Identification of Differentially Regulated Secretome Components During Skeletal Myogenesis

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Myogenesis is a well-characterized program of cellular differentiation that is exquisitely sensitive to the extracellular milieu. Systematic characterization of the myogenic secretome (i.e. the ensemble of secreted proteins) is, therefore, warranted for the identification of novel secretome components that regulate both the pluripotency of these progenitor mesenchymal cells, and also their commitment and passage through the differentiation program. Previously, we have successfully identified 26 secreted proteins in the mouse skeletal muscle cell line C2C12 (1). In an effort to attain a more comprehensive picture of the regulation of myogenesis by its extracellular milieu, quantitative profiling employing stable isotope labeling by amino acids in cell culture was implemented in conjunction with two parallel high throughput online reverse phase liquid chromatography-tandem mass spectrometry systems.

In summary, 34 secreted proteins were quantified, 30 of which were shown to be differentially expressed during muscle development. Intriguingly, our analysis has revealed several novel up- and down-regulated secretome components that may have critical biological relevance for both the maintenance of pluripotency and the passage of cells through the differentiation program. In particular, the altered regulation of secretome components, including follistatin-like protein-1, osteoglycin, spondin-2, and cytokine-induced apoptosis inhibitor-1, along with constitutively expressed factors, such as fibulin-2, illustrate dynamic changes in the secretome that take place when differentiation to a specific lineage occurs. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.004804, 1–20, 2011.

Development, growth, and maintenance of skeletal muscle are a critical feature of all metazoan species (2–9). Skeletal muscle, which forms in the vertebrate body axis, is derived from myoblast cells that acquire their lineage identity in the somites during embryonic development (2, 4, 10–12). Over several decades, myogenesis has proven to be a paradigm for cellular differentiation that has led to many discoveries concerning lineage commitment and the molecular control of tissue-specific gene activation. At the microscopic level, skeletal muscle differentiation or myogenesis is a highly orchestrated process in which mononucleated muscle precursor cells, the myoblasts (MBs)1, undergo proliferation. Upon differentiation, they withdraw from the cell cycle, migrate, align with each other, and subsequently fuse to form terminally differentiated multinucleated myotubes (MTs) (13–16). At the molecular level, each of these steps is regulated by the interplay of intracellular signal transducers and nuclear factors, such as fibulin-2, illustrate dynamic changes in the secretome that take place when differentiation to a specific lineage occurs. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.004804, 1–20, 2011.

1 The abbreviations used are: MBs, myoblasts; 1D-SDS PAGE, One dimensional-SDS PAGE; ALP, alkaline phosphatase ; BSA, bovine serum albumin; CDKs, cyclin-dependent kinases; CIAPIN-1, cytokine-induced apoptosis inhibitor-1; CM, conditioned media; DM, differentiation medium; DMEM, Dulbecco’s modified Eagle’s medium; DMEM/F12, Dulbecco’s MEM/Ham’s Nutrient Mixture F-12 medium; ECM, extracellular matrix; FA, formic acid; FBS, fetal bovine serum; Fstl-1, follistatin like protein-1; GM, growth medium; HS, horse serum; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LIT, linear ion trap; MALDI-MS/MS, matrix-assisted laser desorption/ionization-tandem mass spectrometry; MCK, muscle creatine kinase; MIF, macrophage migration inhibitory factor; MRF, muscle regulatory factor; MTs, myotubes; MyHC, myosin heavy chain; NAC-α, NAC alpha domain containing-protein; OGN, osteoglycin; PBS, phosphate-buffered saline; Prx-1, peroxiredoxin-1; QqTOF, quadrupole/time-of-flight; RP, reverse phase; RT-PCR, reverse transcription-polymerase chain reaction; SILAC, stable isotope labeling by amino acids in cell culture; SLRP, small leucine-rich proteoglycan gene family; SPARC, secreted protein acidic and rich in cysteine; TCTP-1, translationally controlled tumour protein-1; TGF, transforming growth factor; TIMP-2, tissue inhibitor of metalloproteinase-2.
transcription factors. In particular, the muscle regulatory factor (MRF) family, MyoD (17, 18), Myf5 (19, 20), myogenin (21–23), and MRF4 (24) are essential for myoblast lineage commitment (25–27), and, in conjunction with other transcriptional regulators, expression of muscle-specific genes, such as myosin heavy chain (MyHC) and muscle creatine kinase (MCK), to establish and reinforce the terminal myogenic differentiated state (24, 28, 29). For MRFs to function, they dimerize with E protein partners; this heterodimer recognizes and binds to the consensus DNA sequence (CANNTG) named the E-box, a key cis-element lying in the regulatory regions of the majority of muscle-specific genes (30).

During proliferation, cyclin/cyclin-dependent kinases (CDKs), such as cyclin D/CDK4, cyclin D/CDK6, cyclin E/CDK2, and cyclin A/CDK2, are active. These kinases phosphorylate pRb and subject it to degradation. As a result, pRb cannot bind to the E2F transcription factor complex and inhibit its activation of downstream proliferation-associated cellular events, such as chromosome segregation, mitotic spindle formation, and chromatin remodelling (31–37). This allows MBs to proliferate. In addition, CDKs can also phosphorylate MRFs and promote their degradation, thereby further suppressing the differentiation program (38–40). This initial repression of muscle differentiation is important to allow MBs to propagate iteratively until a sufficient amount of muscle precursor cells is attained prior to cell-cycle exit, in order to populate the vast amount of skeletal musculature that has to be constructed to form the mature body plan.

Once the MB population reaches a critical confluence in vivo, a variety of triggers provoke cell-cycle withdrawal such as cell-cell contact and altered competence to respond to autocrine and paracrine signaling events. The connection between cell-cell contact and cell-cycle exit is mediated by transmembrane proteins, such as m-cadherin (41–46). Upon cell-cell contact, m-cadherin is activated and induces the expression of CDK inhibitors such as p21 and p57, which serve as key repressors of the G1 cyclin/CDK complexes leading to a G1 arrest (47, 48). Interestingly, it is known that the G1 cyclin/CDK complexes function as molecular sensors of the nutritional status of the cell and growth factor signaling, thus constituting a link between extracellular signals and the intracellular conditions for continued proliferation or differentiation. Thus G1 arrest is critically dependent on a variety of extracellular cues and is a fundamental prerequisite for differentiation to occur (49).

Although the intracellular events controlling myogenesis have been well characterized, our understanding of the role of the extracellular factors that preside over the decision to exit the cell cycle and induce the differentiation program is much less clear. However, it is apparent that myogenesis is exquisitely sensitive to the extracellular milieu (50, 51). Intriguingly, whereas the effects of the extracellular environment impact myogenesis, it is also acknowledged that skeletal muscle may also function as the largest endocrine organ in humans for secreting extracellular factors, including myokines that regulate muscle development (52, 53). Apart from the well-known myokines, such as members of the insulin-like growth factor-1 (54–63) and transforming growth factor (TGF) families (64–72), there are studies investigating other myokines, such as plasminogen activator (73), collagenase (74), decorin (75), glial growth factor (76), neurocrescin (77), meltrin (78), musculin (52, 79), interleukin-1 beta (80), interleukin-7 (81), ADAMTS-like-2 (82), Fstl-1 (83), and secreted protein acidic and rich in cysteine (SPARC) (84–86). To advance our understanding of secreted proteins in an unbiased manner, we have undertaken a comprehensive “discovery” approach to initially identify and quantify components of the secretome. This served as the first step in elucidating a more complete picture of the extracellular molecular circuitry that regulates this dynamic and essential phenotypic conversion. To address this and follow up on a previous study that we reported (1), we have implemented a quantitative proteomics approach involving stable isotope labeling by amino acids in cell culture (SILAC) (87) to compare the secretomes of MBs and MTs derived from mouse skeletal muscle cells (C2C12) that were metabolically labeled with light [12C6]-lysine (L) and heavy [13C6]-lysine (H), respectively.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and SILAC Labeling** — The workflow of cell culture and SILAC labeling is depicted in Fig. 1. Mitogenic murine C2C12 MBs (American Type Culture Collection, ATCC) were initially seeded on 10-cm gelatin-coated (Sigma) culture plates (Fisher Scientific) containing 10 ml of growth medium (GM) which was composed of Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), 10% fetal bovine serum (FBS) (HyClone), 2 mM L-glutamate (Invitrogen), 50 units/ml penicillin-streptomycin (Invitrogen), and 1 mM sodium pyruvate (Invitrogen) at 37 °C and under an atmosphere of 5% CO2. For SILAC labeling, MBs were switched to either light- or heavy-GM for four cell divisions (i.e. for 192 h); these media comprised SILAC DMEM (Invitrogen) containing 10% dialyzed FBS (Invitrogen), 2 mM L-glutamate, 50 units/ml penicillin-streptomycin, and 1 mM sodium pyruvate.
pyruvate, supplemented with either 0.7 mm $[^{13}C_6]$- or $[^{13}C_6]$-lysine (Invitrogen), respectively (see Step 1 of Fig. 1).

Both the light- and heavy-labeled MBs were then treated with serum-free isotope-labeled differentiation medium (DM). Confluent (90%) MBs were rinsed with versene (Bioshop, Burlington, ON, Canada) and segregated in 1 ml of 0.125% trypsin (Invitrogen) for 1 min. Trypsinization was terminated by the addition of 5 ml of serum-free DM, comprising SILAC DMEM: Ham’s Nutrient Mixture F-12 medium (DMEM/F12) (Invitrogen) supplemented with 2 mM L-glutamate, 50 units/ml penicillin-streptomycin, 1 mM sodium pyruvate, and 0.4 μM bovine insulin (Sigma). This supplemented serum-free medium was empirically determined by us to support normal differentiation of the cells in a manner comparable to the classical DM, i.e. 2% horse serum (HS), for these cells (1). This medium allows differentiation of the cells in a serum protein-free environment, which has proven critical for secretoome analysis (88–95). In addition, supplementation of the media as we have identified above is critical as culture of cells in nonsupplemented DMEM is incompatible with cell survival and leads rapidly to the onset of apoptosis and release of proteins into the medium as a result. The cells were then spun down by centrifugation at 153 x g for 10 min. The pellet was resuspended in 5 ml of the serum-free DM and spun down by centrifugation. The resulting pellet was finally resuspended in 5 ml of either light- or heavy-labeled serum-free DM (serum-free DM supplemented with either 0.7 mm $[^{13}C_6]$- or $[^{13}C_6]$-lysine) in which light- and heavy-labeled MBs were allowed to inoculate for 24 h and 120 h, respectively (see Step 2 of Fig. 1). During the differentiation of heavy-labeled MBs, cells were washed with 10 ml of phosphate-buffered saline (PBS) (Invitrogen) for five times, followed by replenishment of 5 ml of heavy-labeled serum-free DM every 24 h. At 120 h, differentiation into multinucleated MTs was apparent (see Fig. 1).

Preparation of Cell Lysates—Cell lysates were collected to examine the incorporation of $[^{13}C_6]$-lysine and to determine the thresholds for differential expression. For the former, lysates were collected from MBs cultured in heavy-labeled GM every 24 h up to 192 h; for the latter, lysates were collected from MBs cultured in light- and heavy-labeled GM for 192 h (see Step 1 of Fig. 1).

Cells were washed with PBS twice and scraped from culture plates in 1 ml of PBS, followed by centrifugation at 57 x g for 3 min at 4 °C. The supernatant was discarded and the remaining pellet was resuspended in 200 μl lysis buffer composed of 50 mM Tris-Cl (pH 8.0), 150 mM NaCl (Bioshop), 0.5% Nonidet P-40 (BioRad), 2 mM EDTA (Bioshop), 100 mM NaF (Sigma), 10 mM Na₂HPO₄ (Bioshop), 1 mM Na₂VO₄ (Sigma), 1 mM PMSF (BioRad), 1 μg/ml leupeptin (BioRad), 1 μg/ml apronin (BioRad), and 1 μg/ml pepstatin A (BioRad). This was followed by a 30-s vortex and 10-min cooling on ice. This step was repeated twice and completed by a final spin at 457 x g for 5 min at 4 °C. The supernatant was collected.

Preparation of Conditioned Media (CM)—To examine the expression pattern of the secretoome during myogenesis, CM were collected from light-labeled MBs and heavy-labeled MTs cultured in serum-free DM for 24 h and 120 h, respectively (see Step 2 of Fig. 1). The CM collected were purified by two-step centrifugation at 98.2 x g for 30 min at 4 °C, followed by 12,499 x g for 30 min. The clarified media were then syringe-filtered (0.45-μm pore size, Milllex-HV) to remove remaining particles. Proteins in the CM were precipitated with 80% acetone (v/v) (Caledon, Georgetown, Canada) at −20 °C for 24 h. The proteins were then pelleted by centrifugation at 4 °C (15,344 x g for 1 h) and allowed to air dry. The dried proteins were then dissolved in a minimal volume of SDS-gel loading buffer, comprising 50 mM Tris-HCl (BioShop), 2% SDS (BioShop), 25% glycerol (BDH), and 2.93% β-mercaptoethanol (BioShop).

Bradford Assay—Protein concentration was determined by Bradford assay (Bio-Rad). Briefly, standard solutions of bovine serum albumin (BSA) (Sigma), 0 to 8 mg/ml, were analyzed for their optical densities at 595 nm using a spectrophotometer (Beckman). Protein concentrations in the samples were determined by comparing their optical densities with those in the BSA standard curve.

For evaluating the incorporation of $[^{13}C_6]$-lysine, 30 μg of light-labeled cell lysate protein derived from MBs culturing in heavy-labeled GM from 0 to 192 h (see Step 1 of Fig. 1) was subjected to 1D-SDS PAGE. An identical band around 37.1 kDa was excised for analysis. This was followed by trypsin digestion and matrix-assisted laser desorption/ionization-tandem mass spectrometry (MALDI-MS/MS) analyses (see Fig. 2A).

For determining the thresholds for differential expression, cell lysates from MBs cultured in light- and heavy-labeled GM for 192 h (see Step 1 of Fig. 1) were prepared in two identical sets. In each set, 15 μg of light-labeled cell lysate protein was mixed with 15 μg of the corresponding heavy-labeled counterpart and separated by 1D-SDS PAGE. Six identical bands were excised from each set for in-gel digestion and online reverse phase (RP) liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses (see Fig. 2B).

To examine the relative expression of the secretoome at the MT versus MB stage, CM collected from light-labeled MBs and heavy-labeled MTs (see Step 2 of Fig. 1) were prepared in two identical sets. In each set, 15 μg of light-labeled CM was combined with 15 μg of heavy counterpart and fractionated by 1D-SDS PAGE. The entire gel lane was sliced into 16 equal portions for trypsin digestion and online RPLC-MS/MS analyses (see Fig. 2C).

In-gel Digestion With Trypsin—Isolated gel bands were subjected to in-gel reduction, alkylation, and trypsin digestion. Briefly, proteins in the gel slices were reduced with 30 μl of 50 mM ammonium bicarbonate (Sigma)/10 mM dithiothreitol (Sigma) at 56 °C for 15 min. This was followed by alkylation conducted with 30 μl of 100 mM iodoacetamide (Sigma)/50 mM ammonium bicarbonate for 15 min in darkness. Enzymatic digestion was then performed overnight with sequencing-grade trypsin (Promega) at 37 °C.

MALDI-MS/MS Analyses—Tryptic peptides were concentrated and desalted by ZipTip (Millipore) according to the manufacturer’s instructions, and ultimately eluted with 1.5 μl of 10 mg/ml α-cyano-4-hydroxy-cinnamic acid (Sigma) in 60% acetonitrile (Sigma) and 0.3% trifluoroacetic acid (Sigma) onto a MALDI sample plate. All MS and MS/MS analyses were performed on a QSTAR XL hybrid quadrupole/time-of-flight (QqTOF) tandem mass spectrometer (Applied Biosystems/MDS SCIEX) equipped with a nitrogen laser (337 nm) for MALDI. The mass spectra generated were searched against NCBI nr databases (released on February 25, 2008 with a total of 137,762 protein sequences concatenated with a list of common contaminants, e.g. trypsin, keratins, and BSA) using the Mascot search engine 2.2 (Matrix Science). The search parameters were: taxonomy, Mus musculus; enzyme, trypsin; fixed modifications, carboxyamidomethylation of cysteine, and oxidation of methionine; variable modifications, i.e., Cys: 13C(6)(K); missed cleavages allowed, one; peptide and MS/MS tolerance, ±50 ppm and ±100 ppm, respectively, with a peptide charge of 1+.

Online RPLC-MS/MS Analyses—Tryptic peptides were desalted by ZipTip and eluted with 10 μl of 0.3% formic acid (FA) in 60% acetonitrile. The peptide solution was brought to dryness by speed-vac (Thermo Fisher Scientific) and dissolved in 10 μl of buffer A composed of 0.1% FA in 5% methanol. Tryptic peptides from set 1 were subjected to online LC-MS/MS on an Agilent 1100 RPLC-LTQ linear ion trap (LIT) (Thermo Fisher Scientific) tandem mass spectrometer, whereas those from set 2 to an LC Packings RPLC-QSTAR Pulsar hybrid QqTOF (Applied Biosystems/MDS SCIEX) instrument.
Secretome Analysis During Skeletal Myogenesis

A

Examine the incorporation of $^{13}$C$_6$-lysine

Cell lysate proteins of MBs cultured in heavy-GM (30 µg of each)

Cell lysate proteins of MBs cultured in light-GM (5 µg of each)

kDa

B

Determine the thresholds for differential expression

Cell lysate proteins of MBs cultured in light-GM for 192 h (15 µg of each)

Cell lysate proteins of MBs cultured in heavy-GM for 192 h (15 µg of each)

kDa

C

Secretome analysis during skeletal myogenesis

CM proteins of MBs cultured in light-DM for 24 h (15 µg of each)

CM proteins of MTs cultured in heavy-DM for 120 h (15 µg of each)

kDa

Fig. 2. 1D-SDS PAGE: A, for examining the incorporation of $^{13}$C$_6$-lysine, B, for determining the thresholds for differential expression, and C, for secretome analysis during skeletal myogenesis. M shows the protein ladder.

For Agilent 1100 RPLC separation, 2 µl of the tryptic peptide sample was injected using the autosampler and concentrated on a 5 × 0.3-mm precolumn (Zorbax C18 silica beads, 5-µm diameter, 100-Å pore size, Rockland Technologies, Inc., Mount Vernon, NY) at 10 µl/min for 5.8 min. This was followed by LC separation on a 10-cm long fused silica PicoTip emitter (75-µm inner diameter, New Objective) packed in-house with Zorbax C18 silica beads (5-µm diameter, 100-Å pore size, Rockland Technologies, Inc.) at 240 nl/min for 54.2 min using a multisegment linear gradient of buffer B (0.1% FA in 94.9% methanol) as follows: 0–4.2 min, 5–30% B; 4.2–46 min, 30–80% B; 46–49.2 min, 80–100% B; 49.2–54.2 min, 100% of B. Each sample was analyzed in triplicate. Mass spectra were acquired with the LTQ Orbitrap tandem mass spectrometer in data-dependent acquisition mode, beginning with an enhanced MS scan ($m/z$ 400–1500), followed by three MS/MS scans of the top three most abundant precursor ions ($m/z$ 50–2000) at a relative collision energy of 35%. The precursor ion was then subjected to dynamic exclusion for 30 s. The LTQ was operated in the positive ion detection mode with a potential of 2.15 kV. Mass spectra generated were analyzed with Xcalibur 2.0.7 (ThermoFisher Scientific) and submitted to the Mascot search engine with the following search parameters: database, NCBI; species, Mus musculus; enzyme, trypsin; fixed modifications, cysteine carboxamidomethylation; variable modifications, lysine $^{13}$C$_6$ and methionine oxidation; missed cleavages, 0; peptide charge, +1, +2, and +3; peptide and fragment tolerance, 10 Da and 0.8 Da, respectively; and instrument type, electrospray ionization Trap. Peptides with $p < 0.05$ were regarded as significant hits.

For LC Packings RPLC separation, 1 µl of the tryptic peptide sample was injected using the autosampler and desalted on a 5-mm C18 precolumn (LC Packings Vernon Hills, Illinois) at 25 µl/min for 4 min. This was followed by peptide separation on a 15-cm long Integrafract capillary column (75-µm inner diameter, New Objective) packed in-house with C18 beads (3.5-µm diameter, 100-Å pore size, Akzo Nobel/EKA Chemicals inc, NY) at 200 nl/min for 130 min using a multisegment linear gradient of buffer B (0.1% FA in 94.9% methanol): 0–5 min, 5–15% B; 5–65 min, 15–35% B; 65–80 min, 35–80% B; 80–130 min, 80% of B. Each sample was analyzed in triplicate. Mass spectra were acquired with the QSTAR in data-dependent acquisition mode beginning with a 1-s MS scan ($m/z$ 400–1500), followed by five 2-s MS/MS scans of the five most-abundant precursor ions ($m/z$ 80–2000). The precursor ions were then subjected to a 30-s dynamic exclusion window. The QSTAR was operated in the positive ion detection mode with a potential of 2.6 kV. Mass spectra were analyzed with Analyst QS 1.1 (Applied Biosystems/MDS SCIEX) and submitted to ProteinPilot 2.0.1 (96) for analysis with the following search parameters: database, NCBInr; species, Mus musculus; enzyme, trypsin; and submitted to ProteinPilot 2.0.1 (96) for analysis with the following search parameters: database, NCBInr; species, Mus musculus; enzyme, trypsin; and submitted to Mascot for identification.

For sample desalting, 1 µl of the tryptic peptide sample was injected using the autosampler and desalted on a 5-mm C18 precolumn (LC Packings Vernon Hills, Illinois) at 25 µl/min for 4 min. This was followed by peptide separation on a 15-cm long Integrafract capillary column (75-µm inner diameter, New Objective) packed in-house with C18 beads (3.5-µm diameter, 100-Å pore size, Akzo Nobel/EKA Chemicals inc, NY) at 200 nl/min for 130 min using a multisegment linear gradient of buffer B (0.1% FA in 94.9% methanol): 0–5 min, 5–15% B; 5–65 min, 15–35% B; 65–80 min, 35–80% B; 80–130 min, 80% of B. Each sample was analyzed in triplicate. Mass spectra were acquired with the QSTAR in data-dependent acquisition mode beginning with a 1-s MS scan ($m/z$ 400–1500), followed by five 2-s MS/MS scans of the five most-abundant precursor ions ($m/z$ 80–2000). The precursor ions were then subjected to a 30-s dynamic exclusion window. The QSTAR was operated in the positive ion detection mode with a potential of 2.6 kV. Mass spectra were analyzed with Analyst QS 1.1 (Applied Biosystems/MDS SCIEX) and submitted to ProteinPilot 2.0.1 (96) for analysis with the following search parameters: database, NCBInr; species, Mus musculus; enzyme, trypsin; and submitted to ProteinPilot 2.0.1 (96) for analysis with the following search parameters: database, NCBInr; species, Mus musculus; enzyme, trypsin; and submitted to Mascot for identification.

The LTQ was operated in the positive ion detection mode with a potential of 2.15 kV. Mass spectra generated were analyzed with Xcalibur 2.0.7 (ThermoFisher Scientific) and submitted to the Mascot search engine with the following search parameters: database, NCBI; species, Mus musculus; enzyme, trypsin; fixed modifications, cysteine carboxamidomethylation; variable modifications, lysine $^{13}$C$_6$ and methionine oxidation; missed cleavages, 0; peptide charge, +1, +2, and +3; peptide and fragment tolerance, 10 Da and 0.8 Da, respectively; and instrument type, electrospray ionization Trap. Peptides with $p < 0.05$ were regarded as significant hits.

Our threshold determination is based on a 1:1 mix derived from the cell lysates of MBs cultured in light- and heavy-labeled GM for 192 h (see Step 1 of Fig. 1). Because both lysates are
originated from the same cell state (i.e., MB), the protein ratios (H:L) should be 1 in theory. In practice, however, the measured protein ratios exhibit a range because of the experimental uncertainties and/or random errors. The minimum ratio of the proteins in a batch is given by \( \min(p) \) and the maximum ratio by \( \max(p) \), in which \( p \) denotes the expression level of the protein in the batch. In the log-space, we expect the protein ratio distribution range to be close to normal and symmetrical around zero, with minimum log-protein ratio being equal to maximum. \( \min(p) = -\max(\log(p)) \). In linear space, we can express this equation as \( \max(p) = \frac{1}{\min(p)} \). In experiments, we find a set of protein ratios and can determine the minimum and the maximum of them. If the maximum determined protein ratios is larger than one over the minimum, i.e. \( \max(p) > \frac{1}{\min(p)} \), we then use the maximum of detected protein ratios to reconstruct the theoretical distribution range as \( \frac{1}{\max(p)} \cdot \max(p) \). Analogously, if the minimum of the determined protein ratios is less than one over the maximum, i.e. \( \min(p) < \frac{1}{\max(p)} \), we reconstruct the theoretical protein range as \( \frac{1}{\min(p)} \cdot \min(p) \). We then use a very conservative approach to determine the threshold, with the upper threshold equal to the upper boundary of the theoretical range, and the lower threshold equal to the lower boundary. Therefore, we are only considering proteins lying outside of the defined range of the protein ratios as differentially expressed. Summarizing, the lower and upper thresholds are \( \min(\frac{1}{\max(p)}) \) and \( \max(\frac{1}{\min(p)}) \), respectively.

Assignment of Classical and Nonclassical Secreted Proteins—Proteins identified were parsed with two open-source algorithms, SignalP 3.0 (97) and SecretomeP 2.0 (98) for secreted protein prediction. Proteins with SP probability \( \geq 0.5 \) were assigned as classical secreted proteins (C); those with SP probability \( < 0.5 \) and NN-score \( \geq 0.5 \) were regarded as nonclassical secreted proteins (N), and the rest (i.e. both SP probability and NN-score \( < 0.5 \)) were classified as hypothetical intracellular proteins (I).

Western Blot Analyses—Differentially expressed proteins identified with SILAC were verified by quantitative Western blot analyses using the Li-COR Odyssey system. Primary antibodies used included MyHC (Developmental study Hybridoma bank), SPARC (Hematologic Technology Inc., Santa Cruz, CA), OGN (R&D), BSA (Sigma), and cleaved caspase-3 (Cell Signaling Technology, Beverly, MA). In brief, 20 µg of CM proteins derived from light-labeled MBs or heavy-labeled MTs cultured in serum-free DM for 24 h and 120 h, respectively, were resolved by 10-SDS PAGE, followed by an overnight electrophoretic transfer to Immobion-P membranes (Millipore) at 20 V and a 1-h transfer at 50 V. The membranes were then blocked with Odyssey blocking buffer (Li-COR, Inc., Lincoln, NE) for 1 h and probed overnight at 4°C with primary antibodies diluted in Odyssey blocking buffer at various dilution factors: MyHC, 1:50; SPARC, 1:1000; α-actin, 1:2000; OGN, 1:2000; BSA, 1:1000; and cleaved caspase-3, 1:1000. The membranes were washed three times with 0.1% Tween in PBS (v/v) and probed with fluorescenc-conjugated secondary antibody (Li-COR, Inc.) for 1 h in darkness. They were washed three times with 0.1% Tween in PBS and then with PBS. The fluorescent signals from the membranes were detected and quantified with the Odyssey imaging system, which allows quantitative detection of immunoreactive bands on Western blots over many orders of magnitude.

Detecting MyHC with Immunocytochemistry—MyHC subunits are encoded by distinct members of a multigene family expressed at different stages of muscle development and are the archetypical marker proteins used to indicate cellular differentiation of muscle. In order to detect MyHC expression in cultured muscle cells, we used the MF20 monoclonal antibody (99) which recognizes all sarcomeric myosins. Briefly, after fixation (6 min in 90% methanol), the cells were incubated with the primary antibody (monoclonal supernatant produced in our laboratory), followed by incubation with a horseradish peroxidase-conjugated goat antimouse secondary antibody at a dilution of 1:1000 (Bio-Rad). Positively stained cells were visualized using a colorimetric substrate, diaminobenzidine (Sigma), which results in a brown stain of the MyHC positive cells.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Plasmid Construction—Total RNA was isolated from transfected cells using TRIzol reagent (Invitrogen). First-strand complementary DNA was synthesized using SuperScript III reverse transcription kit (Invitrogen), and used for semi-quantitative PCR analysis. Primer sequences are shown in supplemental data S4A. Spondin-2 cDNA was generated by RT-PCR using the following primers: 5′ TAGGATCC-ATGGAAACGTTAGTTG3′ and 5′ ATGGCGGCCCGTATGAGGGAGTTATCTGG3′ (BamH I and NotI sites are underlined). Spondin-2 cDNA was inserted into BamH I and NotI digested pcDNAs (Invitrogen), and the resulting construct was verified by sequencing. Three predesigned MISSION siRNAs (Sigma) with the highest possible ranking were used against each target gene. Some of the primers for semiquantitative RT-PCR include restriction sites for cDNA cloning.

Cell Transfection and Reporter Gene Assays—To knock down the expression of selected genes, we used MISSION predesigned siRNAs (Sigma). Three distinct siRNA sequences were tested for each gene (see supplemental data S4A for siRNA ID numbers). C2C12 MBs were grown in 10% FBS-containing GM. At ~80–90% confluence, cells were cotransfected with an MCK promoter-driven luciferase reporter plasmid (100), a CMV-β-galactosidase control plasmid, and 50 nM siRNA using lipofectamine reagent (Invitrogen) in serum-free DMEM. Cy3-labeled scrambled siRNA (Ambion) was used as negative control. All transfections were performed in triplicates. Eight hours after transfection, cells were replenished with DM containing 2% HS (Atlanta Biologicals, Atlanta, GA). By 2.5 days of differentiation period, cells were rinsed twice with ice-cold PBS, and lysed with 20 µm Tris at pH 7.6 with 0.1% Triton X-100. Luciferase- and β-galactosidase-activity were determined as previously described (101, 102). In brief, relative luciferase unit was calculated by dividing the measured luciferase activity with the respective β-galactosidase activity; the resulting ratio was normalized to the negative control value (i.e. scrambled siRNA).

Cell Culture with Exogenously Added OGN—To examine the role of OGN in myogenesis, GM (10% FBS) and DM (2% HS) supplemented with purified OGN (R&D) at various concentrations (0, 0.02, 0.2, 2, and 10 nM) were employed. C2C12 cells were cultured in supplemented-GM for 48 h, then switched to the supplemented-DM for 120 h. Phase contrast microscopic images of the cells were taken throughout the course of cell culture.

MyHC-Alkaline Phosphatase (ALP) Double-staining—To examine the potential role of OGN in modulating cell commitment to myogenic- and osteogenic-lineages, MyHC-ALP double-staining was implemented. Parallel sets of C2C12 cells cultured in OGN-supplemented DM were washed with PBS three times and blocked with 10% goat serum in PBS at 37°C for 30 min. This was followed by a 1-h incubation at room temperature with primary MyHC antibody diluted 10 times with 1.5% goat serum (Cedarlane) in PBS. Cells were then washed three times with PBS and incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) diluted 1000 times with 1.5% goat serum in PBS at room temperature for 1 h. The cells were then washed three times with PBS, and stained with ALP stain (Sigma) for 20 min at room temperature, followed by three times PBS.
The cells were then developed with AEC Chromogen kit as the chromogenic substrate (Sigma). 0 nM OGN and 8 nM bone morphogenic protein-2 (BMP-2) was used as positive control for MyHC- and ALP-positive cells, respectively; the former were stained brown, whereas the latter purple.

RESULTS

Incorporation of Isotope-labeled Lysine into C2C12 Cells—To assure isotope-labeled lysine was the sole source of lysine incorporated into the cells, dialyzed lysine deficient culture medium was used (87, 103, 104). The morphologies of MBs were monitored throughout the course of SILAC labeling. MBs were observed to maintain a typical mononucleated polygonal shape (Fig. 3A) and their respective lysates were subjected to 1D-SDS PAGE, trypsin digestion, and MALDI-MS/MS analyses for evaluating the incorporation of heavy-labeled lysine. As illustrated in Fig. 3B, a tryptic peptide of β-actin at m/z 1954.1 was present prior to SILAC labeling (i.e. 0 h). This peak commenced to shift with time to m/z 1960.2 because of the incorporation of [13C6]-lysine, as verified by MS/MS analyses in which both peaks at m/z 1954.1 and 1960.2, corresponding to nonlabeled and labeled version of the tryptic peptide, respectively, were monitored (Fig. 3C).

This gave us a preliminary idea on the minimal labeling time, which was 120 h according to Fig. 3B. To be more stringent, cells labeled for 192 h were used. The comprehensiveness of the labeling was indicated by the protein ratios derived from the 1:1 mix of heavy- and light-labeled lysate proteins collected at 192 h labeling time. For complete labeling, protein ratios derived from such a mix should be close to 1. According to Figs. 4A and 4B, protein ratios from both sets (sets 1 and 2) were indeed centralized around 1. These observations were based on 80 proteins and 229 peptides in set 1, as well as 165 proteins and 858 peptides in set 2 (supplemental data S3A and S3B).

Importantly, we empirically determined conditions that allowed the program of differentiation to occur normally under SILAC labeling, as shown from normal proliferation of MBs under growth conditions and the formation of multinucleated MTs expressing MyHC in differentiation conditions (Fig. 1). Taken together, these data indicate that SILAC analysis can be implemented compatibly with our myogenic cell culture model without any observable negative impact on cell growth and differentiation.

Determination of the Thresholds for Differentially Expressed Proteins—To set the thresholds for distinguishing differentially expressed proteins from the nondifferentiated pool, labeled lysate proteins were used by virtue of the fact that a much greater number of proteins could be obtained from this sample representing a large data set of proteins for subsequent statistical analysis to be executed with greater reliability. All of the samples, irrespective of cell lysates or CM, were subjected to 1D-SDS PAGE, followed by in-gel digestion, in which the matrix differences between the two could be attenuated. Essentially, equal amounts of light- and heavy-labeled lysates derived from MBs cultured in their respective isotope-labeled GM for 192 h were combined and subjected to 1D-SDS PAGE and trypsin digestion (Fig. 2B). The resulting tryptic peptides from sets 1 and 2 were analyzed with the Agilent 1100 RPLC-LTQ LIT tandem mass spectrometer and LC Packings RPLC-QSTAR Pulsar hybrid QqTOF tandem mass spectrometer, respectively. As both light- and heavy-labeled lysates were derived from the same cell stage (i.e. MBs cultured in GM for 192 h), a protein ratio (H:L) of 1 was expected in theory and any discrepancy would reveal systematic and experimental error and/or uncertainty. As shown in Fig. 4, the experimental protein ratios from sets 1 and 2 were tightly centralized around 1. After statistical analysis, protein ratios (H:L) > 1.68 and ≤ 0.60 were categorized as up- and down-regulated in MT versus MB respectively, in set 1; whereas in set 2, protein ratios ≥ 1.58 and ≤ 0.63 were designated as up- and down-regulated, respectively.

With regard to the consistency of the peptide ratios from the same protein, their respective coefficient of variation (CV) was examined. In set 1, the average and the median CV of the peptide ratios from the same protein was 8.4% and 8.1%, respectively (supplemental data S3A); whereas those in set 2 was 5.6% and 4.9%, respectively (supplemental data S3B). These numbers demonstrate consistent and acceptable performance of the analytical system. Furthermore, at the protein level, the average protein ratio in set 1 was 1.02 with a CV of 15.4% (supplemental data S3A); whereas in set 2, the average protein ratio was 0.91 with CV of 14.1% (supplemental data S3B). Again these figures of merit are indicative of well-behaving experiments both in terms of accuracy and precision. As the MBs were cultured under identical conditions except for the labels, we expect the average protein ratio to be 1; the determined ratios of 1.02 and 0.91 approached 1 and were deemed to be indicative of good accuracies. CVs of 15.4% and 14.1% were judged to be of good precision in a discovery-based proteomic experiment.

In the discovery phase study reported here, we have invested extensive effort into ensuring that our dataset is relevant to the myogenic program under study by empirically determining culture conditions that are consistent with SILAC, but are also as faithful as possible to traditional methods of culturing these cells in minimizing false positives and invoking confidence in the community of “myogenesis” researchers that the identified secretome is relevant to the normal cell biology of muscle differentiation. One issue that requires careful consideration in studies of this nature is where to draw the line in terms of what can be seen as above threshold and, by adopting a conservative approach in choosing a stringent threshold, it is inevitable that we may have made some Type II errors (i.e. in having some false negatives). However, our philosophy is that we are prepared to lose some genuine hits in favor of excluding many false positives: our view is that the obvious presence of many false positives in these discovery datasets deters subsequent follow up by biologists and we...
Optimization of SILAC experiments: A, Phase-contrast photomicrographs of MBs cultured in heavy-labeled GM from 0 to 192 h. B, Incorporation of heavy-labeled lysine into MBs. At 0 h, a nonlabeled tryptic peptide at m/z 1954.1 (green dashed line) was present in the MS scan. This was followed by a shift with time to m/z 1960.2 (red dashed line) as labeling progressed. C, MS/MS spectrum of m/z 1954.1 at 0 h (upper panel) and that of m/z 1960.2 at 192 h (lower panel) revealed that both peaks originated from the same tryptic peptide (VAEEHPVLLTEAPLNPK) of β-actin. Light- and heavy-labeled y ions were labeled in green and red, respectively.
would rather have a higher confidence that the reported findings are true positives.

**Examination of Relative Expression of the Secretome During Skeletal Myogenesis**—To analyze the secretome of skeletal myogenesis by SILAC, CM proteins derived from light-labeled MBs and heavy-labeled MTs were mixed in equal amounts and subjected to 1D-SDS PAGE, followed by trypsin digestion (Fig. 2C). The resulting tryptic peptides derived from sets 1 and 2 were analyzed by online RPLC-MS/MS as detailed above. In total, 214 proteins were identified, 108 of which were predicted to be secretory with SecretomeP 2.0 (supplemental data S1). The relative expression levels of these secreted proteins were determined manually. Thirty-four secreted proteins were quantified and 30 of these proteins had expression ratios beyond the thresholds and were considered to be differentially expressed. The majority of differentially expressed proteins (28 out of 30) were down-regulated in MT (Table I, supplemental data S2).

**Verification by Western Blot Analyses**—In a proof of principle experiment, differential expression of selected proteins quantified with SILAC were chosen to be tested using classical Western blot analyses to determine the level of agreement between the two approaches. Proteins chosen were based on the availability of well characterized antibodies for Western analysis, which include MyHC, SPARC, α-actin, and OGN (Figs. 5A–5D). There is, in general, very good agreement between the SILAC ratios and the ratios of the quantitated fluorescent signals from the Western blot analyses (shown under the blot images). Notably, SILAC permits the selective identification of proteins produced by the cells and excludes contaminants and/or exogenously added proteins (e.g. BSA), which do not incorporate heavy-labeled lysine. For these proteins, a ratio of zero (H:L) was measured (see BSA in Fig. 5E). In contrast, Western blotting does not (cannot) make this distinction and yields a protein ratio of ~1.

We were also interested in comparing the expression profile of secreted proteins in classical media versus the media we empirically determined and used for SILAC analysis. To do this we assessed protein expression levels by quantitative Western blot analyses in both types of culture conditions. The data were mostly reassuring, because we observed that the relative expression levels of MyHC, SPARC, and α-actin are quite consistent between the serum-free SILAC and 2% HS culture conditions. The only exception in this data set was OGN, which we observed to be down-regulated in SILAC conditions and the opposite in 2% HS (Fig. 5). This could be an effect of HS, which may protect the OGN protein from degradation. This is not unusual for growth factors and there may be growth factor binding proteins in HS that are not present in the SILAC medium. Furthermore, it is also possible that a component of HS is stimulating the cells to produce OGN. Although we have observed a discrepancy in the relative levels of OGN in the two different types of culture conditions between MB and MT, it is nevertheless important to state that we have identified OGN as an important component of the secretome for these cells because siRNA mediated knock down of OGN resulted in a modest decrease in myoblast differentiation as indicated by our MCK-Luc reporter gene system (Fig. 6). The fact that exogenous supplementation of the media with OGN protein did not change the phenotypic differentiation program in a demonstrable way suggests that the endogenous OGN secretion is sufficient or that the effects of supplementation are subtle and not easily discernable by phenotypic observation. Obviously, there are still questions that need to be clarified now that we have identified OGN as a *bona fide* part of the myogenic secretome.

**Functional Assessment of Selective Secretome Components During Myogenesis Using a Reporter Gene-based Differentiation Assay**—To initiate functional studies of the identified secretome components, a set of 10 proteins: spondin-2, fibulin-2, Fstl-1, OGN, macrophage migration inhibitory factor (MIF), SPARC, translationally controlled tumor protein-1 (TCTP-1), NAC α domain containing-protein (NAC-α), CIAPIN-1, and tissue inhibitor of metalloprotei-
Secreted proteins quantified. Down- and up-regulated expression levels (H:L) were shaded in green and red respectively, whereas those in grey represented nondifferential expression. Classical and nonclassical secreted proteins were denoted as C and N respectively.

| gi #          | Protein name                              | Protein group | Set 1 | Set 2 | Total # unique peptides quantified | Total # Peptides identified | Total # distinct peptides identified | # unique Peptides ID | Protein coverage (%) |
|---------------|-------------------------------------------|---------------|-------|-------|-----------------------------------|-----------------------------|------------------------------------|---------------------|---------------------|
| gi 6754976    | Peroxiredoxin 1                           | N             | 0.46  | 0.37  | 26                                | 202                         | 14                                 | 12                  | 80.9                |
| gi 437125     | Insulin-like growth factor binding protein 5 | C             | 0.52  | 0.36  | 34                                | 275                         | 12                                 | 11                  | 58.9                |
| gi 6996913,   | Annexin A2                                | N             | 0.24  | 0.23  | 8                                 | 69                          | 12                                 | 10                  | 40.7                |
| gi 6680924,   | Cofilin 1, nonmuscle                       | N             | 0.41  | 0.35  | 28                                | 227                         | 8                                  | 5                   | 56.0                |
| gi 6755040    | Profilin 1                                | N             | 0.51  | 0.35  | 43                                | 242                         | 9                                  | 8                   | 90.7                |
| gi 6201487,   | Glyceraldehyde-3-phosphate dehydrogenase   | C             | 0.60  | 0.42  | 17                                | 104                         | 11                                 | 10                  | 55.3                |
| gi 6679937,   |                                      | C             | 0.13  | 0.09  | 46                                | 314                         | 12                                 | 10                  | 48.3                |
| gi 668077,    |                                          | C             | 0.99  | 0.70  | 27                                | 292                         | 11                                 | 10                  | 43.5                |
| gi 94390366   |                                          | C             | 1.34  | 0.90  | 27                                | 292                         | 11                                 | 10                  | 48.3                |
| gi 128116574  | NAC α domain containing                    | C             | 0.99  | 0     | NA                                | NA                          | NA                                 | NA                  | 3.5                 |
| gi 1831528    | Cip1 protein                              | N             | 0.99  | 0     | NA                                | NA                          | NA                                 | NA                  | 23.4                |
| gi 585122     | Fibulin-2 precursor                       | C             | 1.25  | 0     | NA                                | NA                          | NA                                 | NA                  | 4.1                 |
| gi 24586721   | Eukaryotic translation                     | C             | 0.82  | 0     | NA                                | NA                          | NA                                 | NA                  | 4.2                 |
| gi 31980922   | Elongation factor 1 β2                    | C             | 0.82  | 0     | NA                                | NA                          | NA                                 | NA                  | 4.2                 |
| gi 7304963    | Chloride intracellular channel 4          | C             | 0.87  | 0.03  | 9                                 | 62                          | 6                                  | 5                   | 42.5                |
| gi 7422953    | Phosphatidylethanolamine binding protein 1 | N             | 0.60  | 0.07  | NA                                | NA                          | NA                                 | NA                  | 50.3                |
| gi 309315,    | Hypoxanthine-guanine phosphoribosyltransferase | N             | 0.59  | 0.06  | NA                                | NA                          | NA                                 | NA                  | 15.6                |
| gi 7305155    | Malate dehydrogenase 1, NAD (soluble), isoform CRA_c | N             | 0.58  | 0.08  | NA                                | NA                          | NA                                 | NA                  | 27.6                |
| gi 148675904  | Histidine triad nucleotide binding protein 1 | N             | 0.57  | 0     | NA                                | NA                          | NA                                 | NA                  | 16.7                |
| gi 12846252   | Peroxiredoxin 2                           | N             | 0.57  | 0.05  | NA                                | NA                          | NA                                 | NA                  | 40.7                |
| gi 4758158    | Small nuclear ribonucleoprotein polypeptide D2 | N             | 0.56  | 0     | NA                                | NA                          | NA                                 | NA                  | 40.7                |
| gi 6754524    | Lactate dehydrogenase A                    | N             | 0.50  | 0     | NA                                | NA                          | NA                                 | NA                  | 60.5                |
| gi 3914434,   | Proteasome subunit β type-5 precursor      | C             | 0.5   | 0.09  | NA                                | NA                          | NA                                 | NA                  | 55.5                |
| gi 6755204    | AP-1, β 1 subunit, isoform CRA_a          | N             | 0.48  | 0.15  | NA                                | NA                          | NA                                 | NA                  | 4.0                 |
| gi 148708561  | Cu/Zn superoxide dismutase                | N             | 0.47  | 0.04  | NA                                | NA                          | NA                                 | NA                  | 32.7                |
| gi 143811353  | Sterile α motif domain-containing protein 3 | N             | 0.4   | 0     | NA                                | NA                          | NA                                 | NA                  | 7.1                 |
| gi 2498391,   | Follistatin-like 1                         | C             | 0.36  | 0     | NA                                | NA                          | NA                                 | NA                  | 18.3                |
nase-2 (TIMP-2) were selected for testing in a functional assay using a myogenic differentiation marker MCK promoter-driven luciferase reporter gene system (Fig. 6A). Six of these proteins had been successfully quantified with SILAC. C2C12 MBs were cotransfected with an MCK promoter-driven luciferase reporter plasmid, a CMV-driven β-galactosidase control plasmid, and siRNA designed to reduce the expression (i.e., knock down) of the selected genes. To quantitatively assess the effect of candidate gene knockdown on myogenic progression, we measured the activity of the muscle-specific MCK promoter at the end of the differentiation period. The efficiency of the target gene knockdown was confirmed by semi-quantitative RT-PCR (supplemental data S4B). Knockdown of several genes, spondin-2, Fstl-1, fibulin-2, and OGN, significantly attenuated MCK-luciferase levels, suggesting a positive role for these proteins in myogenesis, whereas knockdown of TIMP-2 generated an enhancement effect, suggesting a negative role in the differentiation program. For the remainder of the genes tested: SPARC, MIF, TCTP-1, NAC, and CIAPIN-1, no significant alteration of the MCK-luciferase activity was discernible (Fig. 6A). Of all the genes tested with siRNA, spondin-2 showed the most pronounced effect on myogenesis in this cell culture model, in which the MCK-luciferase activity was decreased by 2.6-fold (Fig. 6A). To further address the role of spondin-2 in myogenesis, we carried out gain-of-function analysis by overexpressing spondin-2 in C2C12 cells. By the end of the 2.5-day differentiation period, the MCK-luciferase activity was ramped up by 9.2-fold (Fig. 6B). We suggest that adapting an siRNA-mediated loss-of-function analysis in tandem with a gain-of-function examination by overexpressing candidate proteins are useful to initially assess the role of identified components in the biological process under study. In this case, an MCK luciferase reporter gene provided the output measurement, whereas knocking down spondin-2 was confirmed by semi-quantitative RT-PCR expression (i.e., knock down) of the selected genes. To quantitatively assess the effect of candidate gene knockdown on myogenic progression, we measured the activity of the muscle-specific MCK promoter at the end of the differentiation period. The efficiency of the target gene knockdown was confirmed by semi-quantitative RT-PCR expression (i.e., knock down) of the selected genes.

### Functional Studies of OGN Using Purified OGN and MyHC-ALP Double-Staining

Another functional examination that we initiated was to exogenously provide a purified form of the identified secretome component to cultured cells for observing effects on the differentiation program. During myogenesis, OGN was down-regulated by more than sixfold (Table I); we decided to examine the augmentation of this protein as it was also available in a purified, biologically active form. OGN was originally identified as a bone inductive factor (105). Thus, we speculated that OGN might be a repressor of myogenesis in multipotent mesenchymal cells, whereas promoting the osteogenic-lineage. We had previously reported that C2C12 cells are multipotent mesenchymal cells, whereas promoting the osteogenic-lineage when exposed to osteogenic factors (e.g., BMP-2, 106). To test this, C2C12 cells were treated with purified OGN at various concentrations (0.02, 0.2, 2, and 20 nM). We observed that OGN significantly decreased the expression of myogenic markers (e.g., MyoD, Myogenin), whereas increasing the expression of osteogenic markers (e.g., osteocalcin). The effect of OGN on myogenesis was dose-dependent, with the highest concentration (20 nM) resulting in the most pronounced effect. We also measured the expression levels of key regulatory genes involved in myogenesis (e.g., MyoD, Myogenin) and osteogenesis (e.g., osteocalcin) using qPCR. We observed a significant decrease in MyoD and Myogenin expression and a significant increase in osteocalcin expression with OGN treatment. These results suggest that OGN plays a role in regulating the myogenic-osteogenic switch during myogenesis.

### Table I—continued

| gi #            | Protein name                          | Protein group | Set 1       | Set 2       | Total # unique peptides identified | Total # distinct peptides identified | Total # proteins quantified | Protein coverage (%) |
|-----------------|---------------------------------------|---------------|-------------|-------------|-----------------------------------|--------------------------------------|--------------------------|----------------------|
| gi 30519911,   | Transgelin 2                           | N             | 0.35 0.03   | NA NA       | 3                                 | 106                                   | 118                      | 68.3                 |
| gi 6679166      | Osteoglycin                            | C             | 0.16 0      | NA NA       | 1                                 | 44                                    | 66                       | 11.0                 |
| gi 148706007    | Ubiquitin-activating enzyme E1-like 2, | N             | 0 0         | NA NA       | 2                                 | 12                                    | 55                       | 26.1                 |
| gi 3914439      | Proteasome subunit β type-4            | N             | 0 0         | NA NA       | 3                                 | 12                                    | 55                       | 18.3                 |
| gi 18252782     | Serine (or cysteine) proteinase inhibitor, clade C, member 1 | C             | 0 0         | NA NA       | 4                                 | 65                                    | 113                      | 11.2                 |
| gi 1167907,     | α-1(XVIII) collagen                    | C             | 0 0         | NA NA       | 7                                 | 13                                    | 22                       | 11.6                 |
| gi 50190        | β-tropomyosin                          | N             | 0 0         | NA NA       | 1                                 | 49                                    | 76                       | 49.0                 |
| gi 13435747     | Rho GDP dissociation inhibitor (GDI) α | N             | 0 0         | NA NA       | 1                                 | 11                                    | 77                       | 37.3                 |
| gi 12836985,    | A1m-1(XVIII) collagen                  | N             | 0 0         | NA NA       | 2                                 | 2                                    | 3                         | 8.7                  |
| gi 10946870,    | Annexin A1                            | N             | 0 0         | NA NA       | 2                                 | 2                                    | 3                         | 8.7                  |
| gi 94372279     | Annexin A1                            | N             | 0 0         | NA NA       | 2                                 | 2                                    | 3                         | 8.7                  |
| gi 74183811     | Annexin A1                            | N             | 0 0         | NA NA       | 2                                 | 2                                    | 3                         | 8.7                  |
As shown in Figs. 7A and 7B, the treated cells proliferated and differentiated in a fashion similar to the control (i.e. 0 nM OGN), as indicated by the myogenic differentiation marker MyHC being prominently expressed (Fig. 7C). In addition, there was no detectable osteogenic lineage reprogramming, made apparent by the absence of ALP-positive cells, in which ALP was used as the osteogenic differentiation marker (Fig. 7C).

Thus to fully assess the role of each identified secretome factor, several assays may need to be employed to dissect the multitude of possible biological roles played by these factors on the differentiation program. However, as an initial
screening strategy, the siRNA knock down approach coupled with a gain of function analysis, as exemplified by our analysis of spondin-2, demonstrates a robust and expedient approach to determining a potential role for identified secretome components on myogenesis.

**DISCUSSION**

Differential protein expression analyses using SILAC in tandem with high-throughput online RPLC-MS/MS were implemented in an effort to identify and quantify secreted proteins.
during the muscle differentiation program. In total, 34 secreted proteins were quantified, 30 of which were shown to be differentially expressed. Intriguingly, some of these differentially expressed proteins had hitherto not been identified or connected with the myogenic program, and the following discussion will attempt to highlight the potential roles of some of these proteins based on their properties and the existing literature. One advantage of our experimental strategy is that it has allowed us to identify secreted proteins whose abundance decreases as well as increases during the differentiation program. We make the case below that these down-regulated secretome components may be as instrumental to the onset of differentiation as up-regulated components. Moreover, knowledge of factors that contribute to maintaining cells in an “undifferentiated state” will also be important to our understanding of pluripotency and how it is maintained in various stem-cell populations. Even the C2C12 model used here is designated as a pluripotent mesenchymal cell line because, although the cell line was originally derived from skeletal muscle, under appropriate conditions, the cells can commit to adipocyte and osteoblast lineages. Thus, at the precursor state, the cells are pluripotent and the down-regulation of factors that maintain this permissive state is a prerequisite to differentiation down any lineage. In addition to identifying secreted factors that influence the myogenic program, this study may also have recognized novel general components of the secretome in pluripotent mesenchymal progenitors. We have identified several secreted proteins whose concentration is manifestly reduced in differentiating cells (supplemental data S2).

It has been documented that apoptosis may be a stochastic physiological phenomenon associated with the differentiation program (107, 108). By extension, it is possible that higher levels of intracellular proteins in the secretome of cells in the MB state might be due to the adaptation to the differentiation medium, the fact that some cell death does occur and may actually be important for the differentiation under normal conditions makes it very difficult, without further study, to determine the importance of these observations at this point. However, to gain some insight into this question, we carried out quantitative Western blotting for cleaved (activated) caspase-3 under our specialized SILAC condition and also classical culture conditions, i.e., 2% horse serum. As shown in Fig. 8, there may be a slightly higher level of caspase-3 in the MB under SILAC conditions, although there is not much difference in the ratios. Moreover, we were surprised to observe that there was a small decrease in activated caspase between the MBs and MTs in both sets of conditions because one might have predicted larger differences that were not observed. At this point we cannot rule out that some previously characterized proteins (or peptides derived from them) that have an intracellular function may also have an independent role in the secretome or that these proteins are a consequence of the small degree of cell death that occurs in the normal differ-

![Secretome Analysis During Skeletal Myogenesis](image)

**FIG. 8.** Comparison of apoptosis between SILAC and 2% horse serum (HS) culture condition. Cleaved (activated) caspase-3 was implemented as the apoptotic indicator in both myoblast (MB) and myotube (MT) state.

One important observation for our understanding of the differentiation program is that, for many years, it has been known that replacement of the mitogen-rich medium in myoblast cultures by serum withdrawal is a key initiating factor for the onset of differentiation. Thus the reduction, but not elimination, of extracellular factors is a potent signal for myogenic differentiation, and this may include reduction of myoblast secreted factors as well as exogenously provided serum components. For example, it is known that TGF-β is secreted by MBs, even though it is repressive to the progression of the differentiation program. Therefore, down-regulation of TGF-β in the secretome is a prerequisite to the onset of the differentiation program, and this has been proven using neutralizing antibodies (70, 72, 109–116). Hence, we have carefully considered the biology of some of the confirmed down-regulated as well as up-regulated secreted factors in our analysis.

One of these down-regulated factors is Fstl-1, also known as TSC36; this protein was initially identified as a TGF-β regulated-secreted protein which was derived from mouse osteoblast cells (117). During development, Fstl-1 is first expressed in the mesenchymal component (118), and subsequently regionalized in the dorsomedial compartment of the somite (119). The exact role of Fstl-1 is still unknown, although it should be noted that the general properties of the follistatin gene family protein products are to antagonize TGF-β signaling, and TGF-β is a well-known negative regulator of myogenesis (70, 72, 110, 115, 119–121). It is tempting to postulate that Fstl-1 may function as a positive regulator of myogenesis by counteracting the inhibitory effect of TGF-β. In consensus with this, knock down of Fstl-1 decreased the transcriptional activity of the MCK differentiation marker by 1.7-fold (Fig. 6A). MCK was used as a molecular marker of the muscle differentiation program. In addition, it has been demonstrated that augmenting the circulating level of Fstl-1 promoted muscle growth (122). As Fstl-1 was more abundant in MB (Table I), we speculate that Fstl-1 may be involved in the early phase of myogenesis, such as cell-cycle exit and/or cell migration, which has been documented in other biological models, such as endothelial cells (83), and ovarian and endometrial cancer cells (123). Hence, it would be of interest to examine whether Fstl-1 mediates a similar effect in skeletal muscle.
Another down-regulated factor is OGN (also termed mimecan), which is an extracellular matrix component belonging to the small leucine-rich proteoglycan gene family (SLRP). The prominent role attributed to this factor is collagen fibrillogenesis: this protein is abundant in cartilage connective tissues and bone matrices (105, 124). As discussed earlier, muscle progenitor cells exposed to appropriate cues are permissive to committing toward an osteogenic lineage. It is known that BMP can induce an osteoblast phenotype even in the C2C12 model used in our studies (125–132). Moreover, we have also recently reported that down-regulation of the Men-1 gene product (Menin) is a prerequisite to myogenesis, and maintenance of its expression favors osteoblast, rather than myoblast, commitment in multipotent mesenchymal cells such as C2C12 and C3H10T1/2 cell lines (106). To test this hypothesis, C2C12 cells were treated with purified OGN at various concentrations. As shown in Figs. 7A and 7B, there was no prominent effect on cell proliferation and differentiation in the presence of OGN. In addition, there was no notable osteogenic-lineage commitment in the OGN-treated myogenic cells, as observed by the absence of ALP-positive cells (Fig. 7C). Much to our surprise, knock down of OGN decreased MCK transcriptional activity by 1.6-fold (Fig. 6A). We rationalize that these findings nonetheless provide new insight into the role of OGN, which may only influence the early stages of myogenesis. The observation that purified OGN did not ultimately result in a demonstrable difference in the differentiation of the cells likely reflects that it is not absolutely required for the program to reach its conclusion, even though it can modulate it. This is often seen for developmentally important regulators as redundancy is inherently built into most critical processes.

Peroxiredoxin-1 (Prx-1) is another down-regulated protein of some interest: it is known to orchestrate the cell cycle via interactions with various cellular proto-oncogenes such as c-Myc (133) and c-Abl (134). It has been found to be expressed in proliferative cells and deregulated in some cancer cells, suggesting that it may play a role in dividing cells (135–137). In addition, it was found to be secreted from A549 cells (135). Cell-cycle withdrawal is an essential feature of MB differentiation and, as stated above, the down-regulation of secreted factors that promote cell division is crucial for differentiation to proceed. Studies will need to be designed to dissect the possible extracellular role of Prx-1 because it has a well-demonstrated intracellular role in interacting with proto-oncogenes. At this point, there is no evidence that the secreted form of Prx-1 plays any role in proliferation and we plan to address this by supplementing the media of cells with a Prx-1 neutralizing antibody or purified Prx-1 protein.

The above discussion has focused on down-regulated secretome factors during myogenic differentiation; there are, however, some novel up-regulated secretome factors that also deserve consideration. CIAPIN-1, also known as anamorsin, was originally isolated from a mouse interleukin-3 (IL-3)-independent cell line, which conferred resistance to factor-deprived apoptosis (138). According to Table I, CIAPIN-1 was solely expressed in MT. Knock down of CIAPIN-1, however, did not significantly alter the transcriptional activity of MCK (Fig. 6A). This in part may be attributable to the presence of other functionally redundant factor(s), which could compensate for the loss of CIAPIN-1, and thus maintain the expression of MCK. Furthermore, one might speculate that the abundance of CIAPIN-1 at the later stages of myogenesis (i.e. MT) suggests a postdifferentiation role in maintaining a subset of quiescent muscle stem cells, the satellite cells, which reside along the muscle fibers beneath the basal lamina (139–141). This satellite cell population is crucial for postnatal muscle regeneration (reviewed in 142–148). CIAPIN-1 has been shown to inhibit renal carcinoma cell proliferation by arresting cancer cells in the G1/S phase of the cell cycle (149–151). This suggests that the antiproliferative effect of CIAPIN-1 could promote the survival of satellite cells in the in vivo setting, which may have therapeutic implications for various muscle-wasting syndromes, including Duchenne muscular dystrophy and cachexia.

In addition to differentially expressed proteins, some proteins whose abundances do not change substantially upon myogenesis are also of interest. An example of such a protein is fibulin-2, a 1195-residue polypeptide preceded by a 26-residue signal peptide that mainly resides in the basement membranes and other connective tissue compartments (152). Fibulin-2 is expressed in heart, placenta, and ovarian tissues (153), and has been shown to link the cells to the basement membrane in epithelial (154) and hematopoietic cells (155). This linkage contributes to cell migration in epicardial cells during coronary vasculogenesis and angiogenesis (156), and in smooth muscle cells during atherosclerotic lesions (157). In addition, fibulin-2 was persistently expressed in myocardial cells (156) and the adult heart valves to maintain tensile strength of the cardiac valves (158). This persistent expression was also observed in our skeletal muscle model, in which fibulin-2 was constitutively expressed in both MB and MT (Table I). Taking into account of the fibulin-2 knock down data, in which MCK transcriptional activity was decreased by 1.8-fold (Fig. 6A), we hypothesize that fibulin-2 may be a housekeeping protein entailed in cell-cell interaction, cell migration, and extracellular matrix (ECM) remodelling, which may provide a foundation in myogenesis. Within our siRNA-mediated knock down data, TIMP-2 and spondin-2 exhibited a prominent yet opposite effect on myogenesis: knock down of TIMP-2 enhanced MCK transcription by 1.5-fold, whereas that of spondin-2 inhibited MCK transcription by 2.6-fold (Fig. 6A).

TIMP-2 is an inhibitor of matrix metalloproteinases (MMPs), which are zinc-dependent neutral endopeptidases responsible for releasing growth factors from the ECM and for processing growth factor receptors (159). In addition, MMPs can degrade all ECM constituents to facilitate cell migration and...
tissue remodelling (160). MMP activity is inhibited by interaction with TIMPs in a 1:1 stoichiometric ratio (161). This ratio is critical in modulating muscle cell migration and differentiation, and in maintaining homeostasis of the muscle fibers (162). Intriguingly, overexpression of TIMP-2 decreased myogenin expression, which implies that TIMP-2 may function as a negative regulator of myogenesis (163). This is in accordance with our observation that knock down of TIMP-2 rescues MCK transcription (Fig. 6A). Interestingly, there are multiple E-box consensus sequences in the mouse TIMP-2 promoter (164), which raises the slightly unusual possibility that TIMP-2 expression may be negatively regulated by MRFs in the myogenic cascade.

Spondin-2 knock down diminished the transcription of MCK by 2.6-fold (Fig. 6A). Conversely, MCK transcriptional activity was enhanced by 9.2-fold with exogenous overexpression of spondin-2 (Fig. 6B). These data suggest that spondin-2 is a potent positive regulator of myogenesis, which has hitherto not been observed. In developing hippocampus, mindin-2 (the zebrafish homolog of spondin-2) promotes adhesion and outgrowth of hippocampal embryonic neurons (165, 166). In addition, spondin-2 has also been implicated in innate immunity, in which it bounds to bacteria as an opsonin (165, 166). In addition, spondin-2 has also been implicated in innate immunity, in which it bounds to bacteria as an opsonin for subsequent macrophage phagocytosis (167, 168). In the context of tumor growth, spondin-2 exerts different effects depending on the cell type: in ovarian cancer, spondin-2 was overexpressed and deployed as a new serum diagnostic biomarker (169); analogously, in prostate cancer, spondin-2 was found to be overexpressed in >80% of the prostate cancers metastatic to bone or the lymph nodes, as well as in locally recurrent tumors in androgen-unresponsive patients. Prominent antitumor effects were achieved with a single administration of radiolabeled spondin-2-antibodies to animals (170). In contrast, overexpression of spondin-2 was shown to moderate the invasiveness of hepatocellular carcinoma by attenuating cell migration (171). In view of the extensive data from other cell types, we postulate that spondin-2 may regulate myogenesis with respect to cell proliferation and migration.

During the preparation of this current manuscript, a report by Henningsen et al. (172) was published describing an approach to characterizing the myogenic secretome that was very similar to our current study. In general, there is some agreement between the results of the two studies, and this indeed provides added confidence that proteins identified in both studies are bona fide secretome components. However, there are also significant discrepancies that are probably attributable to a combination of differences in cell culture and also data analyses. These discrepancies underscore the complexity in conducting secretome analysis and should serve as the stimulus for additional studies.

While the discussion above does not consider every individual identified protein, it does illustrate the potential importance of up- and down-regulated secretome components for the biology of the system. It should, however, be acknowl-
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In order to cite this article properly, please include all of the following information: Chan, C. Y. X., Masui, O., Krakovska, O., Belozerov, V. E., Voisin, S., Ghannya, S., Chen, J., Moyez, D., Zhu, P., Evans, K. R., McDermott, J. C., and Siu, K. W. M. (2011) Identification of Differentially Regulated Secretome Components During Skeletal Myogenesis. Mol. Cell. Proteomics 10(5):M110.004804. DOI: 10.1074/mcp.M110.004804.