Bone marrow mesenchymal stem cells (MSCs) can differentiate into different types of cells and have tremendous potential for cell therapy and tissue engineering. Transforming growth factor β1 (TGF-β) plays an important role in cell differentiation and vascular remodeling. We showed that TGF-β induced cell morphology change and an increase in actin fibers in MSCs. To determine the global effects of TGF-β on MSCs, we employed a proteomic strategy to analyze the effect of TGF-β on the human MSC proteome. By using two-dimensional gel electrophoresis and electrospray ionization coupled to quadrupole/time-of-flight tandem mass spectrometers, we have generated a proteome reference map of MSCs. We identified 30 proteins with an increase or decrease in expression or phosphorylation in response to TGF-β. The proteins regulated by TGF-β included cytoskeletal proteins, matrix synthesis proteins, membrane proteins, metabolic enzymes, etc. TGF-β increased the expression of smooth muscle α-actin and decreased the expression of gelsolin. Overexpression of gelsolin inhibited TGF-β-induced assembly of smooth muscle α-actin; on the other hand, knocking down gelsolin expression enhanced the assembly of α-actin and actin filaments without significantly affecting α-actin expression. These results suggest that TGF-β coordinates the increase of α-actin and the decrease of gelsolin to promote MSC differentiation. This study demonstrates that proteomic tools are valuable in studying stem cell differentiation and elucidating the underlying molecular mechanisms.
quadrupole tandem mass spectrometers (MS/MS). MS/MS spectra can be used to determine the peptide sequence with high specificity (18, 22).

In this study, we used ESI-MS/MS to identify proteins in two-dimensional gels. A preliminary two-dimensional reference map of MSCs was generated, and about 30 TGF-β-regulated proteins with changes at the expression level and/or post-translational modifications were identified. We showed that TGF-β coordinated the increase of SM α-actin and the decrease of gelsolin to promote the assembly of α-actin and actin filaments in MSCs. These results from proteomic profiling will not only provide insight into the global responses of MSCs to TGF-β stimulation but will also lead to in-depth studies on the mechanisms of proteomic changes in MSCs.

EXPERIMENTAL PROCEDURES

Cell Culture—Human bone marrow MSCs were obtained from Cambrex Corp. (Walkersville, MD). These MSCs had been well characterized by their surface markers and differentiation potential. They are positive for CD105, CD166, CD29, and CD44 but negative for CD34, CD14, and CD45. MSCs were cultured in MSCGM medium with pre-screened fetal bovine serum (Cambrex Corp.) to allow for cell proliferation without differentiation. The cells were maintained in humidified incubators at 37 °C with 5% CO₂. Cell culture products and other consumable laboratory supplies were purchased from Fisher and VWR International (Brisbane, CA). MSCs up to passage 10 were used in our experiments.

Flow Cytometry—to confirm that MSCs maintain their phenotype after expansion in culture, the cells were subjected to flow cytometry analysis. The cells were detached by trypsin treatment, followed by centrifugation and washing with PBS. After resuspension of the cells, the nonspecific binding sites were blocked by incubation with 1% bovine

| Gene name | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) |
|-----------|---------------------------|--------------------------|
| SM α-actin| ACCCTGCTACGGAGGC          | GTCTCAAACTATTTTGATCATTTTCTC |
| Gelsolin  | TGGACTTTCTGCTAAAGGGATCATTC | GGCCTAAAGCCTGCCCTCCTCAC |
| 18 S      | CSGAGCTAGGAAATAATGAATAGG   | CATGGGCTCAAGTTCGAAA      |

**Table 1**

**Primers used in qPCR**
Upon addition of lysis buffer, cells were immediately pipetted up and incubated with an antibody against gelsolin (from BD Biosciences), α-actin (polyclonal) and FLAG tag (polyclonal) were from Sigma. The antibody against HSP27 was from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada). CD44 antibody was from BIOSOURCE (Camarillo, CA). CD166 antibody was from Serotec (Raleigh, NC). CD29 antibody was from BIOSOURCE (Camarillo, CA).

**Chemicals and TGF-β Treatment**—Chemicals were purchased from Sigma unless otherwise specified. TGF-β1 (Sigma) at 10 ng/ml was used to treat MSCs. Our pilot experiments showed that TGF-β1 at 5 and 20 ng/ml induced similar levels of SM α-actin and collagen I expression in MSCs. For long term culture, TGF-β1 was supplemented when cultured medium was changed (every 2–3 days).

**Cell Staining and Microscopy**—The phase contrast images of MSC morphology were collected by using a Nikon inverted microscope (TE300) with 10× objective and a Hamamatsu Orca100 cooled digital CCD camera. The images were transferred directly from a frame grabber to the computer storage using C-Imageing System software (Compix Inc., Cranberry Township, PA).

Immunostaining and confocal microscopy were used to determine the subcellular distribution and organization of the proteins. MSCs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 10 min. For immunostaining, the specimens were incubated with the primary antibody against gelsolin (from BD Biosciences), α-actin, or FLAG tag (Sigma) for 2 h and with FITC- or rhodamine-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h. To stain F-actin filaments, the specimen was incubated with rhodamine-phaloidin for 30 min, followed by confocal microscopy. The images of the specimen were collected as Z series sections with a Leica TSL confocal microscopy system equipped with argon and He/Ne laser sources, a scanner, and a Leica DM IBZ microscope. Multiple sections (0.3 μm thick for each section) were projected onto one plane for presentation.

**Immunoblotting Analysis of Proteins**—To prepare cell lysates for SDS-PAGE, the cells were lysed in a lysis buffer containing 25 mM Tris, pH 7.4, 0.5 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 mM Na2VO4. The lysates were centrifuged at 12,000 rpm by using a microcentrifuge, and the protein concentration of the supernatants was measured by using a DC protein assay (Bio-Rad). The proteins were run in SDS-PAGE and transferred to a nitrocellulose membrane, which was blocked with 3% nonfat milk and incubated with the primary antibody in TTBS buffer (25 mM Tris-HCl, pH 7.4, 80 mM NaCl, and 0.05% Tween 20) containing 0.1% bovine serum albumin. The bound primary antibodies were detected by using a goat anti-mouse or a goat anti-rabbit IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnologies, Santa Cruz, CA) and the ECL detection system (Amersham Biosciences). The immunoblotting results were scanned with a Hewlett Packard high resolution scanner, and the intensity of protein bands were quantified by using NIH Image software.

The monoclonal antibody against gelsolin was from BD Biosciences. The antibodies against actin (including all isoforms) and tubulin were from Santa Cruz Biotechnologies. The antibodies against α-actin (monoclonal) and FLAG tag (polyclonal) were from Sigma. The antibody against HSP27 was from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada).

**Two-dimensional Gel Electrophoresis**—Cells were washed three times using ice-cold PBS buffer and centrifuged down to 3000 rpm for 5 min. Residual PBS buffer on top was removed by careful pipetting. Cells were then disrupted with lysis buffer, which is a mixture of 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris (Tris base), and 20 mM DTT. Normally 1 ml of lysis buffer was used for 1–2×10^6 cells. Upon addition of lysis buffer, cells were immediately pipetted up and down several times to mix well. Samples were allowed to stand at room temperature for about 1 h and vortexed occasionally. They were transferred to Beckman thick wall tubes (362305) and centrifuged at 66,000 rpm (100,000 g) in a Beckman TLA100.4 rotor for 30 min at 20 °C. Supernatant were aliquoted into siliconized tubes (PGC Scientifics, Frederick, MD) and stored at −80 °C. Modified Bradford assay (Bio-Rad) was used to quantify the total protein amount in the cell lysates.

The first-dimension IEF was performed by using an Ettan IPGphor unit (Amersham Biosciences) with a power supply EIPS 3501XL. Pre-cast 18-cm, pH 3–10, NL IPG strips were obtained from Amersham Biosciences. 100 μg of lysate mixtures in triplicate were supplemented with rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, trace of bromophenol blue, 20 mM DTT, and 0.5% corresponding IPG buffer) to a final volume of 350 μl. IPG strips were then rehydrated with the sample mixture in a strip holder for 24 h. IEF was carried out in three steps under step-n-hold mode as follows: (i) 500 V, 1.0 h; (ii) 1000 V, 3 h; and (iii) 8000 V, 8 h. The total voltage-hour applied was 67,000. The second-dimension SDS-PAGE was carried out in an Ettan DALTsix system (Amersham Biosciences). IPG strips were equilibrated in two consecutive steps: (i) 30 min in 10 mg/ml of DTT, and (ii) 30 min in 25 mg/ml iodoacetamide (Sigma), both dissolved in SDS equilibration buffer (50 mM Tris base, 6 mM urea, 30% glycerol (v/v), 2% SDS (w/v), and trace bromophenol blue). 1-mm-thick 10% polyacrylamide gels with a dimension of 27.5 × 21 cm were cast with 30% Duracryl, 0.65% Bis (Genomic Solutions, Ann Arbor, MI), 10% SDS, 10% ammonium per-sulfate, and 0.375% Tris buffer at pH 8.8. IPG strips were sealed on the top of gels with 0.5% SeaKem LE-agarose (Cambrex Corp.). SDS-PAGE was performed at a constant voltage of 100 V at 10 °C and stopped once the bromophenol blue front disappeared from the gel.

**Silver Staining and Image Analysis**—Proteins on gels were visualized by using silver staining performed with minor modifications to published procedures (27). Briefly, gels were fixed in 50% methanol, 5% acetic acid for at least 2 h followed by a 20-min washing in 50% methanol. Gels were washed twice with ddH2O for 15 min, treated with 0.02% Na2S2O3 for 3 min, and rinsed twice with ddH2O for 1 min before incubation in 0.1% silver nitrate for 30 min. After silver staining the gels were rinsed twice with ddH2O for 2 min and shaken vigorously in developer containing 0.04% formalin (37% formaldehyde in water) and 2% Na2CO3. After 30 s, the developer was discarded, and gels were shaken in fresh developer until the desired intensity was attained (∼3 min). Incubation in 5% acetic acid for 5 min terminated development after which gels were rinsed three times with ddH2O for 2 min prior to imaging. For long term storage, gels were incubated with 1% acetic acid at 4 °C.

Stained gels were imaged with an Umax PowerLook 1100 scanner (Umax Technologies, Dallas, TX) with a defined scan resolution of 250 dpi in the transmissive and gray blue mode. Protein expression with and without TGF-β treatment was compared using Z3 3.0 software.
All gel images were cropped to the same dimensions and auto-contrasted in Photoshop 7.0 prior to image analysis. Multiple gel analysis wizard was applied to compare the two groups of three gels each. Spot detection and matching were achieved initially automatically and were fine-tuned by manual registration. Spurious spots were excluded by manual annotation. To define spots with differential expression, the settings used are as follows: spot contrast of 8, minimum confidence level of 0.95, and minimum spot area (pixels) of 50. Protein spots that were determined to be differentially expressed (n-fold more than 2.0 or less than 0.5) using the automatic analyses were verified manually by local pattern comparison to exclude artifacts.

In-gel Tryptic Digestion and Peptide Extraction—To identify differentially expressed proteins, the spots were excised from the gels manually and digested with trypsin. Gel spots were diced into small pieces (1 mm²) and placed into 0.65-ml siliconized tubes. 100 μl (or enough to cover) of 50 mM NH₄HCO₃, 50% acetonitrile was added, and the tube was vortexed for 10 min. After a brief spin, the supernatant was discarded by pipetting to reduce trypsin autolysis. Finally 30 μl of 25 mM NH₄HCO₃ was gently overlaid on top, and the tubes were incubated at 37 °C overnight (16–20 h).

To extract the peptides from the gel pieces, 30 μl of ddH₂O was added, and the tube was vortexed for 10 min followed by sonication in a water bath for 5 min. The aqueous portion was transferred to a clean siliconized tube. Peptides were further extracted twice with 30 μl of 50% acetonitrile, 5% formic acid, and supernatants were combined. The total volume was reduced to 5 μl by using the SpeedVac. The resultant samples were then subjected to Q-TOF mass spectrometry directly or stored at −20 °C freezer for future analysis.

Protein Identification by LC-MS/MS—A hybrid quadrupole/orthogonal time-of-flight mass spectrometer, Q-TOF API US (Waters) interfaced with a capillary liquid chromatography system (Waters), was used to carry out LC-MS/MS analysis. 1–2 μl of samples were injected through an auto-sampler into the LC system at the flow rate of 20 μl/min and pre-concentrated on a 300-μm × 5-mm PepMap C18 pre-column (Dionex, CA). The peptides were then eluted onto a 75-μm × 15-cm PepMap C18 analytical column. The column was equilibrated with solution A (3% acetonitrile, 97% water, 0.1% formic acid), and the
peptide separation was achieved with a solution gradient from 3 to 40% of solution B (95% acetonitrile, 5% water, 0.1% formic acid) over 35 min at a flow rate of 250 nL/min. This flow rate through the column was reduced from 8 µL/min from pumps A and B by flow splitting. The LC eluent was directed to the electrospray source with a PicoTip emitter (New Objectives, Woburn, MA). The mass spectrometer was operated in positive ion mode with a source temperature of 100 °C and a cone voltage of 40 V. A voltage of 2 kV was applied to the PicoTip. TOF operated in positive ion mode with a source temperature of 100 °C and a cone voltage of 40 V. A voltage of 2 kV was applied to the PicoTip. TOF analyzer was set in the V-mode. The instrument was calibrated with a multipoint calibration by using selected fragment ions from the collision-induced decomposition of Glu-fibrinopeptide B. MS/MS spectra were obtained in a data-dependent acquisition mode in which the three multipletached (±2, ±3, and ±4) peaks with the highest intensity in each MS scan were chosen for collision-induced decomposition. Collision energies were set at 10 and 30 V, respectively, during the MS and MS/MS scans.

Mass spectra were processed by using MassLynx 4.0 software, and proteins were identified by using Protein Global Server 1.02.0 software. The protein identities were further confirmed by Mascot (www.matrixscience.com) by using the MS/MS peak lists exported from MassLynx. The nonredundant data bases in the molecular mass range of 1,000–50,000 Da and pI values between 3.0 and 10.0 were used at the NCBI website. Modifications considered included carboxyamidomethylation of cysteine, amino-terminal acetylation, amino-terminal Gln to pyro-Glu, oxidation of methionine, and phosphorylation of serine, threonine, and tyrosine.

**RNA Isolation and Quantitative PCR (qPCR)—**Cells in each well in 6-well plates were lysed with 0.5 mL of RNA Stat 60 (Tel-Test Inc, Friendswood, TX). RNA was extracted using chloroform and phenol extraction steps. Samples were centrifuged between each of these steps for 15 min at 4 °C at 12,000 rpm. Isopropyl alcohol was added to precipitate the RNA, and the samples were centrifuged for 30 min under the same conditions. 75% ethanol was added to wash the RNA pellet and centrifuged at 7500 rpm for 5 min. The pellet was resuspended in 20 µL of diethyl pyrocarbonate-treated water and quantified by using a RiboGreen® RNA quantification assay (Molecular Probes Inc, Eugene, OR).

Two-step reverse transcription (RT)-PCR was performed by using the ThermoScript RT-PCR system for first-strand cDNA synthesis (Invitrogen). The cDNA was made from equal amounts of total RNA from each sample, and qPCR was performed by using SYBR green kits and the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) (28). Primers for SM α-actin, gelsolin, and 18 S were designed by using the ABI Prism Primer Express™ software version 2.0 (Applied Biosystems). Complete genomic sequences and the mRNA sequence were downloaded from the NCBI website (www.ncbi.nlm.nih.gov/LocusLink) to identify the intron-exon junctions. The primers that span an exon-exon junction were used to ensure the specific amplification of cDNA. The sequences of the designed primers were used to BLAST against nucleotide sequences in the NCBI data base (www.ncbi.nlm.nih.gov/BLAST) to make sure that the primer sequences were unique. The primers used in this study are listed in Table II.

After each experiment, the melting temperature and the dissociation curve of PCR products were obtained to confirm the product specificity. The amount of RNA for each gene was normalized with the amount of 18 S RNA in the same sample.

**Transfection of DNA Plasmids and Small Interfering RNA (siRNA)—**PCR was used to engineer the FLAG-tagged gelsolin construct by inserting the gelsolin cDNA beginning with the second codon immediately following the FLAG nucleotide sequence (GACTACAAGGTAGCATGACCAAG) in the pCMV5b vector.

MSCs were seeded in serum-free medium, and the DNA plasmids (2 µg for 10-cm² culture area) were transfected into MSCs by using the LipofectAMINE PLUS reagent (Invitrogen). After incubation with the mixture of plasmids and LipofectAMINE reagents for 4 h, the cells were cultured for 1 day and treated with TGF-β or kept as control. The expression efficiency of DNA plasmids was about 20%. The siRNA for gelsolin was from Dharmaco Inc. (Lafayette, CO). The FITC-conjugated control siRNA was from Cell Signaling Technology, Inc. (Beverly, MA). The siRNAs (100 nM for 10-cm² culture area) were transfected into MSCs by using LipofectAMINE 2000. The transfection efficiency of siRNA was more than 90%.

**RESULTS**

**Characterization of MSCs—**We used MSCs up to passage 10 in our experiments. To confirm that expanded MSCs maintain their phenotype, MSCs were stained for a set of cell surface markers. As shown in Fig. 1, MSCs at passage 10 were positive for CD105, CD166, CD29, and CD44 but were negative for CD14, CD45, and CD34, suggesting that expanded MSCs maintain their phenotype. To further prove that expanded MSCs had pluripotent differentiation potential, MSCs at pas-
MSCs were either kept as no treatment control or treated with TGF-β for 4 days. The protein lysates were subjected to two-dimensional electrophoresis, followed by silver staining and image analysis. Results were quantified from three sets of two-dimensional electrophoresis. The spots of interest were excised from the gels and digested with trypsin. The resulting peptides were used for LC-MS/MS analysis, and the proteins were identified by searching the databases by using peptide sequences.

### Proteins identified in two-dimensional electrophoresis gel

| Spot no. | TGF-β-induced change | Protein name | Swiss Prot accession no. | Molecular mass kDa (theoretical) | pI (theoretical) |
|----------|----------------------|--------------|--------------------------|---------------------------------|-----------------|
| 1394     | 0.3                  | Gelsolin     | P06396                   | 85.7                            | 6.2             |
| 1144     | 0.1                  | T-plastin (isoform) | P13797                   | 70.4                            | 5.50            |
| 1098     | 0.3                  | T-plastin    | P13797                   | 70.4                            | 5.50            |
| 1324     | 1.0                  | γ-Actin      | P02571                   | 41.8                            | 5.31            |
| 1032     | 1.1                  | α-Cardiac actin | P04270                   | 42                              | 5.23            |
| 1050     | 0.3                  | Annexin A6  | P08133                   | 75.7                            | 5.42            |
| 2198     | 2.0                  | Annexin A2 (isoform) | P07355                   | 38.5                            | 7.56            |
| 2034     | 5.2                  | Annexin A2  | P07355                   | 38.5                            | 7.56            |

**Proteins involved in matrix synthesis**

- Collagen-binding protein 2 (precursor)
- Collagen-binding protein 2 (isoform)
- Unique Collagen-binding protein 2 (precursor) (isoform)
- 47-kDa heat shock protein (precursor) (isoform)

**Metabolic Enzymes**

- Glyceraldehyde-3-phosphate dehydrogenase, liver
- Thioredoxin reductase
- Malate dehydrogenase, cytoplasmic
- Tyrosyl-tRNA synthetase
- UDP-glucose-6-dehydrogenase
- Glucose-6-phosphate 1-dehydrogenase
- Pyruvate kinase, M2 isoform
- Aldehyde dehydrogenase X, mitochondrial (precursor)
- Transaldolase

**Protein synthesis and degradation**

- T-complex protein 1, ε subunit
- Ubiquitin carboxyl-terminal hydrolase isozyme L1
- Proteasome subunit α type 2

**Others**

- Heat shock 27-kDa protein (phosphorylated at Ser-82)
- Heat shock 72-kDa protein
- Programmed cell death 6 interacting protein
- Septin 6
- Peroxiredoxin 2
- Chloride intracellular channel protein 4
- Nicotinamide N-methyltransferase
- Vimentin (fragment)

### Note

- For spot 1468, the digested peptides from the spot were present in both collagen-binding protein 2 and HSP47 (these two proteins have 80% homologous sequences). This spot was only detected in TGF-β/H9251.

### Table II

| Spot no. | Fold change | Protein name |
|----------|-------------|--------------|
| 1176     | 0.1         | Gelsolin     |
| 1776     | 0.3         | Thioredoxin reductase |
| 1540     | 0.4         | Malate dehydrogenase, cytoplasmic |
| 1116     | 0.5         | Tyrosyl-tRNA synthetase |
| 1106     | 2.3         | Glucose-6-phosphate 1-dehydrogenase |
| 1506     | 3.9         | Pyruvate kinase, M2 isoform |
| 1680     | 4.8         | Aldehyde dehydrogenase X, mitochondrial (precursor) |
| 2114     | 4.9         | Transaldolase |

**Table II: Proteins identified in two-dimensional electrophoresis gel**

**Proteins identified in two-dimensional electrophoresis gel**

MSCs were either kept as no treatment control or treated with TGF-β for 4 days. The protein lysates were subjected to two-dimensional electrophoresis, followed by silver staining and image analysis. Results were quantified from three sets of two-dimensional electrophoresis. The spots of interest were excised from the gels and digested with trypsin. The resulting peptides were used for LC-MS/MS analysis, and the proteins were identified by searching the databases by using peptide sequences.

| Spot no. | TGF-β-induced change | Protein name | Swiss Prot accession no. | Molecular mass kDa (theoretical) | pI (theoretical) |
|----------|----------------------|--------------|--------------------------|---------------------------------|-----------------|
| 1394     | 0.3                  | Gelsolin     | P06396                   | 85.7                            | 6.2             |
| 1144     | 0.1                  | T-plastin (isoform) | P13797                   | 70.4                            | 5.50            |
| 1098     | 0.3                  | T-plastin    | P13797                   | 70.4                            | 5.50            |
| 1324     | 1.0                  | γ-Actin      | P02571                   | 41.8                            | 5.31            |
| 1032     | 1.1                  | α-Cardiac actin | P04270                   | 42                              | 5.23            |
| 1050     | 0.3                  | Annexin A6  | P08133                   | 75.7                            | 5.42            |
| 2198     | 2.0                  | Annexin A2 (isoform) | P07355                   | 38.5                            | 7.56            |
| 2034     | 5.2                  | Annexin A2  | P07355                   | 38.5                            | 7.56            |

**Proteins involved in matrix synthesis**

- Collagen-binding protein 2 (precursor)
- Collagen-binding protein 2 (isoform)
- Unique Collagen-binding protein 2 (precursor) (isoform)
- 47-kDa heat shock protein (precursor) (isoform)

**Metabolic Enzymes**

- Glyceraldehyde-3-phosphate dehydrogenase, liver
- Thioredoxin reductase
- Malate dehydrogenase, cytoplasmic
- Tyrosyl-tRNA synthetase
- UDP-glucose-6-dehydrogenase
- Glucose-6-phosphate 1-dehydrogenase
- Pyruvate kinase, M2 isoform
- Aldehyde dehydrogenase X, mitochondrial (precursor)
- Transaldolase

**Protein synthesis and degradation**

- T-complex protein 1, ε subunit
- Ubiquitin carboxyl-terminal hydrolase isozyme L1
- Proteasome subunit α type 2

**Others**

- Heat shock 27-kDa protein (phosphorylated at Ser-82)
- Heat shock 72-kDa protein
- Programmed cell death 6 interacting protein
- Septin 6
- Peroxiredoxin 2
- Chloride intracellular channel protein 4
- Nicotinamide N-methyltransferase
- Vimentin (fragment)

**Note:** For spot 1468, the digested peptides from the spot were present in both collagen-binding protein 2 and HSP47 (these two proteins have 80% homologous sequences). This spot was only detected in TGF-β/H9251.

---

**Induced Morphological Changes, Increased Actin Filaments, and Increased SM α-Actin Expression**—Long term treatment of MSCs with TGF-β significantly changed the cell morphology. As shown in Fig. 2, 2 days after TGF-β treatment, MSCs have a more spread out and myoblast-like morphology, and intracellular fibrous structures were visible (indicated by arrows in Fig. 2B). This cell morphology was maintained as the cells grew and reached confluence after 6 days (Fig. 2F).

To determine whether the intracellular fibrous structure was actin cytoskeleton, MSCs were stained on actin filaments. Indeed, MSCs treated by TGF-β for 4 days showed more actin filaments and thick fibers (Fig. 3). To determine whether TGF-β regulated the amount of actin, immunoblotting analysis was performed. As shown in Fig. 3C, TGF-β specifically increased α-actin expression without significantly affecting total actin amount. Tubulin expression was used as an internal control to show equal loading of the protein samples. At the transcription level, TGF-β increased the gene expression of SM α-actin and SM-22α (data not shown). These results suggest that TGF-β may promote the expression of SM contractile markers in MSCs.

**TGF-β Induced Proteome Changes in MSCs**—Although the effects of TGF-β on various cell types have been widely studied, the effect of TGF-β on MSCs has not been investigated comprehensively. We used a proteomic approach to profile the TGF-β-induced protein expression and modifications. The proteins in the cell lysates were separated by two-dimensional electrophoresis, followed by silver staining. A representative two-dimensional gel image of protein lysates from MSCs without TGF-β treatment is shown in Fig. 4. About 1500 protein spots were resolved and identified with high confidence (>95%). Overall, around 60 protein spots were found consistently up- or down-regulated by over 2-fold in triplicate experiments after TGF-β treatment for 4 days. We have made an initial effort to identify around 30 protein spots, encompassing a wide range of molecular weights, pI values, fold changes, and abundance. All 30 protein spots were identified successfully.
with high confidence by using in-gel trypsin digestion followed by tandem mass spectrometry as described under “Experimental Procedures.” The location of each spot is labeled with a number and an arrow indicating upward or downward regulation by TGF-β. The predicted molecular weights and isoelectric points of unmodified proteins using the Z3 program agree well with their theoretical values (±10%).

Proteins identified so far are listed in Table II and are grouped according to their primary functions. For all the proteins identified, two search engines (ProteinLynx and Mascot) gave the same protein hits with high confident scores, and at least two peptides sequenced with good MS/MS spectra. The majority of these spots has an n-fold value either larger than 2 or smaller than 0.5, defined previously as differentially expressed. Two actin spots 1324 and 1032 and one heat shock protein 27 (HSP27) spot 1018 were also identified as reference spots. For spots 1116 and 1030, more than one protein was identified without ambiguity, suggesting co-migration of these proteins. These TGF-β-regulated proteins are involved in a variety of cellular processes. They include cytoskeleton proteins (i.e., gelsolin and T-plastin), cell membrane proteins (i.e., annexin A2), proteins involved in matrix synthesis (i.e., collagen-binding protein 2 or CBP2), metabolic enzymes (i.e., thiorodoxin reductase), protein synthesis, and degradation (i.e., T-complex protein 1 and proteasome subunit), and stress response proteins (i.e., HSP27), etc. In addition, TGF-β not only modulates protein expression levels but also post-translational modifications, e.g., HSP27 phosphorylation at Ser-82 (data not shown). The TGF-β-induced change in SM α-actin and gelsolin expression were further investigated as described below.

**TGF-β Coordinated the Increase of SM α-Actin and the Decrease of Gelsolin in MSCs—**As shown in Fig. 5A, spot 1394 had a lower level after TGF-β treatment. This protein was identified as gelsolin, an actin-severing protein. Gelsolin has been shown to regulate actin structure, cell motility, and apoptosis (29, 30), but the role of gelsolin in cell differentiation is not clear. To determine the time course of gelsolin expression in response to TGF-β, immunoblotting analysis was performed. As in Fig. 5B, TGF-β significantly decreased gelsolin expression after 4 and 6 days.

To determine whether TGF-β regulated the expression of gelsolin and α-actin at the transcription level, we examined the gene expression at the earlier time points with qPCR (Fig. 6). After 24 h of TGF-β treatment, the gene expression of gelsolin decreased by 50%, whereas α-actin expression increased by 4-fold. After 48 h, the gene expression of gelsolin and α-actin showed the same trend as that at 24 h, suggesting the TGF-β-induced protein expression change is sustained. These results indicate that TGF-β coordinated the increase of SM α-actin expression and the decrease of gelsolin expression at the transcriptional level.

To determine the spatial relationship between gelsolin and actin filaments in MSCs, cells with or without TGF-β treatment for 4 days were double-stained for gelsolin and actin filaments (Fig. 7). Gelsolin mostly co-localized with actin filaments except for a weak background in the cytoplasm. TGF-β decreased gelsolin staining and the co-localization of gelsolin with actin cytoskeleton and increased actin filaments in MSCs. In local areas where the gelsolin level was low (indicated by arrows in Fig. 7B), more actin filaments were assembled (indicated by arrows in Fig. 7D), suggesting that the decrease of gelsolin is correlated with the increased actin filament assembly. Double staining for gelsolin and SM α-actin showed the same results (data not shown).

**The Decrease of Gelsolin Enhanced the Assembly of α-Actin and Actin Filaments but Did Not Affect the Expression of α-Actin—**To determine directly whether the increase of gelsolin expression would inhibit TGF-β-induced α-actin assembly into actin filaments, we overexpressed the FLAG-tagged gelsolin in MSCs, and we treated the cells with TGF-β for 2 days. As shown in Fig. 8, overexpression of gelsolin (Fig. 8, A and B) significantly decreased the incorporation of SM α-actin into actin filaments in comparison with nontransfected cells in the same field (Fig. 8, C and D, respectively). TGF-β increased actin filaments containing α-actin in nontransfected cells, but this increase was blocked in cells overexpressing gelsolin (Fig. 8D). These results suggest that the decrease of gelsolin is required for TGF-β-induced α-actin assembly into filaments.

Because actin polymerization had been shown to increase SM α-actin expression (31), we determined whether the decrease of gelsolin expression would increase actin polymerization thus enhancing the expression of SM α-actin. To test this possibility, gelsolin siRNA was used to knock down the expression level of gelsolin in MSCs to mimic the decrease of gelsolin expression by TGF-β. The transfection efficiency of siRNA in MSCs was more than 90% (Fig. 9A), which allowed us to ana-
lyze the gene and protein expression in the whole cell population. As shown in Fig. 9B, transfection of control siRNA did not affect gelsolin levels, whereas gelsolin siRNA suppressed gelsolin mRNA levels by more than 80% after 1 day. Because the gelsolin protein level was related to many factors such as protein half-life and degradation rate, the protein level of gelsolin did not show a significant change within 2 days but was decreased dramatically by gelsolin siRNA transfection after 4 and 6 days. However, based on immunoblotting, there was no significant change of $\alpha$-H9251-actin protein level at any time point. These results suggest that the decrease of gelsolin expression does not significantly regulate $\alpha$-H9251-actin expression.

To determine whether the decrease of gelsolin expression was sufficient to enhance $\alpha$-actin assembly into actin filaments, MSCs were transfected with gelsolin siRNA and stained on F-actin and $\alpha$-actin. As shown in Fig. 10, knocking down gelsolin increased actin filaments (Fig. 10, A and B), and enhanced $\alpha$-actin assembly into filaments (Fig. 10, C and D).

**DISCUSSION**

A major challenge in the post-genomic era is to decipher the temporal and spatial functions and interactions of proteins in a cell. Although still in its developmental stage, proteomic profiling is poised to play an essential role in this endeavor. Proteome-wide screening may identify unique markers and elucidate interconnections between different cellular signaling pathways. In this study, we have profiled for the first time the global protein expression in human bone marrow MSCs upon TGF-β stimulation. In combination with more traditional biochemical/biophysical methods such as Western blotting and microscopy, we have gained important insights into the mechanisms of TGF-β-regulation of MSCs. By using a proteomic approach, we have generated the first two-dimensional reference map for MSCs. This two-dimensional reference map of MSCs will facilitate future studies on MSC functions and differentiation in response to various environmental factors. Based on this map, higher resolution proteome maps of MSCs with pre-fractionated cell lysates and zoom-in pI range two-dimensional gels can be generated. The information obtained from proteomic profiling will help us to elucidate connections between broad cellular pathways/molecules that were neither apparent nor predictable through traditional biochemical analysis in the past.
Proteomic Profiling of MSCs upon TGF-β Stimulation

We showed that TGF-β induced a sustained increase of SM α-actin expression in MSCs (Fig. 3). This is consistent with the role of TGF-β in angiogenesis and vasculogenesis. It has been shown that TGF-β can enhance SMC differentiation and the recruitment of SMCs to the newly formed blood vessels (32). However, TGF-β induces chondrogenic differentiation of MSCs in the presence of dexamethasone or three-dimensional cell aggregates (16, 17). These results suggest that TGF-β-induced responses in MSCs are context-dependent, e.g. dependent on cell-cell adhesion, other chemical factors, and mechanical factors in the microenvironment. There is evidence that TGF-β-mediated signaling pathways can cross-talk with mechanical force-induced signaling and gene expression in vascular cells (33, 34). For example, for SMCs cultured in a collagen scaffold, TGF-β stimulates the expression of SM α-actin, which is further enhanced by mechanical strain (33). It is possible that mechanical strain and TGF-β may collaborate to induce MSC differentiation into mature SMCs.

A significant finding from this study is that TGF-β coordinates the expression of gelsolin and α-actin to promote the differentiation of MSCs (Figs. 3 and 5–7) and that the decrease of gelsolin is necessary and sufficient for the assembly of α-actin and actin filaments induced by TGF-β (Figs. 8–10). Gelsolin, a protein originally identified as an actin-severing protein, has been shown to regulate actin structure, cell motility, and apoptosis (29, 30). To our knowledge, this is the first report on the regulation of gelsolin expression by TGF-β. Whether this mechanism is MSC-specific and whether the gelsolin pathway synergizes with other muscle-specific pathways remain to be determined. It is interesting that the decrease of gelsolin in MSCs enhanced the assembly of SM α-actin into actin filaments at the post-translational level (Figs. 8 and 10) but did not affect the protein expression of α-actin (Fig. 9). In SMCs, TGF-β increases α-actin gene expression through a TCE element by decreasing KLF4 expression and increasing KLF5 expression (35–37). In addition, TGF-β enhances serum-response factor (SRF) expression/activity and SM marker expression through a CArG element (35, 38). Actin polymerization has been shown to increase SRF activity and thus α-actin expression (31). In our system, the down-regulation of gelsolin expression by TGF-β increases actin filament assembly (Figs. 3 and 7) and that the decrease of gelsolin did not significantly affect α-actin expression. One explanation is that the basal level of actin polymerization was sufficient to maintain SRF activity and that the further increase of actin polymerization by decreasing gelsolin would not enhance SRF activity. Alternatively, other factors such as KLFs could play important roles in α-actin expression in MSCs.

Most interestingly, TGF-β also regulates other molecules involved in actin organization. HSP27 has multiple phosphorylated isoforms (39) and mediates actin polymerization downstream of the p38 mitogen-activated protein kinase pathway (40). Our results indicate a decrease of HSP27 phosphorylation at Ser-82 after TGF-β stimulation (Table II). TGF-β also de-

Fig. 9. Effect of knocking down gelsolin on α-actin expression. MSCs were transfected with gelsolin siRNA or control siRNA (conjugated with FITC) and used for microscopy, gene expression analysis, and immunoblotting analysis. A, to demonstrate the transfection efficiency, 1 day after transfection with control siRNA, each field was subjected to phase contrast and fluorescence microscopy, as shown by the representative field. Bar indicates 100 μm. B, 1 day after transfection, the samples were lysed for qPCR analysis of gelsolin gene expression. The expression of gelsolin in each sample was normalized with the respective 18 S RNA level and normalized with the gelsolin expression in no treatment control. *Bar graphs are mean ± S.D. from three experiments. For statistical analysis, the data was log-transformed, and a one-sample t test was performed. The asterisks indicate significant difference when compared with no treatment controls (p < 0.05). C, immunoblotting analysis of gelsolin and α-actin protein expression after knocking down gelsolin.

Fig. 10. Effect of knocking down gelsolin on actin filament assembly. MSCs were transfected with control siRNA (A and C) or gelsolin siRNA (B and D). Four days after transfection, cells were fixed and stained for F-actin (A and B) or α-actin (C and D), followed by confocal microscopy. Bar indicates 50 μm.
creased T-plastin (fimbrin) expression in MSCs (Table II). T-plastin is normally found in epithelial and mesenchymal cells and is an actin-bundling protein regulating microvilli actin filaments (41–43). The functional consequence of HSP27 dephosphorylation and T-plastin down-regulation needs further investigation.

It needs to be pointed out that some proteome changes induced by TGF-β in MSCs may be cell type-specific, whereas some may be ubiquitous (e.g. CBP2). The distinction between these two cases needs further investigation. The proteomic profiling allowed us to identify many novel targets and effects of TGF-β-induced signaling. The data derived from this study will lead to more focused and in-depth research on the effects of TGF-β on cellular functions and MSC differentiation. The information obtained from this study will not only have significant impact on stem cell biology but also have profound implications in stem cell therapy and tissue regeneration. MSCs have tremendous potential as a cell source for cell transplantation and tissue engineering. The knowledge of MSC responses to environmental factors such as TGF-β will help us to understand MSC differentiation in vivo, e.g. participation of tissue regeneration in ischemic heart after transplantation, and will provide a rational basis for stem cell engineering, e.g. to optimize in vitro culture conditions to expand MSCs and control MSC differentiation for tissue engineering applications.

Acknowledgment—The gelsolin cDNA was a generous gift from Dr. Alan Lader in the Hematology Division at Brigham and Women’s Hospital in Boston. D. W. acknowledges the technical support of Dr. Priscilla K. Cooper.

REFERENCES
1. Caplan, A. I., and Bruder, S. P. (2001) Trends Mol. Med. 7, 259–264
2. Waktiani, S., Saite, T., and Caplan, A. I. (1995) Muscle Nerve 18, 1417–1426
3. Ferrari, G., Cusella-De Angelis, G., Coletta, M., Pausucci, E., Stornaiuolo, A., Cost, G., and Mavilio, F. (1998) Science 279, 1528–1530
4. Jiang, Y., Jahagirdar, B. N., Schwartz, R. E., Keene, C. D., Aldrich, S., Lisberg, A., Low, W. C., Largaespada, D. A., and Verfaillie, C. M. (2002) Science 298, 66–72
5. Green, D. J. (1997) Science 276, 71–74
6. Gojo, S., Gojo, N., Takeda, Y., Mori, T., Abe, H., Kyo, S., Hata, J., and Umezawa, A. (2003) Exp. Cell Res. 288, 51–59
7. Mangi, A. A., Noieux, K., Kong, D., He, H., Rezvani, M., Ingwall, J. S., and Deau, V. J. (2003) Nat. Med. 9, 1195–1201
8. Topper, J. N. (2000) Trends Cardiovasc. Med. 10, 132–137
9. Massague, J., and Wotton, D. (2000) EMBO J. 19, 1745–1754
10. Mountaksas, A., Souchelnytskyi, S., and Holdin, C. H. (2001) J. Cell Sci. 114, 4359–4369
11. Roberts, A. B. (1998) Miner. Electrolyte Metab. 24, 111–119
12. Derynck, R., and Zhang, Y. E. (2003) Nature 425, 577–584
13. Kinner, B., Zaleskas, J. M., and Spector, M. (2002) Exp. Cell Res. 278, 72–83
14. Johnstone, B., Hering, T. M., Caplan, A. I., Goldberg, V. M., and Yoo, J. U. (1998) Exp. Cell Res. 238, 265–272
15. Gojo, S., Gojo, N., Takeda, Y., Mori, T., Abe, H., Kyo, S., Hata, J., and Umezawa, A. (2003) Exp. Cell Res. 288, 51–59
16. Johnstone, B., Hering, T. M., Caplan, A. I., Goldberg, V. M., and Yoo, J. U. (1998) Exp. Cell Res. 238, 265–272
17. Mackay, A. M., Beck, S. C., Murphy, J. M., Barry, F. P., Chichester, C. O., and Pittenger, M. F. (1998) Tissue Eng. 4, 415–428
18. Patterson, S. D., and Aebersold, R. H. (2003) Nat. Genet. 33, (suppl.) 311–323
19. Blackstock, W. P., and Weir, M. P. (1999) Trends Biotechnol. 17, 121–127
20. Mo, W., and Karger, B. L. (2002) Curr. Opin. Chem. Biol. 6, 666–675
21. Rabilloud, T. (2002) Proteomics 2, 3–10
22. MacCoss, M. J., and Yates, J. R., III (2002) Curr. Opin. Clin. Nutr. Metab. Care 4, 369–375
23. Yarmush, M. L., and Jayaraman, A. (2002) Annu. Rev. Biomed. Eng. 4, 349–373
24. Srivivas, P. R., Verma, M., Zhao, Y., and Srivastava, S. (2002) Clin. Chem. 48, 1160–1169
25. Wittmann, F. A., and Li, J. (2002) Am. J. Physiol. 282, G735–G741
26. Lilljeholm, J., Razaq, A., and Dupree, P. (2002) Curr. Opin. Chem. Biol. 6, 46–50
27. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Anal. Chem. 68, 850–858
28. Li, S., Lao, J. M., Chen, B. P. C., Li, Y. S., Zhao, Y. H., Chu, J., Chen, K. D., Tsou, T. C., Peck, K., and Chien, S. (2002) FASEB J. 16, U346–U370
29. Kwiatkowski, D. J. (1999) Curr. Opin. Cell Biol. 11, 103–108
30. Sun, H. Q., Yamamoto, M., Megillano, M., and Yin, H. L. (1999) J. Biol. Chem. 274, 33179–33182
31. Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999) Cell 98, 349–369
32. Hirschi, K. K., Skalak, T. C., Peirce, S. M., and Little, C. D. (2002) Ann. N. Y. Acad. Sci. 961, 223–242
33. Stegmann, J. P., and Nerem, R. M. (2003) Annu. Biomed. Eng. 31, 391–402
34. Topper, J. N., Cai, J., Qu, Y., Anderson, K. R., Xu, Y. Y., Deeds, J. D., Feeley, R., Gimeno, C. J., Woll, E. A., Bayber, O., Jays, G. G., Sampson, B. A., Schoen, F. J., Gimbrone, M. A., Jr., and Fabb, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9314–9319
35. Hautmann, M. B., Madisen, C. S., and Owens, G. K. (1997) J. Biol. Chem. 272, 10948–10956
36. Adams, P. R., Regan, C. P., Hautmann, M. B., and Owens, G. K. (2000) J. Biol. Chem. 275, 37798–37806
37. Liu, Y., Sinha, S., and Owens, G. K. (2003) J. Biol. Chem. 278, 48094–48011
38. Hirschi, K. K., Lai, L., Belaguli, N. S., Dean, D. A., Schwartz, R. J., and Zimmer, W. E. (2002) J. Biol. Chem. 277, 6287–6295
39. Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., and Anderson, C. W. (1992) J. Biol. Chem. 267, 794–803
40. Hedges, J. C., Dechert, M. A., Yamboliev, I. A., Martin, J. L., Hickey, E., Weber, L. A., and Gerthoffer, W. T. (1999) J. Biol. Chem. 274, 24211–24219
41. Glenny, R. J., Jr., Kaulus, P., Matsudaira, P., and Weber, K. (1981) J. Biol. Chem. 256, 9283–9288
42. Aparin, M., Friede, E., Algrain, M., Vernel, F., and Louvard, D. (1994) J. Cell Biol. 123, 1995–2006
43. Fath, K. R., and Burgess, D. R. (1995) Curr. Biol. 5, 591–593