applied sciences

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Screening for Growth-Factor Combinations Enabling Synergistic Differentiation of Human MSC to Nucleus Pulposus Cell-Like Cells

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Abstract: Background: Multiple studies have examined the potential of growth factors (GF) to enable mesenchymal stromal cells (MSC) to nucleus pulposus (NP) cell-like cell differentiation. Here we screened a wide range of GF and GF combinations for supporting NP cell-like cell differentiation. **Methods:** Human MSC were stimulated using 86 different GF combinations of TGF-β1, -2, -3, GDF5, -6, Wnt3a, -5a, -11, and Shh. Differentiation potency was assessed by alcian blue assay and NP cell marker expression (e.g., COL2A1, CD24, etc.). The top four combinations and GDF5/TGF-β1 were further analyzed in 3D pellet cultures, on their ability to similarly induce NP cell differentiation. **Results:** Almost all 86 GF combinations showed their ability to enhance proteoglycan production in alcian blue assay. Subsequent qPCR analysis revealed TGF-β2/Wnt3a, TGF-β1/Wnt3a, TGF-β1/Wnt3a/GDF6, and Wnt3a/GDF6 as the most potent combinations. Although in pellet cultures, these combinations supported NP marker expression, none showed the ability to significantly induce chondrogenic NP matrix production. Only GDF5/TGF-β1 resulted in chondrogenic pellets with significantly enhanced glycosaminoglycan content. **Conclusion:** GDF5/TGF-β1 was suggested as an optimal GF combination for MSC to NP cell induction, although further assessment using a 3D and in vivo environment is required. Wnt3a proved promising for monolayer-based NP cell differentiation, although further validation is required.

Keywords: nucleus pulposus; growth factors; differentiation; mesenchymal stromal cell; growth factor; Wnt; differentiation factor; chondrogenesis; cell therapy

Citation: Morita, K.; Schol, J.; Volleman, T.N.E.; Sakai, D.; Sato, M.; Watanabe, M. Screening for Growth-Factor Combinations Enabling Synergistic Differentiation of Human MSC to Nucleus Pulposus Cell-Like Cells. *Appl. Sci.* **2021**, *11*, 3673. https://doi.org/10.3390/app11083673

Academic Editor: Antonio Scarano

Received: 28 February 2021
Accepted: 15 April 2021
Published: 19 April 2021

1. Introduction

The primary causes of disability worldwide are low back and neck pain [1]. Despite their prevalence and socioeconomic impact, long-term curative treatments for these disorders are lacking. Novel approaches are being explored to resolve this issue, mainly focusing on alleviation or reversal of intervertebral disc (IVD) degeneration [2–4]. IVD degeneration is a progressive pathology, involving a decrease in cell numbers and cell potency, primarily within the central nucleus pulposus (NP) of the IVD, resulting in a switch in extracellular matrix (ECM) production [5,6]. Specifically, the initially proteoglycan-rich and type II collagen NP-ECM progressively turns into a type I collagen fibrotic tissue, undermining the IVD's biomechanical characteristics [7]. The responding loss in IVD integrity alters disc height, requiring compensation in other spinal components, thereby potentially leading to a variety of spinal disorders such as facet joint arthritis and spondylolisthesis. Alternatively,

the outer collagen layers, collectively termed the annulus fibrosus (AF), can similarly lose their integrity, thereby enabling disc bulging or complete AF rupture. Finally, in reaction to these degenerative changes, the native IVD cells secrete inflammatory factors, potentially stimulating nerve ingrowth, vascularization, and immunogenic cell influx in the normally avascular and un-innervated discs or sensitizing nearby nerve structures [8–10]. Collectively, these changes can trigger nociception and in severe cases, might result in debilitating pain and disability in patients [11,12].

This vicious cycle of disc degeneration [6] has proven difficult to combat in a clinical setting. Conservative treatment generally involves pain medication or muscle relaxants, while surgical intervention at later stages of degeneration, involves removal of disc tissue (e.g., microdiscectomy) or replacement of the complete disc (e.g., arthrodesis or arthroplasty). These final stage interventions are costly, invasive, and come with a range of complications such as adjacent disc disease, surgical infections, etc. [13,14]. Moreover, their effectiveness in alleviating pain also remains to be confirmed [15]. Moreover, a large treatment gap exists for disc degeneration associated with LBP in the mild to moderate range, for patients for which analgesics proved ineffective but are not yet indicated for surgery.

The field of regenerative medicine promises a wide range of therapeutics that have been theorized to be effective against disc degeneration. A variety of therapeutic strategies are being explored, each aimed to support cells to regenerate the IVD, e.g., gene therapy [16], growth factor injection [17], biomaterial injection [18,19], or tissue engineering [20,21]. Farthest in its clinical development is cell therapy, a regenerative strategy in which *de novo* cells are introduced directly into the IVD [2,3]. There, transplanted cells can either integrate within the IVD and contribute to appropriate ECM production, or otherwise modulate endemic cells to change the catabolic/inflammatory state to a more anabolic one. Multiple animal studies [22] have suggested the ability to modulate disc degeneration and induce regeneration of its matrix composition [23,24] and inflammatory environment [25]. In the last decade, a multitude of clinical trials reported initial results, confirming the safety of cell therapy and have suggested their ability to reduce pain and disability [2]. However, large-scale placebo-controlled trials are still highly anticipated [2].

The primary cell source being investigated for IVD regeneration are MSC [26]. MSC are advantageous due to their accessibility, expandability, and suggested limited immunogenicity [27]. These, in contrast to NP cells or other chondrogenic cells, which lose their potency during culture [28] and have limited and compromised tissue sources that present low cell yields [29]. Furthermore, MSC possess immunomodulatory features, as well as multipotent differentiation capacity, and could thus potentially target IVD degeneration from two approaches. Nevertheless, the hypoxic and acidic IVD environment is foreign to MSC and questions remain about their ability to survive and thrive in such a demanding niche [30,31]. A recent report from the Mesoblast phase II/III clinical trial suggested their MSC-based product was able to alleviate some pain-associated outcomes, but no regenerative changes were observed on MRI modalities [32]. As such, a range of studies has attempted to induce the differentiation of MSC toward an NP cell-like cell phenotype. MSC can be subjected to environmental conditions [33], hydrogel systems [34,35], genetic manipulation [36,37], etc.; aimed to induce more NP cell-like features. Particularly, the application of growth and differentiation factors to support differentiation towards an NP cell-like cell, as indicated by enhanced proteoglycan production rates or enhanced NP cell marker expression levels; e.g., aggrecan, type II collagen, Brachyury, and CD24 [38]. Multiple growth factors are suggested to be able to support NP cell differentiation, including Wnt3a [39], Wnt5a [40], TGF-β1 [41], TGF-β3 [42], GDF5 [43], and GDF6 [44]. Very few bodies of work have compared different growth factor potency for NP cell differentiation. One study by Clarke et al. [44] suggested GDF6 media supplementation optimal for MSC to NP cell differentiation, following assessment of GDF5, GDF6, or TGF-β1 media supplementation. Moreover, work by Hodgkinson et al. revealed quite eloquently, the potential of GDF6 to differentiate adipose stem cells toward NP cell-like cells [45], and

subsequently highlighted a requirement of the SMAD1/5/8 pathway [46]. Alternatively, work from Colombier et al. [47] revealed the ability of growth factors to synergistically enhance NP cell differentiation. In their work, they found that only combined application of TGF-β1 and GDF5 was able to create MSC that formed fully chondrogenic pellets, as well as stringently enhance expression of NP cell markers; e.g., ACAN, COL2A1, CD24, PAX1, and OVOS2. Review work has also suggested the application of both Wnt3a or Wnt5a as promising chondrogenic factors, particularly combining Wnt3a with other chondrogenic factors was speculated to hold potential [48]. A wide range of factors has been proposed to enable MSC to NP cell differentiation. However, a large-scale assessment or screening to determine which factors, or even more, which combination of factors, are most potent to induce NP cell features, has not been reported. To optimize growth factor-mediated MSC differentiation, particularly for the creation of cell therapy products, we initiated this study to screen a wide range of growth factor combinations to assess which combination showed most potent in inducing proteoglycan production and NP cell-associated expression levels as indicators of MSC to NP cell-like cell differentiation.

2. Materials and Methods

Study protocols and design, which involve the collection and application of human tissue samples, were reviewed and approved by the institutional ethics review committee of the Tokai University School of Medicine. (application number No. 06I-49 and 16R-051). Informed consent was obtained for the collection and usage of tissue materials from the respective patients.

2.1. Cell Isolation and Culture

As part of adult spinal fusion surgery procedures, bone marrow cells were collected and isolated using gradient centrifugation as previously described [49]. The cells were collected and cryopreserved in a commercial cryopreservation medium (Cellbanker®-1, Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan) until further usage. At the start of the experiment, the mononuclear cells were rapidly thawed, gently washed, and seeded in αMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 20% (*v*/*v*) FBS (Sigma-Aldrich, St. Louis, MO, USA), 1% (*v*/*v*) Penicillin/Streptomycin (P/S; 10,000 U/mL; Gibco, Gaithersburg, MD, USA) and cultured for 2 weeks uninterrupted at approximately 21% O_2, 5% CO_2 at 37 °C. The unattached cells were aspirated, and media was refreshed. Alternatively, MSC were obtained commercially from LONZA Group AG (Basel, Switzerland) and similarly cultured. MSC were further expanded up to a confluency of ~80% upon which 0.25% Trypsin/EDTA (Thermo-Fisher, Waltham, MA, USA) mediated passaging was performed.

Additionally, human NP cells were obtained from surgical tissue material during lumbar disc herniation microdiscectomy. NP cells were isolated as previously described [29]. In brief, IVD tissue was collected in saline and macroscopically glossy NP tissue was separated. The tissues were fragmented by a scalpel and digested in Tryple Express (Thermo-Fisher) for 1 h. The subsequent suspension was further digested in collagenase P solution. The resulting cell suspension was filtered and cultured at 2–5% O_2, 5% CO_2 within 10% (*v*/*v*) FBS, 1% (*v*/*v*) P/S, αMEM (Thermo-Fisher).

2.2. Growth Factor-Induced Differentiation Screening

Differentiation assays in monolayer cultures were performed in Dulbecco's Modified Eagle Medium (DMEM; Wako Pure Chemical Industries) containing 10% (*v*/*v*) FBS and 1% (*v*/*v*) P/S, 50 nM Ascorbic Acid (Wako Pure Chemical Industries), 1% (*v*/*v*) Insulin, Transferrin, Selenium, Ethanolamine Solution (ITS-X; Beckton Dickinson, Franklin Lakes, NJ, USA), and 10 nM dexamethasone (Sigma-Aldrich), further referred to as differentiation medium [47]. Differentiation medium was further supplemented with different combinations of TGFβ-1 (Peprotech, Rocky Hill, NJ, USA), TGFβ-2 (Peprotech), and TGFβ-3 (Peprotech) at concentrations of 10 ng mL^{-1} following the work of Colombier et al. [47]

and Clarke et al. [44], GDF5 (Peprotech) and GDF6 (Peprotech) at concentrations of 100 ng mL^{-1} following the work of Colombier et al. [47] and Clarke et al. [44], Wnt3a (Peprotech) at a concentration of 250 ng mL^{-1}, Wnt5a (Peprotech) at a concentration of 50 ng mL^{-1} based on the work of Gibson et al. [50], Wnt11 (Peprotech) at a concentration of 50 ng mL^{-1}, and Sonic hedgehog (Shh; Peprotech) at a concentration of 100 ng mL^{-1}. Maximally three growth factors were combined. A negative sample, involving identically cultured MSC without growth factor supplementation was included. The induced MSC were cultured under 2% O_2 at 37 °C culture environment, with media replenishment every 2–3 days.

2.3. Alcian Blue Assay for Quantification of Glycosaminoglycan Content

Alcian blue assay was performed in triplicate by seeding unstimulated MSC (22-year-old) at a density of 6000 cells/cm^2 on a 96-wells plate and cultured with differentiation media supplemented with indicated growth factor combinations for 3 weeks. Subsequently, the cells were fixed in 10% formaldehyde (Wako Pure Chemical Industries) for 15 min at room temperature. Cells were thoroughly washed and subjected to 50 µL 1% (*w*/*v*) alcian blue (MERCK & Co., Kenilworth, NJ, USA), 0.1 M HCL for 60 min. Stained samples were thoroughly rinsed and digested overnight by 150 µL 6 M Guanidine (WAKO) HCL. The resulting samples were analyzed by measurement of absorbance at 650 nm using SpectraMax® i3 (Molecular Devices®, San Jose, CA, USA). Absorbance values were calculated relative to the negative control of MSC cultured without growth factors.

2.4. Chondrogenic Pellet Cultures

For chondrogenic pellet cultures, MSC (n = 2; 23- and 31-year-old) were seeded at a density of 3000 cells/cm^2 in T-75 culture flasks (IWAKI, Japan) with αMEM containing 20% FBS, 1% P/S. Upon 70–80% confluency, MSC were harvested and aliquoted at 2.5×10^5 cells per 15 mL polypropylene falcon tubes (Corning Inc., Midland, NC, USA). Chondrogenic pellet culture was initiated by spinning down the tubes at 500 g for 5 min. The resulting cellular pellet was cultured for 1 day at 21% O_2 at 37 °C. The next day, the medium was replaced by differentiation media as previously described. Based on the alcian blue assay the growth factor combinations; GDF5/TGF-β1, TGF-β2/Wnt3a, TGF-β1/Wnt3a, TGF-β1/Wnt3a/GDF6, GDF6/Wnt3a were applied. As a negative control, unstimulated MSC were identically cultured but were not stimulated with growth factors. The pellets were incubated with media replenishment thrice a week, at 37 °C under 5% CO_2 and hypoxic conditions of 2% O_2. Additionally, human NP cells obtained from surgical waste material were similarly cultured in differentiation media without growth factors (n = 3).

2.5. Wnt3a Mediated Pre-Conditioning

A recent literature review [48] suggested the potential ability of Wnt3a to maintain the differentiation potency of MSC as opposed to inducing chondrogenic differentiation, which could help explain the discrepancies in monolayer and pellet culture outcomes. To test this hypothesis, we applied Wnt3a at 250 ng mL^{-1} during the MSC (n = 1, 23-year-old) expansion culture and subsequently subjected these cells to chondrogenic pellet culture supplemented with GDF5/TGF-β1 or commercial pre-defined Mesenchymal Stem Cell Chondrogenic Differentiation Medium (Chon.M.: PromoCell, Heidelberg, Germany).

2.6. PicoGreen Assay for Quantification of DNA Content

Following 3 weeks of chondrogenic pellet culture, cells were washed and subjected to overnight papain digestion solution (125 µg mL^{-1} Papain-enzyme (Sigma-Aldrich), 8 mg mL^{-1} sodium acetate (Wako Pure Chemical Industries), 4 mg mL^{-1} Ethylene diamine tetra acetic acid disodium salt (EDTA2Na; Sigma-Aldrich), 0.8 mg mL^{-1} L-Cysteine (Sigma-Aldrich) 0.2 sodium phosphate buffer (pH 6.4)) at 65 °C. The resulting suspension was centrifuged for 10 min at 8400 rpm, after which the supernatant was collected and stored at −80 °C until further use. Thereafter, a fraction of the suspensions were analyzed using a QUANT-IT PicoGreen Kit (ThermoFisher) following the manufacturer's instruction. Briefly,

the suspension was transferred to a 96-well assay microplate (black, transparent bottom; Corning Inc.). 100 µL of PicoGreen solution was added to each sample, and agitated in an environment devoid of light, for 3 min. 520 nm fluorescence emission was measured under 480 nm excitation using a SpectraMax® i3. DNA concentrations were determined by interpolating the fluorescence values from a standard curve obtained by adding solutions with known concentrations of DNA.

2.7. DMMB Assay for Glycosaminoglycan Content

Glycosaminoglycan content was measured by 1,9-dimethyl methylene blue (DMMB) staining, using the Glycosaminoglycan assay Blycan™ kit (Biocolor, Carrickfergus, UK) following the manufacturer's instructions. In short, the papain digested solution was mixed with Blycan DMMB and incubated for 30 min at room temperature. Samples were spun down for 10 min. The supernatant was discarded, and 0.5 mL dissociation reagent was added followed by 2-h incubation. The resulting suspension was centrifuged for 10 min and 150 µL of each sample was transferred to a 96-wells plate. Absorbance values were measured at 656 nm using the SpectraMax® i3 and concentration values were interpolated from a standard curve obtained from solutions with known concentrations of glycosaminoglycans.

2.8. RNA Isolation and Gene Expression Analysis

Monolayer cultures (n = 2; 23- and 31-year-old) were processed using SV total RNA System (Promega, Madison, WI, USA) following the manufacturer's instruction. DNAse treatment was employed to digest genomic DNA. Chondrogenic 3D pellet cultures were harvested for RNA isolation, by digestion in 1 mL Trizol (ThermoFisher) and subsequent grinding using RNase free stainless-steel balls and 3 min 3000 rpm shakes using a Shake Master NEO (Hirata, Kita, Japan). Subsequent suspensions were processed by Qiagen RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction to isolate the respective RNA molecules.

Quality and quantity of RNA were determined via NanoDropTM Lite spectrophotometer (Thermo Scientific) Subsequently, RNA was reverse transcribed into cDNA using a High-Capacity RNA-to-cDNA kit (Applied Biosystems, Beverly, MA, USA). Relative quantification of mRNA was performed using an SYBR Green Master Mix approach via QuantStudio3 (Applied Biosystems) employing custom primer sequences (Table A1). qPCR analysis of each sample was performed in duplicates. Obtained C_T values were compared via $2^{-\Delta\Delta CT}$ analysis, calculating gene expressions level relative to the housekeeping gene GAPDH and the average ΔC_T value of the growth-factor unstimulated MSC.

2.9. Tissue Processing and Histology

Chondrogenic pellets were washed and fixed using 10% formalin solution for 15 min. Samples were thereafter processed by incubating them for 1 h in subsequent 10% (w/v), 20% (w/v), and 30% (w/v) sucrose solutions. Finally, the pellet samples were submerged in an OCT TEK compound (Sakura Finetek, Torrance, USA) and snap-frozen in liquid nitrogen. Obtained cryo-samples were cut in 8 µm sections. Sections were stained by hematoxylin/eosin (HE) staining. Additionally, cryo-sections were submerged in 60% ethanol, 30% chloroform, 10% acetic acid solution for 30 min. Samples were thereafter counterstained by hematoxylin (5 s), followed by 800 mg/L Fast Green FCF staining (Merck; 10 min), 1% (v/v) acetic acid wash, and 1 g/L Safranin O (Merck) solution (10 min). Finally, stained sections were captured using Olympus IX70 converted microscope (Olympus, Tokyo, Japan).

2.10. Statistical Analysis

All data were processed, analyzed, and illustrated using GraphPad Prism (MacOS v9.0.1 (128), GraphPad Software, LLC, San Diego, CA, USA). Pictures and images were formatted using Adobe Illustrator (Adobe, San Diego, CA, USA). All values are presented as

mean ±standard deviation. Statistical differences were determined via one-way ANOVA, followed by Dunnett's multiple or Tukey's comparison test. A p-value < 0.05 was considered statistically significant. Standard comparisons (as indicated by a *) involves the comparison of MSC samples cultured in identical conditions with the respective growth factor combination and the negative control of MSC cultured without growth factors.

3. Results

3.1. Alcian Blue Mediated Screening for Proteoglycan Production Rate

Human MSC were seeded in triplicate and subjected to culture with different growth factor combinations as a screening assay to identify the most promising combinations. (Figure 1) Specifically, GDF5 or GDF6 were combined with one or two factors from the selection; TGF-β1, TGF-β2, TGF-β3, Wnt3a, Wnt5a, Wnt11, and Shh. Following 3 weeks of culture, samples were examined via alcian blue staining. All growth factors except single factor supplementation of Shh and Wnt5a resulted in enhanced proteoglycan production (Figure 1). All other factors and factor combinations enhanced alcian blue deposition rates, although the rates diverged largely. Specifically, the combinations of TGF-β2/Wnt3a, TGF-β1/Wnt3a, GDF6/TGF-β1/Wnt3a, TGF-β3/Wnt3a, GDF6/TGF-β2/Wnt3a, TGF-β3/Wnt5a, GDF6/Wnt3a, GDF5/TGF-β3, GDF5/Wnt3a, and TGF-β1/Wnt5a proved most potent, respectively. The most potent growth factor combinations, as well as the Colombier GDF5/TGF-β1 combination [47], were further analyzed.

3.2. Nucleus Pulposus Cell Marker Expression Analysis

Stimulated MSC were cultured for 2 weeks. The differentiated cells were subsequently harvested and processed for mRNA expression analysis. Expression of NP cell maker [38] CD24 (Figure 2A), T (Figure 2B), SOX9 (Figure 2C), and COL2A1 (Figure 2D) all increased in MSC stimulated with growth factors, and approached expression levels of naturally obtained NP cells. Particularly the combinations TGF-β2/Wnt3a ($p = 0.0522$) showed strong enhancement in COL2A1 expression; however, no combinations resulted in a significant increase of COL2A1 levels. SOX9 expression was significantly higher in all conditions and highest in MSC treated with TGF-β1/Wnt3a ($p < 0.0001$) and TGF-β1/Wnt3a/GDF6 ($p < 0.0001$) combinations. Specifically, the combination GDF5/TGF-β1, GDF5/TGF-β3, TGF-β1/Wnt5a, and TGF-β3/Wnt5a were found to be inferior compared to GDF6/TGF-β1/Wnt3a, GDF6/TGF-β2/Wnt3a, TGF-β1/Wnt3a, and TGF-β3/Wnt3a. CD24 expression was significantly higher in all (but TGF-β3/Wnt5a) conditions, specifically, GDF6/Wnt3a ($p < 0.0001$) treated MSC. However, GDF6/Wnt3a showed significantly higher expressions compared to GDF5/TGF-β1, GDF5/TGF-β3, TGF-β1/Wnt5a, and TGF-β3/Wnt5a. TGF-β2/Wnt3a ($p = 0.0009$) and GDF6/Wnt3a ($p = 0.0066$) showed highest enhancement of T expression. TGF-β2/Wnt3a also showed superior T expression compared to GDF6/TGF-β2/Wnt3a and GDF5/Wnt3. As a result, we continued work with the combinations TGF-β2/Wnt3a, TGF-β1/Wnt3a, GDF6/TGF-β1/Wnt3a, and GDF6/Wnt3a, as these overall showed the strongest potency of NP cell marker induction.

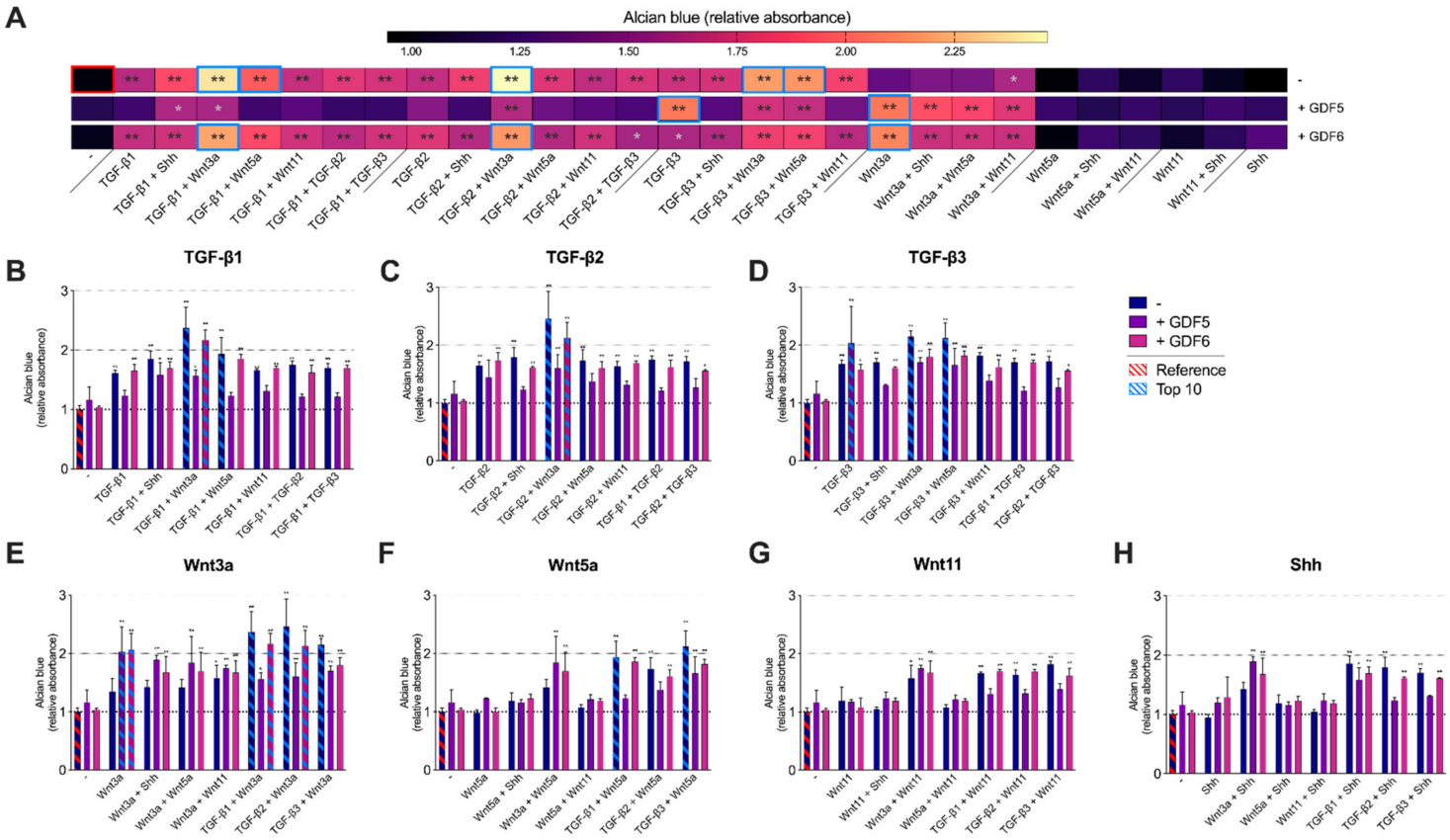

Figure 1. Representation of alcian blue screening assay for the assessment of different growth factor combinations to stimulate proteoglycan production in human MSC. Following 3 weeks of culture, alcian blue staining and subsequent absorbance measurement were used to determine the rate of proteoglycan production. (**A**) A large range of growth factor combinations without (top row) or with either GDF5 (middle row) or GDF6 (bottom row) to form a set of 1 to 3 growth factor media supplements were screened on their ability to stimulate MSC to produce proteoglycans. The top 10 combinations (highlighted with a blue border) were further assessed in subsequent assays. (**B**–**H**) Bar graph representation of heatmap data (to present variability in outcomes) of all combinations that contained (**B**) TGF-β1, (**C**) TGF-β2, (**D**) TGF-β3, (**E**) Wnt3a, (**F**) Wnt5a, (**G**) Wnt11, and (**H**) Shh. Additional supplementation of GDF5 (purple bars), GDF6 (pink bars), or without GDF (blue bars). The top 10 combinations are indicated by a blue pattern. Note that some combinations are repeatedly presented. All bars present mean values with error bars indicating standard deviations. Statistics were performed by comparing the average (n = 3) alcian blue absorbance values using one-way ANOVA followed by Dunnett's multiple comparison test where * indicates a $p < 0.05$, and ** a $p < 0.005$, comparing the values of the growth-factor-stimulated MSC to unstimulated MSC (-; indicated by red cell border or red pattern).

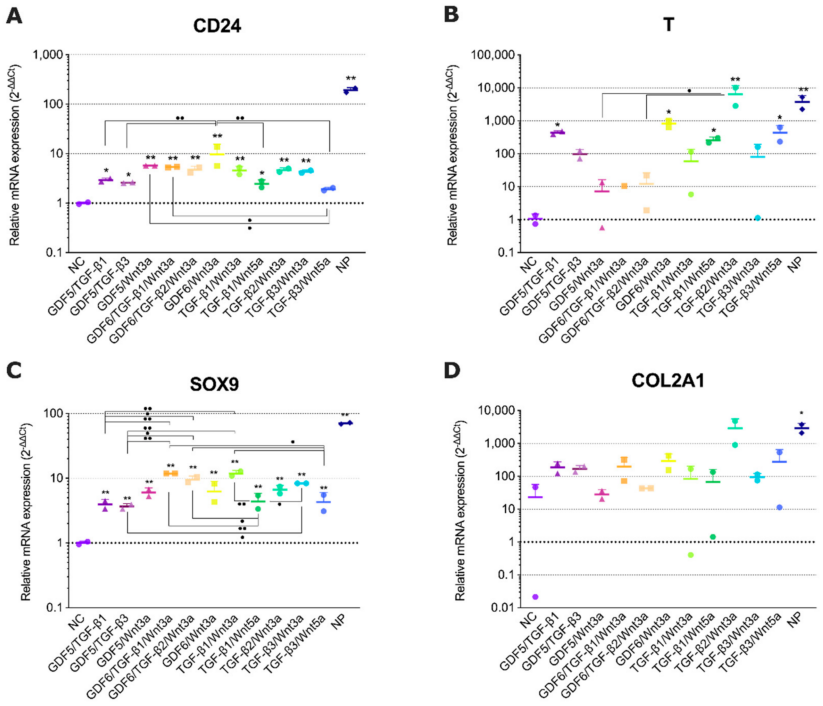

Figure 2. Gene expression assessment of NP cell markers in monolayer culture of MSC stimulated by different combinations of growth factors. (**A**) Relative expression levels of CD24 mRNA, (**B**) T (Brachyury) mRNA, (**C**) SOX9 mRNA, and (**D**) COL2A1 mRNA. Values are presented for individual donors (shapes) and as average values (bar), with error bars representing standard deviations. Statistical analysis was performed by one-way ANOVA followed by * Dunnett's multiple comparison test, which compared growth-factor-stimulated MSC to unstimulated MSC and • Tukey's multiple comparisons test comparing stimulated conditions. * or • indicates a $p < 0.05$, and ** or •• a $p < 0.005$. NC: negative control involving unstimulated MSC cultured in identical 2D culture conditions and media, but without growth factor supplementation, NP: natural human nucleus pulposus cells obtained from surgical waste tissue.

3.3. Chondrogenic Pellet Culture NP Cell Marker Expression

Next, the four top hitters in qPCR analysis, as well as the Colombier factors [47] of GDF5/TGF-β1, were applied for MSC differentiation in a standard chondrogenic 3D-pellet culture, to assess their ability to produce an NP-like chondrogenic matrix. Following 2 weeks of culture, pellets ($n = 3$) were harvested, RNA was isolated, and prepared for qPCR analysis. Expression levels were compared to MSC cultured without growth factor stimulation. CD24 expression (Figure 3A) revealed enhanced expression for all cases, with a significant increase for GDF5/TGF-β1 ($p < 0.0001$), TGF-β1/Wnt3a ($p = 0.0001$), and GDF6/TGF-β1/Wnt3a ($p < 0.0001$). Comparing the different combinations, we found GDF5/TGF-β1, GDF6/TGF-β1/Wnt3a, and TGF-β1/Wnt3a statistically outperforming GDF6/Wnt3a and TGF-β2/Wnt3a. SOX9 expression (Figure 3B) was similarly enhanced in all conditions, but most prominently for GDF5/TGF-β1 ($p = 0.0003$). COL2A1 (Figure 3C) showed similar results as the SOX9 assessment, with GDF5/TGF-β1 demonstrating the most prominent increase ($p < 0.0001$) and a significantly higher expression than all other combinations. Additionally, TGF-β1/Wnt3a and GDF6/TGF-β1/Wnt3a outperformed GDF6/Wnt3a and TGF-β2/Wnt3a. ACAN expression (Figure 3D) showed a trend of reduced expression in all conditions except GDF5/TGF-β1 and GDF6/TGF-β1/Wnt3a. Specifically, GDF5/TGF-β1 was the only combination that demonstrated an increase compared to the negative control, although not significant ($p = 0.0594$).

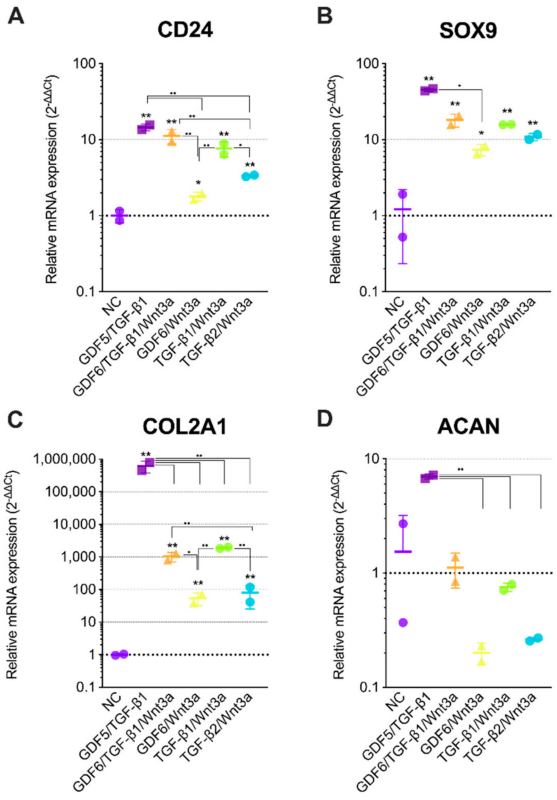

Figure 3. Gene expression assessment of NP cell markers within chondrogenic pellet culture containing human MSC stimulated by different combinations of growth factors. Relative expression levels of (**A**) CD24 mRNA, (**B**) SOX9 mRNA, (**C**) COL2A1 mRNA, and (**D**) ACAN mRNA. Values are presented for individual donors (shapes) and as average values (bar), with error bars representing standard deviations. Statistics were performed by one-way ANOVA followed by * Dunnett's multiple comparison test comparing growth-factor-stimulated to MSC cultured in identical conditions without growth factors (NC) and • Tukey's multiple comparisons test comparing stimulated conditions. * or • indicates a $p < 0.05$, and ** or •• a $p < 0.005$. NC: negative control involving unstimulated MSC cultured in identical pellet conditions and media, but without growth factor supplementation.

3.4. Chondrogenic Matrix Assessment Following MSC Differentiation

Following 3 weeks of culture, pellets were harvested either by papain digestion ($n = 3$) for DMMB and PicoGreen assay or processed for cryo-sectioning and subsequent histological analysis ($n = 3$). PicoGreen assay revealed a limited change in the number of cells compared to the negative control. (Figure 4B) Generally, most conditions showed a trend of a small increase in cell content, except for GDF6/TGF-β1/Wnt3a stimulated pellets. DMMB assay (Figure 4A) revealed an increase in sulfated glycosaminoglycans compared to the negative control, although the differences were not very large compared to the values obtained from NP cell-derived pellets. The GDF5/TGF-β1 combination showed the most potent in inducing glycosaminoglycan production, (5.974 (±1.850) times higher than the negative control; $p = 0.0496$). Finally, the ratio of glycosaminoglycans per DNA content (Figure 4C) showed the highest values for GDF5/TGF-β1 (4.926 ± 1.608) and GDF6/TGF-β1/Wnt3a (4.380 ± 2.461) stimulated pellets. However, only the GDF5/TGF-β1 combination involved a statistically significant increase with a $p = 0.0466$, while GDF6/TGF-β1/Wnt3a presented a $p = 0.1043$. No statistically significant differences were found between the growth factor conditions. Histological observations (Figure 4D) generally confirmed the DMMB findings. Safranin-O/Fast green stained sections revealed a matrix completely devoid of proteoglycans in the negative control (Figure 4D). Some safranin-O positive staining could be observed in the TGF-β2/Wnt3a, TGF-β1/Wnt3a, and GDF6/Wnt3a stimulated pellets (Figure 4D). This opposed to the pellet stimulated with

GDF5/TGF-β1 (Figures 4D,E and 5A), which showed regions of high-intensity safranin-O positive staining.

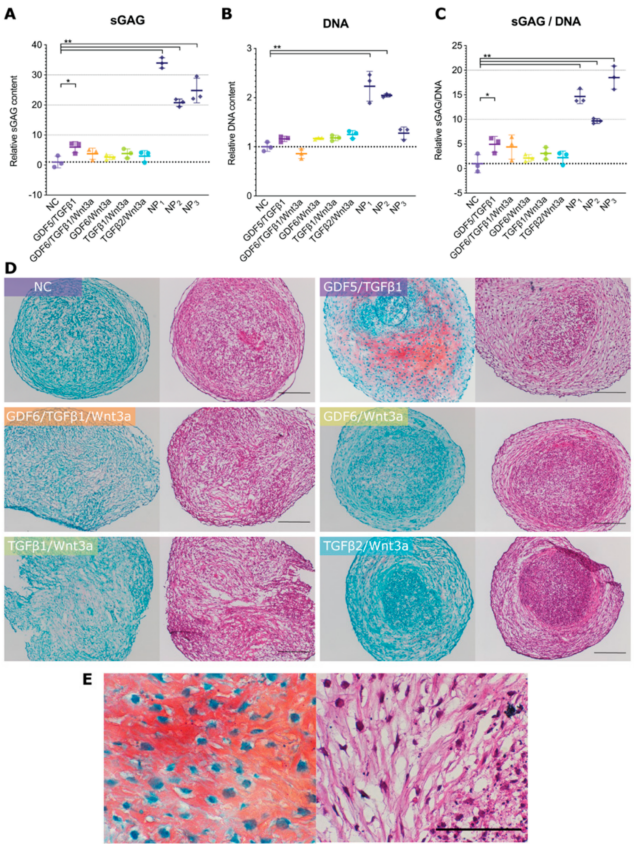

Figure 4. Assessment of growth factor combinations on extracellular matrix production of human MSC cultured in chondrogenic pellets. Via DMMB and Picogreen determined (**A**) glycosaminoglycan (sGAG), (**B**) DNA, and (**C**) the sGAG/DNA content was measured and calculated relative to non-stimulated MSC following 3 weeks of pellet culture (n = 3). (**D**) Histological examination of MSC-derived chondrogenic pellets stimulated with indicated growth factor combinations. Images represent pellets at 10× magnification stained with safranin-O/fastgreen (left) and hematoxylin/eosin (right). Scale bars represent 250 µm. (**E**) 40× magnification images of GDF5/TGF-β1. The scale bar equates to 125 µm. Statistics were performed by one-way ANOVA followed by Dunnett's multiple comparison test, where * indicates a $p < 0.05$ and ** a $p < 0.005$, relative to the NC (identically cultured, but without growth factors). No statistically significant differences were found between the different growth factor combinations. NC: negative control involves unstimulated MSC that were cultured in identical pellet conditions and media without growth factors, sGAG: sulfated glycosaminoglycans, NP: naturally obtained human nucleus pulposus cells from different donors.

3.5. Wnt3a Preconditioning to Enhance MSC Chondrogenesis

Human MSC expanded with or without 250ng mL^{-1} Wnt3a up to passage 1 and subsequently cultured for 3 weeks in GDF5/TGF-β1 containing differentiation media (Figure 5A,C) or commercial Chon.M. (Figure 5B,D). Histological observations did not reveal a clear benefit for MSC expanded in Wnt3a, as safranin-O staining was present at a similar intensity in pellets derived from both conditions.

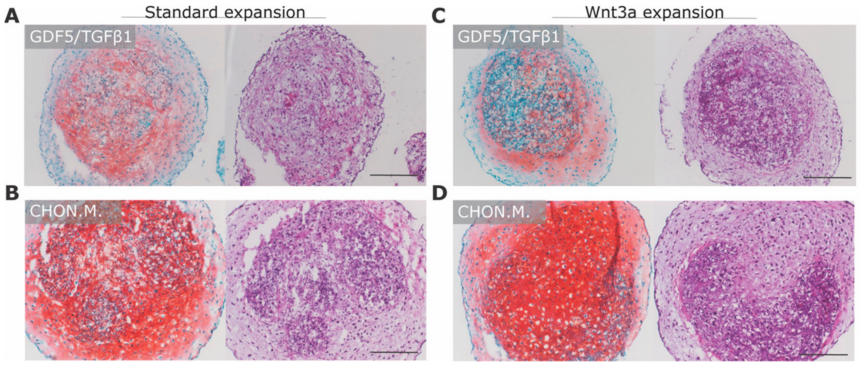

Figure 5. Assessment of Wnt3a pre-culturing for enhancement of chondrogenesis. Human MSC were expanded in standard expansion media without Wnt3a (**A**,**B**) or with 250 ng mL^{-1} Wnt3a (**C**,**D**). Subsequently obtained MSC were cultured in chondrogenic pellets and media supplemented with GDF5/TGF-β1 (**A**,**C**) or commercial predefined chondrogenic medium (**B**,**D**). Subsequent pellets were sectioned and stained by safranin-O/fast-green (left) or hematoxylin/eosin (right) staining. Scale bars represent 250 µm. Chon.M.: Mesenchymal stem Cell Chondrogenic Differentiation Medium.

4. Discussion

In this study, we aimed to identify growth factors or factor combinations that showed the highest potency in enhancing MSC to NP cell-like cell differentiation. Starting with monolayer cultures, a large array of factor combinations (86 different growth factors and factor combinations) were screened on their ability to stimulate proteoglycan production, as indicated by alcian blue staining. Unsurprisingly, almost all factor combinations showed the ability to at least enhance proteoglycan production compared to unstimulated MSC, as all factors were selected based on their general chondrogenic potential. Interestingly, of all the combinations identified as high hitters, a large fraction involved a combination with Wnt3a. A selection of the 10 most potent growth factor combinations, plus the combination of GDF5/TGF-β1, were further analyzed on their ability to induce NP cell marker expression. Here we showed the ability of particularly TGF-β2/Wnt3a, TGF-β1/Wnt3a, GDF6/TGF-β1/Wnt3a, and GDF6/Wnt3a to strongly enhance the expression of NP markers [38] CD24 and SOX9. A trend of enhancement in COL2A1 and T was observed for most conditions. Contrary to the impactful findings of Colombier et al. [47], our initial screening outcomes (in monolayer culture) did not indicate GDF5/TGF-β1 as a potent chondrogenic factor-combination.

Nevertheless, at this stage of the study, differentiation culture was still within the 2D monolayer set-up. MSC tend to change their characteristics relatively quickly in monolayer culture and lose their multipotency capacity [51–53]. Moreover, for induction of differentiation, the extracellular micro and macro-environment are critical as they can promote or support specific cellular behavior by providing mechanotransduction cues [54], anchor points, and chemical cues through the matrix proteins or by growth factor sequestering [55]. As such, we further validated the findings observed in monolayer cultures in a 3D environment, using a common pellet culture method [47]. Pellet cultures revealed an opposing trend, in which none of the Wnt3a combinations were able to significantly enhance glycosaminoglycan production rates. Although an increase was seen in the expression levels of some NP markers, i.e., CD24, SOX9, and COL2A1, increases in the ACAN expression level were not observed. This in contrast with the pellet cultures treated with GDF5/TGF-β1, which resulted in a significant increase in glycosaminoglycan production rates, and large proteoglycan positive regions within the pellet as indicated by safranin-O staining. Additionally, GDF5/TGF-β1 showed continuously most potent in enhancing NP cell marker expressions following the 3D pellet culture conditions. Although the relative glycosaminoglycan content of the other 4 growth factor combinations did not result in a significant increase, a trend of enhanced glycosaminoglycan production was observed both

by the DMMB assay, as well as on histological observation. The negative control revealed no pink/red staining in the safranin-O stained section, the growth factor-stimulated pellets did present lightly pink areas, suggesting the presence of proteoglycans. Interestingly, the combination GDF6/TGF-β1/Wnt3a showed the second-highest rate of glycosaminoglycan production/DNA but did not strongly show safranin-O positive regions. The GDF6/TGF-β1/Wnt3a combination did present an overall lower DNA content, suggesting that the GDF6/TGF-β1/Wnt3a combination might have limited cellular proliferation or otherwise comprised cellular viability.

Although our initial screening failed to validate GDF5/TGF-β1 as a potent inducer of a chondrogenic and NP cell phenotype, subsequent assessment within a 3D environment did confirm the observations from Colombier et al. [47] that the synergy of GDF5/TGF-β1 was able to induce a desirable cell product. Even more, GDF5/TGF-β1 was the only combination able to result in fully chondrogenic pellets. The disagreement between 2D and 3D culture outcomes is not surprising. Cell behavior is strongly altered by such biomechanical cues [56]. Moreover, TGF-β1 is not a specific chondrogenic factor as it has similarly been shown to induce MSC differentiation to other cell lineages, e.g., cardiomyocytes, osteocytes, or adipocytes [57,58]. The ability to promote a chondrogenic NP cell-like phenotype through TGF-β1 is therefore likely context-dependent. This suggestion is supported by the original work of Colombier et al. [47], who showed the requirement of dexamethasone supplementation for a complete NP-like differentiation for MSC. That said, unlike the results of Colombier et al., our cells were unable to attain a final expression profile or matrix composition that matched those of naturally obtained human NP cells [47]. This might in part be due to the shorter culture periods that we employed. Colombier et al. maintained their pellet cultures for up to 4 weeks, whereas we cultured our pellets for 2 or 3 weeks dependent on the analysis performed. Additionally, while Colombier et al. applied adipogenic-derived MSC [47], our study employed bone marrow-derived MSC for the analysis. Distinct differentiation potential of differently sourced MSC is a well-reported phenomenon [46,59].

Wnt signaling is critical in general joint development, homeostasis, and has also been determined to have a role in disc degeneration [60–62]. Regardless, the role of Wnt3a in chondrogenesis is less clear [48]. Wnt3a has been shown able to enhance MSC proliferation rates and BMP2-mediated chondrogenesis, within 2D monolayer cultures [63]. Other studies have shown mixed results when applying Wnt3a on MSC for the enhancement of chondrogenic differentiation in 3D conditions [48]. Similarly, our initial screening confirmed these findings, where the highest-ranking growth factor combinations often included Wnt3a in monolayer culture. However, these enhancements were not observed in 3D culture. This could in part be ascribed to the potential role of Wnt3a and its maintenance of a more undifferentiated state, as suggested by Volleman et al. [48]. Where 2D culture could induce maturation or loss of potency with extended periods of culture, Wnt3a could potentially play a role in mitigating this loss of cell potency, thereby supporting the chondrogenic potential of additionally added growth factors. This beneficial role is not observed in 3D cultures. To assess this potential application of Wnt3a, we reapplied a pellet culture system, in which MSC were expanded with Wnt3a containing media to possibly maintain their potency, and subsequent differentiation in 3D pellet cultures with chondrogenic factors but without Wnt3a. The histological results were unable to confirm this theory, as the application of Wnt3a during expansion, did not appear to enhance the deposition rate of proteoglycan in the matrix.

Another aspect of our study is the direct comparison of growth factor combinations. For example, from our alcian blue assay (Figure 1), we observed that the addition of GDF5 appears to temper alcian blue rates compared to identical combinations without GDF5 or with GDF6 instead. Combinations that combined either Wnt11, Wnt5a, or Shh with GDF5 or GDF6 also did not present strong stimulation of proteoglycan production. Our screening assay also allows for further examination of specific combinations and single growth factor addition benefits or otherwise. For example, we found that the addition of either TGF-β1 or

TGF-β2 to GDF6/Wnt3a resulted in similar alcian blue production rates and subsequently did not appear to enhance NP marker expressions either. This suggested that TGF-β1 or TGF-β2 have no added value to the GDF6/Wnt3a combination. However, subsequent pellet cultures revealed that GDF6/Wnt3a with TGF-β1 resulted in significantly higher CD24 and COL2A1 expression levels, and showed a trend of enhanced ACAN and SOX9 expression levels. These observations further highlighting the context-dependent nature of cell culture and the effects of growth factors on the respective cells.

Notwithstanding, our results require some consideration before interpretation. One limitation of our study is the limited number of donor samples applied, particularly for the initial screening assay. Due to the high cost of applied growth factors and relatively long culture period, the initial screening of 86 different combinations was assessed on one single-sourced MSC. Moreover, the initial alcian blue screening was performed without correction for cell numbers or DNA content. Differences could in theory be caused by differences in cell content. However, we believe this is unlikely, as the long (3 weeks) culture period led all conditions to a confluent culture condition, and the differences at the time of assessment were likely minimal. Another study limitation to consider is that we did not assess outcomes of our monolayer and pellet cultures at different time points. Such particular combinations might have revealed more beneficial outcomes at shorter or longer stimulation periods. This requires further examination.

Overall, a need exists for novel regenerative strategies to treat intervertebral disc diseases. Currently, cell therapy [26] and growth factor injection [17] is gaining momentum, however, further validation and optimization of different regenerative products will likely be needed [64]. One potential approach for enhancing cellular products is priming the cells before transplantation [65], to promote an optimal anabolic state, such that the transplanted cells have the highest potential to contribute to the reorganization of the disc matrix or otherwise direct regional cells to do so [30]. Growth factors could be a potent tool in this regard. However, caution is required. Growth factors often function in context-dependent manners, and as such not every MSC population sourced from different donors might respond similarly. For clinical application and marketability, cell-product quality control is key, and this aspect should be taken into consideration [3,4,66]. Moreover, growth factors are generally quite expensive, particularly when applied over longer culture periods and for large-scale production. Thus, their application could hinder commercialization, by potentially increasing the price of cellular therapy production. Again, care should be taken that the benefits of growth factor stimulation outweigh the additional expense of the cellular product. From our screening of 86 different growth factor combinations for MSC to NP cell differentiation, we found that Wnt3a could potentially be an impactful factor when applied in 2D monolayer, although the mechanisms and requirements for these effects compel further exploration. Of all combinations analyzed, only the combination of GDF5 and TGF-β1 was able to result in fully chondrogenic pellets when applied in 3D cultures, and it might be the most potent combination for MSC to NP cell differentiation.

Author Contributions: D.S. and J.S.; conceptualization, T.N.E.V., K.M., J.S.; methodology, T.N.E.V., K.M., J.S.; formal analysis, D.S., M.S., M.W.; resources, J.S.; writing—original draft preparation, J.S., T.N.E.V., K.M., D.S., M.S., M.W.; writing—review and editing, J.S.; visualization, D.S., M.S., M.W.; supervision, D.S., M.S., M.W.; funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the Tokai University School of Medicine (protocol code 06I-49 and 16R-051: 15 July 2016).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data can be requested to the corresponding authors upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Tabular overview of primer sequences used for gene expression analysis.

Target	Primer Sequence (Forward)	Primer Sequence (Reverse)
GAPDH	5′-AATCAAGTGGGGCGATGCTG-′3	5′-GCAAATGAGCCCCAGCCTTC-′3
CD24	5′-GCACTGCTCCTACCCACGCAGATTT-′3	5′-GCCTTGGTGGTGGCATTAGTTGGAT-′3
Brachyury	5′-GGGTCCACAGCGCATGAT-′3	5′-TGATAAGCAGTCACCGCTATGAA-′3
COL2A1	5′-GGAAGAGTGGAGACTACTGGATTGAC-′3	5′-TCCATGTTGCAGAAAACCTTCA-′3
SOX9	5′-CAGACAGCCCCCTATCGACT-′3	5′-CGTTGACATCGAAGGTCTCG-′3
ACAN	5′-GTGCCTATCAGGACAAGGTCT-′3	5′-GATGCCTTTCACCACGACTTC-′3

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