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Proteomic Comparison of the Secreted Factors of Mesenchymal Stem Cells from Bone Marrow, Adipose Tissue and Dental Pulp

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Abstract

Mesenchymal stem cells (MSCs) have therapeutic potential in various diseases, including myocardial infarction. Recently, the importance of the therapeutic effects of secreted factors from MSCs is increasing, but their identification has not progressed. In this study, we uncover the secreted protein profiles of MSCs derived from bone marrow, adipose tissue and dental pulp. Shotgun proteome analysis of conditioned MSC media by mass spectrometry identified 1533 proteins totally and 124 secreted proteins commonly produced among all three MSCs. The commonly secreted factors include already well-known factors whose functions are linked to MSCs' biological effects, such as CTGF, SERPINE1, TGFB1, DKK3 and MYDGF, and also include newly identified factors whose roles are not well investigated, for example AIMP1, CLEC11A, GAS6, HDGF, INHBA, and PCSK5. Computational biological pathway analysis revealed that these common factors strongly relate to tissue regeneration pathways such as angiogenesis, migration, and inflammatory response. Further analysis showed enrichment of ossification, sprouting, and organ survival factors, suggesting connection to the functions closely related to MSCs' therapeutic effects. This list of commonly secreted proteins could provide a reliable resource of biological factors which explain various effects of MSCs and would be useful for identifying new therapeutic factors produced from MSCs.

Keywords: Cardioprotection; Mass spectrometry; Mesenchymal stem cells; Secretome

Abbreviations: ASCs: Adipose-Derived Mesenchymal Stem Cells; BMSCs: Bone Marrow-Derived Mesenchymal Stem Cells; CM: Conditioned Media; DPSCs: Dental Pulp-Derived Mesenchymal Stem Cells; IPA: Ingenuity Pathway Analysis; MSCs: Mesenchymal Stem Cells

Introduction

Mesenchymal stem cells (MSCs), which are isolated from various kinds of tissues, including bone marrow, adipose tissue, and dental pulp, are multipotent cells capable of proliferating and differentiating into mesenchymal lineage cells [1]. MSCs are one of the promising cell types for regenerative medicine, particularly in the treatment of bone and cartilage disorders, skin wounds, neuronal injury, and cardiovascular diseases [2]. Therapeutic effects of transplanted MSCs were initially considered to be mediated by the homing of MSCs to a damaged site and differentiating into the appropriate cells. However, recently many reports have proposed that MSCs exert their therapeutic effects by secreting trophic factors [3]. These factors are assumed to regulate angiogenesis, immune response, and tissue protection, and cause acceleration of wound healing, reduction of inflammation, and protection against ischemic tissue injury [1,3].

MSC-based cell therapy has been applied to various diseases such as bone and cartilage disorder, autoimmune disease, and cardiovascular disease [4]. Cell therapy is a potential therapeutic approach, but there remain unsolved issues attributable to using cells including xenozootic infection, immune compatibility, and waiting time for *ex vivo* expansion of autologous cell preparations. Therefore, there would be great advantages if MSC-based therapeutic effects are substituted with MSC-derived trophic factor(s). Although several secreted factors such as vascular endothelial growth factor A (VEGFA), interleukin 6 (IL6), fibroblast growth factor 2 (FGF2), and TNF alpha induced protein 6 (TNFAIP6) were identified in conditioned media from MSCs as candidates for being such therapeutic factors, infusion of any single factor did not completely substitute the effects of MSCs [5,6]. This suggests that there remain important unidentified factors, or multiple factors are necessary for a full substitution of MSC therapy. For an alternative MSC therapy, it is therefore critically important to understand the profiles of secreted factors produced from MSCs.

Many gene expression analyses, typically using microarrays, have been performed to identify secreted factors from MSCs [7]. Although the strength of analyzing gene expression profiles of cells is in its comprehensiveness, mRNA expression profiles moderately correlate but not completely reflect protein expression profiles because of various post-transcriptional regulations, such as mRNA stability, translation regulation by miRNA, localization/secretion control, and post-translational protein modifications/cleavage [8]. Thus analysis of protein level would be straight-forward and desired. Actually, proteome analysis of conditioned media from MSCs has been reported [9]. However, the number of identified proteins is limited to less than 1000

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proteins [9-11]. Furthermore, there are few studies which compare the secreted protein profiles among different types of MSCs.

In this study, we analyzed the secretory proteome of MSCs by performing shotgun analysis by mass spectrometry on cultured media conditioned by bone marrow derived-MSCs (BMSCs), adipose tissue derived-MSCs (ASCs), and dental pulp derived-MSCs (DPSCs). Recently, DPSCs have come into focus as stem cell sources because of their ready availability, easy preparation, and strong therapeutic ability, as is the case of other MSCs [12,13]. However, the secreted factors from DPSCs have not been fully analyzed at the protein level. The aim of this study is to elucidate secreted protein profiles of MSCs, including DPSCs, to characterize the function of common secreted proteins, and to provide a list of proteins which might serve as novel indicators for the definition of MSCs.

Material and Methods

Isolation and culture of rat BMSCs, ASCs, and DPSCs

MSCs were isolated from bone marrow of 6- to 8-week-old Wistar rats (SLC, Shizuoka, Japan). All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

BMSCs were prepared as described [14] with slight modifications. Briefly, femoral and tibial bone marrow was flushed with α-minimum essential medium (αMEM; Gibco, New York, NY, USA). The whole marrow cells were cultured at 37°C in a humidified atmosphere, 5% CO_2 and 5% O_2 for 7 days and adherent cells were harvested. The collected cells were immunodepleted with CD11b/c with biotinylated anti-rat CD11b/c antibody (BioLegend; San Diego, CA, USA) and M-280 Streptavidin super-paramagnetic polystyrene beads (Life Technologies, Carlsbad, California, USA). The immunodepleted cells were plated and used for further experiments.

ASCs were isolated from the lateral epididymis region in 6- to 8-week-old Wistar male rats. After visible blood vessels were removed from the adipose tissue, they were minced by scalpel and digested by 0.1% collagenase type I (Worthington; Lakewood, NJ, USA) for 1 h at 37°C. Whole separated cells were washed with PBS and plated.

To isolate DPSCs, dental pulp was extracted from the incisors of neonatal Zucker rats. Pulp tissue was incubated at 37°C with 0.2% collagenase type I for 1 h, filtered, and washed in the same way as the ASC preparation. Finally, cells were cultured at 37°C in a humidified atmosphere, 5% CO_2 and 5% O_2, and were used for experiments until passage 6.

Immunophenotyping

BMSCs, ASCs and DPSCs were trypsinized with TrypLE select (Life Technologies) containing 1 mM EDTA and washed with PBS. After, these cells were suspended in Fc block (Becton Dickinson, San Jose, CA, USA) diluted DPBS 1% FBS at 1:50 ratio before labeling with the following monoclonal antibodies: CD31 (Becton Dickinson), CD11b/c, CD44, CD90, CD45, CD29 (BioLegend). More than 10,000 labeled cells were analyzed using a FACS CantoII Cytometer System running Diva software (Becton Dickinson).

Preparation of conditioned medium from BMSCs, ASCs and DPSCs

Eighty to ninety percent confluent MSC cultures were washed three times with PBS and cultured for 3 d in serum free αMEM at 37°C in a humidified atmosphere, 5% CO_2 and 5% O_2. The collected medium was filtered by 0.22 μm filters and was concentrated 50 times using Amicon Ultra 3k Da centrifugal filters (Millipore, Billerica, MA, USA). The samples were prepared with the same culture area, the same culture period (3 days), and the same enrichment factor (50 folds).

In vitro ischemia model

H9c2 rat cardiomyoblasts were obtained from ATCC (Manassas, VA, USA) and cultured in high glucose DMEM containing 10% FBS. H9c2 cells were stimulated by performing oxygen glucose deprivation (OGD). Cells were incubated in glucose-free DMEM bubbled by nitrogen gas in an atmosphere of 1% O_2 and 5% CO_2 for 6 h before transfer to high glucose DMEM and addition of conditioned medium from the MSCs. To assess cell viability we used CellTiter-Glo (Promega, Madison, WI, USA) to count the cell number, calcein-AM (Life Technologies) to observe live cells, and ethidium homodimer-1 (Life Technologies) to stain dead or damaged cells.

Mass spectrometry

Ten microliters of the conditioned media was diluted to 100 μL with purified water. As an internal control, 250 fmol of apomyoglobin from equine skeletal muscle (Sigma-Aldrich, St. Louis, MO, USA) was added to 30 μL of the diluted conditioned media. Proteins were analyzed by mass spectrometry as described [15] with slight modifications and detailed procedures are described in Supplementary Method. Briefly, the proteins were precipitated twice by methanol chloroform precipitation, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin. Samples were desalted and concentrated by using a slightly modified StageTips protocol [16]. All protein digestion was conducted three times independently. The dissolved peptides were analyzed two times independently with liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA USA) equipped with an EASY-nLCII liquid chromatography system (Thermo Fisher Scientific). The tandem mass spectra (MS/MS) were searched against the NCBI reference sequence rat database (downloaded in Jan. 2015) by Andromeda search engine in the MaxQuant suite (version 1.5.3.8, Max Plank Institute) [17,18] Database search parameters are listed in Supplementary Table 1. Protein quantity was determined by LFQ intensity of the protein normalized to that of apomyoglobin. We are submitting all MS data to jPOST (Japan Proteome Standard Repository/Database, http://jpost.org/).

Data analysis

Normalized signal intensity of identified protein data was log-transformed and then hierarchical clustering was performed using R software (http://www.R-project.org/). Cellular location and function of the identified proteins were analyzed through the use of Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). The listed common secreted proteins from MSCs were classified by Panther (http://pantherdb.org/).

Western blot analysis

Proteins in conditioned media (CM) were separated by SDS-PAGE and electrotansferred to Immobilon-FL PVDF membrane (Millipore). The blots were blocked with Odyssey Blocking Buffer (LI-COR Biosciences; Lincoln, NE, USA) for 1 h, and were probed overnight at 4°C with these primary antibodies at the final concentrations of 1 μg per mL: goat anti-CTGF, rabbit anti-SERPINE1, rabbit anti-FMOD, mouse anti-INHBA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), sheep anti-HTRA1 (R&D systems, Minneapolis, MN, USA), rabbit

anti-FN1 (Millipore), mouse anti-THBS1 (Neomarkers, Fremont, CA, USA) and rabbit anti-PCSK5 (ProteinTech, Chicago, IL, USA). After incubation with the donkey anti-rabbit, anti-goat, anti-sheep or goat anti-mouse secondary antibodies conjugated with IRDye800CW (LI-COR Biosciences GmbH, Bad Homburg, Germany) for 1 h, detection was carried out using LI-COR's Odyssey Infrared Imaging System (LI-COR Biotechnology).

Results

Characteristics of BMSCs, ASCs and DPSCs

MSCs secrete many factors that have various kinds of therapeutic effects. To compare protein expression profiles of CM from different types of MSCs, bone marrow MSCs (BMSCs), adipose-derived MSCs (ASCs), and dental pulp-derived MSCs (DPSCs) were prepared. BMSCs, ASCs and DPSCs showed a characteristic fibroblastic spindle-like shape (data not shown). The surface marker analysis by flow cytometry showed that these cells are immunologically positive for CD29, CD44 and CD90 and negative for CD45, and CD11b/c (Figure 1A), confirming these cells had the known immunophenotypic characteristics of MSCs [19]. We next performed an OGD induced cardiomyocyte cell death assay to ascertain cardioprotective efficiency of each CM type. Dual staining assessment with calcein-AM and ethidium homodimer-1 showed the CM from DPSCs recovered cell viability (Figure 1B - D) and similar effects were observed by the CM from BMSCs and ASCs (data not shown). In the quantitative cell counting assay, the CM from BMSCs, ASCs and DPSCs restored survival rate of H9c2 cardiomyocyte from 66.1% to 81.5%, 83.4% and 89.3%, respectively (Figure 1E). Therefore, both immunophenotype and functional assays of CM demonstrated the BMSC, ASC and DPSC preparations shared previously reported MSC-characteristics.

Proteome analysis of conditioned media from BMSCs, ASCs and DPSCs by mass spectrometry

Next, we performed shotgun proteome analysis of CM from BMSCs, ASCs, and DPSCs by mass spectrometry. We identified 1533 proteins in total and 999 proteins as commonly expressed proteins in all of the three CM (Figure 2A and Supplementary Table 2). Hierarchical clustering analysis showed a good reproducibility of sample preparation, and that the protein set of BMSCs was closer to ASCs than DPSCs (Figure 2B). To focus on the proteins commonly secreted by BMSCs, ASCs and DPSCs, the 700 proteins which were identified in all 18 datasets were regarded as reliable datasets and were annotated by Ingenuity Pathway Analysis (IPA) software. Cell component classification showed that 124 proteins (18%) were identified as extracellular space proteins (Figure 2C, Table 1). The percentage of extracellular component was almost equal to other secretome analysis [20]. This protein list contained many known trophic factors reported to be secreted by MSCs, for example, connective tissue growth factor (CTGF), serpin peptidase inhibitor, clade E (SERPINE1), HtrA serine peptidase 1 (HTRA1), thrombospondin 1 (THBS1), dickkopf WNT signaling pathway inhibitor 3 (DKK3), and myeloid-derived growth factor (MYDGF) [9,21]. The list also contained aminoacyl tRNA synthetase complex-interacting multifunctional protein 1 (AIMP1), C-type lectin domain family 11, member A (CLEC11A), growth arrest specific 6 (GAS6), hepatoma-derived growth factor (HDGF), inhibin beta A (INHBA), and proprotein convertase subtilisin/kexin type 5 (PCSK5) which are not related to MSC functions. The results of secretory proteome analysis were validated by Western blotting of eight representative proteins. Expression of all CTGF, fibromodulin (FMOD), SERPINE1, THBS1, fibronectin 1 (FN1), INHBA, HTRA1, and PCSK5A (secreted

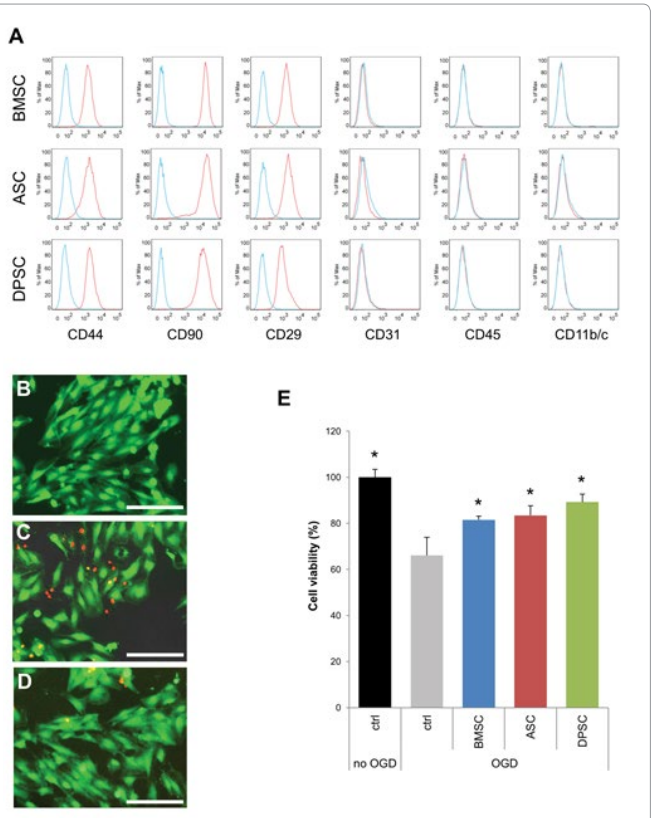

Figure 1: Characterization of three types of MSCs. (A) BMSCs, ASCs, and DPSCs were stained with antibodies against indicated antigens (red line) and isotype control (blue line), and analyzed by flow cytometry. (B-D) H9c2 were stained by calcein-AM and EthD-1. In the no OGD condition, living H9c2 cells were positive for calcein-AM and negative for EthD-1 (B). OGD/reperfusion induced cell death (C), and the conditioned medium from DPSCs increased living cardiomyocytes (D). (E) Cell viability was determined using the CellTiter-Glo luminescent cell viability assay. Data are shown as the means ± SD (N=3, **P*<0.05 versus control by Student's t test). Bar=50μm.

type isoform of PCSK5) was confirmed in CM from MSCs (Figure 3). In addition, we have measured SERPINE1 by Western blotting using recombinant protein as standard, demonstrating 100-200 ng/mL in ASC after 50-fold concentration. Thus, we estimated SERPINE1 concentration in ASC as 2-4 ng/mL range, which was functional range of the protein. These data revealed that the identified protein list provides a reliable set of genes secreted in common among the three MSC types.

Biological function of the proteins commonly secreted by BMSCs, ASCs and DPSCs

To understand the character of the 124 secreted proteins in common to BMSCs, ASCs and DPSCs, these proteins were first classified according to Protein Class based on molecular function in Panther under the major subgroups of extracellular matrix, signal molecule, and receptor (Figure 4A). Enzymes such as hydrolase, protease, oxidoreductase are also main subgroups. Then, the 124 proteins were classified according to protein functional annotation based on IPA software. This analysis showed that these factors are most closely associated with tissue regeneration, such as angiogenesis, migration, and inflammatory response (Figure 4B). The analysis also revealed the connection to ossification, sprouting, and organ survival, which are known as therapeutic effects of MSCs [22,23] (Figure 4B). The IPA

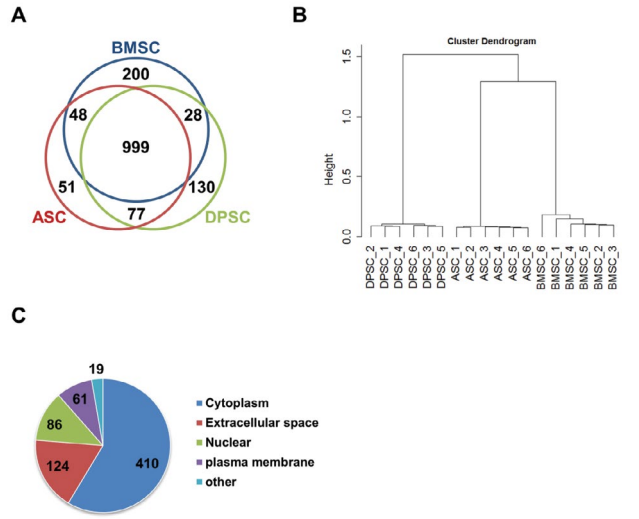

Figure 2: Proteome analysis of the conditioned media from BMSCs, ASCs and DPSCs by mass spectrometry. (A) Venn-diagram of the detected proteins in the identified datasets. (B) Unsupervised hierarchical clustering of 18 LC-MS/MS datasets. (C) The 700 proteins detected in all 18 datasets were classified by their cellular locations.

upstream regulator analysis, which predicted the cascade of common transcriptional regulators of the list, revealed that these secreted factors are regulated by twist family bHLH transcription factor 1 (TWIST1), hypoxia inducible factor 1, alpha subunit (HIF1a), and early growth response 1 (EGR1) (Figure 4C), which maintain mesenchymal stem cell properties [24-26]. These data suggest that the candidate proteins that exert therapeutic effects of MSCs, such as regenerative effects and tissue protective effects, are contained in the protein list identified as the secreted factors common to MSCs.

Discussion

Many experiments and clinical trials have reported that infusion of MSCs beneficially affects various diseases, but the exact mechanism in MSC therapies has not been fully uncovered. Recently it has been regarded that MSCs might exert their therapeutic effects through producing secreted factors which regenerate and protect damaged tissues [3]. And actually, our study shows that CM from MSCs suppressed the cardiomyocyte cell death in an *in vitro* OGD assay that well characterized myocardial infarction (Figure 1E). Although the importance of MSCs' secreted factors is gaining more attention, not many factors have been proved to substitute for MSCs' functions because of several hurdles.

The first of such hurdles is the limited number of identified secreted

Major Protein ID	Gene symbol	Protein name	Signal intensity		
			BMSC	ASC	DPSC
XP_003749876.1	A2M	alpha-2-macroglobulin	0.0279	0.0597	16.3715
XP_006248295.1	Abi3bp	ABI gene family, member 3 (NESH) binding protein	0.2176	0.0028	0.0009
NP_077376.2	ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.0574	0.0151	0.0166
NP_001099890.2	ADAMTS12	ADAM metallopeptidase with thrombospondin type 1 motif, 12	0.0486	0.0234	0.0064
NP_446209.1	AIMP1	aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	0.0181	0.0256	0.0352
NP_112641.1	ATRN	attractin	0.0371	0.0285	0.0339
NP_058783.1	BGN	biglycan	8.0566	11.4552	2.7284
NP_112613.1	BMP1	bone morphogenetic protein 1	0.0089	0.0080	0.0212
XP_008769221.1	BTD	biotinidase	0.0312	0.0378	0.0410
NP_001127908.1	C1QTNF3	C1q and tumor necrosis factor related protein 3	0.6521	0.0051	0.0713
NP_001128027.1	C1R	complement component 1, r subcomponent	0.2321	0.5779	0.6503
NP_620255.1	C1S	complement component 1, s subcomponent	0.0290	0.0571	0.0703
NP_757376.2	C2	complement component 2	0.4788	0.2556	0.7073
NP_001012477.1	CLEC11A	C-type lectin domain family 11, member A	0.1712	0.0219	0.1242
NP_037249.1	COL11A1	collagen, type XI, alpha 1	4.2557	1.6553	2.4899
XP_001060689.2	COL12A1	collagen, type XII, alpha 1	3.0851	1.6315	24.2579
NP_001124020.1	COL14A1	collagen, type XIV, alpha 1	0.0185	0.0187	0.0213
XP_003749981.1	Col15a1/ LOC100909752	collagen, type XV, alpha 1	0.4020	2.3500	0.2256
XP_006239015.1	COL16A1	collagen, type XVI, alpha 1	0.0020	0.0042	0.0159
NP_445756.1	COL1A1	collagen, type I, alpha 1	47.7847	23.1591	74.0662
NP_114474.1	COL3A1	collagen, type III, alpha 1	5.7285	5.3784	7.0606
NP_001128481.1	COL4A1	collagen, type IV, alpha 1	0.2338	0.5403	0.1713
XP_008769639.1	COL4A2	collagen, type IV, alpha 2	0.0320	0.1698	0.0649
XP_006257375.1	Col4a5	collagen, type IV, alpha 5	0.0114	0.0210	0.0281
NP_604447.1	COL5A1	collagen, type V, alpha 1	1.5493	0.9353	1.5618
NP_445940.1	COL5A2	collagen, type V, alpha 2	3.5963	1.7975	4.3577
XP_008764201.1	COL5A3	collagen, type V, alpha 3	0.0113	0.0069	0.0122
XP_215375.5	COL6A1	collagen, type VI, alpha 1	1.3206	0.3482	0.7185
NP_001094211.1	COL6A2	collagen, type VI, alpha 2	0.6199	0.2134	0.4512
XP_006227002.1	COL6A3	collagen, type VI, alpha 3	0.7366	0.7000	0.4342
XP_233542.4	COL8A2	collagen, type VIII, alpha 2	0.0136	0.0075	0.0118
XP_008763639.1	CPQ	carboxypeptidase Q	0.2642	0.4984	0.2369
NP_001099776.1	CPXM2	carboxypeptidase X (M14 family), member 2	0.0288	0.0091	0.0115
NP_076471.3	CSF1	colony stimulating factor 1 (macrophage)	0.0272	0.0632	0.0325

NP_036969.1	CST3	cystatin C	0.2887	0.4258	0.3429
NP_071602.1	CTGF	connective tissue growth factor	0.1518	0.4688	0.0308
NP_112617.2	CYR61	cysteine-rich, angiogenic inducer, 61	0.0287	0.1708	0.0546
XP_006241347.1	DCN	decorin	0.1304	0.0527	0.1963
XP_006230071.1	DKK3	dickkopf WNT signaling pathway inhibitor 3	1.1948	0.9247	1.4676
NP_446334.1	ECM1	extracellular matrix protein 1	0.1242	0.8841	0.1032
XP_006230806.1	EFEMP2	EGF containing fibulin-like extracellular matrix protein 2	0.0864	0.2105	0.2969
NP_001100180.1	EMILIN1	elastin microfibril interfacer 1	0.0506	0.0465	0.1624
XP_008758824.1	ERAP1	endoplasmic reticulum aminopeptidase 1	0.0315	0.0344	0.1193
XP_008768399.1	FAM114A1	family with sequence similarity 114, member A1	0.0136	0.0088	0.0211
XP_008763723.1	FAM49B	family with sequence similarity 49, member B	0.0079	0.0099	0.0187
XP_008774018.1	FBLN2	fibulin 2	0.0929	0.8117	0.2353
NP_062026.2	FBLN5	fibulin 5	0.0051	0.0227	0.2153
XP_008760425.1	FBN1	fibrillin 1	0.1030	0.5587	1.0240
NP_114014.1	FBN2	fibrillin 2	0.0032	0.0013	0.0790
NP_542429.1	FMOD	fibromodulin	0.0551	0.0215	0.0597
XP_006245214.1	FN1	fibronectin 1	18.0532	11.9231	7.4117
XP_006232016.1	FST	follistatin	0.0103	0.0179	0.0271
NP_077345.1	FSTL1	follistatin-like 1	2.2374	2.6397	3.2233
NP_001004218.1	FUCA2	fucosidase, alpha-L- 2, plasma	0.0041	0.0093	0.0045
NP_476441.2	GAS6	growth arrest-specific 6	0.1123	0.0756	0.0333
NP_997475.1	GPI	glucose-6-phosphate isomerase	0.5396	0.7572	0.8015
XP_008766203.1	GRN	granulin	0.0106	0.0357	0.0131
XP_006234102.1	GSN	gelsolin	0.0708	0.0695	0.1030
NP_446159.1	HDGF	hepatoma-derived growth factor	0.0351	0.0294	0.0864
NP_001258221.1	HMCN1	hemicentin 1	0.0811	0.1225	0.1697
NP_113909.1	HTRA1	HtrA serine peptidase 1	0.1468	0.2708	0.3753
NP_036720.2	IGFBP3	insulin-like growth factor binding protein 3	0.0381	0.1236	0.0256
NP_001004274.1	IGFBP4	insulin-like growth factor binding protein 4	0.0194	0.0241	0.1587
NP_037236.1	IGFBP6	insulin-like growth factor binding protein 6	0.0187	0.0930	0.0672
NP_001013066.1	IGFBP7	insulin-like growth factor binding protein 7	1.1682	0.9196	0.7393
XP_008769938.1	INHBA	inhibin, beta A	0.0649	0.0647	0.0385
XP_003753537.1	LAMA4	laminin, alpha 4	0.3678	0.5865	0.2223
XP_006240059.1	LAMB1	laminin, beta 1	0.3529	0.7615	0.3332
NP_446418.1	LAMC1	laminin, gamma 1 (formerly LAMB2)	0.4637	0.9408	0.3888
NP_063969.1	LGALS1	lectin, galactoside-binding, soluble, 1	1.0603	1.1178	1.8905
NP_114020.1	LGALS3	lectin, galactoside-binding, soluble, 3	0.0060	0.0300	0.0306
XP_008763191.1	LOC103692716	heat shock protein HSP 90-alpha	0.3629	0.6495	1.1855
NP_001099517.2	LOXL2	lysyl oxidase-like 2	0.1879	0.4050	0.2144
NP_067598.1	LTBP1	latent transforming growth factor beta binding protein 1	0.0776	0.1057	0.1217
XP_006240412.1	LTBP2	latent transforming growth factor beta binding protein 2	0.2818	1.3109	0.2345
NP_112312.1	LUM	lumican	0.5346	0.0021	3.1152
XP_006243837.1	Manf	mesencephalic astrocyte-derived neurotrophic factor	0.0134	0.0108	0.0300
XP_006248588.1	Masp1	mannan-binding lectin serine peptidase 1	0.0611	0.2367	0.1086
NP_001008346.1	MESDC2	mesoderm development candidate 2	0.0054	0.0077	0.0215
NP_036943.1	MFGE8	milk fat globule-EGF factor 8 protein	0.1749	0.7081	0.3723
NP_001100629.1	MMP19	matrix metallopeptidase 19	0.0083	0.0194	0.0407
NP_112316.2	MMP2	matrix metallopeptidase 2	2.4294	1.4391	6.5126
XP_008756141.1	MYDGF	myeloid-derived growth factor	0.0168	0.0172	0.0399
NP_001002851.1	NENF	neudesin neurotrophic factor	0.0118	0.0129	0.0226
XP_213954.4	NID1	nidogen 1	1.5978	2.8422	1.1877
NP_001012005.2	NID2	nidogen 2 (osteonidogen)	0.0965	0.3051	0.0779
NP_110495.1	NOV	nephroblastoma overexpressed	0.1144	1.0559	0.0114
NP_775141.2	NPC2	Niemann-Pick disease, type C2	0.3482	0.7171	0.5390
NP_001099573.1	OGN	osteoglycin	0.3674	0.2553	0.2353
XP_006233139.1	OLFML3	olfactomedin-like 3	0.0212	0.0213	0.0368
XP_006238331.1	PAPPA	pregnancy-associated plasma protein A, pappalysin 1	0.0534	0.0054	0.0123
XP_006221574.1	PAPPA2	pappalysin 2	0.1094	0.1008	0.9496
NP_062110.1	PCOLCE	procollagen C-endopeptidase enhancer	1.8591	2.8958	3.1640
NP_446275.1	PCSK5	proprotein convertase subtilisin/kexin type 5	0.0146	0.0373	0.0872
NP_640348.2	PLBD2	phospholipase B domain containing 2	0.0737	0.1281	0.0550

NP_001162015.1	PLTP	phospholipid transfer protein	0.0296	0.0358	0.0322
NP_001101892.1	PLXDC2	plexin domain containing 2	0.0690	0.0444	0.0453
XP_006232398.1	POSTN	periostin, osteoblast specific factor	3.2539	0.5683	0.4740
NP_112348.2	PROS1	protein S (alpha)	0.0740	0.0919	0.1198
NP_001177167.1	PSAP	prosaposin	0.2440	0.5507	0.3535
NP_001258190.1	PXDN	peroxidasin	0.3106	0.6939	0.2733
XP_006251969.1	RNASE4	ribonuclease, RNase A family, 4	0.2844	0.5855	0.3817
NP_001100048.1	SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	0.0163	0.0473	0.0433
NP_001098103.1	SEMA3D	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	0.1098	0.0325	0.3249
XP_008760920.1	SEMA3E	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E	0.0419	0.0236	0.0416
NP_036752.1	SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	2.0028	7.4714	0.2493
NP_808788.1	SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	2.9882	3.9966	2.3547
NP_058869.1	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	0.1892	0.2190	1.1739
NP_001005539.1	SMPDL3A	sphingomyelin phosphodiesterase, acid-like 3A	0.0640	0.0199	0.0141
NP_037012.1	SOD3	superoxide dismutase 3, extracellular	0.1297	0.0874	0.0123
XP_008765885.1	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	15.7040	19.1240	15.7550
NP_612542.1	SPON2	spondin 2, extracellular matrix protein	0.0143	0.0101	0.0422
XP_008768218.1	SPP1	secreted phosphoprotein 1	0.5236	0.0787	0.1310
XP_006251432.1	TCN2	transcobalamin II	0.3218	0.4342	0.6680
NP_067589.1	TGFB1	transforming growth factor, beta 1	0.0225	0.0140	0.0215
XP_006250510.1	TGFB2	transforming growth factor, beta 2	0.0796	0.0928	0.0260
XP_006240428.1	TGFB3	transforming growth factor, beta 3	0.0361	0.0779	0.0105
NP_001013080.1	THBS1	thrombospondin 1	2.6504	1.1193	0.3630
XP_008759460.1	THBS3	thrombospondin 3	0.0206	0.0104	0.0131
XP_008771256.1	TIMP1	TIMP metallopeptidase inhibitor 1	0.7176	1.3352	0.5195
NP_068824.1	TIMP2	TIMP metallopeptidase inhibitor 2	0.8361	0.7614	1.4598
XP_008761979.1	TNC	tenascin C	0.2648	6.2157	0.4773
NP_001164030.1	VCAN	versican	0.0188	0.0417	0.0439
NP_001014157.1	WDR1	WD repeat domain 1	0.3081	0.3749	0.4758

Signal intensity shows LFQ intensity of the protein normalized to that of apomyoclobin. Full list of identified protein list with more detailed information is provided as Supplementary Table 2.

Table 1: Common secreted proteins identified by secretory proteome analysis of BMSCs, ASCs and DPSCs.

factors. Although MSC-secreted factors have been intensively analyzed using gene expression analysis, mass spectrometry-based proteomics, and cytokine arrays, the exact profiles of secreted proteins influencing the therapeutic effects are still unclear. We identified as many as 1533 proteins totally in the CM from BMSCs, ASCs and DPSCs by using shotgun proteomics. Compared to the previous study that identified less than 1000 proteins [9-11], this study showed much more coverage. We provided a common list of secreted factors by comparing MSCs, including DPSCs whose secreted factors have not yet been analyzed in a comprehensive manner. This list contained not only therapeutic factors already identified in past reports, but also new bioactive factors that have not been connected to the MSC effects. It could provide important clues to understanding underlying mechanisms of MSCs' therapeutic effects mediated by secreted factors from MSCs.

Another hurdle to identifying biologically important factors is the prospect that multiple, not single, factors are required to fully substitute for MSCs. For instance, it has reported that combination of chemokine (C-C motif) ligand 2 (CCL2) and sialic acid binding Ig-like lectin 9 (SIGLEC9), which are produced from DPSCs, is needed to enhance repair of the spinal cord [27]. We identified several secreted factors which have been reported as cardioprotective, including CTGF, MYDGF, DKK3, and follistatin like 1 (FSTL1) [21,28-30]. However, single treatment of CTGF, DKK3 and FSTL1, did not enhance cell viability in an *in vitro* OGD-assay (data not shown). This comprehensive list of factors secreted in common from three different types of MSCs could be useful to uncover the therapeutic mechanisms of MSC therapies by using it to narrow down the possible combinations of biological factors.

The list also contains several factors not yet linked to MSC functions, including AIMP1, C1q and tumor necrosis factor related protein 3 (C1QTNF3), CLEC11A, INHBA, and PCSK5, and this information could provide a new perspective on uncovering MSCs' therapeutic mechanisms. For example, PCSK5 belongs to the proprotein convertase subtilisin/kexin (PCSK) family and cleaves various precursor proteins at the site after unique paired basic amino acids within the motif (R/K Xn R/K↓, downward arrow indicates the cleavage site) [31]. PCSK5 exists as two isoforms, secreted type PCSK5A and membrane-bound type PCSK5B [31]. In the CM from MSCs, PCSK5A was detected by Western blotting (Figure 3). A number of proteins identified in CM of the MSCs in our analysis possess putative PCSK cleavage sites (data not shown). It is possible that PCSK5 regulates release of these secreted proteins from MSCs. Superoxide dismutase 3, extracellular (SOD3), which catalyzes superoxide radicals into hydrogen peroxide, has the

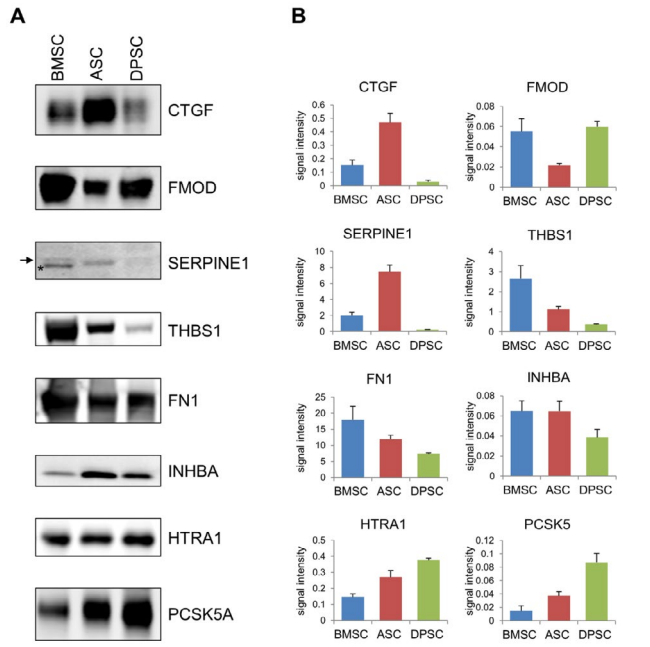

Figure 3: Validation of the mass spectrometry-based proteome result by Western blotting. (A) The concentrated CM from BMSCs, ASCs and DPSCs were subjected to SDS-PAGE analysis, and CTGF, FMOD, SERPINE1, THBS1, FN1, INHBA, HTRA1, and PCSK5A were detected by Western blotting using each antibody. Arrow indicates SERPINE1, and asterisk indicates non-specific band. (B) The bar charts show the signal intensity of each protein in BMSCs, ASCs and DPSCs. Data are shown as the means ± SD (N=6).

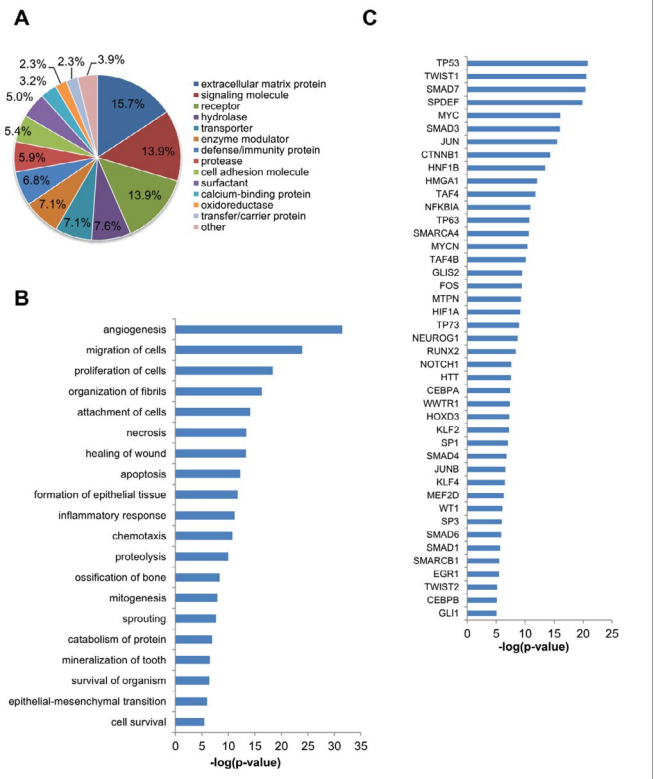

Figure 4: Characterization of 124 proteins identified as common secreted factors by secretory proteome analysis of BMSCs, ASCs and DPSCs. (A) Protein classification of 124 secreted factors based on Panther. (B), (C) The list of protein functions (B) and the list of the cascade of upstream transcriptional regulators (upstream regulators) (C) significantly altered. The both lists were generated using the Ingenuity Pathway Analysis software.

PCSKs recognition sequence (RKKR↓R[235]) at the heparin binding site and is produced as intact and cleaved form [32]. Because the intact form of SOD3 binds to extracellular matrix mediated by a heparin binding site, cleavage of SOD3 by PCSKs is important to its release from the cell surface [33]. In addition, fibronectin, which forms a complex with transforming growth factor beta (TGFβ), latent TGF beta binding protein and fibrillin and keeps TGFβ in an extracellular matrix [34], also has the PCSK recognition sequence (RRAR↓R[1911]). Therefore, SOD3 and TGFβ complex may be promoted to release from the extracellular matrix surrounding MSCs to the medium conditioned by PCSK5. Since the anti-oxidant function of SOD3 and pro-regenerative function of TGFβ are major effects of MSCs, it is possible that cleavage of SOD3 and/or fibronectin explains the cardioprotective function of the CMs. PCSK5 produced from MSCs may control the function and localization of multiple proteins produced from not only MSCs themselves but also surrounding cells and cause various biological effects. It is tempting to speculate that this kind of regulation could explain a wide range of MSCs' therapeutic effects, although further investigations are needed.

This list of secreted factors could also have the potential to expand MSC therapy into new target diseases. We identified several secreted proteins related to genetic diseases, which include bone morphogenetic protein 1 (BMP1), serpin peptidase inhibitor, clade F (SERPINF1), serpin peptidase inhibitor, clade H (SERPINH1), and Niemann-Pick disease, type C2 (NPC2). Mutation of BMP1, SERPINF1, and SERPINH1 causes osteogenesis imperfecta, which is characterized by bone fragility, deformity of the spine and long bones, and growth deficiency [35]. Infusion of allogenic MSCs to osteogenesis imperfecta promotes osteoblast differentiation and improvement of symptoms [36,37]. In addition to providing a source of osteoblasts, MSCs that secrete BMP1, SERPINF1, and SERPINH1 may be able to supplement these proteins in osteogenesis imperfecta patients. Mutations in Niemann-Pick disease, type C 1 (NPC1) and NPC2 cause Niemann–Pick type C (NP-C) disease, a fatal neurodegenerative disease associated with abnormal cholesterol accumulation. Several reports have demonstrated that transplantation of MSCs contributes to improvement of neurological functions in NP-C model mice that contain mutated NPC1 [38]. Recombinant NPC2 restores cholesterol accumulation in human NPC2-mutated fibroblasts [39]. Therefore, the CM from MSCs including NPC2 may have benefits for treatment of NP-C caused by NPC2. Likewise, MSCs or CM from MSCs may have similar effects on other genetic diseases by replenishing the lacking proteins. Accordingly, it may be an interesting approach to uncover new curable diseases currently reported as untreatable by MSCs but able to take advantage of the information of the MSC secreted factors.

We have now identified secreted factors of three different types of MSCs, including DPSCs. These factors contain the cytokines and growth factors reported as therapeutic factors of MSCs, and their global analysis predicted functions associated with the therapeutic effects of MSCs. This protein list would be beneficial for identification of new alternative factors for MSC therapy, definition of the mechanism of action of MSCs, and prediction of new diseases for the application of MSC therapy. More detailed analysis of these secreted factors will clarify further therapeutic potential of MSCs and promote their clinical application.

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Conflict of Interest

The authors have declared no conflict of interest.

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