Shotgun label-free proteomic analysis for identification of proteins in HaCaT human skin keratinocytes regulated by the administration of collagen from soft-shelled turtle

Tetsushi Yamamoto, Saori Nakanishi, Kuniko Mitamura, Atsushi Taga
Pathological and Biomolecule Analyses Laboratory, Faculty of Pharmacy, Kindai University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan

Received 14 September 2017; accepted 13 October 2017
Published online 28 November 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.34034

Abstract: Soft-shelled turtles (Pelodiscus sinensis) are widely distributed in some Asian countries, and we previously reported that soft-shelled turtle tissue could be a useful material for collagen. In the present study, we performed shotgun liquid chromatography (LC)/mass spectrometry (MS)-based global proteomic analysis of collagen-administered human keratinocytes to examine the functional effects of collagen from soft-shelled turtle on human skin. Using a semiquantitative method based on spectral counting, we were able to successfully identify 187 proteins with expression levels that were changed more than twofold by the administration of collagen from soft-shelled turtle. Based on Gene Ontology analysis, the functions of these proteins closely correlated with cell–cell adhesion. In addition, epithelial–mesenchymal transition was induced by the administration of collagen from soft-shelled turtle through the down-regulation of E-cadherin expression. Moreover, collagen-administered keratinocytes significantly facilitated wound healing compared with nontreated cells in an in vitro scratch wound-healing assay. These findings suggest that collagen from soft-shelled turtle provides significant benefits for skin wound healing and may be a useful material for pharmaceuticals and medical care products.

Key Words: collagen, soft-shelled turtle, E-cadherin, epithelial–mesenchymal transition, wound healing

INTRODUCTION
Collagen is a ubiquitous structural protein in both invertebrates and vertebrates, comprising >20 different types based on the function in each tissue.1,2 These proteins are involved in the formation of fibrillar and microfibrillar networks of extracellular matrix and basement membranes to maintain the extracellular matrix environment.3–7 Recent reports have demonstrated that collagen is able to interact with several cell surface receptors and regulate cell proliferation or apoptosis.8,9 In addition, collagen is used for skin substitutes and drug delivery.10–15 Therefore, collagen is an important material for cosmetics, pharmaceuticals, and medical care products.

Most of the collagen presently in use is derived from bovine and porcine skin. However, allergic reactions and connective tissue disorders, such as arthritis and lupus, have been reported with the use of collagen from these animals.16 Moreover, these materials can potentially carry animal diseases, such as bovine spongiform encephalopathy and foot and mouth disease. Thus, these animals have been reconsidered as the main source for collagen products. In addition, many Muslims and Jews do not consume pig-derived food products, and many Hindus do not consume cow-derived products.17 Therefore, collagen of marine origin, such as fish, sponges, and mollusks, was recently considered as a useful alternative to mammalian sources because of its high availability.18–25 In addition, we previously reported that soft-shelled turtle (Pelodiscus sinensis) tissue could be a useful alternative for collagen.26 Recently, several reports demonstrated its usefulness,27,28 making collagen from soft-shelled turtle a useful material for cosmetics, pharmaceuticals, and medical care products.

However, collagen from soft-shelled turtle may differ greatly from that of mammalian resources in regards to physicochemical properties, amino acid compositions, and physiological functions due to the difference in the habitat environment. Therefore, further research is needed before using collagen from soft-shelled turtle as a source for collagen products. In the present study, we performed shotgun liquid chromatography (LC)/mass spectrometry (MS)-based...
global proteomic analysis of collagen-administered human keratinocytes to examine the functional effects of collagen from soft-shelled turtle on human skin. We found that 187 proteins were differentially expressed in the collagen-administered keratinocytes compared with nontreated cells, and these proteins may be involved in wound healing in human skin.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were of the highest grade available and purchased from Wako Pure Chemical Industries (Osaka, Japan).

Turtles

Emperor tissue, a soft tissue in the region around the shell of soft-shelled turtles (*P. sinensis*), was provided by Shin-uei (Osaka, Japan).

Collagen extraction

Collagen extraction was performed in accordance with our previous study.26 Briefly, emperor tissue was treated with 0.1M formic acid at a ratio of 1:10 (w/v) for 24 h for demineralization. The sample was then treated with 0.1M sodium hydroxide (NaOH) at a ratio of 1:10 (w/v) for 3 days to remove noncollagenous proteins, including endogenous proteases. The NaOH solution was changed every day. Finally, the sample was incubated with 0.03M citric acid for 24 h. After incubation, the solution was centrifuged at 6500g for 20 min at 4°C and the supernatant collected as the collagen solution.

Cell culture

HaCaT immortalized human keratinocytes were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany). The cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) in an atmosphere containing 5% CO2.

Cell growth assay

Cells were plated at a density of 5×10³ cells per well in a 96-well plate and grown in culture medium. The next day, the medium was changed and cells grown in collagen-containing culture medium. After 72 h, the cells were incubated with WST-8 cell counting reagent (Wako) and the optical density of the culture solution in the plate measured using an ELISA plate reader.

Protein preparation

HaCaT cells were plated in a 60-mm dish at a density of 2×10³ cells per dish and grown in culture medium. The next day, the medium was changed and the cells grown in collagen-containing culture medium. After 72 h, the cells were solubilized in urea lysis buffer (7M urea, 2M thiourea, 5% CHAPS, 1% Triton X-100). The protein concentration was measured using the Bradford method.

In-solution trypsin digestion

A gel-free digestion approach was performed in accordance with a previously described protocol.29 Briefly, 10 μg of protein extract from each sample was reduced by the addition of 45 mM dithiothreitol and 20 mM **tris**(2-carboxyethyl)phosphine, and then alkylated using 100 mM **iodoacetic acid**. After alkylation, the samples were digested with trypsin gold, mass spectrometry grade (Promega Corp., Madison, WI, USA) at 37°C for 24 h. Next, the digests were purified using PepClean C-18 Spin Columns (Thermo, Rockford, IL, USA) according to the manufacturer’s protocol.

LC-MS/MS analysis for protein identification

Peptide samples (~2 μg) were injected into a peptide L-trap column (Chemicals Evaluation and Research Institute, Tokyo, Japan) using an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) and further separated through a Paradigm MS4 (AMR, Tokyo, Japan) using a reverse-phase C18-column (L-column, 3 μm diameter gel particles and 120 Å pore size, 0.2 × 150 mm, Chemicals Evaluation and Research Institute). The mobile phase consisted of 0.1% formic acid in
| No. | ID               | Accession Number and Description                                                                 | Number of Amino Acids | Fold Change |
|-----|------------------|--------------------------------------------------------------------------------------------------|-----------------------|-------------|
| 1   | H2B1K_HUMAN      | Histone H2B type 1-K                                                                             | 126                   | -3.690      |
| 2   | EF1A3_HUMAN      | Putative elongation factor 1-alpha-like 3                                                         | 462                   | -3.080      |
| 3   | H2B1M_HUMAN      | Histone H2B type 1-M                                                                             | 126                   | -2.698      |
| 4   | K2C3_HUMAN       | Keratin, type II cytoskeletal 3                                                                 | 628                   | -2.178      |
| 5   | H2A1H_HUMAN      | Histone H2A type 1-H                                                                             | 128                   | -1.611      |
| 6   | RL10_HUMAN       | 60S ribosomal protein L10                                                                       | 214                   | -1.611      |
| 7   | ARF3_HUMAN       | ADP-ribosylation factor 3                                                                        | 181                   | -1.611      |
| 8   | DYC1_HUMAN       | Cytoplasmic dynein 1 heavy chain 1                                                                | 4646                  | -1.477      |
| 9   | TBAL3_HUMAN      | Tubulin alpha chain-like 3                                                                       | 446                   | -1.359      |
| 10  | ENOB_HUMAN       | Beta-enolase                                                                                      | 434                   | -1.359      |
| 11  | FLNB_HUMAN       | Filamin-B                                                                                        | 2602                  | -1.359      |
| 12  | PDL11_HUMAN      | PDZ and LIM domain protein 1                                                                      | 329                   | -1.359      |
| 13  | FLNA_HUMAN       | Filamin-A                                                                                        | 2647                  | -1.359      |
| 14  | M1H4_HUMAN       | Myosin-14                                                                                        | 1995                  | -1.359      |
| 15  | K2C80_HUMAN      | Keratin, type II cytoskeletal 80                                                                   | 452                   | -1.053      |
| 16  | K2C72_HUMAN      | Keratin, type II cytoskeletal 72                                                                   | 511                   | -1.053      |
| 17  | POTE_HUMAN       | POTE ankyrin domain family member F                                                                 | 1075                  | -1.053      |
| 18  | GDI1_HUMAN       | Rab GDP dissociation inhibitor alpha                                                                 | 447                   | -1.053      |
| 19  | RS27A_HUMAN      | Ubiquitin-40S ribosomal protein S27a                                                                | 156                   | -1.053      |
| 20  | CAH2_HUMAN       | Carbonic anhydrase 2                                                                               | 260                   | -1.053      |
| 21  | SEPT9_HUMAN      | Septin-9                                                                                        | 586                   | -1.053      |
| 22  | PRK6_HUMAN       | Pre-mRNA-processing-splicing factor 8                                                               | 2335                  | -1.053      |
| 23  | IM1_HUMAN        | Importin subunit beta-1                                                                           | 876                   | -1.053      |
| 24  | HS105_HUMAN      | Heat shock protein 105 kDa                                                                         | 858                   | -1.053      |
| 25  | PLST_HUMAN       | Plastin-3                                                                                        | 630                   | -1.053      |
| 26  | H2AV_HUMAN       | Histone H2A type 1-D                                                                              | 130                   | -1.036      |
| 27  | ALAS3_HUMAN      | Aldehyde dehydrogenase family 1 member A                                                           | 512                   | 1.020       |
| 28  | HNR1_HUMAN       | Heterogeneous nuclear ribonucleoprotein H                                                           | 449                   | 1.102       |
| 29  | PEPL_HUMAN       | Periplakin                                                                                        | 1756                  | 1.102       |
| 30  | LDH2_HUMAN       | L-lactate dehydrogenase B chain                                                                     | 334                   | 1.102       |
| 31  | TPM4_HUMAN       | Tropomyosin alpha-4 chain                                                                          | 248                   | 1.102       |
| 32  | 2AAH_HUMAN       | Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform                    | 589                   | 1.102       |
| 33  | EZR_HUMAN        | Ezrin                                                                                             | 586                   | 1.102       |
| 34  | CORC_HUMAN       | Coronin-1C                                                                                         | 474                   | 1.102       |
| 35  | SPTX1_HUMAN      | Spermin alpha chain, nonerythrocytic 1                                                               | 2472                  | 1.182       |
| 36  | H2AV_HUMAN       | Histone H2A                                                                                       | 128                   | 1.245       |
| 37  | ARPS3_HUMAN      | Actin-related protein 3                                                                            | 418                   | 1.245       |
| 38  | TCP8_HUMAN       | T-complex protein 1 subunit beta                                                                     | 535                   | 1.245       |
| 39  | AHSA1_HUMAN      | Activator of 90 kDa heat shock protein ATPase homolog 1                                             | 338                   | 1.245       |
| 40  | KMT2A_HUMAN      | Histone-lysine N-methyltransferase 2A                                                                 | 3969                  | 1.245       |
| 41  | SYLC2_HUMAN      | Leucine-tRNA ligase, cytoplasmic                                                                    | 1176                  | 1.245       |
| 42  | PGAM2_HUMAN      | Phosphoglycerate mutase 2                                                                           | 254                   | 1.245       |
| 43  | ICAL_HUMAN       | Calpain                                                                                           | 708                   | 1.245       |
| 44  | CISY_HUMAN       | Citrate synthase, mitochondrial                                                                    | 466                   | 1.245       |
| 45  | LIMA1_HUMAN      | Lim domain and actin-binding protein 1                                                               | 759                   | 1.245       |
| 46  | CAPR1_HUMAN      | Caspin-1                                                                                          | 709                   | 1.245       |
| 47  | MYADM_HUMAN      | Myeloid-associated differentiation marker                                                          | 322                   | 1.245       |
| 48  | PDCC4_HUMAN      | Programmed cell death protein 4                                                                     | 469                   | 1.245       |
| 49  | APEX1_HUMAN      | DNA-(apurinic or apyrimidinic site) lyase                                                          | 318                   | 1.245       |
| 50  | MARE1_HUMAN      | Microtubule-associated protein RP/EB family member 1                                               | 268                   | 1.408       |
| 51  | NACAM_HUMAN      | Nascent polypeptide-associated complex subunit alpha, muscle-specific form                          | 2078                  | 1.562       |
| 52  | SHLB2_HUMAN      | Endophilin-B2                                                                                      | 395                   | 1.562       |
| 53  | LIM1_HUMAN       | Lim domain and senescent cell antigen-like-containing domain protein 1                              | 325                   | 1.562       |
| 54  | EHD1_HUMAN       | EH domain-containing protein 1                                                                      | 534                   | 1.562       |
| 55  | TNPO1_HUMAN      | Transportin-1                                                                                      | 898                   | 1.562       |
| 56  | PYGB_HUMAN       | Glycogen phosphorylase, brain form                                                                  | 843                   | 1.562       |
| 57  | BZV1_HUMAN       | Basic leucine zipper and W2-domain-containing protein 1                                             | 419                   | 1.562       |
| 58  | AP2B1_HUMAN      | AP-2 complex subunit beta                                                                          | 937                   | 1.562       |
| No. | ID            | Accession Number and Description                              | Number of Amino Acids | Fold Change (R<sub>sc</sub>) |
|-----|---------------|----------------------------------------------------------------|-----------------------|-----------------------------|
| 59  | CMC1_HUMAN    | O75746 Calcium-binding mitochondrial carrier protein Aralar1   | 678                   | 1.562                       |
| 60  | SKAP_HUMAN    | Q9Y448 Small kinetochore-associated protein                    | 316                   | 1.562                       |
| 61  | CD9_HUMAN     | P21926 CD9 antigen                                             | 228                   | 1.562                       |
| 62  | P4HA1_HUMAN   | P13874 Prolyl 4-hydroxylase subunit alpha-1                    | 534                   | 1.562                       |
| 63  | PPAC_HUMAN    | P24666 Low molecular weight phosphotyrosine protein phosphatase |                      |                             |
| 64  | FUBP2_HUMAN   | Q92945 Far upstream element-binding protein 2                  | 711                   | 1.562                       |
| 65  | RGPD2_HUMAN   | P0DJD1 RANBP2-like and GRIP domain-containing protein 2         | 1756                  | 1.562                       |
| 66  | RAB1C_HUMAN   | Q92928 Putative Ras-related protein Rab-1C                     | 201                   | 1.562                       |
| 67  | HUWE1_HUMAN   | Q7Z6Z7 E3 ubiquitin-protein ligase HUWE1                        | 4374                  | 1.562                       |
| 68  | IPYR2_HUMAN   | Q9H2U2 Inorganic pyrophosphatase 2, mitochondrial               | 334                   | 1.562                       |
| 69  | CERS2_HUMAN   | Q96G23 Ceramide synthase 2                                     | 380                   | 1.562                       |
| 70  | IRS4_HUMAN    | O14854 Insulin receptor substrate 4                            | 1257                  | 1.562                       |
| 71  | DDX3_HUMAN    | O00571 ATP-dependent RNA helicase DDX3X                        | 662                   | 1.562                       |
| 72  | PARP1_HUMAN   | P09874 Poly(ADP-ribose) polymerase 1                           | 1014                  | 1.562                       |
| 73  | MAP4_HUMAN    | P27816 Microtubule-associated protein 4                        | 1152                  | 1.562                       |
| 74  | LAT1_HUMAN    | Q01850 Large neutral amino acids transporter small subunit 1   | 507                   | 1.562                       |
| 75  | CARD6_HUMAN   | Q9BX69 Caspase recruitment domain-containing protein 6          | 1037                  | 1.562                       |
| 76  | PCD16_HUMAN   | Q6J900 Protopatherin-16                                        | 3298                  | 1.562                       |
| 77  | CP250_HUMAN   | Q9BV73 Centrosome-associated protein CEP250                     | 2442                  | 1.562                       |
| 78  | MCM3_HUMAN    | P25205 DNA replication licensing factor MCM3                    | 808                   | 1.562                       |
| 79  | SYS_C_HUMAN   | P49591 Serine-tRNA ligase, cytoplasmic                          | 514                   | 1.562                       |
| 80  | EPHA4_HUMAN   | P54764 Ephrin type-A receptor 4                                | 986                   | 1.562                       |
| 81  | NT5D1_HUMAN   | Q5TE4 5-nucleotidase domain-containing protein 1                | 455                   | 1.562                       |
| 82  | GIPC3_HUMAN   | Q8TF64 PDZ domain-containing protein GIPC3                      | 312                   | 1.562                       |
| 83  | MRKAS_HUMAN   | Q9NR99 Matrix-remodeling-associated protein 5                   | 2826                  | 1.562                       |
| 84  | CO4A4_HUMAN   | P53420 Collagen alpha-4 (IV) chain                             | 1690                  | 1.562                       |
| 85  | POTEB_HUMAN   | Q6SHH4 POTE ankyrin domain family member B                      | 581                   | 1.562                       |
| 86  | MYH1_HUMAN    | P12882 Myosin-1                                               | 1939                  | 1.562                       |
| 87  | NFRKB_HUMAN   | Q6P4R8 Nuclear factor related to kappa-B-binding protein        | 1299                  | 1.562                       |
| 88  | NAC2_HUMAN    | Q9PR5 Sodium/calcium exchanger 2                               | 921                   | 1.562                       |
| 89  | NRK2_HUMAN    | Q9PNIP Nicotinamide riboside kinase 2                          | 230                   | 1.562                       |
| 90  | BRM1L_HUMAN   | Q5PS5V4 Breast cancer metastasis-suppressor 1-like protein      | 323                   | 1.562                       |
| 91  | SAP3_HUMAN    | P17900 Ganglioside GM2 activator                               | 193                   | 1.562                       |
| 92  | APBA1_HUMAN   | Q02410 Amyloid beta A4 precursor protein-binding family A member 1 |            |                             |
| 93  | RS14_HUMAN    | P62263 40S ribosomal protein S14                               | 151                   | 1.562                       |
| 94  | ENDOV_HUMAN   | Q8BOQ3 Endonuclease V                                          | 282                   | 1.562                       |
| 95  | UBE4B_HUMAN   | Q95155 Ubiquitin conjugation factor E4 B                        | 1302                  | 1.562                       |
| 96  | F134C_HUMAN   | Q8VR2 Protein FAM134C                                          | 466                   | 1.562                       |
| 97  | ACSM5_HUMAN   | Q6UNU0 Acyl-coenzyme A synthetase ACSM5, mitochondrial          | 579                   | 1.562                       |
| 98  | DPOE1_HUMAN   | Q7964 DNA polymerase epsilon catalytic subunit A                | 2286                  | 1.562                       |
| 99  | SRTT_HUMAN    | Q9BX95 Serrate RNA effector molecule homolog                    | 876                   | 1.562                       |
| 100 | EXOC1_HUMAN   | Q9NV70 Exocyst complex component 1                             | 894                   | 1.562                       |
| 101 | GDE1_HUMAN    | Q9NZC3 Glycerophosphodiester phosphodiesterase 1               | 331                   | 1.562                       |
| 102 | CAMP3_HUMAN   | Q9P1Y5 Calmodulin-regulated spectrin-associated protein 3       | 1249                  | 1.562                       |
| 103 | BCAS2_HUMAN   | Q9HEU6 Breast carcinoma-amplified sequence 3                    | 926                   | 1.562                       |
| 104 | NXF2_HUMAN    | Q9GZYO Nuclear RNA export factor 2                              | 626                   | 1.562                       |
| 105 | HIC1_HUMAN    | Q14826 Hypermethylated in cancer 1 protein                      | 733                   | 1.562                       |
| 106 | VP13C_HUMAN   | Q708C8 Vacuolar protein sorting-associated protein 13C          | 3753                  | 1.562                       |
| 107 | DCE1_HUMAN    | Q99259 Glutamate decarboxylase 1                               | 594                   | 1.562                       |
| 108 | RUVB2_HUMAN   | Q9Y230 RuvB-like 2                                             | 463                   | 1.562                       |
| 109 | UBA1_HUMAN    | P22314 Ubiquitin-like modifier-activating enzyme 1              | 1058                  | 1.562                       |
| 110 | ANX11_HUMAN   | P50995 Annexin A11                                            | 505                   | 1.562                       |
| 111 | 2AAB_HUMAN    | P30154 Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform | 601                   | 1.562                       |
| 112 | TFG_HUMAN     | Q92734 Protein TFG                                             | 400                   | 1.562                       |
| 113 | 1432Z_HUMAN   | P63104 14-3-3 protein zeta/delta                               | 245                   | 1.562                       |
| 114 | C1TC_HUMAN    | P11586 C-1-tetrahydrofolate synthase, cytoplasmic              | 935                   | 1.562                       |
| 115 | PRD4X_HUMAN   | Q13162 Peroxisomal redoxin-4                                   | 271                   | 1.562                       |
| 116 | TENA_HUMAN    | P24821 Tenascin                                                 | 2201                  | 1.562                       |
| No. | ID         | Accession Number and Description                                              | Number of Amino Acids | Fold Change ($R_{sc}$) |
|-----|------------|-------------------------------------------------------------------------------|-----------------------|------------------------|
| 117 | MIF_HUMAN  | P14174 Macrophage migration inhibitory factor                                 | 115                   | 1.562                  |
| 118 | NIPS2_HUMAN| O75323 Protein NipSnap homolog 2                                               | 286                   | 1.562                  |
| 119 | CTN1_B_HUMAN| P3222 Catenin beta-1                                                           | 781                   | 1.562                  |
| 120 | ADIRF_HUMAN | Q15847 Adipogenesis regulatory factor                                          | 76                    | 1.562                  |
| 121 | COASY_HUMAN| Q13057 Bifunctional coenzyme A synthase                                        | 564                   | 1.562                  |
| 122 | TF_HUMAN    | P13726 Tissue factor                                                           | 295                   | 1.562                  |
| 123 | MATR3_HUMAN | P43243 Matrin-3                                                                | 847                   | 1.562                  |
| 124 | RabA4A_HUMAN| P20338 Ras-related protein Rab-4A                                              | 218                   | 1.562                  |
| 125 | IF4H_HUMAN  | Q15056 Eukaryotic translation initiation factor 4H                             | 248                   | 1.562                  |
| 126 | ER29_HUMAN  | P30040 Endoplasmic reticulum resident protein 29                               | 261                   | 1.562                  |
| 127 | RL30_HUMAN  | P62888 60S ribosomal protein L30                                                | 115                   | 1.562                  |
| 128 | Ppce_HUMAN  | P48147 Prolyl endopeptidase                                                    | 710                   | 1.562                  |
| 129 | UBFL1_HUMAN | P0CB47 Putative upstream-binding factor 1-like protein 1                       | 393                   | 1.562                  |
| 130 | HGB1A_HUMAN | B2RPK0 Putative high mobility group protein B1-like 1                          | 211                   | 1.562                  |
| 131 | TM163_HUMAN | Q8TC26 Transmembrane protein 163                                               | 289                   | 1.562                  |
| 132 | DCK_HUMAN   | P27707 Deoxycytidine kinase                                                    | 250                   | 1.562                  |
| 133 | PSB6_HUMAN  | P28072 Proteasome subunit beta-type-6                                          | 239                   | 1.562                  |
| 134 | Glyc_HUMAN  | P34896 Serine hydroxymethyltransferase, cytosolic                             | 483                   | 1.562                  |
| 135 | ETFB_HUMAN  | P31117 Electron transfer flavoprotein subunit beta                             | 255                   | 1.562                  |
| 136 | SEPT2_HUMAN | Q15019 Septin-2                                                                | 361                   | 1.562                  |
| 137 | Ig2AS_HUMAN | Q6U949 Putative insulin-like growth factor 2 antisense gene protein            | 168                   | 1.562                  |
| 138 | SYEP_HUMAN  | P07814 Bifunctional glutamate/proline-tRNA ligase                              | 1512                  | 1.562                  |
| 139 | GGH_HUMAN   | Q92820 Gamma-glutamyl hydrolase                                                | 318                   | 1.562                  |
| 140 | SMCS5_HUMAN | Q8Y18 Structural maintenance of chromosomes protein 5                           | 1101                  | 1.562                  |
| 141 | 3Bhs2_HUMAN | P26439 3 beta-hydroxysteroid dehydrogenase/Delta 5-4-isomerase type 2         | 372                   | 1.562                  |
| 142 | Sias_HUMAN  | Q9NR45 Sialic acid synthase                                                     | 359                   | 1.562                  |
| 143 | Dyh7_HUMAN  | Q8WXX0 Dynine heavy chain 7, axonemal                                          | 4024                  | 1.562                  |
| 144 | Gm2_HUMAN   | Q14416 Metabolotropic glutamate receptor 2                                     | 872                   | 1.562                  |
| 145 | Plcb_HUMAN  | Q15120 1-acyl-sn-glycerol-3-phosphate acyltransferase beta                      | 278                   | 1.562                  |
| 146 | Pnpo_HUMAN  | Q9NVS9 Pyridoxine-5-phosphate oxidase                                           | 261                   | 1.562                  |
| 147 | Gftp1_HUMAN | Q60210 Glutamine-fructose-6-phosphate aminotransferase [isomerizing] 1          | 699                   | 1.562                  |
| 148 | Inadl_HUMAN | Q8NI35 InaD-like protein                                                        | 1801                  | 1.562                  |
| 149 | Cpm8_HUMAN  | Q8IZJ3 C9 and P2P-like alpha-2-macroglubulin domain-containing protein 8        | 1895                  | 1.562                  |
| 150 | Cosa1_HUMAN | P20849 Collagen alpha-1(I) chain                                               | 921                   | 1.562                  |
| 151 | Dnja2_HUMAN | O68084 Dnaj homolog subfamily A member 2                                       | 412                   | 1.562                  |
| 152 | Gasp1_HUMAN | Q5JY77 G-protein coupled receptor-associated sorting protein 1                 | 1395                  | 1.562                  |
| 153 | Birc3_HUMAN | Q13489 Baculoviral IAP repeat-containing protein 3                              | 604                   | 1.562                  |
| 154 | Il2rg_HUMAN | P31785 Cytokine receptor common subunit gamma                                   | 369                   | 1.562                  |
| 155 | Fucm_HUMAN  | A2VDFO Fucose mutarotase                                                        | 154                   | 1.562                  |
| 156 | Kad3_HUMAN  | Q9UJ7 GTP:AMP phosphotransferase AK3, mitochondrial                            | 227                   | 1.562                  |
| 157 | Gsx2_HUMAN  | Q9BZM3 GS homeobox 2                                                            | 304                   | 1.562                  |
| 158 | Mimt_HUMAN  | Q8N183 Mimitin, mitochondrial                                                   | 169                   | 1.562                  |
| 159 | Cyx_HUMAN   | P99999 Cytochrome c                                                             | 105                   | 1.562                  |
| 160 | Cc141_HUMAN | Q6ZP82 Coiled-coil domain-containing protein 141                                 | 1450                  | 1.562                  |
| 161 | Zn503_HUMAN | Q96F45 Zinc finger protein 503                                                   | 646                   | 1.562                  |
| 162 | Chd7_HUMAN  | Q9P2D1 Chromodomain helicase DNA binding protein 7                              | 2997                  | 1.562                  |
| 163 | Radi_HUMAN  | P35241 Radixin                                                                  | 583                   | 1.633                  |
| 164 | Can1_HUMAN  | P07384 Calpain-1 catalytic subunit                                              | 714                   | 1.633                  |
| 165 | Catb_HUMAN  | P07858 Cathepsin B                                                              | 339                   | 1.660                  |
| 166 | Ef1g_HUMAN  | P26641 Elongation factor 1-gamma                                                | 437                   | 1.875                  |
| 167 | Cn2_HUMAN   | Q94439 Calponin-2                                                               | 309                   | 1.938                  |
| 168 | Gels_HUMAN  | P68396 Gelsolin                                                                  | 782                   | 1.938                  |
| 169 | Krtb1_HUMAN | Q14533 Keratin, type II cuticular Hb1                                            | 505                   | 2.094                  |
| 170 | Eif3e_HUMAN | P60228 Eukaryotic translation initiation factor 3 subunit E                     | 445                   | 2.094                  |
| 171 | Dazp1_HUMAN | Q96EP5 DAZ-associated protein 1                                                 | 407                   | 2.094                  |
| 172 | Surf4_HUMAN | O15260 Surfeit locust protein 4                                                 | 269                   | 2.094                  |

TABLE I. Continued
water as solution A and acetonitrile as solution B. The column flow rate was 1 μL/min with a concentration gradient of 5% B to 40% B over 120 min. Gradient-eluted peptides were analyzed using an LTQ ion-trap mass spectrometer (Thermo). The results were acquired in a data-dependent manner in which MS/MS fragmentation was performed on the two most intense peaks of every full MS scan.

All MS/MS spectral data were searched against the Swiss-Prot Homo Sapiens database using Mascot (version 2.4.01, Matrix Science, London, UK). The search criteria were set as follows: enzyme, trypsin; allowance of up to two missed cleavage peptides; mass tolerance ±2.0 Da and MS/MS tolerance ±0.8 Da; and modifications of cysteine carboxymethylation and methionine oxidation.

Semiquantitative analysis of identified proteins
The fold changes in expressed proteins on a base 2 logarithmic scale were calculated using the Rsc based on spectral counting. Relative amounts of identified proteins were calculated using the normalized spectral abundance factor (NSAF). Differentially expressed proteins were chosen so that their Rsc was >1 or ≤1, which correspond to fold changes of >2 or <0.5.

Bioinformatics
Functional annotations for proteins identified to be regulated by collagen administration were processed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 (http://david.abcc.ncifcrf.gov/home.jsp). Functional annotations for proteins identified to be regulated by collagen administration were processed using the DAVID Functional Annotation tool (http://david.abcc.ncifcrf.gov/home.jsp). The data were analyzed using one-way analysis of variance followed by Dunnett's test or the unpaired t test.

Western blot analysis
A total of 5 μg of cell extract was added to each well and subjected to SDS-PAGE under reducing conditions. The separated proteins were transferred to polyvinylidene fluoride transfer membranes. Following blocking in TBS-Tween-20 (0.1%) buffer with 5% skim milk for 2 h at room temperature, the membranes were incubated at 4°C overnight with an anti-E-cadherin antibody (1:5,000; Cell Signaling Technology, Beverly, MA), antivimentin antibody (1:1000; Cell Signaling Technology), or antisnail antibody (1:1000; Cell Signaling Technology). The membranes were washed and incubated with HRP-conjugated antirabbit IgG antibody (American Qualex, San Clemente, CA). Following washing, the blots were visualized using SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific) and bands detected using the myECL Imager system (version 2.0; Thermo Fisher Scientific).

Statistical analysis
All data are presented as the mean ± standard error of the mean. The data were analyzed using one-way analysis of variance followed by Dunnett's test or the unpaired t test. P < 0.01 was considered significant in all analyses. Computations were performed in GraphPad Prism version 5.1 (GraphPad Software, La Jolla, CA, USA).

RESULTS
Cytotoxicity of collagen against HaCaT cells
To examine the cytotoxic effect of collagen on HaCaT cells, we assessed the cell growth rate when cells were grown in collagen-containing culture medium. A microscope system was used to take photographs from the scratch area 0 and 8 h after scratching (Olympus, Tokyo, Japan).

TABLE I. Continued

| No. | ID          | Accession Number and Description                             | Number of Amino Acids | Fold Change (Rsc) |
|-----|-------------|--------------------------------------------------------------|-----------------------|-------------------|
| 173 | GGCT_HUMAN  | O75223 Gamma-glutamylcysteine transferase                     | 188                   | 2.094             |
| 174 | HNRH2_HUMAN | P55795 Heterogeneous nuclear ribonucleoprotein H2             | 449                   | 2.094             |
| 175 | AT1A1_HUMAN | P05023 Sodium/potassium-transporting ATPase subunit alpha-1  | 1023                  | 2.094             |
| 176 | OLA1_HUMAN  | QNF55 Obg-like ATPase 1                                       | 396                   | 2.094             |
| 177 | RL1D1_HUMAN | O76021 Ribosomal L1 domain-containing protein 1               | 490                   | 2.094             |
| 178 | IF4A3_HUMAN | P38919 Eukaryotic initiation factor 4A-III                    | 411                   | 2.094             |
| 179 | MESD_HUMAN  | Q14696 LDLR chaperone MESD                                    | 234                   | 2.094             |
| 180 | K1C27_HUMAN | Q729Y Keratin, type I cytoskeletal 27                        | 459                   | 2.094             |
| 181 | CNPD2_HUMAN | Q66K4 Cytoplasmic non-specific dipeptidase                     | 475                   | 2.191             |
| 182 | H2A2A_HUMAN | Q5613 Histone H2A type 2-A                                    | 130                   | 2.481             |
| 183 | PYGL_HUMAN  | P6737 Glycogen phosphorylase, liver form                       | 847                   | 2.481             |
| 184 | H2A1C_HUMAN | Q93077 Histone H2A type 1-C                                    | 130                   | 2.481             |
| 185 | ADT1_HUMAN  | P12235 ADP/ATP translocase 1                                  | 298                   | 3.039             |
| 186 | VPPA_HUMAN  | Q93074 V-type proton ATPase 116 kDa subunit a isoform 4       | 840                   | 3.049             |
| 187 | H2B1H_HUMAN | Q93079 Histone H2B type 1-H                                   | 126                   | 4.672             |
inhibited at 72 h compared with nontreated cells (Fig. 1). Therefore, we used 100 µg/mL collagen in the following experiments.

Protein identification and semiquantitative comparison of identified proteins in collagen-administered HaCaT cells
To investigate the effect of collagen on the cells in the basal layer of the skin, we determined the molecular profile of proteins in HaCaT cells whose expression levels were regulated by collagen using shotgun proteomics. We performed a label-free semiquantitative method based on spectral counting to determine the proteins whose expression levels were regulated by collagen. In Figure 2, each $R_{sc}$ value is plotted against the corresponding protein ($x$-axis) in increasing order from left to right for proteins identified in collagen-administered HaCaT cells (collagen) and nontreated cells (nontreatment). A positive value indicates increased

FIGURE 3. Gene ontology (GO) analysis for identified proteins. (A) Proteins assigned to biological process, (B) cellular component, and (C) molecular function GO term categories. Only significant categories ($p < 0.05$) are shown.
expression in the collagen-treated cells and a negative value decreased expression in the collagen-treated cells. The NSAF value (Fig. 2, bar) was also plotted on the X-axis for each corresponding protein with collagen treatment above the X-axis and control below. Proteins with a high positive or negative $R_{sc}$ value would be candidates for proteins regulated by collagen.

As a result of semiquantification, a total of 187 differentially expressed proteins were identified (Table I). The expression levels of housekeeping proteins β-actin, GAPDH, and histone H4 were not changed by collagen administration.

Functional annotation of proteins regulated by collagen
Gene ontology (GO) analysis was performed with the candidate proteins for each biological process [Fig. 3(A)], cellular component [Fig. 3(B)], and molecular function [Fig. 3(C)] using DAVID. Some of the differentially expressed proteins were related to cell adhesion, and we focused on the function of proteins classified as cadherin binding involved in cell–cell adhesion (Table II).

Effect of collagen administration on the expression level of E-cadherin and EMT marker proteins in HaCaT cells
To investigate whether collagen administration affected the level of cadherin expression, we examined the expression of E-cadherin in collagen-administered HaCaT cells. The expression of E-cadherin clearly decreased with collagen administration compared with nontreated cells (Fig. 4). Next, we examined the expression levels of vimentin and snail to investigate whether epithelial–mesenchymal transition (EMT) was induced in correlation with the downregulation of E-cadherin. The expression of vimentin and snail clearly increased with collagen administration compared with nontreated cells (Fig. 4).

| No. | Accession Number and Description | Fold Change $(R_{sc})$ |
|-----|---------------------------------|------------------------|
| 1   | O00151 PDZ and LIM domain protein 1 | -1.359 |
| 2   | P21333 Filamin-A                  | -1.359 |
| 3   | O75369 Filamin-B                  | -1.359 |
| 4   | Q9UHD8 Septin-9                   | -1.053 |
| 5   | P15311 Ezrin                      | 1.102 |
| 6   | O60437 Periplakin                  | 1.102 |
| 7   | Q13813 Spectrin alpha chain, nonerythrocytic 1 | 1.182 |
| 8   | Q9UHB6 LIM domain and actin-binding protein 1 | 1.245 |
| 9   | O95433 Activator of 90 kDa heat shock protein ATPase homolog 1 | 1.245 |
| 10  | P20810 Calpastatin                | 1.245 |
| 11  | Q15691 Microtubule-associated protein RP/EB family member 1 | 1.408 |
| 12  | Q7L1Q6 Basic leucine zipper and W2 domain-containing protein 1 | 1.562 |
| 13  | Q9NR46 Endophilin-B2              | 1.562 |
| 14  | O00571 ATP-dependent RNA helicase DDX3X | 1.562 |
| 15  | P35222 Catenin beta-1             | 1.562 |
| 16  | Q9H4M9 EH domain-containing protein 1 | 1.562 |
| 17  | Q15056 Eukaryotic translation initiation factor 4H | 1.562 |
| 18  | P63104 14-3-3 protein zeta/delta  | 1.562 |
| 19  | Q15019 Septin-2                   | 1.562 |
| 20  | P28072 Proteasome subunit beta type-6 | 1.562 |
| 21  | P35241 Radixin                    | 1.633 |
| 22  | P26641 Elongation factor 1-gamma  | 1.875 |
| 23  | Q99439 Calponin-2                 | 1.938 |
| 24  | P60228 Eukaryotic translation initiation factor 3 subunit E | 2.094 |
| 25  | Q9NTK5 Obg-like ATPase 1          | 2.094 |
| 26  | O76021 Ribosomal L1 domain-containing protein 1 | 2.094 |
Effect of collagen administration on keratinocyte migration in a scratch-wound healing process

To investigate whether EMT affected the migration capability of HaCaT cells, we performed an in vitro wound healing study using the HaCaT scratch model. Photographs were taken before treatment and after 8 h of incubation at 37°C in 5% CO₂ [Fig. 5(A)]. Collagen-administered cells significantly facilitated wound healing compared with nontreated cells [Fig. 5(B)].

**DISCUSSION**

In this study, we used a gel-free LC-MS-based proteomics approach to examine the functional effects of collagen from soft-shelled turtle on human skin. Although spectral counting may not accurately reflect the quantity information, it is useful and has been used in many studies, including those searching for novel diagnostic biomarkers. We were able to successfully identify several proteins whose expression levels were changed >2-fold in HaCaT cells by the administration of collagen using a semiquantitative method based on spectral counting.

To examine the role of these identified proteins, we performed GO analysis. The functional category that directly relates to cell–cell adhesion was obtained from among the GO terms on molecular function, biological process, and cellular component. We focused on the functions of proteins classified as cadherin binding involved in cell–cell adhesion because they play important roles in cadherin-mediated cell adhesion; thus, changes in the expression levels of these proteins with the administration of collagen from soft-shelled turtle may affect the expression of cadherin. To evaluate this hypothesis, we examined the expression of a major cadherin protein in epithelial cells, E-cadherin; its expression level was decreased with the administration of collagen from soft-shelled turtle. As down-regulation of E-cadherin is an important factor in EMT induction, we examined the expression of EMT markers in HaCaT cells to investigate whether EMT was induced in keratinocytes by the administration of collagen. The increase in expression of vimentin, a mesenchymal marker, and snail, a major inducer of EMT via suppression of E-cadherin expression, in collagen-administered HaCaT cells compared with nontreated cells suggests that the administration of collagen from soft-shelled turtle induces EMT in human keratinocytes. Recent studies reported that human collagen type I can induce EMT in some cell types, and collagen from soft-shelled turtle as used in this study may have a similar effect.

**FIGURE 5.** Wound healing assay. (A) Microscopic images of wound healing over 8 h. (B) The percentage of wounded area in collagen-administered HaCaT cells was significantly larger than in nontreated cells. *p < 0.01.
EMT was originally described as a phenomenon observed during gastrulation in the early embryo. Recently, EMT was considered to be associated with tissue repair responses to injuries in parenchymal organs, including skin. Therefore, we performed an in vitro wound healing assay using a cell scratch model to clarify the effect of EMT of HaCaT cells induced by the administration of collagen from soft-shelled turtle on the wound healing process. The significant promotion of wound healing in HaCaT cells administered collagen suggests that administration of collagen from soft-shelled turtle enhances the wound healing ability of keratinocytes through the induction of EMT. However, the mechanism of the induction of EMT of keratinocytes upon administration of collagen from soft-shelled turtle is unclear. In this study, we focused on the function of proteins listed in Table II, in which the expression level of $\beta$-catenin was increased with collagen administration. A previous report demonstrated that overexpression of $\beta$-catenin induced cell migration and invasion through the induction of EMT via up-regulation of mesenchymal markers, including vimentin, and down-regulation of epithelial markers, including E-cadherin. Therefore, increased expression of $\beta$-catenin may be one of the mechanisms underlying the induction of EMT after the administration of collagen from soft-shelled turtle. Further studies are necessary to clarify the mechanism of increased $\beta$-catenin expression and the other mechanisms for EMT induction.

In conclusion, we measured the changes in protein expression in HaCaT cells administered collagen from soft-shelled turtle using a shotgun LC/MS-based global proteomic analysis and found that the administration of collagen induced the EMT of keratinocytes and facilitated wound healing. Therefore, collagen from soft-shelled turtle may provide significant benefits for skin wound healing and be a useful material for pharmaceuticals and medical care products.

ACKNOWLEDGMENTS

We are grateful to Mr. T. Aboshi for providing the soft-shelled turtles used in the study (Shin-uoel, Inc.).

REFERENCES

1. Gelse K, Pochli E, Aigner T. Collagens-structure, function, and biosynthesis. Adv Drug Deliv Rev 2003;55(12):1531–1548.
2. Mihlyhary J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 2004;20(1):33–43.
3. Birk DE, Trelstad RL. Extracellular compartments in tendon morphogenesis: Collagen fibril, bundle, and macroaggregate formation. J Cell Biol 1986;103(1):231–240.
4. Adachi E, Hayashi T. Anchoring of epithelia to underlying connective tissue: Evidence of frayed ends of collagen fibrils directly merging with meshwork of lamina densa. J Electron Microsc (Tokyo) 1994;43(5):264–271.
5. Park KH, Bae YH. Phenotype of hepatocyte spheroids in Arg-GLY-Asp (RGD) containing a thermo-reversible extracellular matrix. Biosci Biotechnol Biochem 2002;66(7):1473–1478.
6. Liu B, Weinizer SA, Gibson TB, Mascarenhas D, Cohen P. Type I alpha collagen is an IGFBP-3 binding protein. Growth Horm IGF Res 2003;13(2–3):89–97.
7. Di Lullo GA, Sweeney SM, Korkko J, Ala-Kokko L, San Antonio JD. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem 2002;277(6):4223–4231.
8. Saby C, Buache E, Brassart-Pasco S, El Btaouri H, Courageot MP, Van Gulic L, Garnotel R, Jeannesson P, Morjani H. Type I collagen aging impairs discoildin domain receptor 2-mediated tumor cell growth suppression. Oncotarget 2016;7(18):24908–24927.
9. Maquoi E, Assent D, Delilleux J, Pequeux C, Foidart JM, Noel A. MT1-MMP protects breast carcinoma cells against type I collagen-induced apoptosis. Oncogene 2012;31(4):480–493.
10. Goorel ES, Leung TH, Khuh P, Lane AT. Purified type I collagen wound matrix improves chronic wound healing in patients with recessive dystrophic epidermolysis bullosa. Pediatr Dermatol 2015;32(2):220–225.
11. Shevchenko RV, Silbonds PD, Sharpe JR, James SE. Use of a novel porcine collagen paste as a dermal substitute in full-thickness wounds. Wound Repair Regen 2006;14(2):198–207.
12. Wollina U, Meseg A, Weber A. Use of a collagen-elastin matrix for hard to treat soft tissue defects. Int Wound J 2011;8(3):291–296.
13. Barthoumi A, Salvador-Culla B, Kohane DS. NIR-triggered drug delivery by collagen-mediated second harmonic generation. Adv Healthc Mater 2015.
14. Wallace DG, Rosenblatt J. Collagen gel systems for sustained delivery and tissue engineering. Adv Drug Deliv Rev 2003;55(12):1631–1649.
15. Fries W. Collagen–biomaterial for drug delivery. Eur J Pharm Biopharm 1998;45(2):113–136.
16. Lynn AK, Yannas IV, Bonfield W. Antigenicity and immunogenicity of collagen. J Biomed Mater Res B Appl Biomater 2004;71(2):343–354.
17. Pranoto Y, Lee CM, Park HJ. Characterizations of fish gelatin films added with gelan and kappa-carrageenan. LWT Food Sci Technol 2007;40(5):766–774.
18. Muradilaharan N, Jey Shakila R, Sukumar D, Jeyasekaran G. Skin, bone and muscle collagen extraction from the trash fish, leather jacket (Odonus niger) and their characterization. J Food Sci Technol 2013;50(6):1106–1113.
19. Wang Y, Regenstein JM. Effect of EDTA, HCl, and citric acid on Ca salt removal from Asian (silver) carp scales prior to gelatin extraction. J Food Sci 2009;74(6):C426–C431.
20. Wang C, Zhan CL, Cai QF, Du CH, Liu GM, Su WJ, Cao MJ. Expression and characterization of common carp (Cyprinus carpio) matrix metalloproteinase-2 and its activity against type I collagen. J Biotechnol 2014;177:45–52.
21. Benjaksit S, Thiansilaluk Y, Visessanguan W, Roytrakul S, Kishimura H, Prodpran T, Meesane J. Extraction and characterisation of pepsin-solubilised collagens from the skin of bigeye snapper (Priacanthus tayenus and Priacanthus macracanthus). J Sci Food Agric 2010;90(1):132–138.
22. Nalinanon S, Benjaksit S, Kishimura H. Collagens from the skin of arabesque greenling (Pleuragrammus azonus) solubilized with the aid of acetic acid and pepsin from albacore tuna (Thunnus alalunga) stomach. J Sci Food Agric 2010;90(9):1492–1500.
23. Tziveleka LA, Ioannou E, Tsiourvas D, Berillis P, Foufa E, Roussis V. Collagen from the Marine sponges Axinella cannabina and Suberites carnosus: Isolation and morphological, biochemical, and biophysical characterization. Mar Drugs 2017;15(6).
24. Pallela R, Venkatesan J, Janapala VR, Kim SK. Biophysicochemical evaluation of chitosan-hydroxyapatite-marine sponge collagen composite for bone tissue engineering. J Biomed Mater Res A 2012;100(2):486–495.
25. Coelho RCG, Marques ALP, Oliveira SM, Digo GS, Pirracio RP, Moreira-Silva J, Xavier JC, Reis RL, Silva TH, Mano JF. Extraction and characterization of collagen from Antarctic and Sub-Antarctic squid and its potential application in hybrid scaffolds for tissue engineering. Mater Sci Eng C Mater Biol Appl 2017;78:787–795.
26. Yamamoto T, Uemura K, Sawashi Y, Matamura K, Taga A. Optimization of method to extract collagen from “emperor” tissue of soft-shelled turtles. J Oleo Sci 2016;65(2):169–175.
27. Zou Y, Wang L, Cai P, Li P, Zhang M, Sun Z, Sun C, Xu W, Wang D. Effect of ultrasound assisted extraction on the physicochemical and functional properties of collagen from soft-shelled turtle cali-pash. Int J Biol Macromol 2017.
Yang Y, Li C, Song W, Wang W, Qian G. Purification, optimization and physicochemical properties of collagen from soft-shelled turtle calipash. Int J Biol Macromol 2016;89:344–352.

Bluemlein K, Raizer M. Monitoring protein expression in whole-cell extracts by targeted label- and standard-free LC-MS/MS. Nat Protoc 2011;6(6):859–869.

Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Sevinsky JR, Resing KA, Ahn NG. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics 2005;4(10):1487–1502.

Zybailov B, Coleman MK, Flores L, Washburn MP. Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. Anal Chem 2005;77(19):6218–6224.

Lundgren DH, Hwang SI, Wu L, Han DK. Role of spectral counting in quantitative proteomics. Expert Rev Proteomics 2010;7(1):39–53.

Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009;37(1):1–13.

Liang CC, Park AY, Guan JL. In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007;2(2):329–333.

51. Chen L, Mai W, Chen M, Hu J, Zhuo Z, Lei X, Deng L, Liu J, Yao N, Huang M, Peng Y, Ye W, Zhang D, et al. Arenobufagin inhibits prostate cancer epithelial–mesenchymal transition and metastasis by down-regulating beta-catenin. Pharmacol Res 2017;123:130–142.

In vitro and inexpensive method for analysis of cell migration scratch assay: A convenient