BMB
reports

Comparative secretome analysis of human follicular dermal papilla cells and fibroblasts using shotgun proteomics

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The dermal papilla cells (DPCs) of hair follicles are known to secrete paracrine factors for follicular cells. Shotgun proteomic analysis was performed to compare the expression profiles of the secretomes of human DPCs and dermal fibroblasts (DFs). In this study, the proteins secreted by DPCs and matched DFs were analyzed by 1DE/LTQ FTICR MS/MS, semi-quantitatively determined using emPAI mole percent values and then characterized using protein interaction network analysis. Among the 1,271 and 1,188 proteins identified in DFs and DPCs, respectively, 1,529 were further analyzed using the Ingenuity Pathway Analysis tool. We identified 28 DPC-specific extracellular matrix proteins including transporters (ECM1, A2M), enzymes (LOX, PON2), and peptidases (C3, C1R). The biochemically-validated DPC-specific proteins included thrombospondin 1 (THBS1), an insulin-like growth factor binding protein3 (IGFBP3), and, of particular interest, an integrin beta1 subunit (ITGB1) as a key network core protein. Using the shotgun proteomic technique and network analysis, we selected ITGB1, IGFBP3, and THBS1 as being possible hair-growth modulating protein biomarkers. [BMB reports 2012; 45(4): 253-258]

INTRODUCTION

Dermal papilla cells (DPCs) are specialized fibroblasts that form at the base of the hair follicle. DPCs are essential not only in the control of hair growth, formation and cycling but also in the pathogenesis of androgenetic alopecia (1-3). DPCs secrete diverse growth factors and stimulate the proliferation and differentiation of the follicular epithelium (4, 5). The regulation of hair follicle regeneration depends on a complex series of interactions, mediated by DPCs through a paracrine mechanism (6). It is interesting that DPC-derived factors have been shown to influence surrounding cells, which leads, in turn, to hair growth promotion (7). Because of this, proteomic profiling of the secretome of DPCs can provide understanding of hair follicle development and the clinical application of relevant protein biomarkers. In spite of the benefits that could derive from proteomic techniques, little is yet known about the functional proteins involved in hair biology. A previous proteomic study investigating extracellular matrix (ECM) proteins secreted by two types of fibroblasts from the scalp revealed that thrombospondin1 (THBS1) and fibronectin (FN) were over-expressed in DPCs (8). Furthermore, proteins involved in the aggregative property of cultured DPCs in the early-passage stage have been analyzed using comparative proteomic strategies, through which heat shock protein 70 and mitochondrial ribosomal protein S7 were identified (9).

The present study aimed to examine a whole range of secretome protein profiling, using samples extracted from cultured human DPCs in the third to fourth-passage, compared with matched dermal fibroblast scalp cells (DFs), using large-scale shotgun proteomics to do this. In addition, we characterized the identified secretomes by using protein interaction network analysis, particularly looking at the growth modulating factors of DPCs

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http://dx.doi.org/10.5483/BMBRep.2012.45.4.253

Received 19 September 2011, Revised 8 October 2011, Accepted 19 November 2011

Keywords: Dermal fibroblast, Dermal papilla cell, Extracellular matrix, Proteome, Secretome

RESULTS

Identification and comparison of the secreted proteins using shotgun proteomics

To identify the major proteins secreted by human DPCs, as compared with those secreted by matched DFs, we employed a shot-

gun proteomic method, using a SDS-PAGE/LTQ FT-MS/MS analysis of secreted proteins, as has been performed previously (10). After SDS-PAGE analysis, the secreted proteins were fractionated into 20 bands according to their molecular weight and band intensity, using 1-DE. Next, the separated proteins were subjected to in-gel digestion with trypsin and the resultant tryptic digests were further separated by reverse phase-liquid chromatography prior to FT-MS/MS analysis. We obtained similar numbers of MS/MS spectra (91,679 spectra for CM-DFs; 91,593 for CM-DPCs) from tryptic digests, resulting in 11,124 and 9,871 peptides from CM-DFs and CM-DPCs, respectively. Next, MASCOT search from these peptide sequences revealed 1,271 (83%) and 1,188 (77%) proteins from CM-DFs and CM-DPCs, respectively. Some proteins (930, 61%) were identified in both culture media. A semi-quantitative analysis of the secreted proteins from both cell types was performed using the exponentially modified protein abundance index (emPAI)-based mole percentage (mol%) of each identified protein (11).

Vimentin (54 kDa) was the most dominantly expressed protein in CM-DFs, with a 3.5 mol%, and a peptide hit score (PHS) of 590. The principal protein in CM-DPCs was also vimentin, at 3.2 mol% and with a PHS of 627 (Table S1).

Table 1 shows a complete list of identified ECM proteins with PHS ≥ 2, based on gene ontology and emPAI-mol%. Most of the ECM proteins identified were skeletal proteins, such as serpin, galectin, collagen, and secreted protein acidic and rich in cysteine (SPARC), which were previously found to be the ECM components of hair follicular fibroblasts (12). There were 28 DPC-specific ECM proteins including transporters (ECM1, A2M, APOA1), enzymes (LOX, PON2, DNASE1L1), and peptidases (C3, C1R, C1S, LTF, HTRA1, MMP2). A total of 47 ECM proteins were common to both groups, including cytokines (C19ORF10,

Table 1. List of directly linked extracellular matrix and core plasma membrane proteins identified from the secretomes of cultured DPCs and DFs, quantified by Mol% and peptide hit score (PHS)

IPI Acc. No	Protein Description*	Protein name	Function†	SignalP‡	DFs		DPCs	
					Mol%	PHS	Mol%	PHS
IPI00478003	Alpha-2-macroglobulin	A2M	Transporter	24	164,600/6	N.D.	N.D.	0.010%
IPI00022434	Putative uncharacterized protein ALB	ALB	Transporter	19	73,881/6.33	0.024%	25	0.040%
IPI00021841	Apolipoprotein A-I	APOA1	Transporter	19	30,759/5.56	N.D.	N.D.	0.014%
IPI00297160	Isoform 12 of CD44 antigen	CD44	Other	21	39,904/5.2	0.034%	11	0.052%
IPI00297646	Collagen alpha-1(I) chain	COL1A1	Other	23	139,853/5.6	0.155%	55	0.094%
IPI00304962	Collagen alpha-2(I) chain	COL1A2	Other	25	129,723/9.08	0.128%	34	0.171%
IPI00021033	Isoform 1 of Collagen alpha-1(III) chain	COL3A1	Other	24	139,733/6.21	0.022%	7	0.017%
IPI00291136	Collagen alpha-1(VI) chain	COL6A1	Other	20	109,602/5.26	0.129%	47	0.169%
IPI00304840	Isoform 2C2 of Collagen alpha-2(VI) chain	COL6A2	Other	21	109,709/5.85	0.078%	17	0.071%
IPI00022200	Isoform 1 of Collagen alpha-3(VI) chain	COL6A3	Other	26	345,163/6.26	0.077%	110	0.087%
IPI00027547	Dermcidin	DCD	Other	20	11,391/6.08	0.038%	2	0.041%
IPI00026314	Isoform 1 of Gelsolin	GSN	Other	28	86,043/5.9	N.D.	N.D.	0.107%
IPI00646773	Isoform 2 of Gelsolin	GSN	Other	-	80,876/5.58	0.098%	18	N.D.
IPI00018305	Insulin-like growth factor-binding protein 3	IGFBP3	Other	28	32,680/9.03	N.D.	N.D.	0.013%
IPI00217561	Isoform Beta-1C of Integrin beta-1	ITGB1	Treceptor	21	94,981/5.31	N.D.	N.D.	0.056%
IPI00217563	Isoform Beta-1A of Integrin beta-1	ITGB1	Treceptor	21	91,664/5.27	0.046%	14	N.D.
IPI00013976	Laminin subunit beta-1	LAMB1	Other	22	20,5178/4.84	0.004%	2	0.011%
IPI00296922	Laminin subunit beta-2	LAMB2	Enzyme	33	202,982/6.07	0.002%	1	0.004%
IPI00298281	Laminin subunit gamma-1	LAMC1	Other	36	18,3191/5.01	0.016%	9	0.021%
IPI00219219	Galectin-1	LGALS1	Other	18	15,048/5.34	0.254%	37	0.277%
IPI00465431	Galectin-3	LGALS3	Other	18	26,193/8.57	0.182%	7	0.156%
IPI00020986	Lumican	LUM	Other	19	38,747/6.16	N.D.	N.D.	0.090%
IPI00218398	Matrix metalloproteinase-14	MMP14	Peptidase	29	66,184/7.63	0.019%	4	0.021%
IPI00027780	72 kDa type IV collagenase	MMP2	Peptidase	30	74,918/5.26	N.D.	N.D.	0.012%
IPI00299738	Procollagen C-endopeptidase enhancer 1	PCOLCE	Other	26	48,797/7.41	0.009%	1	0.040%
IPI00015614	Isoform A of Trypsin-3	PRSS3	Peptidase	59	33,306/7.46	0.012%	11	0.013%
IPI00006114	Pigment epithelium-derived factor	SERPINF1	Other	20	46,484/5.97	N.D.	N.D.	0.031%
IPI00032140	Serpin H1	SERPINH1	Other	19	46,525/8.75	0.823%	96	0.737%
IPI00014572	SPARC	SPARC	Other	18	35,465/4.73	0.093%	10	0.101%
IPI00296099	Thrombospondin-1	THBS1	Other	19	133,291/4.71	0.030%	27	0.056%

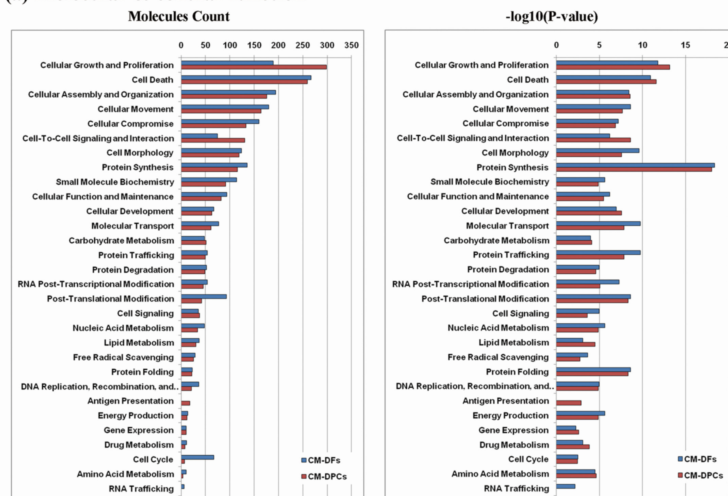
*To identify the peptides, MASCOT (version 2.2) operated on a local server was used to search the human sequences within the IPI database (version 3.63). MASCOT searching was performed with the monoisotopic masses selected with a precursor mass error of 50 ppm, and a fragment ion mass error of 0.8 Da. Trypsin was selected as the enzyme with one potential missed cleavage. Carbamidomethylated cysteine and oxidized methionine were chosen as variable modification. The qualified scoring peptides were considered as satisfied above the threshold dictated by the MASCOT search (confidence, $P < 0.05$; MASCOT score > 28). †Protein function was assigned by the annotated information from IPA (version 7.1, http://www.ingenuity.com). ‡Signal sequences of ECM proteins were predicted by the Signal P (version 3.0, http://www.cbs.dtu.dk/SignalP/).

FAM3C, AIMP1), growth factors (HDGF, TYMP), transporters (IGFBP3, AFP, ALB), peptidases (PRSS3, MMP14, TRF, ERAP1), and an enzyme (GPI). There were 13 secreted proteins exclusively found in DFs, of which cytokines (TXLN1, C5), peptidase (IDE), and enzyme (LAMB2) were newly found in our study.

Gene ontology and functional network analysis of the secretomes

To address the biological function of the proteins found to be secreted from cultured DFs and DPCs, the 1,529 proteins identified were subjected to a gene ontology assessment via Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Inc., Redwood City, CA). Based on a statistical assessment of significance, functional categories were grouped and classified according to molecular and cellular function, and according to their roles in physiological system development and function. Proteins secreted from DPCs showed significant involvement in cellular growth and proliferation ($P = 6.8 \times 10^{-14}$), cell death ($P = 2.6 \times 10^{-12}$), and cellular assembly and organization ($P = 2.7 \times 10^{-9}$), compared to those from DFs (Fig. 1A and Supplementary Table S2). Besides this, the proteins involved in cell-to-cell signaling and interaction (262-fold), cellular development (4-fold), and lipid metabolism (29-fold) were expressed at higher levels in DPCs compared to DFs, suggesting that DPCs might interact via lipid metabolic processes during hair growth. In the categories of physiological system development and function, embryonic and connective tissue development, and immune cell trafficking, the proteins from DPCs were more prominent than those from DFs, as might have been expected. In contrast, cell morphology (111-fold), molecular transport (77-fold), RNA post-transcriptional modification (173-fold), and the cell signaling (23-fold) activities of DFs were higher than those of DPCs. These results imply that DFs are metabolically more active than DPCs.

The principal biological network of DPCs-secreted proteins was cellular growth and proliferation, with a molecular count of 299; while that of DFs was cell death, with a molecular count of 267 (Fig. 1B). This suggests that programmed cell death occurs in the lower part of hair follicles (13). In the physiological system development and function category, a principal network of connective tissue development and function was identified from DPC-secreted proteins, with strong statistical significance (P =



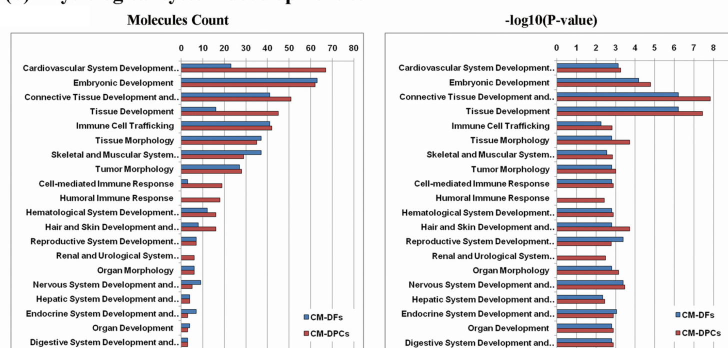


Fig. 1. Distribution of all identified proteins according to categories of biological functions. The secreted proteins identified from conditioned media (CM) of human primary cultured dermal papilla cells (DPCs) and dermal fibroblasts (DFs) were classified as proteins associated with (a) molecular and cellular function, or (b) physiological system development and function.

1.5×10^{-8}), followed by the network cluster of tissue development ($P = 3.6 \times 10^{-8}$; supplementary Table S2).

Of the secretomes of DPCs and DFs, ECM and core plasma membrane proteins were selected to construct the possible protein-protein interaction network based on the IPA. As shown in Fig. 2, matrix metalloproteinase 2 (MMP2, called 72kDa type IV collagenase) from DPCs and collagen type 1α1 (COL1A1) from DFs act as a hub in the extracellular space, converging to the integrin β1 subunit (ITGB1, known as fibronectin receptor) in the plasma membrane. It is notable that the key receptor protein, ITGB1, was also functionally connected with putative hair growth regulating factors such as MMP2, THBS1, and insulin-like growth factor binding protein 3 (IGFBP3), which acted as satellite hub proteins.

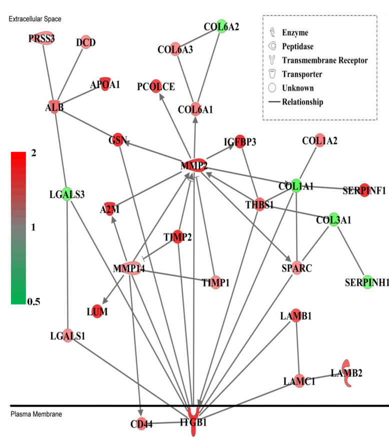


Fig. 2. The protein network of the secretomes of cultured human DPCs and DFs. The directly interacting network of the secretomes containing extracellular matrix and core plasma membrane proteins from DFs (green) and DPCs (red) was demonstrated. The biological functions, in terms of gene ontology and interaction network, were analyzed by Ingenuity Pathways Analysis. Based on the local networks by computational algorithms, identified extracellular proteins and/or all the identified proteins were connected with the hub proteins, forming a functional protein cluster. Solid arrows represent known physical interactions. A fold change (mol% of DPCs/mol% of DFs) with more than two-fold was considered as the maximum red (DPC dominant) or green (DF dominant).

Western blot analysis of CM-DPC-specific proteins

Using the shotgun proteomic technique aided by systematic network analysis of protein identification on a large scale, we selected IGFBP3, ITGB1, and THBS1 as putative hair growth-regulating protein biomarkers, as well as MMP2, a hub protein, from the secretomes of DPCs. All of the selected proteins showed significantly more expression in DPCs, compared with DFs (Fig. 3). In contrast, in DFs, IGFBP3 and ITGB1 were expressed at a basal level, and THBS1 was completely absent. Thus, it can be suggested these three selected proteins are biologically relevant as putative protein markers regulating hair growth.

DISCUSSION

We analyzed the proteins present in CM from cultures of DPCs and DFs using high throughput tandem mass spectrometry. From the available secretomes of DPCs and DFs, ECM and core plasma membrane proteins were selected to construct a possible protein-protein interaction network based on the IPA database. If secretomes in CM can modulate hair growth, then the mixture may contain factors with this activity.

A previous *in vivo* study showed that cultured DPCs from balding follicles secrete distinct mitogenic factors, and that the CM from balding DPCs delayed anagen onset in mice (14). Thus, the identification of paracrine factors in the secretomes of DPCs may be of significant value in the regulation of hair growth. Moreover, further understanding of these factors may enable the development of novel therapeutic approaches for hair loss.

There may be a limitation to our study related to the extent to which the cultured cells reflect anagen DPCs fully and secrete

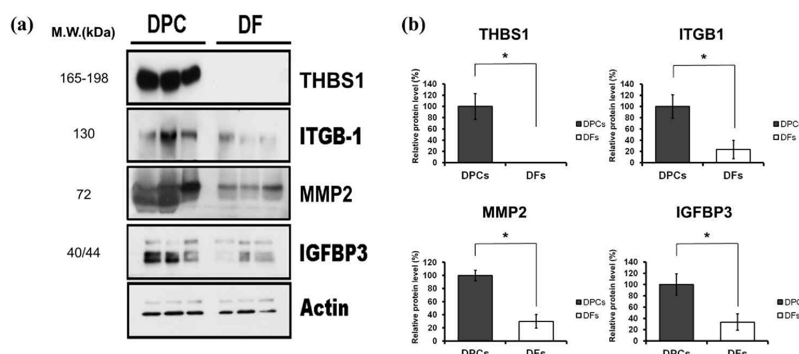


Fig. 3. Biochemical validation of hair growth regulating protein expression by immunoblot. The predominant expressions of thrombospondin1 (THBS1), integrin-b1 (ITGB1), and the insulin-like growth factor binding protein 3 (IGFBP3) as well as matrix metalloproteinase2 (MMP2) in conditioned media (CM) from DPCs, compared to matched CM-DFs, was demonstrated by (A) Western blotting and (B) densitometric analysis. β-actin was used as a control. Values represent the mean ± SEM of data from three independent human volunteers. $^{*}P < 0.05$ between DPCs and DFs. DPC, dermal papilla cell; DF, dermal fibroblast.

factors relevant to hair growth *in vivo*.

Compared to previous studies, our study has strength, however, in its more thorough analysis of sectretomes. Our analysis contains a detailed list of proteomic analysis. We identified 1,271 and 1,188 proteins in DF and DPC respectively, and further analyzed 1,529 using IPA-based network analysis. This kind of work has not been performed by any previous studies, to the best of our knowledge.

Possible explanations for the fact that factors secreted by anagen DP seem to be absent from the list may be as follows: a lack of information about specific cytokines or hormones in the available database, the production of peptide which is beyond MS analysis, the possibility of protein identification as another isoform, or omissions from analysis due to the pattern of classification of the proteins used in this study.

In this study, three secreted proteins were identified as biomarkers of DP, based on convincing proteomics work. The identification of reliable follicular dermal papilla markers is an important step in hair research.

ITGB1 is required for cell adhesion, migration and survival (15). β1 integrin-mediated signaling is also important in human hair growth control (16). It is notable that the key receptor protein ITGB1 was functionally connected with potential hair follicle-modulating factors, probably involving catagen induction, such as IGFBP3, and THBS1 which form satellite protein networks as another hub. DPCs could also regulate the proliferation of follicular epithelial cells through the secretion of IGFBPs, including IGFBP3, possibly by facilitating the binding interaction between IGF-1 and its receptor (17). The association of THBS1 with angiogenesis and hair cycling has been well documented (18). When trying to determine the selected DPC-specific secreted proteins responsible for hair growth modulation, Western blot analysis confirmed the protein expression levels (Fig. 3). Using the shotgun proteomic technique in large scale protein identification, aided by systematic network analysis, we selected ITGB1, IGFBP3, and THBS1 as the possible hair-growth modulating protein biomarkers. Although the practical significance and clinical implication of this proteomic analysis require further investigation, the proteins identified in the present study could be used as biomarkers of DPC growth regulation and, when more specific target proteins are identified, may enable future development of novel hair growth control agents.

In summary, we have identified the secreted proteins of DPCs, validated hair growth-regulating protein markers biochemically, analyzed ECM protein networks, and compared identified ECM proteins with those from DFs. This comparative study of secreted proteomes may thus provide a basis for a deeper understanding of hair growth and activity.

MATERIALS AND METHODS

Culture of scalp cells

Human follicular DPCs were cultured as previously described (19). Human DPCs were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (FBS; Invitrogen-Gibco-BRL), 2 mM L-glutamine, 1X antibiotic antimycotic solution (100 units/ml of penicillin G sodium and 2.5 μg/ml of fungizone) in a 5% CO_2 incubator at 37°C. Third to fourth-passage DPCs were used. DFs from the same individuals were cultured in DMEM with 5% FBS.

Preparation of secreted proteins from DPCs and DFs

The primary cultured DPCs and DFs (3×10^6 cells) were seeded onto 100 mm culture dishes in DMEM with 10% FBS. After 24 h, the cells were washed and incubated in serum-free media. Subsequently, the CM of each cell type was collected after 48 h in serum-free DMEM, centrifuged at 2,000 × g for 10 min, and filtered using a 0.22 μm syringe filter.

SDS-PAGE and in-gel trypsin digestion

For proteomic analysis, the CM-DPCs and CM-DFs were further concentrated approximately 50 fold, using SpeedVac, followed by the use of Centriplus YM-3 centrifugal filter devices (Millipore, Billerica, MA; 3 kDa cut-off). The prepared proteins of 50 μg per lane were loaded onto a 12% SDS-PAGE gel (18 × 16 cm). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250. The deep blue-stained protein gel bands were then cut into a total of 20 slices for each separate lane. Each gel slice was transferred into an Eppendorf tube and in-gel digestion with trypsin, as previously reported (10).

nano-LC-FT-MS/MS analysis

The *nano*-LC/MS system used in the present study was composed of a Surveyor HPLC system and 7-Tesla LTQ-FT MS (Thermo, San Jose, CA) equipped with a *nano*-electrospray ion source (ESI). Each sample (10 μl) was loaded onto a C_{18} trapping column (300 μm × 5 mm, C_{18} silica particle size 5 mm, LC Packings) and desalted. Peptides were eluted from the trap, separated at a flow rate of 20 μl/min, and sprayed into the FT-ICR MS analyzer. The LTQ parameters were as follows: spray voltage, 2.2 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 220°C; collision gas pressure, 1.3 mTorr; normalized collision energy using wide band activation mode; 35% for MS/MS. A 72-min gradient run (5% B for 15 min, ramped to 20% B for 3 min, 50% B for 47 min, to 95% B for 2 min, and finally, to 95% B for 5 min) was used with 0.02% formic acid and 0.5% acetic acid in water (buffer A) and in 80% acetonitrile (buffer B). A mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Xcalibur software was enabled for each MS/MS spectra. Target ions selected for MS/MS were dynamically excluded for 60 sec. The ion selection thresholds were 500 counts for MS/MS. An activation q = 0.25 and activation time of 30 ms was applied in MS/MS acquisitions.

Bioinformatic analysis

To identify the peptides, MASCOT (version 2.2, Matrix Science,

London, UK) was used to search the human sequences within the IPI database (version 3.63). The database contained 84,118 protein sequences. A MASCOT search was performed with the monoisotopic mass selected, a precursor mass error of 50 ppm, and a fragment ion mass error of 0.8 Da. Trypsin was the selected enzyme, with one potential missed cleavage. Carbamidomethyl cysteine and oxidized methionine were chosen as variable modifications. The qualified scoring peptides were considered as satisfied above the designated threshold (confidence, $P < 0.05$; peptide expect ≤ 0.05; false discovery rate of decoy database < 0.05). For the prediction of cleavable sites of ECM proteins, possible signal sequences were analyzed by the SignalP 3.0 (http://www.cbs.dtu. dk/SignalP/) program. The biological functions, in terms of gene ontology and interaction network, were analyzed using Ingenuity Pathways Analysis (IPA, version 7.1; http://www.ingenuity.com). Based on the local networks by computational algorithms, identified proteins were connected with hub proteins, forming a functional protein cluster.

Western blot analysis

Cells were extracted with an extraction buffer (50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 100 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 mM NaCl). Each lysate (40 μg) was run on 10% SDS/PAGE and transferred to PVDF membranes. Primary antibodies against MMP2, IGFBP3, ITGB1, and THBS1 (Santa Cruz Biotechnology, Inc.) were used. Signals were detected using an enhanced chemiluminescent substrate (Amersham International, Little Chalfont, UK). The relative signal strengths as normalized by β-actin were quantified using a densitometry program (ImageJ, NIH, USA), and statistical analyses were performed using a Student's T test. A P value of less than 0.05 was deemed statistically significant.

Acknowledgements

This study was supported by a grant from the Ministry of Health & Welfare, Republic of Korea (A060199) to H.C. Eun, by research agreement with AmorePacific Corporation, Republic of Korea. This study was financially supported by a Korea Basic Science Institute NAP grant (T3178B) and GRAST fund (PUB028) to J.S. Choi.

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