Quantitative proteomic analysis and comparison of two bone marrow stromal cell lines using SILAC method

Xiang Li¹, Ting Wan¹, Sijie Zhang², Dongliang Li¹, Xiaofeng Han^{1 *}

¹ Wuxi Medical School, Jiangnan University, Wuxi, China.

² The Key Laboratory of Carbohydrate Chemistry & Biotechnology, Ministry of Education; School of Biotechnology, Jiangnan University, Wuxi, China.

* Corresponding author. Mailing address: Wuxi Medical School, Jiangnan University, Wuxi, China.

Fax: +86-0510-85918126. E-mail: xfenghan@126.com.

ABSTRACT

Two human bone marrow stromal cell lines, HS5 and HS27a, co-cultured with myeloid cells, have been frequently used in studies of crosstalk between cells in the bone marrow microenvironment and hematopoietic cells. Altered expression of proteins is typically associated with cell-cell signal transduction and regulation of cellular functions. Many studies have focused on key proteins that contribute to functional differences in cell co-culture models, but global quantitative proteome of HS5 and HS27a has not been performed. We applied the SILAC method using two stable isotopes each of arginine and lysine for labeling of proteins in the two cell lines. Labeled proteins were by 2D ultrahigh-resolution liquid chromatography LTQ Orbitrap mass spectrometry. Among 4213 unique identified and annotated proteins in the cell lines, 1462 proteins were detected in two independent experiments. Of these, 69 showed significant upregulation and 48 showed significant downregulation (>95% confidence) in HS27a relative to HS5. Gene ontology (GO) term and pathway analysis indicated that the differentially regulated proteins were involved in cellular movement, cell-to-cell signaling and interaction, and hematological system development and function. A total of 55 items were identified in both genomic and proteomic databases. Quantitative RT-PCR and western blotting were performed on 7 proteins randomly selected from 28 differentially expressed proteins that were identified in both databases and were involved in the top networks/pathways. **We observed that the decreased apoptosis in co-cultured KG1a cells when integrin α V (ITGAV) was inhibited in HS27a cells, which suggested the functional role of ITGAV in co-culture system.** The integrated genomic/ proteomic approach described here, and the identified proteins, will provide a useful basis for further elucidation of molecular mechanisms in the bone marrow microenvironment, and for ongoing studies of crosstalk among stromal cells and myeloma cells in co-culture systems.

INTRODUCTION

Bone marrow stromal cells, the major component of bone marrow, have many important functions besides acting as "silent partners" of hematopoietic cells. They secrete growth factors and insoluble matrix factors that promote clonal cell survival, and recruit endothelial, inflammatory, and mesenchymal stem cells [1]. According to the "Seed and Soil" theory, homing factors from stromal cells in the bone marrow microenvironment promote the growth of clonal cells, *e.g.*, myelodysplastic syndromes (MDS) cells, and are involved in clonal diseases of hematopoietic stem/ precursor cells [2-5]. Increasing evidence indicates that bone marrow stromal cells are enriched in growth factors, cytokines, and proteases, and provide a favorable environment for docking of circulating cells such as prostate cancer cells [6, 7]. Bone marrow stromal cell lines HS5 and HS27a represent two major types of cells related to early cancer development and are useful for deciphering crosstalk between stromal cells and clonal cells. HS27a supports "cobblestone area" formation by early hematopoietic progenitor cells, whereas HS5 secretes multiple cytokines that support proliferation of committed progenitor cells [8]. Apoptosis-resistant clonal MDS progenitor cells from patients with advanced MDS became sensitive to TNF α -induced apoptosis through contact with HS5 or HS27a cells [9-11].

Co-administration of HS27a (but not HS5) cells with hematopoietic progenitor cells from MDS patients facilitated engraftment of clonal CD34⁺ cells of any karyotype [5], indicating that the two stromal cells have differing functions in support of primitive clonal MDS cells. A study of the mechanism of preferential bony metastasis of human prostate cancer (PCa) cells suggested that HS5 and HS27a cells activate trans differentiation apoptotic resistant PCa cells, promoting their adaptation to the bone microenvironment and eventual development of lethal disease [6]. These two stromal cell lines, available from the American Type Culture Collection (ATCC; Manassas, VA, USA), thus provide a very useful model for studies of tumor cell/ stromal cell interactions [12-14].

Over 17,000 genes in the HS5 and HS27a cell lines have been characterized and compared using DNA microarray techniques [8]. However, whole-proteome information is not available for either cell line, and integrated analysis using differential genomic and proteomic techniques has not been performed. Recent advances in mass spectrometry (MS) for protein identification and quantification provide several approaches for in-depth analysis of a large number of proteins [15-17]. The SILAC ("Stable Isotope Labeling by Amino acids in Cell culture") method has many advantages over other methods; *e.g.*, it reduces sample-to-sample variability and is highly suitable for protein quantification [18]. In the present study, proteomic differences between HS5 and HS27a cells were analyzed using two stable isotopes each of arginine (Arg) and lysine (Lys) in two separate cultures: "light" (K0R0, L) and "heavy" (K8R10, H) (Fig. 1). The distinctive "L" and "H" forms of each peptide pair, having *m/z* difference 8 or 10 as detected by MS, reflected relative amounts of the

corresponding protein in each of the two isotopically encoded stromal cells. Labeled proteins with >95% label incorporation were analyzed and quantified by 2D-HPLC-LTQ Orbitrap MS. We integrated information on differentially expressed proteins with genomic data available from open access, validated these data by RT-PCR and western blotting of the two cell lines, and evaluated functions of the differentially expressed proteins using an Ingenuity Systems software program. We observed upregulation (at both protein and mRNA levels) of proteins such as HCLS1, COL1A2, and ALCAM in HS27a, and proteins such as HSP90 and CSE1L in HS5. These findings increase our knowledge of these two stromal cell lines, and provide a useful basis for future functional studies.

MATERIALS AND METHODS

Cell culture

KG1a cells (derived from AML) and bone marrow stromal cell lines HS5 and HS27a were grown, propagated, and subjected to experiments between passages 8 and 24, as described previously [11]. For SILAC labeling, **stroma** cells were cultured in SILAC-labeled RPMI 1640 medium with 10% dialyzed FBS and 1% penicillin/ streptomycin containing “light” (K0R0), or “heavy” (K8R10) Lys and Arg, respectively [19]. L-proline (200 mg/L) was added to medium to prevent Arg-to-proline conversion [20, 21]. Labeled cells were cultured in SILAC medium for ≥ 6 passages to eliminate the presence of native Lys and Arg.

Genomic analysis using open access data

Differential gene expression of HS5 and HS27a was analyzed by Beverly Torok-Storb's group (Fred Hutchinson Cancer Research Center; Seattle, WA, USA) as described previously, and data were made available by open access at ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE463 [8]. In brief, a table populated with sample characteristics is shown on the web interface. We designate up to 8 sample groups for comparison, choose parameters, and click the "GEOquery" button. The results table presents various categories of statistics (P-values, t-statistics, fold change values) and gene annotations (gene symbols, gene names) [22].

Cell lysis and protein extraction

Total proteins were extracted from cells using T-PER reagent (Thermo Scientific; San Jose, CA, USA). In brief, 1×10^7 cells were detached with trypsin, washed with ice-cold $1 \times$ PBS (0.01 M phosphate buffer containing 0.15 M NaCl, pH 7.4), lysed with 0.5 mL T-PER reagent containing 1 mM PMSF and 0.1% aprotinin, incubated for 30 min on ice, homogenized, and centrifuged at 12,000

rpm for 15 min. Protein concentration was measured by BCA assay (Beyotime; Haimen, China).

SDS-PAGE and in-gel digestion

Proteins were separated by 10% SDS-PAGE and visualized by Coomassie staining. Gel slices were washed with 25 mM ammonium bicarbonate/ 50% acetonitrile (ACN), dried, incubated with 20 μ L of 10 mM dithiothreitol (DTT) at 56 °C for 1 hr, transferred to equal volumes of 20 mM iodoacetamide (IAM) at room temperature in the dark for 45 min, washed, trypsinized using 20 μ L of 0.05 μ g/ μ L trypsin (Promega; Madison, WI, USA) at 4 °C for 30 min, subjected to removal of excess trypsin solution, added with 20 μ L of 25 mM NH_4HCO_3 , and incubated at 37 °C for 10 hr. Extracted products were dried using a SpeedVac concentrator (CentriVac Cold Trap, Labconco; Kansas, MO, USA). Dried samples were dissolved in 2 μ L 0.5% trifluoroacetic acid and subjected to MALDI-TOF/TOF-MS analysis (UltrafleXtreme, Bruker Daltonics; Bremen, Germany) for evaluation of amino acid substitutions.

In-solution digestion

Proteins from stable isotope labeled cells were mixed at 1:1, reduced and alkylated by incubation with equal amounts of 10% DTT and 10% IAM, digested by lysyl endopeptidase (Wako; Osaka, Japan), and then digested by trypsin at 37 °C for 4 or 12 hr [23]. Total peptides were concentrated and desalted using a 10 kDa size-exclusion spin ultrafiltration unit, eluted with varying concentrations of ACN, and dried using the SpeedVac concentrator.

LC-MS/MS analysis

2D-LC-MS was performed using LTQ Orbitrap MS (Thermo Fisher; Waltham, MA, USA) as described previously [24]. In brief, digested peptides (100 μ g) were injected onto a biphasic capillary column (internal diameter 200 μ m) packed with C_{18} resin (ReproSil-Pur, 5 μ m, Dr. Maisch GmbH; Ammerbuch, Germany) and strong cation exchange resin (Luna, 5 μ m, SCX 100A, Phenomenex; Torrance, California, USA). Peptide effluents of the biphasic column at each step were transferred to a 15-cm C_{18} analytical column (i.d. 75 μ m; ReproSil-Pur, 3 μ m) at flow rate 500 nL/min. Nano electrospray ionization (ESI) was performed with spray voltage 2.0 kV and heated capillary at 200 °C. One full MS scan (300-1800) using the Orbitrap was followed by five MS/MS scans of the five most intense ions selected from the MS spectrum in LTQ. Charge state screening was enabled for +2, +3, +4, and above.

Data analysis

Raw MS data were analyzed using the MaxQuant software program (V. 1.2.2.5) [25, 26]. A false discovery rate (FDR) of 0.01 for proteins and peptides and a minimum peptide length of 6 amino acids were required. MS/MS spectra were searched by Andromeda [27] against the International Protein Index (IPI) human database (V. 3.85). SILAC states of peptides were determined by the MaxQuant program from mass differences between SILAC peptide pairs, and this information was used for searches with fixed Arg10 or Lys8 modifications, as appropriate. Quantification in MaxQuant was performed as described previously [25].

Differential regulation within each experimental H/L (ratio of “heavy” to “light”) of identified proteins was normalized using $\log_2(\text{ratio}) = \pm 1$ as threshold and z-score analysis, as described previously [28, 29]. In brief, H/L ratios were converted into $\log_2$ space to determine geometric means, and average ratios and standard deviations were determined for each data set. The $\log_2$ H/L ratio for each protein was converted to a z-score using the formula shown below, in which b represents a single protein in a data set population (a ... n). The z-score indicates the number of standard deviation units (σ) the protein’s $\log_2$ H/L ratio is away from the population mean. Thus, a z-score $\geq 1.960\sigma$ indicates that the protein’s differential expression lies outside the 95% confidence interval, $\geq 2.576\sigma$ indicates that differential expression is outside the 99% confidence interval, and $\geq 3.291\sigma$ indicates 99.9% confidence. A z-score $\geq 1.960\sigma$ was considered significant. Extracted data from the two experiments were subjected to further functional analysis.

$$\text{z-scores}(\sigma) \text{ of } [b] = \frac{\log_2 \frac{X(M \text{ or } H)}{L} [b] - \text{Average of } (\log_2 \text{ of each number, } a \dots n)}{\text{Standard deviation of } (\log_2 \text{ of each number, } a \dots n)}$$

Functional annotation and Ingenuity Pathway Analysis

Identified proteins were analyzed using the SWISS-PROT database for classification of biological process, cellular component, and molecular function [30]. Significantly overrepresented gene ontology (GO) terms were identified using the Database for Annotation, Visualization and Integrated Discovery (DAVID) gene bioinformatics resources [31, 32]. Proteins determined to be differentially regulated by statistical analysis were arranged in an Excel spreadsheet, their IPI numbers were uploaded to the Ingenuity System application (www.ingenuity.com), and each number was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of the proteins were generated algorithmically based on their connectivity. Fisher’s exact test was used to calculate p-values reflecting the probability that assignment of a particular biological function and/or disease to a network was due solely to chance.

Western blot analysis

Total cell proteins were prepared and quantified by bicinchoninic acid assay (Pierce Biotechnology; Chicago, IL, USA), and equal amounts of protein (20 µg) from each lysate were diluted in Laemmli SDS sample buffer and resolved by electrophoresis on 4% to 12% Bis-Tris precast NuPAGE gels (Invitrogen; Camarillo, CA, USA) in running buffer (50 mM 2-(N-morpholino) ethane sulfonic acid, 50 mM Tris [tris(hydroxymethyl)aminomethane]) base, 0.1% SDS, and 1 mM EDTA) as per the manufacturer's instructions. Proteins were transferred to PVDF membranes for immunoblotting. Membranes were blocked in 5% nonfat dry milk diluted in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 hr at room temperature, and incubated overnight at 4 °C in 5% nonfat dry milk /TBS-T containing rabbit anti-HCLS1 antibody (1:1000; Abcam; Cambridge, MA, USA), rabbit anti-COL1A2 antibody (1:500; ABclonal; Boston, MA, USA), rabbit anti-ALCAM antibody (1:5000; Abcam), rabbit anti-ITGAV antibody (1:1000; Cell Signaling Technology; Danvers, MA, USA), rabbit anti-MAP4 antibody (1:500; ABclonal), rabbit anti-HSP90 antibody (1:5000; Abcam), rabbit anti-CSE1L antibody (1:1000, Abcam), or rabbit anti-tubulin antibody (1:1000; Cell Signaling). Secondary goat anti-rabbit antibodies (1:2000; Santa Cruz Biotechnology; Santa Cruz, CA, USA) conjugated to horseradish peroxidase were used for enhanced chemiluminescence (Pierce Biotechnology), and membranes were exposed to film.

Quantitative real-time PCR

Total RNA from 1×10^5 cells was isolated using an RNAPure Tissue Kit (CWBiotech; Beijing, China). First-strand cDNA was synthesized from total RNA using ReverTra Ace- α -[®] (Toyobo; Osaka, Japan). Primers were designed using the DNAMAN program (V. 6.0.3; Lynnon Biosoft, Canada). Quantitative real-time PCR was performed by LightCycler-based SYBR Green I dye detection with UltraSYBR Mixture (CWBiotech). Gene expression was quantified by the $2^{-\Delta\Delta CT}$ method [11].

Assessment of apoptosis

KG1a cells were cocultured with unmodified or modified HS27a cells in the presence of TNF α at 25 ng/mL for 24 hrs. Apoptosis was determined in KG1a cells, which were removed with the stroma after trypsin treatment. Cells were then labeled with FITC-conjugated anti-CD45 antibody to distinguish CD45⁺ cells from CD45⁻ cells (stroma cells) and analyzed by FACS for apoptosis using annexin V–phycoerythrin and propidium iodide.

RESULTS

Determination of isotope incorporation efficiency

The SILAC method was used to analyze functional differences of HS5 and HS27a cells at the proteome level. Because incomplete labeling results in errors in quantification, and quantitation of low-abundance proteins may be masked by contaminated “light” peptides, cells were cultured in SILAC medium for a period of time sufficient to allow full incorporation of the stable isotope. To ensure complete labeling of proteins, particularly for labeling efficiency >95%, we performed a pilot experiment to determine incorporation efficiency of labeled Lys and Arg, “light” (HS5) and “heavy” (HS27a) proteins. MALDI-TOF/TOF-MS results for peptide GVVDS~~E~~DLPLNISR of heat shock protein 90- β (HSP90, P08238) indicated that complete incorporation of isotopically labeled Arg and Lys was achieved in HS5 and HS27a, and no Arg-to-Pro conversion occurred (Fig. 2A,B). LC-ESI-MS/MS analysis of the triply-charged peptide HLEINPDHPIVETLR of HSP90 and YGGPYHIGGSPFK of filamin-A (P21333) showed that these doublets of actual peak clusters were from HS5 and HS27a cells, respectively (Fig. 2C,D).

SILAC cell model for quantification of proteome in HS5 and HS27a

Peptides from HS5 and HS27a were analyzed by ultrahigh-resolution liquid chromatography-tandem MS (nLC-ESI-MS/MS) on a hybrid linear ion trap LTQ Orbitrap instrument. A total of 4218 unique proteins were identified in two independent experiments (Fig. 3A). Of these, 1462 proteins (34.7% of the total) that were identified in both experiments and satisfied the criteria for protein quantitation were subjected to further bioinformatic analysis. Distribution histograms of H/L log ratios fit a Gaussian distribution (Fig. 3B,C). Most of the identified proteins were within the ± 1 range of log ratios. Using ± 1 as threshold $\log_2$ ratio, we found that expression of 131 proteins was upregulated and that of 96 proteins was downregulated in HS27a in comparison with HS5 (Fig. 3D). Population distribution-based z-scores allowed direct comparison of proteins from different experiments. Differing confidence level cutoffs were applied to the data in z-score analysis to determine which proteins showed significant differential regulation. The cutoffs applied were 95%, 99%, and 99.9%, corresponding respectively to z-scores of ± 1.960 , ± 2.576 , and ± 3.291 . Using 95% as cutoff, significant differential regulation of 117 proteins (69 upregulated, 48 downregulated) was observed in HS27a as compared with HS5. Using 99% as cutoff, differential regulation of 60 proteins (34 upregulated, 26 downregulated) was observed in HS27a. Using 99.9% as cutoff, only 28 proteins (16 upregulated, 12 downregulated) showed differential regulation in HS27a. These results are summarized in Table 1. Downregulated and upregulated proteins determined in the two experiments using 95% as cutoff are listed respectively in Tables 2 and 3. All proteins differentially regulated with >95% confidence had a >2-fold alteration of SILAC ratio ($\log_2$ ratio >1 or <-1).

Functional classification and pathway analysis of identified proteins

Functional interpretation is a crucial step in data analysis when extensive functional annotation of the data sets is not available. Taking into account their nonexclusive localization in GO, the identified proteins were linked to at least one annotation term each within the GO molecular function, biological process, and molecular component categories. The most common molecular functions were binding (49.0%), and catalytic activity (23.1%) (Fig. 4A). The major biological process categories were cellular (14.2%) and single-organism (13.2%) (Fig. 4B). The major cellular component categories were cell part (17.9%) and organelle (16.8%) (Fig. 4C).

Proteins with $\log_2$ ratio ± 1 were further analyzed, and metabolic and canonical pathways and interconnecting proteins were generated by Ingenuity Pathway Analysis. Proteins identified in the top network were involved in cellular movement, cell-to-cell signaling and interaction, hematological system development and function, scored as 44 (28 proteins; Fig. 5A). Proteins identified in other networks were involved in infectious diseases, post-translational modification, protein folding (23 proteins; Fig. 5B), and connective tissue disorders, dermatological diseases and conditions, and developmental disorders (23 proteins; Fig. 5C).

Integrated analysis of proteomic and genomic data in HS5 and HS27a

The next step was to perform integrated analysis of proteomic and genomic data from HS5 and HS27a cells. A total of 55 items were identified in both databases. Among these, 32 genes/proteins were upregulated in HS27a relative to HS5, including insulin-like growth factor-binding protein 7 (IGFBP7; SILAC $\log_2$ ratio 3.591; Gene array $\log_2$ ratio 4.536), collagen alpha-2(V) chain (COL5A2; SILAC $\log_2$ ratio 3.328; Gene array $\log_2$ ratio 2.494), hematopoietic lineage cell-specific protein (HCLS1; SILAC $\log_2$ ratio 2.979; Gene array $\log_2$ ratio 1.685), and cysteine and glycine-rich protein 2 (CSRP2; SILAC $\log_2$ ratio 2.644; Gene array $\log_2$ ratio 1.653). The other 23 genes/proteins were downregulated in HS27a relative to HS5 cell; these included ankyrin repeat and SOCS box protein 1 (ASB1; SILAC $\log_2$ ratio -3.065; Gene array $\log_2$ ratio -2.236), band 4.1-like protein 3 (EPB41L3, SILAC $\log_2$ ratio -2.930; Gene array $\log_2$ ratio -6.749), transmembrane glycoprotein NMB (GPNMB, SILAC $\log_2$ ratio -2.576; Gene array $\log_2$ ratio -5.310), and plasminogen activator inhibitor 2 (SERPINB2, SILAC $\log_2$ ratio -2.050; Gene array $\log_2$ ratio -5.542). We further compared the 69 upregulated and 48 downregulated proteins with the gene expression profiling that showed 10-fold higher and 10-fold lower expression, in HS27a cells than in HS5 cells [8]. We found Insulin-like growth factor binding protein 7 (IGFBP7) was significantly upregulated in both proteomic and gene expression database. In addition, band 4 like protein 3 (EPB41L3) and transmembrane glycoprotein NMB (GPNMB) were downregulated in both proteomic and gene expression database.

Validation of MS results by real-time PCR (RT-PCR) and western blotting

To confirm the accuracy of protein identifications made by nLC-ESI-MS/MS, we randomly selected 7 out of the 28 differentially expressed proteins that were identified from both genomic and proteomic data and involved in the top networks shown in Fig. 5A, for validation of mRNA and protein levels. Annotations of the genes encoding these proteins are shown in Fig. 6A. We validated the genomic and proteomic data by RT-PCR and western blotting (Fig. 6B,C). Relative to HS5, mRNA and protein levels of HCLS1, COL1A2, and ALCAM were higher in HS27a, whereas mRNA and protein levels of MAP4, HSP90, and CSE1L were lower in HS27a. These findings are consistent with proteomic and genomic data. However, in the case of ITGAV, expression from the genomic database was slightly lower in HS27a relative to HS5, whereas protein level from the proteomic database was higher (Fig. 6A). Validation by RT-PCR and western blotting showed that ITGAV levels were higher in HS27a at both mRNA and protein levels. **We then inhibited the expression of integrin α V (ITGAV) in HS27a cells by specific siRNAs, and observed the decreased apoptosis in KG1a cells after co-cultured with modified HS27a cells under TNF α induction (Fig. 6 D and 6 E).**

DISCUSSION

Human marrow stromal cell lines HS27a and HS5 have been used in studies of MDS as well as various types of cancer. We previously characterized glycan expression levels in these two cell lines using an integrated strategy combining gene microarray, proteomic, and lectin microarray analyses [33]. However, integrated analysis using differential genomic and proteomic techniques has not yet been performed. In the present study, we combined SILAC proteomic techniques with genomic data to elucidate additional signaling pathways related to cell-cell interactions in these two cell lines. A total of 1462 proteins out of 4218 unique proteins were identified in both experiments and met the criteria established for protein quantitation. Using +1 as the threshold $\log_2$ ratio, we found that expression of 131 proteins was upregulated and that of 96 proteins was downregulated in HS27a relative to HS5. Using 95% as cutoff, significant differential regulation was observed for 117 proteins: 69 upregulated and 48 downregulated in HS27a relative to HS5.

We then integrated our proteomic data with available genomic data for the two cell lines. A total of 55 items were identified in both genomic and proteomic databases. **The differential presentation between gene and protein expression may be resulted by, 1) most of protein are posttranslational modified; 2) the trace expression of certain protein were undetectable by current proteomic technique; and 3) the differential technique and analysis strategy used in genome and**

proteome research. GO analysis revealed that most of the common molecular functions were involved in binding and catalytic activity. The major biological process categories were cellular and single-organism, and the major cellular component categories were cell part and organelle. We were interested to find additional molecules that may facilitate cell-cell signaling pathways. Ingenuity Pathway Analysis indicated that identified proteins in the top network were involved in cellular movement, cell-to-cell signaling and interaction, and hematological system development and function; this was consistent with our criteria for supportive and commutative functions of stromal cells. We selected proteins HCLS1, COL1A2, ALCAM, MAP4, HSP90, and CSE1L, which were in the top network, for validation by RT-PCR and western blotting. HCLS1, a well-known signaling protein, is regulated by phosphorylation and interacts with various partners (including Grb2, Syk phosphatidyl-inositol-3 kinase, Lck, Lyn, c-Fgr, Arp2/3, and F-actin) for regulation of immunological synapses, STAT protein activity, and clonal selection of lymphocytes [34]. COL1A2 (collagen alpha-2(I) chain) is present in most connective tissues [35]. ALCAM, also known as CD166 antigen, is a 100-105 kDa type I transmembrane glycoprotein belonging to the immunoglobulin superfamily. ALCAM is expressed on activated T cells, activated monocytes, epithelial cells, fibroblasts, neurons, and melanoma cells. It is an important mediator of adhesion interactions and has been used as a cancer stem cell marker [36, 37]; these findings may be relevant to its function in co-cultured stromal cells. MAP4 (microtubule-associated protein 4), a major non-neuronal MAP, promotes microtubule assembly and counteracts destabilization of interphase microtubule catastrophe promotion. MAP4 interacts with cyclin B, which targets cell division cycle 2 (CDC2) kinase to microtubules [38, 39]. Phosphorylation of MAP4 affects microtubule properties and cell cycle progression [39]. HSP90 (heat shock protein 90) is a chaperone protein that facilitates proper folding of other proteins, stabilizes other proteins against heat stress, and contributes to protein degradation. It is among the most highly expressed cellular proteins across all mammalian species, and stabilizes a number of proteins involved in tumor growth [40]. CSE1L, also known as Exportin-2, mediates re-export of importin-alpha from the nucleus to the cytoplasm after import substrates (cargos) have been released into the nucleoplasm [41]. We observed higher expression of ITGAV (integrin alpha-V) in HS27a relative to HS5 at both mRNA and protein levels, although genomic data indicated lower expression in HS27a. ITGAV is able to combine with multiple integrin beta chains to form different integrins, each of which can interact with extracellular matrix ligands. Besides its role in adhesion, ITGAV facilitates signal transduction [42]; **We then observed the decreased apoptosis in co-cultured KG1a cells when inhibited ITGAV in HS27a cells, which suggested the functional role of ITGAV in co-culture system. Our further work will focus on the functional role of ITGAV in primary bone marrow stroma cells as well as the microenvironmental niche.**

Stromal cells function as important sensors of cell microenvironmental conditions, and help regulate a variety of signaling pathways in response to structural alterations of extracellular matrix or to cell interactions. We used quantitative proteomic analysis of SILAC-labeled proteins integrated with a free-access genomic database for global expression profiling of human bone marrow stromal cell lines HS5 and HS27a, which are frequently used for investigations of the bone marrow microenvironmental niche. Our findings provide a useful basis for our ongoing studies of crosstalk among stromal cells and hematopoietic cells in a co-culture system.

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Author Contributions

X.L. and X.H. designed the research. X.L., T.W., S.Z., and D.L. performed the research and analyzed the data. X.L. wrote the paper. All authors read and approved the final manuscript.

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Table 1: Protein number, log₂ ratio, and z-scores of SILAC-labeled proteins.

Cell line type	Protein	Z-score ^b		
	number ^a	±1.960σ	±2.576σ	±3.291σ
HS27a/HS5 (H/L)	1462	69, 48	34, 26	16, 12

^a Proteins were analyzed in two independent experiments.

^b Z-score value is the average log₂ ratio (HS27a/HS5). Numbers of upregulated and downregulated proteins outside the indicated confidence level are shown.

Table 2: Downregulated proteins in HS27a cells in comparison with HS5 (>95% confidence).

Protein ID	Protein name	Gene name	Log ₂ ratio	Z-score ^a
H0Y8D1	Putative trypsin-6;Trypsin-2;Trypsin-1;Alpha-trypsin chain 1; Alpha-trypsin chain 2	PRSS1	-4.896	-5.461
P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2	-3.680	-4.113
P04264	Keratin, type II cytoskeletal 1	KRT1	-3.469	-3.879
P12277	Creatine kinase B-type	CKB	-3.207	-3.588
Q96FQ6	Protein S100-A16	S100A16	-3.082	-3.450
P35527	Keratin, type I cytoskeletal 9	KRT9	-3.076	-3.443
B9A047	Ankyrin repeat and SOCS box protein 1	ASB1	-3.065	-3.430
P02769	Serum albumin	ALB	-3.061	-3.426
P81644	Apolipoprotein A-II	APOA2	-2.976	-3.332
Q9Y2J2	Band 4.1-like protein 3	EPB41L3	-2.930	-3.281
Q5TA02	Glutathione S-transferase omega-1	GSTO1	-2.829	-3.169
P13645	Keratin, type I cytoskeletal 10	KRT10	-2.664	-2.986
D3DR31	Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	-2.633	-2.951
P12763	Alpha-2-HS-glycoprotein	AHSG	-2.593	-2.907
F6SKP1	Transmembrane glycoprotein NMB	GNPMB	-2.576	-2.888
Q99541	Perilipin-2	PLIN2	-2.481	-2.782
H7C3P7	Ras-related protein Ral-A	RALA	-2.437	-2.733
O14879	Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	-2.400	-2.693
P55060	Exportin-2	CSE1L	-2.395	-2.687
P03956	Interstitial collagenase;22 kDa interstitial collagenase;27 kDa interstitial collagenase	MMP1	-2.351	-2.638
P15924	Desmoplakin	DSP	-2.342	-2.629
Q9GZL7	Ribosome biogenesis protein WDR12	WDR12	-2.309	-2.591
Q14141	Septin-6	41888	-2.295	-2.577
P09913	Interferon-induced protein with tetratricopeptide repeats 2	IFIT2	-2.102	-2.363
Q5SW79	Centrosomal protein of 170 kDa	CEP170	-2.097	-2.356
P35610	Sterol O-acyltransferase 1	SOAT1	-2.075	-2.332
P05120	Plasminogen activator inhibitor 2	SERPINB2	-2.050	-2.304
P01130	Low-density lipoprotein receptor	LDLR	-1.922	-2.163
P05161	Ubiquitin-like protein ISG15	ISG15	-1.830	-2.060
P17096	High mobility group protein HMG-I/HMG-Y	HMGA1	-1.828	-2.058
P29992	Guanine nucleotide-binding protein subunit alpha-11	GNA11	-1.816	-2.045
Q9BZV1	UBX domain-containing protein 6	UBXN6	-1.800	-2.028
P07858	Cathepsin B; Cathepsin B light chain; Cathepsin B heavy chain	CTSB	-1.790	-2.016
Q53EL6	Programmed cell death protein 4	PDCD4	-1.790	-2.016
P40189	Interleukin-6 receptor subunit beta	IL6ST	-1.785	-2.011
E9PDL6	Protein NDRG1	NDRG1	-1.767	-1.991
P18031	Tyrosine-protein phosphatase non-receptor type 1;Tyrosine-protein phosphatase non-receptor type	PTPN1	-1.757	-1.979
B4DGD3	Uridine-cytidine kinase 2	UCK2	-1.749	-1.971
Q9BZQ8	Protein Niban	FAM129A	-1.749	-1.971
Q86TI2-4	Dipeptidyl peptidase 9	DPP9	-1.705	-1.922
Q9NQZ5	StAR-related lipid transfer protein 7, mitochondrial	STARD7	-1.689	-1.904
C9JG97	Angio-associated migratory cell protein	AAMP	-1.661	-1.873
Q5T3Q7	HEAT repeat-containing protein 1	HEATR1	-1.643	-1.853
P51570	Galactokinase	GALK1	-1.627	-1.835
Q16643	Drebrin	DBN1	-1.602	-1.807
Q9NYY8	FAST kinase domain-containing protein 2	FASTKD2	-1.584	-1.788
K7EIG1	Clustered mitochondria protein homolog	CLUH	-1.576	-1.779
P13639	Elongation factor 2	EEF2	-1.568	-1.770

^a Z-score: average log₂ ratio = 0.027. SD= 0.901; 95% confidence cutoff: -1.960σ = -1.766.

Table 3: Upregulated proteins in HS27a in comparison with HS5 (>95% confidence).

Protein ID	Protein name	Gene name	Log ₂ ratio	Z-score ^a
P11047	Laminin subunit gamma-1	LAMC1	1.622	1.769
Q7KZF4	Staphylococcal nuclease domain-containing protein 1	SND1	1.633	1.781
O60701	UDP-glucose 6-dehydrogenase	UGDH	1.643	1.793
B7Z2S5	Thioredoxin reductase 1, cytoplasmic	TXNRD1	1.666	1.817
Q9UEY8	Adducin 3	ADD3	1.678	1.831
P49257	Protein ERGIC-53	LMAN1	1.685	1.839
Q16352	Alpha-internexin	INA	1.697	1.852
P13667	Protein disulfide-isomerase A4	PDIA4	1.732	1.891
P42765	3-ketoacyl-CoA thiolase, mitochondrial	ACAA2	1.746	1.907
Q96AY3	Peptidyl-prolyl cis-trans isomerase FKBP10	FKBP10	1.749	1.910
Q86UV5	Ubiquitin carboxyl-terminal hydrolase 48	USP48	1.751	1.912
H7BXI1	Extended synaptotagmin-2	ESYT2	1.761	1.923
P04792	Heat shock protein beta-1	HSPB1	1.784	1.949
Q8WX93	Palladin	PALLD	1.788	1.953
P60903	Protein S100-A10	S100A10	1.801	1.968
Q9P0L0	Vesicle-associated membrane protein-associated protein A	VAPA	1.812	1.980
Q9UDY4	DnaJ homolog subfamily B member 4	DNAJB4	1.817	1.985
Q96HC4	PDZ and LIM domain protein 5	PDLIM5	1.825	1.994
B4DKB2	Endothelin-converting enzyme 1	ECE1	1.831	2.001
P06396	Gelsolin	GSN	1.856	2.028
P21964	Catechol O-methyltransferase	COMT	1.892	2.068
F5GXJ9	CD166 antigen	ALCAM	1.899	2.076
P00167	Cytochrome b5	CYB5A	1.903	2.081
P54687	Branched-chain-amino-acid aminotransferase, cytosolic	BCAT1	1.932	2.113
P41226	Ubiquitin-like modifier-activating enzyme 7	UBA7	1.941	2.123
Q99715	Collagen alpha-1(XII) chain	COL12A1	1.943	2.125
P50454	Serpin H1	SERPINH1	1.994	2.182
P14550	Alcohol dehydrogenase [NADP(+)]	AKR1A1	1.996	2.184
Q15555	Microtubule-associated protein RP/EB family member 2	MAPRE2	2.014	2.204
Q86SF2	N-acetylgalactosaminyltransferase 7	GALNT7	2.033	2.225
P07951	Tropomyosin beta chain	TPM2	2.039	2.232
P32455	Interferon-induced guanylate-binding protein 1	GBP1	2.068	2.264
Q9BX68	Histidine triad nucleotide-binding protein 2, mitochondrial	HINT2	2.092	2.290
P21266	Glutathione S-transferase Mu 3	GSTM3	2.098	2.298
F8VZY9	Keratin, type I cytoskeletal 18	KRT18	2.103	2.303
P29966	Myristoylated alanine-rich C-kinase substrate	MARCKS	2.145	2.349
Q6EMK4	Vasorin	VASN	2.191	2.400
B4DIT7	Protein-glutamine gamma-glutamyltransferase 2	TGM2	2.223	2.436
O43175	D-3-phosphoglycerate dehydrogenase	PHGDH	2.288	2.508
P15121	Aldose reductase	AKR1B1	2.333	2.558
P30740	Leukocyte elastase inhibitor	SERPINB1	2.337	2.563
P40261	Nicotinamide N-methyltransferase	NNMT	2.388	2.619
P07942	Laminin subunit beta-1	LAMB1	2.391	2.622
Q8NBJ7	Sulfatase-modifying factor 2	SUMF2	2.404	2.636
Q92896	Golgi apparatus protein 1	GLG1	2.424	2.659
Q9Y6H1	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2, Putative coiled-coil-helix-coiled-coil-helix domain-containing protein CHCHD2P9, mitochondrial	CHCHD2	2.446	2.683
Q13642	Four and a half LIM domains protein 1	FHL1	2.452	2.690
A6NCT7	Collagen alpha-1(XVI) chain	COL16A1	2.463	2.702
O43852	Calumenin	CALU	2.582	2.834
P20908	Collagen alpha-1(V) chain	COL5A1	2.588	2.841
Q13636	Ras-related protein Rab-31	RAB31	2.605	2.860
P02452	Collagen alpha-1(I) chain	COL1A1	2.623	2.879
F8VQR7	Cysteine and glycine-rich protein 2	CSRP2	2.644	2.902
Q15113	Procollagen C-endopeptidase enhancer 1	PCOLCE	2.763	3.035
P07996	Thrombospondin-1	THBS1	2.882	3.166
E7EVW7	Hematopoietic lineage cell-specific protein	HCLS1	2.979	3.275

Q9UGI8	Testin	TES	3.012	3.311
P13796	Plastin-2	LCP1	3.091	3.399
Q9Y680	Peptidyl-prolyl cis-trans isomerase FKBP7;Peptidyl-prolyl cis-trans isomerase	FKBP7	3.093	3.400
Q96D15	Reticulocalbin-3	RCN3	3.205	3.525
P05997	Collagen alpha-2(V) chain	COL5A2	3.328	3.662
P12111	Collagen alpha-3(VI) chain	COL6A3	3.387	3.727
Q01995	Transgelin	TAGLN	3.552	3.910
Q16270	Insulin-like growth factor-binding protein 7	IGFBP7	3.591	3.954
P15151	Poliovirus receptor	PVR	3.602	3.965
P02461	Collagen alpha-1(III) chain	COL3A1	3.620	3.986
P08123	Collagen alpha-2(I) chain	COL1A2	3.741	4.120
Q9UPQ0	LIM and calponin homology domains-containing protein 1	LIMCH1	3.778	4.161
O75459	P antigen family member 1	PAGE1	4.507	4.969

^a Z-score: average $\log_2$ ratio = 0.027. SD= 0.901; 95% confidence cutoff: $1.960\sigma = 1.766$.

Table 4: Comparison of SILAC and Gene array log₂ ratios.

No.	Protein ID	Protein name	Gene name	Mol. weight [kDa]	PEP	SILAC log ₂ ratio	Gene array log ₂ ratio
1	P08123	Collagen alpha-2(I) chain	COL1A2	129.3	1.293E+02	3.741	1.217
2	P02461	Collagen alpha-1(III) chain	COL3A1	138.6	1.386E+02	3.620	1.589
3	Q16270	Insulin-like growth factor-binding protein 7	IGFBP7	28.9	2.886E+01	3.591	4.536
4	P05997	Collagen alpha-2(V) chain	COL5A2	144.9	1.449E+02	3.328	2.494
5	E7EVW7	Hematopoietic lineage cell-specific protein	HCLS1	49.7	4.970E+01	2.979	1.685
6	F8VQR7	Cysteine and glycine-rich protein 2	CSRP2	20.7	2.068E+01	2.644	1.653
7	P20908	Collagen alpha-1(V) chain	COL5A1	183.6	1.836E+02	2.588	1.500
8	O43852	Calumenin	CALU	37.1	3.711E+01	2.582	1.821
9	Q13642	Four and a half LIM domains protein 1	FHL1	31.9	3.190E+01	2.452	1.163
10	Q92896	Golgi apparatus protein 1	GLG1	134.6	1.346E+02	2.424	1.043
11	P07942	Laminin subunit beta-1	LAMB1	198.0	1.980E+02	2.391	1.578
12	P40261	Nicotinamide N-methyltransferase	NNMT	29.6	2.957E+01	2.388	2.431
13	F8VZY9	Keratin, type I cytoskeletal 18	KRT18	43.8	4.377E+01	2.103	2.887
14	P07951	Tropomyosin beta chain	TPM2	33.0	3.299E+01	2.039	1.634
15	Q15555	Microtubule-associated protein RP/EB family member 2	MAPRE2	30.7	3.069E+01	2.014	1.075
16	F5GXJ9	CD166 antigen	ALCAM	59.5	5.953E+01	1.899	2.651
17	Q9UDY4	DnaJ homolog subfamily B member 4	DNAJB4	37.8	3.781E+01	1.817	1.917
18	P60903	Protein S100-A10	S100A10	11.2	1.120E+01	1.801	1.011
19	P42765	3-ketoacyl-CoA thiolase, mitochondrial	ACAA2	41.9	4.192E+01	1.746	1.091
21	O60701	UDP-glucose 6-dehydrogenase	UGDH	55.0	5.502E+01	1.643	1.338
22	P20962	Parathymosin	PTMS	11.5	1.153E+01	1.579	1.097
23	P47755	F-actin-capping protein subunit alpha-2	CAPZA2	32.9	3.295E+01	1.567	1.096
24	B4DEY6	LIM and cysteine-rich domains protein 1	LMCD1	28.5	2.851E+01	1.529	1.997
25	O60568	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	PLOD3	84.8	8.478E+01	1.512	1.027
26	P61981	14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed	YWHAG	28.3	2.830E+01	1.343	1.450
27	O15344	Midline-1	MID1	75.3	7.525E+01	1.333	1.989
28	Q99805	Transmembrane 9 superfamily member 2	TM9SF2	75.8	7.578E+01	1.225	1.435
29	A8MWK3	Cadherin-2	CDH2	97.0	9.704E+01	1.216	2.574
30	P30043	Flavin reductase (NADPH)	BLVRB	22.1	2.212E+01	1.199	1.222
31	E7ETU9	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	PLOD2	81.2	8.117E+01	1.157	1.598
32	Q03135	Caveolin-1;Caveolin	CAV1	20.5	2.047E+01	1.099	1.937
33	P30520	Adenylosuccinate synthetase isozyme 2	ADSS	50.1	5.010E+01	-0.995	-1.542
34	P12109	Collagen alpha-1(VI) chain	COL6A1	108.5	1.085E+02	-1.006	-1.694
35	B8ZZQ6	Prothymosin alpha; Thymosin alpha-1	PTMA	11.8	1.176E+01	-1.077	-1.094
36	P21399	Cytoplasmic aconitate hydratase	ACO1	98.4	9.840E+01	-1.256	-2.563
37	P19367	Hexokinase-1;Putative hexokinase HKDC1	HK1	101.1	1.011E+02	-1.313	-1.232
38	P19338	Nucleolin	NCL	76.6	7.661E+01	-1.324	-1.321
39	Q9H2P0	Activity-dependent neuroprotector homeobox protein	ADNP	123.6	1.236E+02	-1.375	-1.431
40	P08473	Neprilysin	MME	85.5	8.551E+01	-1.381	-2.609
41	P17301	Integrin alpha-2	ITGA2	129.3	1.293E+02	-1.537	-3.835
42	C9JG97	Angio-associated migratory cell protein	AAMP	44.7	4.469E+01	-1.661	-1.146
43	P18031	Tyrosine-protein phosphatase non-receptor type 1;Tyrosine-protein phosphatase non-receptor type	PTPN1	50.0	4.997E+01	-1.757	-1.373
44	P40189	Interleukin-6 receptor subunit beta	IL6ST	103.5	1.035E+02	-1.785	-1.300
45	Q53EL6	Programmed cell death protein 4	PDCD4	50.6	5.058E+01	-1.790	-1.476
46	P05161	Ubiquitin-like protein ISG15	ISG15	17.9	1.789E+01	-1.830	-2.258
47	P05120	Plasminogen activator inhibitor 2	SERPIN B2	46.6	4.660E+01	-2.050	-5.542
48	P35610	Sterol O-acyltransferase 1	SOAT1	64.7	6.473E+01	-2.075	-1.096

49	P15924	Desmoplakin	DSP	260.1	2.601E+02	-2.342	-2.289
50	P55060	Exportin-2	CSE1L	107.8	1.078E+02	-2.395	-1.572
51	F6SKP1	Transmembrane glycoprotein NMB	GPNMB	51.1	5.113E+01	-2.576	-5.310
52	D3DR31	Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	51.7	5.171E+01	-2.633	-2.335
53	Q9Y2J2	Band 4.1-like protein 3	EPB41L3	96.5	9.651E+01	-2.930	-6.749
54	B9A047	Ankyrin repeat and SOCS box protein 1	ASB1	26.2	2.617E+01	-3.065	-2.236
55	P12277	Creatine kinase B-type	CKB	42.6	4.264E+01	-3.207	-1.468

Log₂ ratios are HS27a/HS5. MS data are consistent with gene array data.

Figure Legends

Figure 1: Schematic procedure for quantitative analysis of proteins and genes in HS5/ HS27a comparison.

Figure 2: MS analysis of stable isotope-labeled proteins (SILAC method).

(A), (B): Determination of incorporation efficiency by MALDI-TOF/TOF-MS. Peaks annotated as normal vs. R0 (left) and normal vs. R10 (right) are peptide GVVDSEDLPLNISR of HSP90 β from HS5 and HS27a cells, respectively.

(C), (D): Identification and quantification of proteome in HS5 and HS27a by 2D-HPLC LTQ Orbitrap MS. Peaks annotated as R0 and R10 (left) and K0 and K8 (right) are triply-charged peptide HLEINPDHPIVETLR of HSP90 β (P08238) and YGGPYHIGGSPFK of filamin-A (P21333), respectively.

Figure 3: Distributions of proteins identified in various experiments described in the text.

(A): Venn diagrams of numbers of identified proteins from two experiments.

(B), (C): Distribution of SILAC H/L ratios from two experiments.

(D): Ratios of HS27a/HS5 (H/L) for the set of 1462 proteins. Log₂ of the SILAC ratio and z-score for each protein (n= 2) reflect differences in relative expression between HS27a and HS5.

Figure 4: Functional classification of identified proteins using the SWISS-PROT database, based on universal GO annotation terms. Proteins shown were linked to at least one annotation term within the GO molecular function (A), biological process (B), and cellular component (C) categories.

Figure 5: Functional network analysis of differentially regulated proteins with z-score cutoff 95% observed in HS5 and HS27a using Ingenuity Pathway Analysis (IPA). Top network functions include cellular movement, cell-to-cell signaling and interaction, hematological system development and function (A); infectious diseases, post-translational modification, protein folding (B); connective tissue disorders, dermatological diseases and conditions, developmental disorders (C). Red icons: upregulation of protein in HS27a relative to HS5. Green icons: downregulation of protein in HS27a relative to HS5. Color intensity indicates degree of variation.

A

Gene symbol	Gene annotation	Gene array $\text{Log}_2(\text{HS27a}/\text{HS5})$	SILAC ratio $\text{Log}_2(\text{HS27a}/\text{HS5})$
HCLS1	Hematopoietic lineage cell-specific protein	1.685	2.979
COL1A2	Collagen alpha-2(I) chain	0.309	3.741
ALCAM	CD166 antigen	2.651	1.899
ITGAV	Integrin alpha-V	-0.459	1.510
MAP4	Microtubule-associated protein	-0.098	-1.076
HSP90	Heat shock protein 90-beta	-0.005	-0.364
CSE1L	Exportin-2	-1.572	-2.395

B

Figure 6: Validation of MS results by RT-PCR.

(A) Randomly selected genes in the two cell lines (see text).

(B) Gene expression for randomly selected proteins was analyzed by quantitative RT-PCR. Relative expression in comparison with control samples was analyzed by the $2^{-\Delta\Delta C_t}$ method and represented as $\log_2$. Expression of genes above $\log_2(2)$ or below $\log_2(1/2)$ was significantly upregulated or down-regulated, respectively.

(C) Protein expression of HCLS1, COL1A2, ALCAM, ITGAV, MAP4, HSP90, and CSE1L was analyzed by western blotting.

(D) Expression of ITGAV in HS27a cells transfected with scrambled siRNA (SCR) or ITGAV-specific siRNA (siITGAV1 and siITGAV2). ITGAV expression was determined by Western blot. Tubulin served as control.

(E) Early-stage apoptosis induced by $TNF\alpha$ (25 ng/mL) in KG1a cells in contact with or without HS27a or HS27a modified by transfection with a scrambled siRNA sequence (scr) or siRNA specific for ITGAV (siITGAV1 and siITGAV2). Apoptosis was determined by flow cytometry. Only $CD45^+$ (KG1a) cells were considered. Error bars indicate SEM.