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Investigation of the Effect of Secreted Factors from Mesenchymal Stem Cells on Disc Cells from Degenerated Discs

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Keywords
Human mesenchymal stem cells · Intervertebral disc cells · Conditioned media · Low back pain · Chondrogenesis

Abstract
Low back pain is experienced by a large number of people in western countries and may be caused and influenced by many different pathologies and psychosocial factors including disc degeneration. Disc degeneration involves the increased expression of proinflammatory cytokines and matrix metalloproteinases (MMPs) in the disc environment, which leads to the loss of extracellular matrix (ECM) and the viability of the native disc cells (DCs). Treatment approaches using growth factors and cell therapy have been proposed due to the compelling results that growth factors and mesenchymal stem cells (MSCs) can influence the degenerated discs. The aim of this study was to investigate the effects of conditioned media (CM) from human MSCs (hMSCs) and connective tissue growth factor (CTGF) and TGF-β on disc cells, and hMSCs isolated from patients with degenerative discs and severe low back pain. The aim was also to examine the constituents of CM in order to study the peptides that could bring about intervertebral disc (IVD) regeneration. DCs and hMSC pellets (approx.. 200,000 cells) were cultured and stimulated with hMSC-derived CM or CTGF and TGF-β over 28 days. The effects of CM and CTGF on DCs and hMSCs were assessed via cell viability, proteoglycan production, the expression of ECM proteins, and chondrogenesis in 3D pellet culture. To identify the constituents of CM, CM was analyzed with tandem mass spectrometry. The findings indicate that CM enhanced the cellular viability and ECM production of DCs while CTGF and the control exhibited nonsignificant differences. The same was observed in the hMSC group. Mass spectrometry analysis of CM identified >700 peptides, 129 of which showed a relative abundance of ≥2 (CTGF among them). The results suggest that CM holds potential to counter the progression of disc degeneration, likely resulting from the combination of all the substances released by the hMSCs. The soluble factors released belong to different peptide families. The precise mechanism underlying the regenerative effect needs to be investigated further, prior to incorporating peptides in the development of new treatment strategies for low back pain that is potentially caused by IVD degeneration.

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Introduction

Low back pain is experienced by a large number of populations in the West where the lifetime prevalence is >84% [Airaksinen et al., 2006]. Low back pain ranks higher than most fatal diseases in terms of years lived with disability according to the Global Burden of Disease Study [van Hooff et al., 2014]. The cause of low back pain is multifactorial in nature. In individuals with chronic low back pain, disc bulging and degenerative changes in the intervertebral discs (IVDs) have been shown to be more common [Brinjikji et al., 2015] than in control populations. Degenerative disc disease is associated with long-standing severe back pain. The architecture of the IVD involves 2 main tissues, the annulus fibrosus (AF) and the nucleus pulposus (NP).The AF is the thick lamellae ring made up of organized collagen fibers that surround the gelatinous structure of the NP within the end plates [Humzah and Soames, 1988]. The NP is made up of proteoglycan and collagen, crucial for the water content of the IVDs [Roughley, 2004]. The AF is mainly populated by fibroblast-like cells while chondrocyte-like cells inhabit the NP [Adams and Roughley, 2006; Minogue et al., 2010]. In degenerated discs, the collagen fibers in the tissue becomes gradually more and more disorganized, and the 2 tissues become hard to distinguish from each other [Adams and Roughley, 2006]. Increased expression of proinflammatory cytokines and matrix metalloproteinases (MMPs) in degenerated IVDs leads to a loss of proteoglycan and extracellular matrix (ECM), affecting the viability of the disc cells (DCs) in the IVDs [Ekman et al., 2005; Airaksinen et al., 2006]. Current surgical treatments are symptomatic and do not tackle the degenerative process or promote regeneration [Phillips et al., 2013]. Thus, new treatment strategies with effective therapeutic agents that can be clinically applied as a minimally invasive regenerative therapy are warranted. Several treatment approaches for degenerated disc disease, including growth factors and cell therapy, have been suggested [Peroglio et al., 2013; Zeckser et al., 2016]. Growth factors such as transforming growth factor β (TGF-β) and connective tissue growth factor (CTGF) have been reported as major focal points in protein interaction networks that act as the main inducer of chondrogenesis. In fact, in vitro stimulation with these factors demonstrated enhanced cell proliferation and ECM production in rat-tail disc cells [Matta et al., 2017]. Meanwhile, cell therapy with mesenchymal stem cells (MSCs) also show promise in promoting regenerative effects in many disease models [Gou et al., 2014; Somoza et al., 2014]. It has been reported that transplanted cells do not survive for long, but the therapeutic effect observed was due to the vast array of bioactive substances secreted by the cells that played an important role in regulating key biological processes [Vizoso et al., 2017]. DCs have previously been reported to have regenerative outcomes when cocultured with human (h)MSCs, indicating that substances secreted from hMSCs might have therapeutic potential [Svanvik et al., 2010; Hingert et al., 2020]. Studies have also shown that conditioned media (CM) derived from MSCs promotes tissue repair in arthritis and cartilage models [Toh et al., 2014; Kay et al., 2017].

All these reports provide compelling evidence that growth factors (CTGF and TGF-β) and/or proteins secreted from MSCs may have the ability to restore the integrity of the discs and/or arrest degeneration processes. To validate this hypothesis, the aim of this study was to compare the effects of CM from hMSCs with the effects of the growth factors CTGF and TGF-β on DCs and hMSCs isolated from patients with low back pain in a 3D in vitro model. The aim was to further identify the constituents of CM to gain an insight into the network of proteins secreted by the hMSCs.

Materials and Methods

Patients

Disc tissues and bone marrow aspirates (BMA) from the iliac crest were collected from patients with long-standing low back pain without radicular pain, who had been diagnosed with degenerated-disc disease and were undergoing spinal fusion surgery including the removal of the disc(s). The discs were graded according to the Pfirrmann grading system on magnetic resonance imaging (MRI) as grade 3/4. Disc tissue was obtained from the center of the disc. However, the obtained tissue was a mixture of AF and NP tissue due to the disintegration of the discs. Due to limited samples, no characterization of the cells was performed. Both disc tissues and BMA were obtained from 2 females and 1 male donor aged 29—49 years.

DC Isolation and Cell Culture

Disc tissues were cut into small pieces, transferred to culture flasks (CORNING, NY, USA), and incubated with 1 mg/mL type II collagenase at 37 °C and 5% CO_2 overnight. Cell suspensions were centrifuged at 1,500 rpm for 5 min and the cells were thereafter cultured at 37 °C and 5% CO_2 in DC medium (DMEM-low glucose with 110 mg/L sodium pyruvate, 4 mmol/L L-glutamine [Thermo Fisher Scientific, MA, USA], and 1% penicillin/streptomycin, and supplemented with 10% human serum albumin [HSA]). The medium was changed every 48 h and the DCs were passaged at 90% confluency. All DCs used in the study were in passage 5.

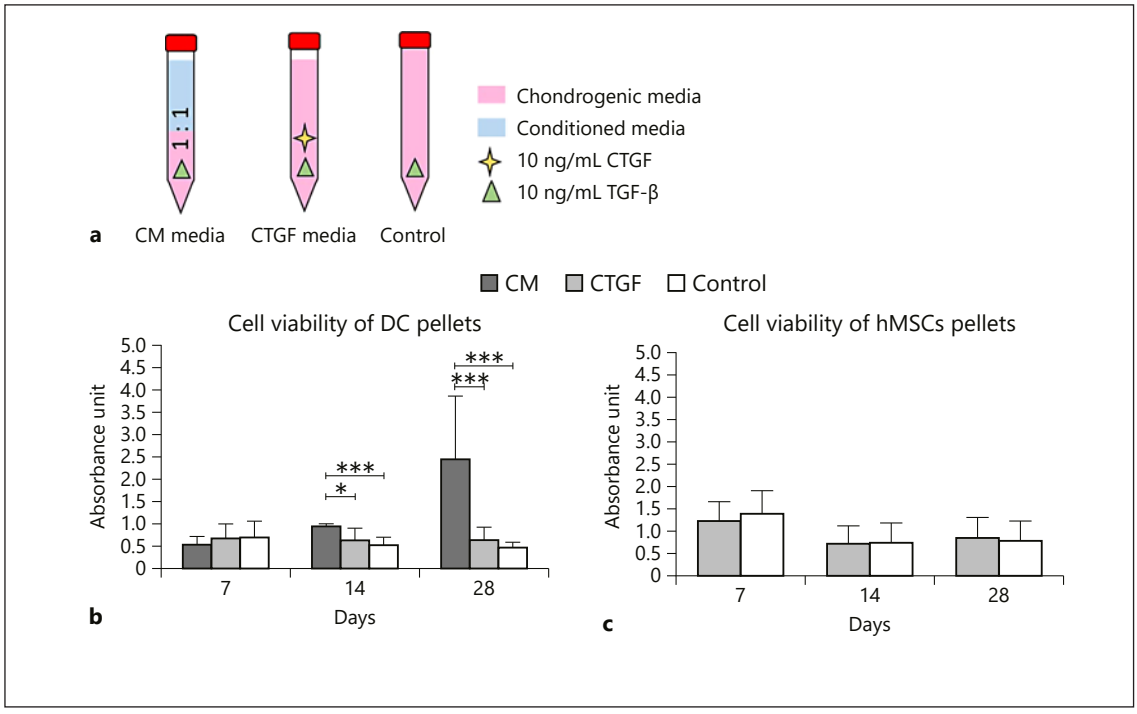

Fig. 1. a Schematic illustration of different stimulations used on hMSC and DC pellets. Illustration of cell viability and proliferation on days 7–28 in DC pellets treated with conditioned media (CM) and 10 ng/mL CTGF (CTGF) (**b**) and hMSC pellets treated with 10 ng/mL CTGF (CTGF) (**c**). Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

hMSC Isolation and Cell Culture

BMA collected in 3.2% sodium citrate (Greiner Bio One, Kremsmuenster, Austria) were transferred to a Ficoll tube (BD Vacutainer® CPT™, Biosciences, NJ, USA) and centrifuged at 1,500 rpm for 20 min. hMSCs were isolated and cultured at 37 °C and 5% CO_2 in MSC media (DMEM low glucose with 110 mg/L sodium pyruvate and 4 mmol/L L-glutamine [Thermo Fisher Scientific]), together with 1% penicillin/streptomycin, 10% HSA, and 4 ng/mL β-fibroblast growth factor (FGF). The medium was changed every 48 h and cell passaging was done at 90% confluency. All hMSCs used in the study were in passage 5.

Collection of hMSC CM

hMSCs from 3 patients were cultured in MSC media at a density of approximately 10,000 cells/cm^2 for 14 days, and CM was harvested every 48 h. The collected CM was centrifuged at 1,500 rpm at 4 °C for 5 min. The supernatants (from all 3 patients) were then pooled, sterile-filtered (SARSTEDT, Nümbrecht, Germany), and stored at –80 °C until further use. All the hMSCs used in the study were in passage 4–5.

3D Pellet Culture

Approximately 200,000 DCs or hMSCs were distributed into each conical tube with 0.5 mL of relevant medium, centrifuged at 1,500 rpm at 4 °C for 5 min, and then incubated at 37 °C and 5% CO_2. The medium was changed every 2–3 days and the pellets were harvested on days 7, 14, and 28. The experiments were conducted separately with each cell type from each donor.

Pellet Stimulation

DC pellets were stimulated with CM (CM in chondrogenic media at a 1:1 ratio) or 10 ng/mL CTGF (R&D Systems, MN, USA) in chondrogenic media (DMEM high glucose with insulin, transferrin, and selenium [ITS-G; Thermo Fisher Scientific], 5 mg/mL linoleic acid, 10 ng/mL TGF-β_1; R&D Systems), 14 mg/mL ascorbic acid , 10^{-7} M dexamethasone [Sigma-Aldrich, MS, USA], 1.0 mg/mL HSA [Equitech-Bio Inc., KV, USA] and 1% penicillin/streptomycin) (Fig. 1a). As the hMSCs were able to produce the same factors that are in CM, hMSC pellets were only stimulated with 10 ng/mL CTGF. Chondrogenic medium (contains TGF-β) without any additives served as a control group. Four replicates of pellets were cultured for each stimulation group per donor (i.e., 12 pellets in total for each stimulation group), where 2 pellets were used for histological evaluation and 2 for the biochemical analysis of each stimulation group.

Cell Proliferation/Viability Measurement

50 µL of cell-counting kit 8 (CCK-8) solution (Dojindo, Munich, Germany) was added to each pellet and incubated for 4 h at 37 °C and 5% CO_2. The supernatant was then collected, and the color intensity measured at 450 nm with a microplate reader (BioTek, VT, USA). CCK-8 assay was performed on days 7, 14, and 28 before harvesting the pellets. The supernatant was collected in duplicate from 4 pellets for each stimulation group (i.e., 8 replicates/stimulation group).

Histological Staining

DC and hMSC pellets were fixed with 4% formaldehyde (Histolab products, Gothenburg, Sweden), sectioned, and then stained with Alcian blue van Gieson. The histology sections were later examined for proteoglycan and collagen deposition on light microscopy (Nikon Eclipse E600). This was conducted on 2 sections from each of the 2 pellets for both cell types (i.e., 4 sections investigated/stimulation group).

Glycosaminoglycan and DNA Assays

The pellets were digested with papain (1.5 mg papain/mL [Sigma-Aldrich, MS, USA]), 20 mM sodium phosphate buffer, 1 mM EDTA, and 2 mM dithiothreitol prior to incubation at 60 °C overnight. Glycosaminoglycan (GAG) and DNA were quantified with the glycosaminoglycans assay kit (Chondrex, WA, USA) and the DNA assay kit (Chondrex), respectively. The GAG and DNA content was then measured at 525 nm and an excitation of 360 nm/emission 460 nm for GAG and DNA, respectively, with a microplate reader (BioTek). The analysis was conducted in 4 replicates for each stimulation group, and GAG content was normalized to the total amount of DNA in each pellet.

Immunohistochemistry

Immunohistochemistry was carried out to verify the characteristics of chondrocyte-like cells in the DCs and chondrogenic differentiation in the hMSCs. The expression of keratin-19 (KRT19), aggrecan (ACAN), and collagen type II (COLIIA1) was studied in DC pellets, and the expression of Sox9, ACAN, and COLIIA1 in hMSC pellets. Briefly, paraffin-embedded sections were deparaffinized and rehydrated; antigen retrieval (citrate buffer, pH 6, 90 °C for 20 min) was then carried out. Primary antibodies used include anti-KRT19 (1:100), anti-Sox9 (1:1,000), anti-ACAN (1:500), and anti-COLIIA1 (1:100, Abcam, Cambridge, MA, USA). After incubation at 4 °C overnight, the sections were blocked with blocking solution (0.1% Triton X-100, 2% BSA, and 100 mM glycine in PBS). COLIIA1 sections only were blocked with 3% BSA. Secondary antibodies include donkey anti-rabbit IgG Alexa Fluor 546 (1:200, Thermo Fisher Scientific) against KRT19, Sox9, and ACAN, and goat anti-rabbit IgG Alexa Fluor 546 (1:200, Thermo Fisher Scientific) against COLIIA1. To complement the detection of COLIIA1, the sections were incubated with SA-HRP (1:100, TSA plus cyanine 3 system kit, PerkinElmer, MA, USA) for 30 min, and cyanine 3 tyramide (1:100, TSA plus cyanine 3 system kit) prior to nuclei counterstaining with ProLong® gold antifade mountant (DAPI; Thermo Fisher Scientific). The samples were examined under fluorescence microscope (Nikon Eclipse E600, Japan). NIS Elements software (Nikon Metrology NV, Europe) was used to perform fluorescence intensity quantification and determine the area of the pellets. The levels of protein expression were presented as pixel/μm^2. The analysis was conducted in 4 replicates for each stimulation group.

CM Preparation for Secretome Analysis

Proteins in CM were digested using the filter-aided sample preparation (FASP) method [Wiśniewski et al., 2009]. Briefly, protein samples were concentrated using 30 kDa MWCO Pall Nanosep centrifugal filters (Sigma-Aldrich). Digestion was performed in 50 mM TEAB and 1% sodium deoxycholate (SDC) overnight at 37 °C after the addition of trypsin (Pierce trypsin protease, MS grade, Thermo Fisher Scientific) in a ratio of 1:100 relative to the amount of protein. Digested peptides were labeled using TMT 10-plex isobaric mass tagging reagents (Thermo Scientific) according to the manufacturer's instructions. MSC medium served as a control.

Mass Spectrometry Analysis

Liquid chromatography-mass spectrometry analysis was performed by the Proteomics Core Facility, Sahlgrenska Academy, Gothenburg University. Mass spectrometric analysis was performed with a Q Exactive mass spectrometer (Thermo Fisher Scientific). Briefly, the CM was analyzed for its peptide content and compared to control medium. Samples were fractioned into 40 fractions based on hydrophobicity, in order to increase analytical resolution. Tandem-mass tags (TMT) were used to quantify the relative amounts of peptides in the CM compared to the control media. Data analysis was performed utilizing Proteome Discoverer v1.4 (Thermo Fisher Scientific) against the Human Swissprot Database version March 2015 (Swiss Institute of Bioinformatics, Switzerland). Mascot 2.3.2.0 (Matrix Science) was used as a search engine. Ratio and fold-changes were calculated by using control (MSC) media as a denominator. Identified peptides were grouped based on literature findings.

Statistical Analysis

All statistical data are presented as the mean ± standard error of the mean (SEM). Data were analyzed via SPSS v25.0 software (IBM SPSS Statistics, NY, USA). Two-tailed Student's t test was used for CCK-8 and GAG assays, as well as fluorescence intensity quantification for hMSC groups (comparing the means of 2 groups) while ANOVA with the Tukey post hoc test was used for that of DC groups (multiple comparisons). $p < 0.05$ was considered statistically significant.

Results

Cell Proliferation/Viability Measurement

In DC pellets, the CM stimulated group demonstrated a continuous cell proliferation on days 7—28, and the number of viable cells was significantly higher than in the CTGF group at 2 time points, i.e., day 14 ($p < 0.05$) and day 28 ($p < 0.001$) (Fig. 1b). However, there was no significant difference between the CTGF and control groups. The cell viability of hMSC pellets was highest on day 7 for both the CTGF and control groups, compared to the other time points. Nevertheless, throughout the experiment, the CTGF group showed no significant difference from controls ($p > 0.05$) (Fig. 1c).

Histological Staining

Alcian blue van Gieson staining was carried out to examine proteoglycan and collagen deposition within the cell pellets. In DC pellets, compared to the other groups, CM demonstrated the highest proteoglycan accumulation at all time points, starting on day 7 and being maintained until day 28 (Fig. 2a). The CTGF and control

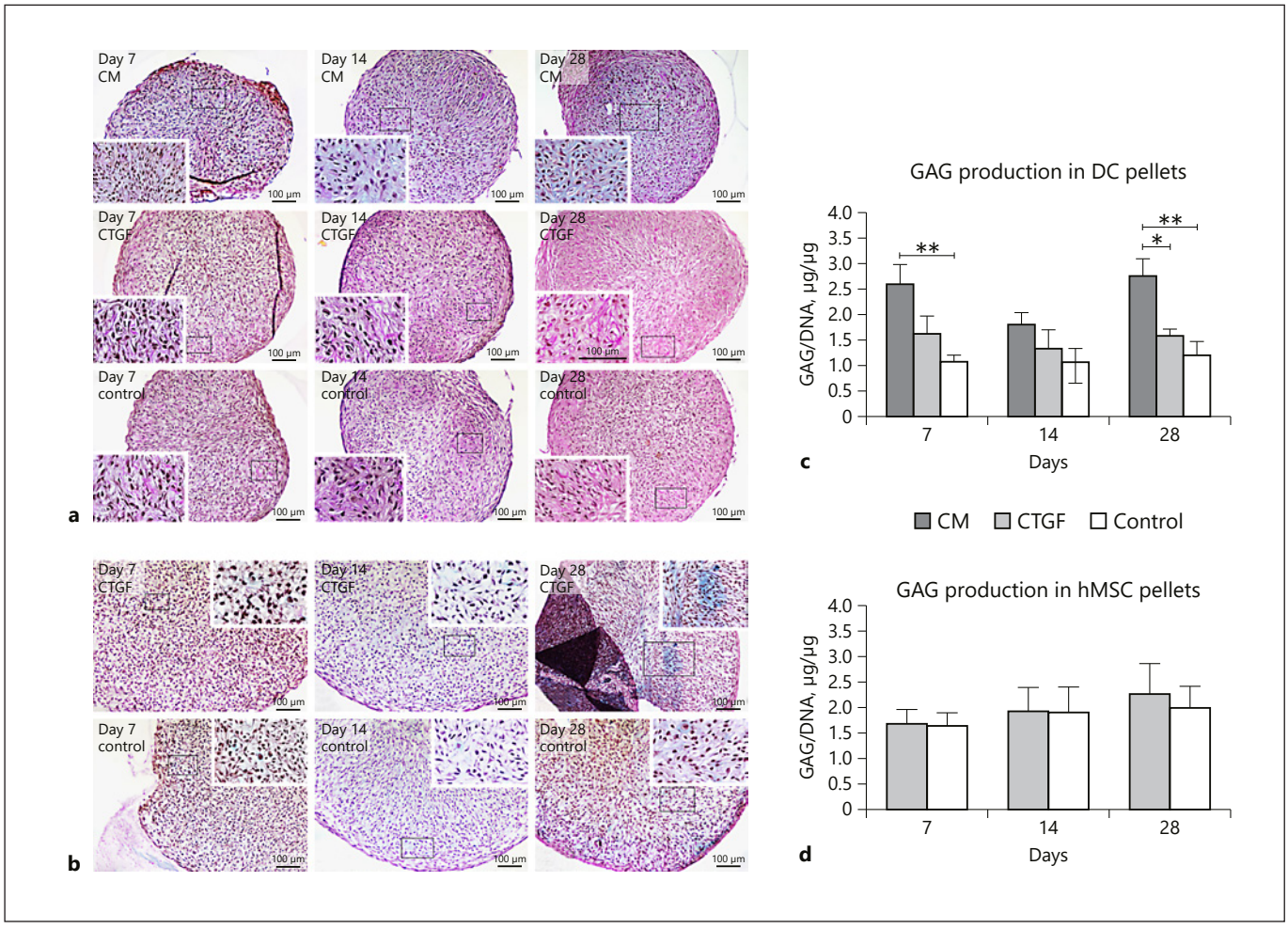

Fig. 2. Histological staining and GAG content of DC pellets stimulated with conditioned media (CM) and 10 ng/mL CTGF and hMSC pellets treated with 10 ng/mL CTGF on days 7–28. Alcian blue van Gieson staining of DC pellets (**a**) and hMSC pellets (**b**). Histology sections present proteoglycan (blue) and collagen (pink). Black dots represent the nuclei of the cells. Quantification of GAG production in DC pellets (**c**) and hMSC pellets (**d**). Values presented in bar charts are the mean ± SEM.* $p < 0.05$, ** $p < 0.01$.

groups revealed no proteoglycan accumulation in DC pellets. However, collagen deposition was observed. For the hMSC pellets, CTGF stimulation demonstrated proteoglycan accumulation on days 7 and 14. A similar pattern was observed in the control group (Fig. 2b).

Glycosaminoglycan and DNA Assays

In DC pellets, CM yielded the highest GAG production at all time points and the levels were significantly higher on day 7 ($p < 0.01$) and day 28 ($p < 0.001$) compared to the CTGF and control groups (Fig. 2c). In hMSC pellets, GAG production gradually increased from day 7 to day 28, but no significant difference was observed between the CTGF and control groups ($p > 0.05$) (Fig. 2d).

Immunohistochemistry

KRT19

The expression of KRT19 protein was observed in DC pellets at all time points regardless of the stimulations (Fig. 3a), confirming the chondrocyte-like cell properties of DC in the experiment. There was, however, no significant difference between the groups (Fig. 3c).

Sox9

The expression of Sox9 protein was observed in hMSC pellets as early as day 7, and the expression was elevated at later time points for both the CTGF stimulated and control groups (Fig. 3b, d), indicating the occurrence of chondrogenesis.

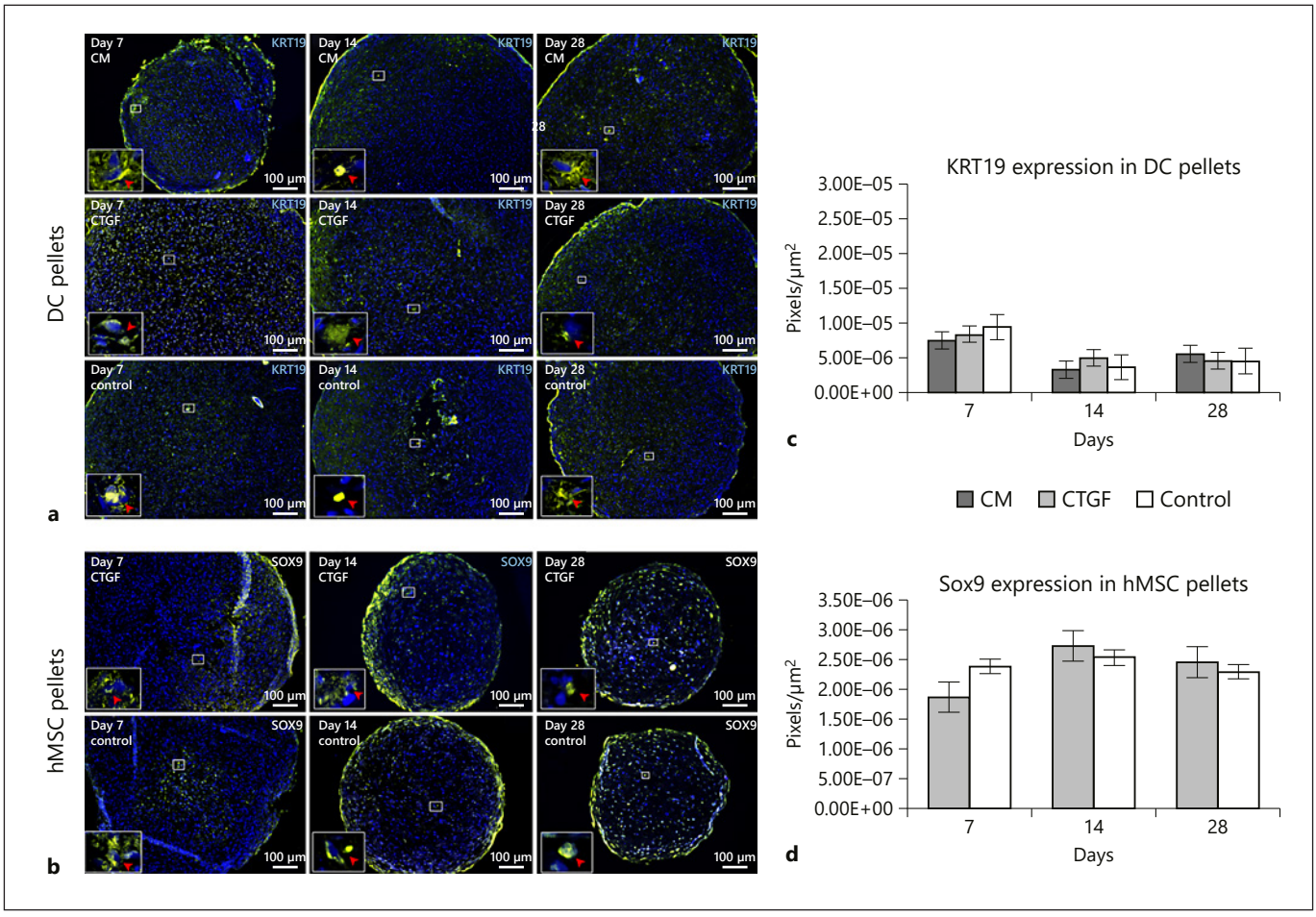

Fig. 3. a KRT19 expressions in DC pellets treated with conditioned media (CM) and 10 ng/mL CTGF on days 7–28. Yellow color indicated by red arrows shows the positive expressions of KRT19 in the pellets. Blue dots are the nuclei of DCs. **b** Expressions of Sox9 in hMSC pellets treated with 10 ng/mL CTGF on days 7–28. Yellow color indicated by red arrows shows the positive expressions of Sox9 in the pellets. Blue dots are the nuclei of hMSCs. **c**, **d** Bar graphs illustrate the level of KRT19 and Sox9-positive cells in DC and hMSC pellets, respectively. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

Aggrecan

In DC pellets, significantly higher expression of ACAN was observed in pellets treated with CM. An increase in expression was detected with time compared to in the CTGF and control groups. No significant difference was observed between the CTGF and control groups (Fig. 4a, c). Similarly, the expression of ACAN protein was observed in hMSC pellets at all time points, with no significant difference between CTGF and control groups (Fig. 4b, d).

COLIIA1

In DC pellets, significantly higher COLIIA1 expression was found in the CM group on days 14 and 28, whereas CTGF showed low COLIIA1 expression and was not significantly different from the control (Fig. 5a, c). Although COLIIA1 expression was detected at all time points in hMSC pellets (Fig. 5b), the expression was relatively low with no significant difference in the CTGF and control groups (Fig. 5d).

Mass-Spectrometry Analysis of CM

Mass spectrometry analysis identified 753 unique peptides present in CM; 129 of these showed a relative abundance (CM/MSC media) of ≥2, and 275 peptides showed <2 (for a complete list of proteins, see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000506350). The 129 peptides comprised

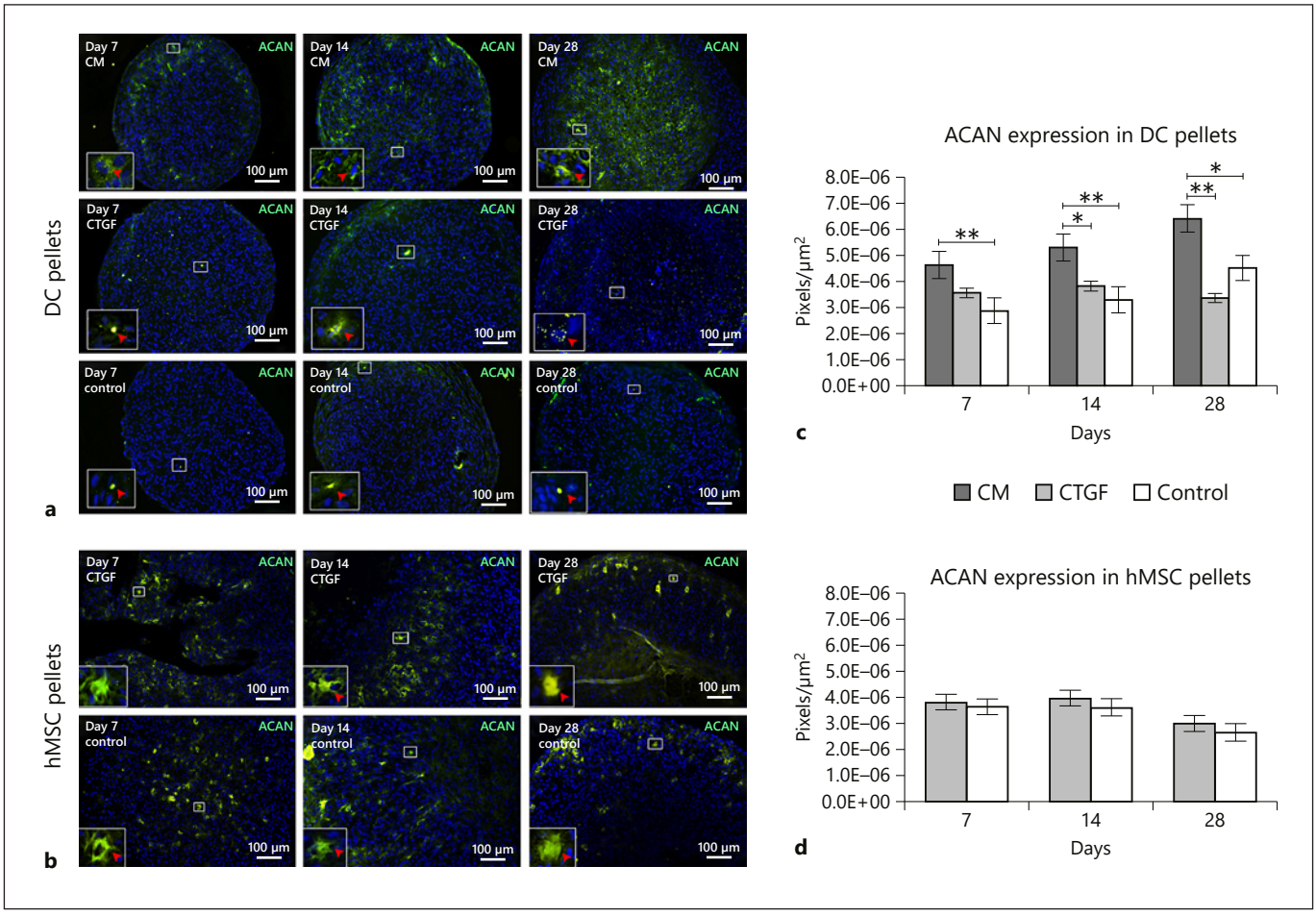

Fig. 4. Expressions of ACAN in DC pellets treated with conditioned media (CM) and 10 ng/mL CTGF (**a**) and hMSC pellets with 10 ng/mL CTGF (**b**) on days 7–28. Yellow color indicated by red arrows represents the expression of ACAN in the pellets. Blue dots are the nuclei of the cells. **c**, **d** Bar graphs illustrate the level of ACAN-positive cells in DC and hMSC pellets. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

ECM protein, enzymes, growth factors, tissue inhibitors of metalloproteinase 1 (TIMP-1), MMPs, MMP regulators, transporters, etc. (Fig. 6). Among the growth factors, vascular endothelial growth factor (VEGF), bone morphogenetic protein-1 (BMP-1) and CTGF were identified (Table 1).

Discussion

The findings in this study demonstrated that the stimulation of DCs with CM enhanced cellular viability as well as promoting ECM production, crucial for maintaining the structural integrity and biomechanical properties of the spine. The stimulation with CTGF did not add to the normal effect of chondrogenic media (containing TGF-β), in promoting chondrogenesis in either hMSCs or DCs, and was less effective than the hMSC CM.

Disc tissues collected from patients were a mixture of AF and NP due to disintegration of the discs; this made it difficult to distinguish between AF and NP. Suggested markers for chondrocyte-like/NP cells are ACAN, COLIIA1, KRT19, NCAMI, etc. [Minogue et al., 2010; Rutges et al., 2010; Risbud et al., 2015; Tang et al., 2016]. KRT19, ACAN, and COLIIA1 expression in DC pellets confirmed the chondrocyte-like cell characteristic of the DCs isolated. One of the limitations of the study was that no characterization of the cells in the disc tissues was performed to determine the ratio of AF and NP cells in these experiments due to limited sample availability. Sox9, ACAN, and COLIIA1 expression in hMSC pellets vali-

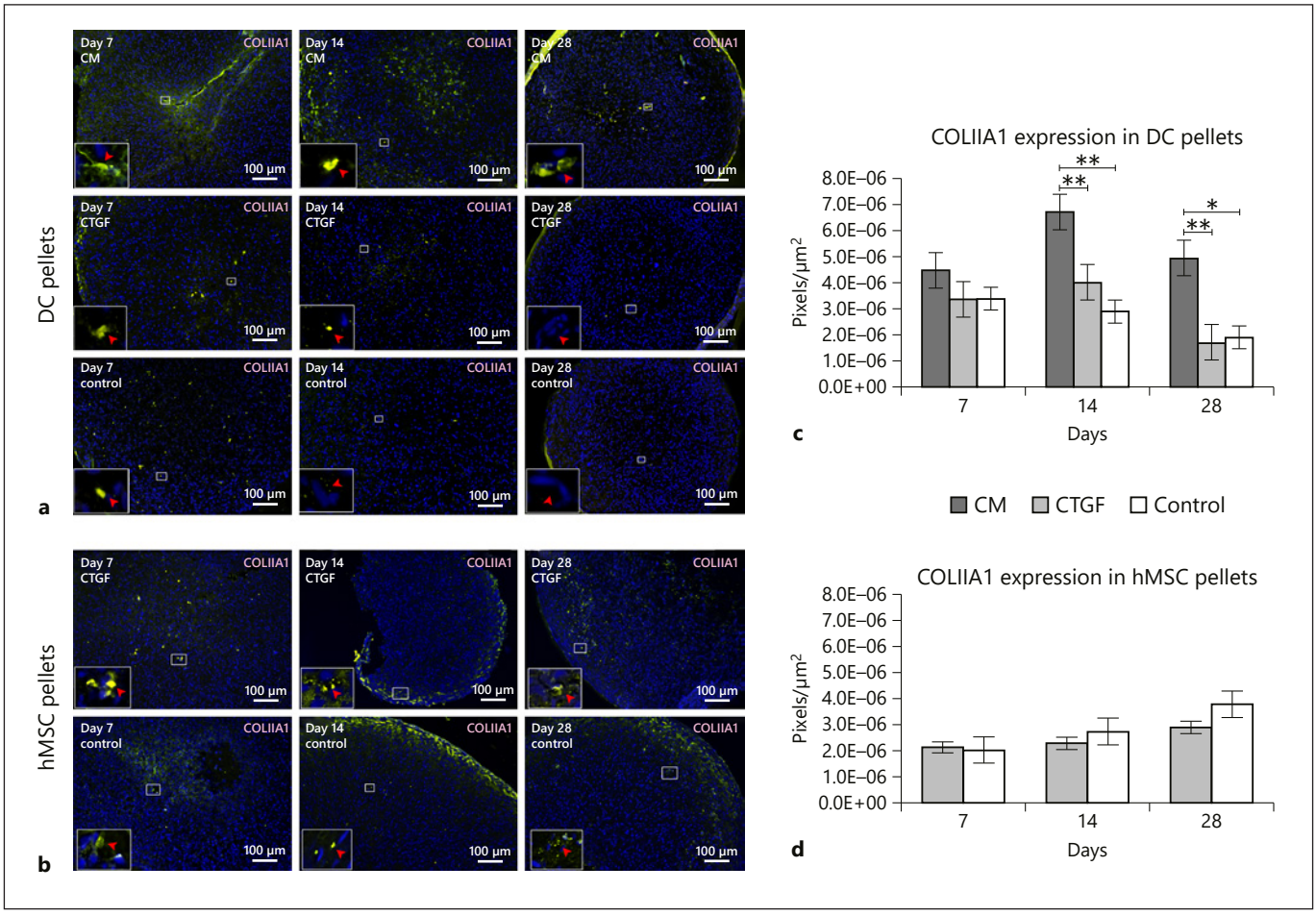

Fig. 5. Expressions of COLIIA1 in DC treated with conditioned media (CM) and 10 ng/mL CTGF (**a**) and hMSC pellets with 10 ng/mL CTGF (**b**) on days 7–28. Yellow color indicated by red arrows represents the expressions of COLIIA1 in the pellets. Blue dots are the nuclei of DCs. **c**, **d** Bar graphs illustrate the level of COLIIA1-positive cells in DC and hMSC pellets. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

dated the chondrogenic differentiation of the hMSCs [Chimal-Monroy et al., 2003].

Mass spectrometry analysis revealed 129 secreted peptides, which included modulators of growth factors, anti-apoptotic factors, antiangiogenic factors, MMP inhibitors, and several proteoglycans including collagens and small leucine-rich proteoglycans (SLRPs). CTGF was among the growth factors identified. It is known as a vital component of the IVD microenvironment where it interacts with several growth factors and ECM proteins [Abreu et al., 2002; Leask and Abraham, 2006; Nishida et al., 2007; Tran et al., 2010]. Like TGF-β, CTGF plays a role in the development of cartilage and IVD in the embryonic stage of the spine [Jin et al., 2011]. The different domains in CTGF mediate physical interaction with bone morphogenetic proteins (BMPs) and TGF-β ligands. The interaction is assisted by growth factor modulators such as insulin-like growth factor (IGF)-binding proteins, latent TGF-β-binding protein 3, follistatin, decorin, and EMILIN-1 [Abreu et al., 2002; Leask and Abraham, 2006; Munjal et al., 2014]. BMP-1, a member of the TGF-β superfamily that interacts with CTGF, along with VEGF, a growth factor that promotes cartilage repair and regeneration [Gugjoo et al., 2016; Schorn et al., 2017; Vadalà et al., 2018], was also identified. These protein networks illustrate the complexity of the signaling mechanisms required to maintain a healthy IVD.

Even though CM was supplemented with chondrogenic media containing TGF-β (at a 1:1 ratio; Fig. 1), the concentration of TGF-β might have been lower than that in the CTGF and control groups. However, stimulation with CM resulted in greater cell proliferation and ECM production than in the CTGF and control groups in DC pellets. Detec-

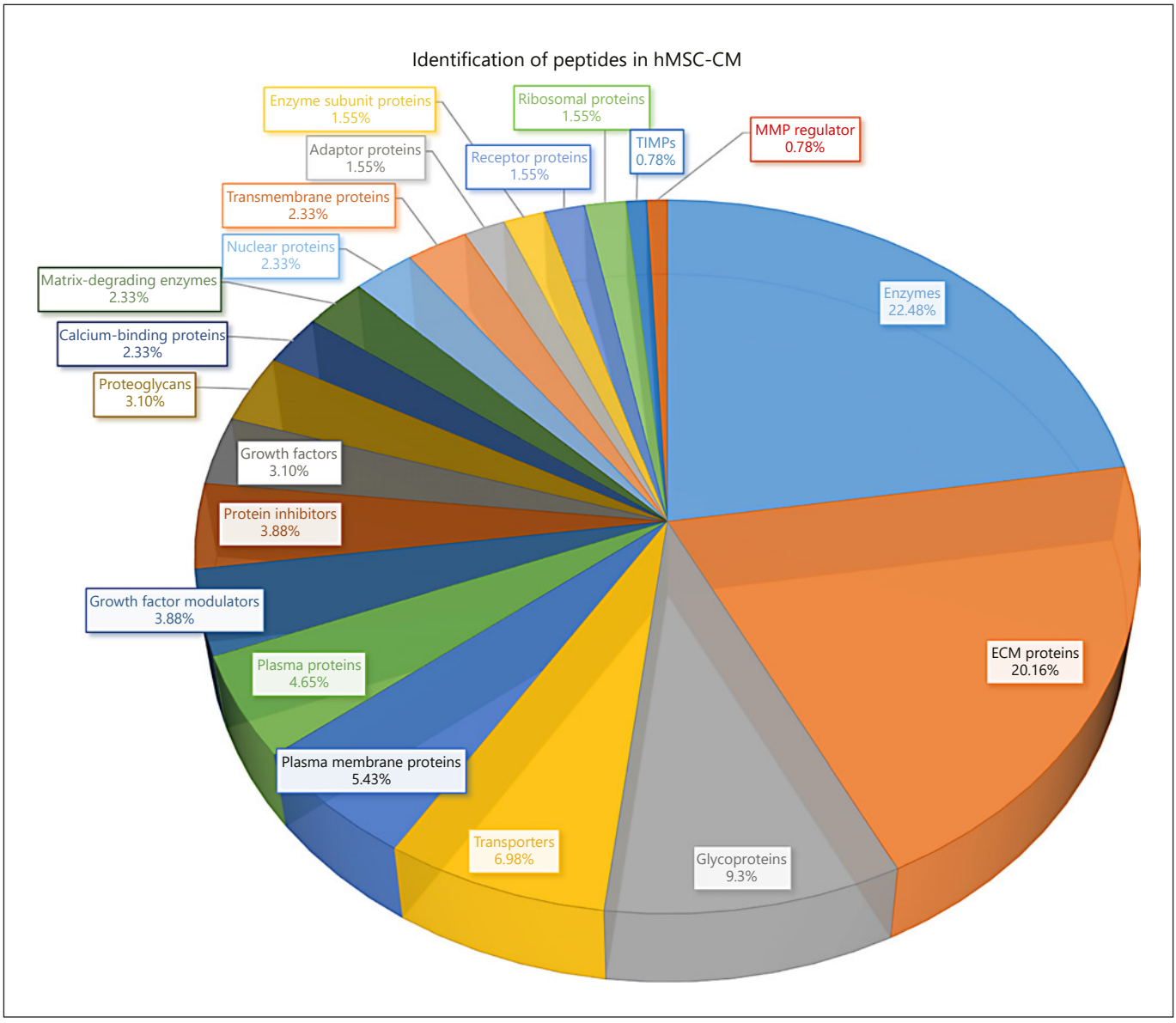

Fig. 6. Pie chart illustrating the distribution of different groups of peptides identified in CM derived from hMSCs.

tion of significantly higher cell proliferation in DCs could be triggered synergistically by the growth factors, the ECM, and the plasma proteins in the CM. VEGF, BMP-1, and CTGF are known to promote proliferative activities [Kim et al., 2012]. Several ECM and plasma proteins, such as EGF-containing fibulin-like extracellular matrix protein 2 and prosaposin, regulate cell proliferation, while stanniocalcin-1 acts as an antiapoptotic factor in response to the activation by apoptotic cells [Misasi et al., 2004; Block et al., 2009; Sun et al., 2010]. This may account for the increased viability observed in DCs when stimulated with CM.

CM induced significantly higher proteoglycan accumulation and GAG production, and it displayed higher expression of ACAN and COLIIA1 in DCs than in the CTGF and control groups. These findings are supported by previous findings that MSCs elevated ACAN and COLIIA1 expression and CM lowered ACAN degradation in a murine model of arthritis [Kay et al., 2017; Soetjahjo et al., 2018]. The observed effects of CM could possibly be caused by the presence of growth factors and several ECM proteins (cathepsin, peroxidasin, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1, which are involved

Table 1. Representative list of proteins identified in CM of hMSCs along with their classes and functions

No.	Accession No.	Name	Class	Function
1	P49767	Vascular endothelial growth factor	growth factor	involved in cartilage repair and regeneration
2	P13497	Bone morphogenetic protein 1	growth factor	cartilage proliferation and collagen synthesis
3	P29279	Connective tissue growth factor	growth factor	cartilage and bone development
4	P22692	Insulin-like growth factor-binding protein	growth factor modulator	regulates CTGF
5	Q9NS15	Latent-transforming growth factor β-binding protein 3	growth factor modulator	key regulator of TGF-β
6	P09382	Galectin-1	growth factor modulator	suppresses proinflammatory cytokine synthesis
7	P19883	Follistatin	protein inhibitor	regulates TGF-β
8	P05121	Plasminogen activator inhibitor 1	protein inhibitor	involved in cartilage repair
9	Q9BY76	Angiopoietin-related protein 4	precursor protein	Wnt signaling antagonist
10	P07602	Prosaposin	precursor protein	regulates cell proliferation and apoptosis
11	O95967	EGF-containing fibulin-like extracellular matrix protein 2	ECM protein	regulates cell proliferation and apoptosis
12	Q92626	Peroxidasin	ECM protein	plays a role in ECM modification
13	Q14112	Nidogen-2	ECM glycoprotein	involved in ECM assembly and cell invasion
14	Q9NRN5	Olfactomedin-like protein 3	ECM glycoprotein	binds to BMP-4 and involved in angiogenesis
15	Q9Y6C2	EMILIN-1	ECM glycoprotein	negatively regulates TGF-β signaling
16	P52823	Stanniocalcins	plasma protein	antiapoptotic factor
17	O75326	Semaphorin	transmembrane protein	inhibits angiogenesis and nerve in-growth in IVDs
18	P07585	Decorin	proteoglycan	negatively regulates TGF-β signaling
19	P01033	Metalloproteinase inhibitor 1	TIMP-1	inhibitor of MMP-1
20	P00749	Urokinase-type plasminogen activator	enzyme	involved in angiogenesis and cartilage remodeling
21	P07339	Cathepsin D	enzyme	ECM remodeling

in ECM assembling, remodeling, and modification [Campbell et al., 1991; Péterfi et al., 2009; Bonnans et al., 2014; Mao et al., 2014; Popa et al., 2014]. The multiple proteins in CM most likely worked hand in hand to bring about the chondrogenic effects observed in this study.

In addition, the presence of angiopoietin-related protein 4, an antagonist of Wnt-signaling in CM, may have been beneficial as the Wnt-signaling pathway regulates the expression of proinflammatory cytokines involved in IVD degeneration [Hiyama et al., 2013]. Metalloproteinase inhibitor-1, that can combat the activities of MMPs, was also identified. However, more investigations into these factors are needed to elucidate the exact role they play in combating IVD degeneration.

Other proteins such as semaphorin, nidogen-2, and olfactomedin-like protein 3 were also identified and their exact role remains unknown [Matta et al., 2017]. However, semaphorin is reported to be a key regulator of a specific signaling mechanism through several receptor complexes essential for cell migration, morphology, and motility [Casazza et al., 2007; Alto and Terman, 2017]. Semaphorin has been demonstrated to be highly expressed in healthy DCs [Alto and Terman, 2017].

Stimulation with CTGF in this study had no significant effect on DC and hMSC proliferation compared to the control group as we have hypothesized, implying that CTGF was not solely responsible for the effect seen by CM even in the presence of TGF-β. Furthermore, the concentration

used in this study might have been too low. The concentration of 100 ng/mL of CTGF has been reported to promote cell viability in the monolayers of NP cells [Matta et al., 2017] and articular chondrocytes [Xing et al., 2018]. However, the motivation for using 10 ng/mL CTGF here was that chondrogenic/control medium contained 10 ng/mL TGF-β, which has been reported to induce the expression of CTGF [Kothapalli et al., 1997; Chen et al., 2000]. CTGF at 5 ng/mL has also been shown to coordinate ECM synthesis in the presence of TGF-β [Wu et al., 2018]. The notion behind not testing hMSC pellets with CM was that the hMSCs could produce their own secretome, and that CTGF should boost proliferation and chondrogenesis. The fact that CM was effective on DCs implies that the secretome was perhaps meant for other cell types [Baraniak and McDevitt, 2010]. CM from other cell types has also been reported to show regenerative effects [Bach et al., 2016]. The presence of complex protein networks in the CM explains why a therapeutic effect was observed in coculture models. Enhanced chondrogenic activity was previously reported in cocultured DCs and hMSCs, and it was believed to be due to cell-to-cell communication or the secretion of substances through paracrine signaling [Svanvik et al., 2010; Hingert et al., 2020]. In addition to signaling peptides, hMSCs are reported to secrete extracellular vesicles, including exosomes that act as vehicles transporting various biological factors to targeted cells [Cheng et al., 2018]. These exosomes have been reported to have regenerative effects on disc degeneration [Bach et al., 2016]; however, further investigations are needed.

In conclusion, CM holds the potential to influence DCs from degenerated discs. The therapeutic effect observed was most probably caused by a combination of all the substances released by the hMSCs (not just by the growth factors, e.g., CTGF and TGF-β). These signaling peptides belong to different peptide families. The identification of the key factors needs to be carried out before suggesting peptide combinations should be used as a new treatment strategy for IVD degeneration that leads to low back pain.

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Statement of Ethics

All human tissues/cells were collected with permission from the Regional Ethics Review Board at Sahlgrenska Academy, Gothenburg University, Gothenburg, Sweden (Dnr. 532-04) and with the informed consent of the patients.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

P.N. performed cell cultures, biochemical analysis, histology, and image analysis, and contributed to writing the manuscript. D.H. designed the studies, supervised P.N. with the experiments, analyzed and compiled the results, and was a major contributor to writing the manuscript. J.A. performed conditioned media collection and compiled the data from mass spectrometry analysis. A.B. collected tissue samples for the experiment and provided input for the study. H.B. collected tissue samples from patients and provided the samples for the studies, helped design the studies, interpreted the results, and contributed to writing the manuscript. All authors read and approved the manuscript.

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