating room was performed in a similar fashion as in our experiments; that is, the NP cells were cryopreserved before transplantation.

The purpose of the current study was to identify the most appropriate cryopreservation solutions and freezing procedures by investigating their effects on NP cells over time. Of the 10 patients treated in the previous clinical trial [16], an average of 4.6×10^6 NP cells were administered following harvest from their respective coculture conditions. At the time of surgery, the cryostored NP cells were recovered, thawed, and transported from a cell-processing center to the operating room, and the waiting time before transplantation was on average 122.9 min, which meant that the cells were exposed to the cryopreserving agents for about 2 h. Our current results show that NP cells subjected to cryopreserving agents for up to 3 h had survival rates of $\leq 70\%$ in SCB and SCBD-Free. Interestingly, cells kept on ice in CB1 retained a cell viability of 90% for up to 5 h.

Although CB1 has been shown to be optimal, for clinical translation, it is desirable to preserve and administer cells in solutions devoid of animal-derived serum or DMSO. Therefore, we examined six additional commercial cryopreserving preparations. We found that cell viability after thawing was maintained at $\geq 80\%$ with CB1, CS10, and CP-1. CS10 was also able to maintain a high Tie2-expressing fraction. In addition, CS10 is an animal component-free GMP-certified formula, making it potentially safer to administer directly to patients than CB1 when used as an OTS [23]. However, SCB is a product with an identical composition as CB1, but without animal components, and this resulted in a sharp decrease in viability at room temperature or on ice with time. Moreover, the CS10 formula does contain 10% DMSO, which showed a decrease in the number of cells after an incubation of ≥ 3 h incubation, which suggests that the collection and freezing of harvested cells should be performed in the shortest possible time. If we select one type of cell preservation solution for industrialization, CS10 is considered to be most optimal because it showed similar levels of cell viability maintenance as CB1 but does not contain any animal components.

The limitation of our study is that procurement of human-derived cell sources was limited, and some experiments had a limited sample size. Moreover, in this experiment, the cryopreservation period was only 24 h and long-term storage might have a more impactful effect on NP cells. Still, freezing and thawing process is likely the most important and damaging phase in cryopreservation, as such we believe this experiment is sufficient to compare the usefulness of solutions. Additionally, we did not further assess the impact of cryopreservatives on NP cell potency. Furthermore, a recent study by Croft et al. suggested that the type of cryopreservation medium, including CS10, had limited effects on NP cell multipotency [33]. The work by Hiraishi et al. suggested no effect on the *in vivo* regenerative potency of NP cells [24]. However, the effects on the potentially more sensitive Tie2 cells remains unclear [13]. Further advances in culture, storage, and logistics for NP cell-based cell therapeutics are needed to expand their potential as effective and marketable products in the clinic [17,25].

5. Conclusions

EZSPHERE culture plates enabled us to examine colony formation in cell culture without the use of methylcellulose or other hydrogel products. EZSPHERE culture methods produced high colony formation and increased proliferation rates, which could be augmented by FGF supplementation. Continued examination of the optimal culture methods is required for further development and commercialization. The derived cells should be

assessed in vivo for their potential as a regenerative medicinal product. Our study showed that CS10 is presently, the best cryopreservation medium for retaining NP progenitor cell phenotype and viability when cells are kept on ice or even at room temperature for up to 3 h. Our findings provide information that may be useful for the translation of NP cell-based therapeutics to the clinic.

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References

1. Strine, T.W.; Hootman, J.M. US national prevalence and correlates of low back and neck pain among adults. *Arthritis Rheum.* **2007**, *57*, 656–665. [[CrossRef](#)]
2. Vos, T.; Allen, C.; Arora, M.; Barber, R.M.; Bhutta, Z.A.; Brown, A.; Carter, A.; Casey, D.C.; Charlson, F.J.; Chen, A.Z.; et al. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: A systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **2016**, *388*, 1545–1602. [[CrossRef](#)]
3. Oichi, T.; Taniguchi, Y.; Oshima, Y.; Tanaka, S.; Saito, T. Pathomechanism of intervertebral disc degeneration. *JOR Spine* **2020**, *3*, e1076. [[CrossRef](#)]
4. Frost, B.A.; Camarero-Espinosa, S.; Foster, E.J. Materials for the Spine: Anatomy, Problems, and Solutions. *Materials* **2019**, *12*, 253. [[CrossRef](#)]
5. O'Halloran, D.M.; Pandit, A.S. Tissue-engineering approach to regenerating the intervertebral disc. *Tissue Eng.* **2007**, *13*, 1927–1954. [[CrossRef](#)]
6. Antoniou, J.; Steffen, T.; Nelson, F.; Winterbottom, N.; Hollander, A.P.; Poole, R.A.; Aebi, M.; Alini, M. The human lumbar intervertebral disc: Evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, ageing, and degeneration. *J. Clin. Investig.* **1996**, *98*, 996–1003. [[CrossRef](#)]
7. Weiler, C.; Nerlich, A.G.; Zipperer, J.; Bachmeier, B.E.; Boos, N. 2002 SSE Award Competition in Basic Science: Expression of major matrix metalloproteinases is associated with intervertebral disc degradation and resorption. *Eur. Spine J.* **2002**, *11*, 308–320. [[CrossRef](#)]
8. Le Maitre, C.L.; Freemont, A.J.; Hoyland, J.A. Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. *J. Pathol.* **2004**, *204*, 47–54. [[CrossRef](#)] [[PubMed](#)]
9. Sakai, D.; Nakamura, Y.; Nakai, T.; Mishima, T.; Kato, S.; Grad, S.; Alini, M.; Risbud, M.V.; Chan, D.; Cheah, K.S.; et al. Exhaustion of nucleus pulposus progenitor cells with ageing and degeneration of the intervertebral disc. *Nat. Commun.* **2012**, *3*, 1264. [[CrossRef](#)] [[PubMed](#)]
10. Guerrero, J.; Häckel, S.; Croft, A.S.; Albers, C.E.; Gantenbein, B. The effects of 3D culture on the expansion and maintenance of nucleus pulposus progenitor cell multipotency. *JOR Spine* **2020**, *4*, e1131.
11. Ishii, T.; Sakai, D.; Schol, J.; Nakai, T.; Suyama, K.; Watanabe, M. Sciatic nerve regeneration by transplantation of in vitro differentiated nucleus pulposus progenitor cells. *Regen. Med.* **2017**, *12*, 365–376. [[CrossRef](#)]
12. Li, X.C.; Tang, Y.; Wu, J.H.; Yang, P.S.; Wang, D.L.; Ruan, D.K. Characteristics and potentials of stem cells derived from human degenerated nucleus pulposus: Potential for regeneration of the intervertebral disc. *BMC Musculoskelet. Disord.* **2017**, *18*, 242. [[CrossRef](#)] [[PubMed](#)]
13. Sakai, D.; Schol, J.; Bach, F.C.; Tekari, A.; Sagawa, N.; Nakamura, Y.; Chan, S.C.W.; Nakai, T.; Creemers, L.B.; Frauchiger, D.A.; et al. Successful fishing for nucleus pulposus progenitor cells of the intervertebral disc across species. *JOR Spine* **2018**, *1*, e1018. [[CrossRef](#)] [[PubMed](#)]
14. Tekari, A.; Chan, S.C.W.; Sakai, D.; Grad, S.; Gantenbein, B. Angiopoietin-1 receptor Tie2 distinguishes multipotent differentiation capability in bovine coccygeal nucleus pulposus cells. *Stem Cell Res. Ther.* **2016**, *7*, 75. [[CrossRef](#)]

15. Schol, J.; Sakai, D. Cell therapy for intervertebral disc herniation and degenerative disc disease: Clinical trials. *Int. Orthop.* **2019**, *43*, 1011–1025. [[CrossRef](#)] [[PubMed](#)]
16. Mochida, J.; Sakai, D.; Nakamura, Y.; Watanabe, T.; Yamamoto, Y.; Kato, S. Intervertebral disc repair with activated nucleus pulposus cell transplantation: A three-year, prospective clinical study of its safety. *Eur. Cell Mater.* **2015**, *29*, 202–212, discussion 212. [[CrossRef](#)]
17. Sakai, D.; Schol, J. Cell therapy for intervertebral disc repair: Clinical perspective. *J. Orthop. Translat.* **2017**, *9*, 8–18. [[CrossRef](#)]
18. Gruber, H.E.; Ingram, J.A.; Norton, H.J.; Hanley, E.N., Jr. Senescence in cells of the aging and degenerating intervertebral disc: Immunolocalization of senescence-associated beta-galactosidase in human and sand rat discs. *Spine* **2007**, *32*, 321–327. [[CrossRef](#)]
19. Jiang, L.; Zhang, X.; Zheng, X.; Ru, A.; Ni, X.; Wu, Y.; Tian, N.; Huang, Y.; Xue, E.; Wang, X.; et al. Apoptosis, senescence, and autophagy in rat nucleus pulposus cells: Implications for diabetic intervertebral disc degeneration. *J. Orthop. Res.* **2013**, *31*, 692–702. [[CrossRef](#)]
20. Zhang, X.; Guerrero, J.; Croft, A.S.; Albers, C.E.; Häckel, S.; Gantenbein, B. Spheroid-Like Cultures for Expanding Angiopoietin Receptor-1 (aka. Tie2) Positive Cells from the Human Intervertebral Disc. *Int. J. Mol. Sci.* **2020**, *21*, 9423. [[CrossRef](#)]
21. Frauchiger, D.A.; Tekari, A.; May, R.D.; Dzafo, E.; Chan, S.C.W.; Stoyanov, J.; Bertolo, A.; Zhang, X.; Guerrero, J.; Sakai, D.; et al. Fluorescence-Activated Cell Sorting Is More Potent to Fish Intervertebral Disk Progenitor Cells Than Magnetic and Beads-Based Methods. *Tissue Eng. Part C Methods* **2019**, *25*, 571–580. [[CrossRef](#)]
22. Tanaka, M.; Sakai, D.; Hiyama, A.; Arai, F.; Nakajima, D.; Nukaga, T.; Nakai, T.; Mochida, J. Effect of cryopreservation on canine and human activated nucleus pulposus cells: A feasibility study for cell therapy of the intervertebral disc. *Biores. Open Access* **2013**, *2*, 273–282. [[CrossRef](#)]
23. Hiraishi, S.; Schol, J.; Sakai, D.; Nukaga, T.; Erickson, I.; Silverman, L.; Foley, K.; Watanabe, M. Discogenic cell transplantation directly from a cryopreserved state in an induced intervertebral disc degeneration canine model. *JOR Spine* **2018**, *1*, e1013. [[CrossRef](#)]
24. Morris, T.J.; Picken, A.; Sharp, D.M.C.; Slater, N.K.H.; Hewitt, C.J.; Coopman, K. The effect of Me(2)SO overexposure during cryopreservation on HOS TE85 and hMSC viability, growth and quality. *Cryobiology* **2016**, *73*, 367–375. [[CrossRef](#)]
25. Smith, L.J.; Silverman, L.; Sakai, D.; Le Maitre, C.L.; Mauck, R.L.; Malhotra, N.R.; Lotz, J.C.; Buckley, C.T. Advancing cell therapies for intervertebral disc regeneration from the lab to the clinic: Recommendations of the ORS spine section. *JOR Spine* **2018**, *1*, e1036. [[CrossRef](#)] [[PubMed](#)]
26. Yamaguchi, Y.; Ohno, J.; Sato, A.; Kido, H.; Fukushima, T. Mesenchymal stem cell spheroids exhibit enhanced in-vitro and in-vivo osteoregenerative potential. *BMC Biotechnol.* **2014**, *14*, 105. [[CrossRef](#)]
27. Lee, J.Y.; Hall, R.; Pelinkovic, D.; Cassinelli, E.; Usas, A.; Gilbertson, L.; Huard, J.; Kang, J. New use of a three-dimensional pellet culture system for human intervertebral disc cells: Initial characterization and potential use for tissue engineering. *Spine* **2001**, *26*, 2316–2322.
28. Risbud, M.V.; Schoepflin, Z.R.; Mwale, F.; Kandel, R.A.; Grad, S.; Iatridis, J.C.; Sakai, D.; Hoyland, J.A. Defining the phenotype of young healthy nucleus pulposus cells: Recommendations of the Spine Research Interest Group at the 2014 annual ORS meeting. *J. Orthop. Res.* **2015**, *33*, 283–293. [[CrossRef](#)] [[PubMed](#)]
29. Aszodi, A.; Chan, D.; Hunziker, E.; Bateman, J.F.; Fassler, R. Collagen II is essential for the removal of the notochord and the formation of intervertebral discs. *J. Cell Biol.* **1998**, *143*, 1399–1412. [[CrossRef](#)] [[PubMed](#)]
30. Silverman, L.I.; Flanagan, F.; Rodriguez-Granrose, D.; Simpson, K.; Saxon, L.H.; Foley, K.T. Identifying and Managing Sources of Variability in Cell Therapy Manufacturing and Clinical Trials. *Regen. Eng. Transl. Med.* **2019**, *5*, 354–361. [[CrossRef](#)]
31. Francois, M.; Copland, I.B.; Yuan, S.; Romieu-Mourez, R.; Waller, E.K.; Galipeau, J. Cryopreserved mesenchymal stromal cells display impaired immunosuppressive properties as a result of heat-shock response and impaired interferon-gamma licensing. *Cytotherapy* **2012**, *14*, 147–152. [[CrossRef](#)] [[PubMed](#)]
32. Moll, G.; Alm, J.J.; Davies, L.C.; von Bahr, L.; Heldring, N.; Stenbeck-Funke, L.; Hamad, O.A.; Hinsch, R.; Ignatowicz, L.; Locke, M.; et al. Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem Cells* **2014**, *32*, 2430–2442. [[CrossRef](#)] [[PubMed](#)]
33. Croft, A.S.; Guerrero, J.; Oswald, K.A.C.; Häckel, S.; Albers, C.E.; Gantenbein, B. Effect of different cryopreservation media on human nucleus pulposus cells' viability and trilineage potential. *JOR Spine* **2021**, *4*, e1140. [[CrossRef](#)] [[PubMed](#)]