Identification and quantification of the secretome during skeletal myogenesis

Chi Yuet Chan

A dissertation submitted to the Faculty of Graduate Studies

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Programme in Biology

York University

Toronto, Ontario, Canada

August 2011

Your file Votre référence
ISBN: 978-0-494-80525-1
Our file Notre référence
ISBN: 978-0-494-80525-1

Abstract

Myogenesis, the formation of skeletal muscle, is a multi-step event that commences with myoblast proliferation, followed by cell-cycle arrest, and ultimately the formation of multinucleated myotubes via fusion of mononucleated myoblasts. Each step is orchestrated by well-documented intracellular factors, such as cytoplasmic signaling molecules and nuclear transcription factors. Regardless, the key step in obtaining a more comprehensive understanding of the regulation of myogenesis is to explore the extracellular factors that are capable of eliciting the downstream intracellular factors. This could further provide valuable insight into the acute cellular response to extrinsic cues in maintaining normal muscle development.

Intriguingly, the effects exerted by the 'conditioned' media on the development of muscle cells have been documented, illustrating the phenomena that myogenic cells modify their own extracellular milieu by secreting factors that exert autocrine and paracrine effects on the differentiation program. Furthermore, the skeletal muscle has been recognized as the largest endocrine organ in humans for secreting extracellular factors, the myokines that orchestrate muscle development in an autocrine fashion. To make progress on the characterization of the 'secretome' (i.e., profile of secreted factors) in an unbiased manner, we launched a study using a mass spectrometry-based proteomics approach to identify secreted proteins of a mammalian skeletal muscle cell line (Chapter 3). Subsequently, we implemented a high throughput quantitative proteomics approach using stable isotope labelling by amino acids in cell culture (SILAC) in conjunction with online reverse phase (RP) liquid chromatography-tandem mass spectrometry (LC-MS/MS), to identify differentially expressed secreted proteins during myogenesis (Chapter 4). The studies described herein contribute to a further understanding of the regulation of myogenesis by the extracellular milieu.

Dedications

To my beloved parents, and sister Jenny.

In memory of my loyal brother Joseph Chan.

Acknowledgements

For my seven years of stay here in Canada, one word to conclude is 'extraordinary'. Everything is unexpected started from day 1 in York when I enrolled as a graduate student. Even at this moment, I am still amazed by my supervisors, Prof. John McDermott and Prof. Michael Siu for their 'guts' to have me in their research groups. Prof. McDermott, thank you for always being an encouraging mentor with a super high EQ and patience with students (I do not think anyone will disagree on this). Prof. Siu, you are a very sharp, persevere and insightful person. For all the honest advices, passion and care, I will bring them along with me wherever I go.

On top of that, I would also like to thank all the past and current fellows in both research groups for their support and participation in various events, in particular the Chinese New Year lunch buffet!

Last but definitely not the least; I would like to express my sincere gratitude to my parents who are always supportive, caring and awesome. Thank you for allowing me to fly halfway around the world and expose in a different continent which gives me a very unique lesson of life. Needless to say, my elder sister Jenny, you are my real best friend as always! Thank you for taking care of everything while I am away from home. We will soon be reunited, and I am looking forward to it, as well as the next chapter of my journey.

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Abbreviations

1D-SDS PAGE	one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
ACN	acetonitrile
ALP	alkaline phosphatase
bHLH	basic helix-loop-helix
BMP-2	bone morphogenetic protein-2
bp	base pair
BSA	bovine serum albumin
C	classical secreted proteins
CBP	CREB-binding protein
CDKIs	CDK inhibitors
CDKs	cyclin-dependent kinases
CIAPIN-1	cytokine-induced apoptosis inhibitor-1
CID	collision-induced dissociation
CM	conditioned media
CREB	cAMP response element-binding

CRM	charge residue model
CV	coefficient of variation
DC	direct current
DDA	data-dependent acquisition
DM	differentiation medium
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle's medium
DMEM/F12	Dulbecco's MEM:Ham's Nutrient Mixture F-12 medium
DML	dorsal medial lip
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECM	extracellular matrix
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
ESI	electrospray ionization
FA	formic acid
FBS	fetal bovine serum

FDR	false discovery rate
FGF	fibroblast growth factor
Fstl-1	follistatin like protein-1
GM	growth medium
H	heavy [$^{13}C_6$]-lysine
HATs	histone acetyltransferases
HDACs	histone deacetylases
HRP	horseradish peroxidase
HS	horse serum
I	intracellular proteins
ICATs	isotope-coded affinity tags
ICC	immunocytochemistry
ID	identification
IEM	ion evaporation model
IGF1	insulin-like growth factor-1
IL-3	interleukin-3
IPS	induced pluripotent stem cells

iTRAQ	isobaric tag for relative and absolute quantitation
L	light [$^{12}C_6$]-lysine
LIT	linear ion trap
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MBs	myoblasts
MCK	muscle creatine kinase
MEF2	myocyte enhancer factor-2
MIF	macrophage migration inhibitory factor
MMPs	matrix metalloproteinases
MRFs	muscle regulatory factors
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTs	myotubes

MyHC	myosin heavy chain
N	non-classical secreted proteins
NAC-α	NAC alpha domain containing-protein
OD	optical density
OGN	osteoglycin
PBS	phosphate-buffered saline
PCEP	procollagen C-proteinase enhancer protein
PEDF	pigment epithelium-derived factor
PI3K	phosphatidylinositol-3-kinase
PMF	peptide mass fingerprinting
pol II	RNA polymerase II
pRb	retinoblastoma protein
Prx-1	peroxiredoxin-1
q	high pass mass filter
Q	selective mass filter
QqTOF	quadrupole/time-of-flight
Quan	quantification

RF	radio frequency
RLU	relative luciferase unit
RPLC	reverse phase liquid chromatography
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
Serpin	serine protease inhibitors
SILAC	stable isotope labelling with amino acids in cell culture
SLRP	small leucine-rich proteoglycan gene family
SMCs	smooth muscle cells
SPARC	secreted protein acidic and rich in cysteine
TCTP-1	translationally controlled tumour protein-1
TFA	trifluoroacetic acid
TGF	transforming growth factor
TIMP-2	tissue inhibitor of metalloproteinase-2
TOF	time-of-flight
VLL	ventral lateral lip

Chapter 1:

Introduction

Embryogenesis

The process by which an embryo forms and develops is called embryogenesis. It commences with the fusion of an egg and a sperm during fertilization by which a zygote is generated. This is followed by a series of successive cell cleavages which results in a vesicular blastocyst comprised of trophoectoderm encasing primitive endoderm and epiblast **(Figure 1)**.

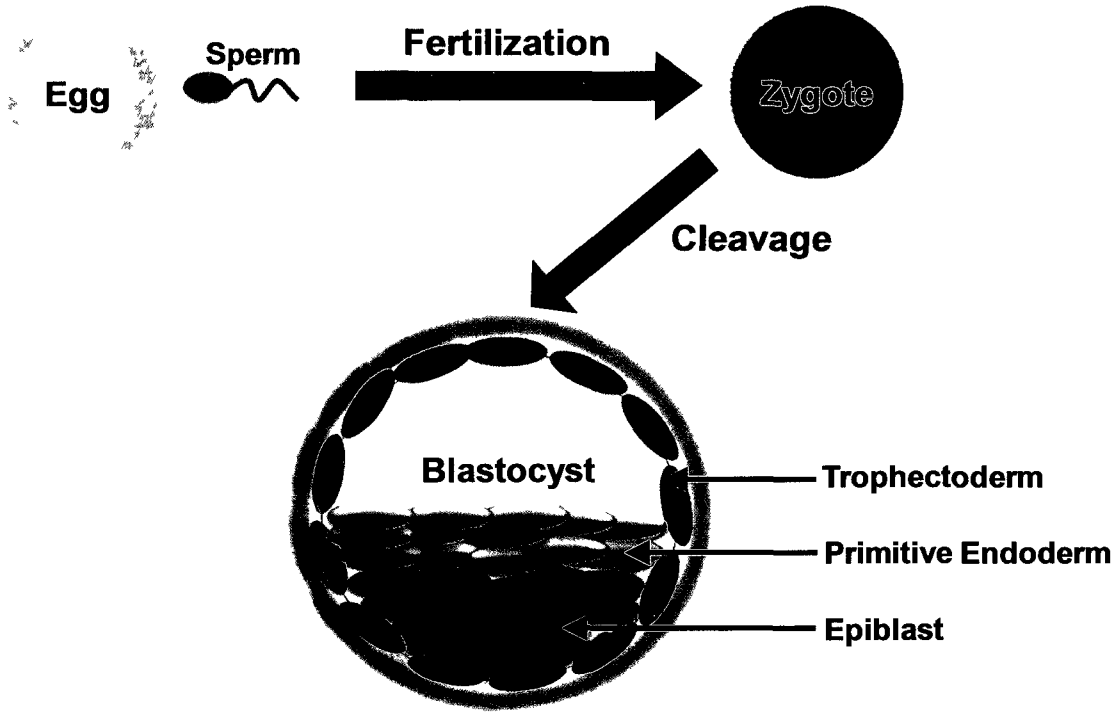


Figure 1. Blastocyst formation during embryogenesis.

Only cells from the epiblast will give rise to the tissues of the embryo, while trophoectoderm and primitive endoderm are engaged in the formation of the extra-embryonic structures to support the intra-uterine development of the embryo (Murry, C.E. and Keller, G., 2008; Rossant, J. and Tam, P.P., 2009).

Prior to embryo formation, gastrulation takes place by which epiblast cells are transformed into three primary germ layers: ectoderm, mesoderm, and endoderm, each of which is committed and differentiated into diverse cell types **(Figure 2)**.

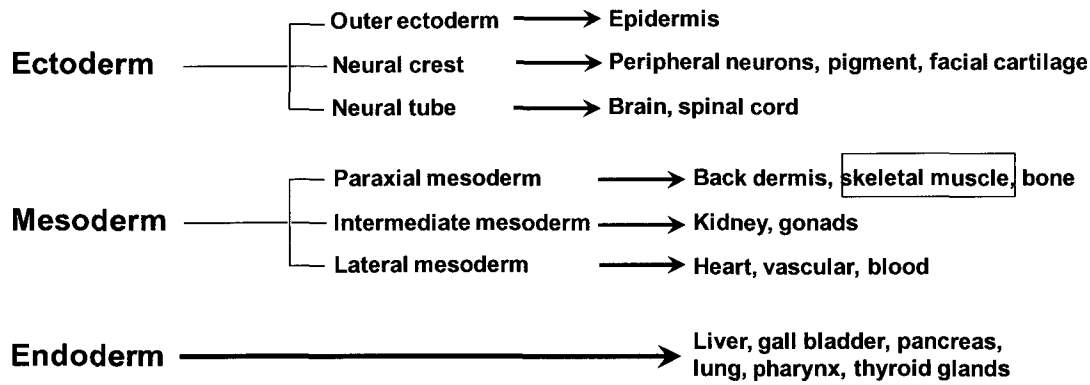


Figure 2. Cell lineages of ectoderm, mesoderm, and endoderm.

Somitogenesis

As shown in **Figure 2**, skeletal muscle is derived from the paraxial mesoderm, which is composed of loosely packed mesenchymal cells formed lateral to the neural tube and notochord on both sides beneath the ectoderm. Upon differentiation or somitogenesis, these cells undergo epithelialization and segregation in a cranio-caudal order (i.e., from head to tail) to generate somites. Various compartments of the somite are specified and committed to distinct cell lineages: the ventral medial compartment is the sclerotome which forms the vertebrae and ribs; whereas the double-layered structure remaining is the dermomyotome which encompasses the dermatome and myotome, in which the former gives rise to the back epidermis, whereas the latter is divided into two muscle lineages: (1) the epaxial myotome located at the dorsal medial lip (DML) which corresponds to the back muscle; (2) the hypaxial myotome at the ventral lateral lip (VLL) which gives rise to the muscles of the body wall, limbs, and tongue (Ordahl, C. P. and Le Douarin, N.M., 1992; Pourquie, O. *et al.*, 1993; Christ, B. and Ordahl, C.P., 1995; Kato, N. and Aoyama, H., 1998) **(Figure 3).**

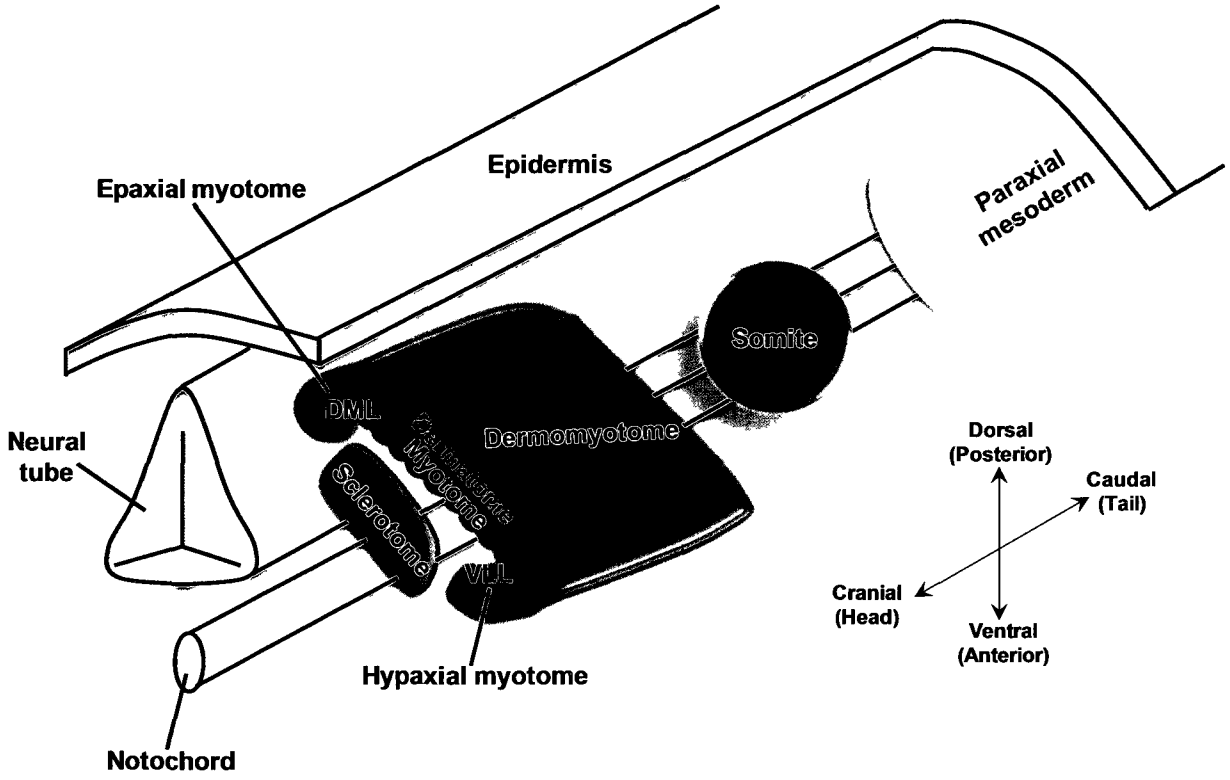


Figure 3. Somitogenesis. Somites are originated from the paraxial mesoderm which epithelializes and segregates in a cranio-caudal order during somitogenesis. Various compartments of the somite are committed to distinct cell lineages: sclerotome (bone), dermatome (skin), and myotome (muscle). Dorsal medial lip and ventral lateral lip are denoted as DML and VLL, respectively.

Myogenesis at the microscopic- and molecular-level

After somitogenesis, the primary wave of muscle precursor cells or myoblasts (MBs) undergoes myogenesis, in which well-differentiated muscle fibres are generated. This process is a multi-step event which commences with iterative proliferation of MBs, followed by cell-cycle exit, in which the mononucleated MBs stop propagating and ultimately fuse with each other to form multinucleated myotubes (MTs), the building blocks of the contractile muscle fibres. This is accompanied by the concomitant expression of muscle-specific genes, such as myosin heavy chain (MyHC), muscle creatine kinase (MCK) **(Figure 4A)**.

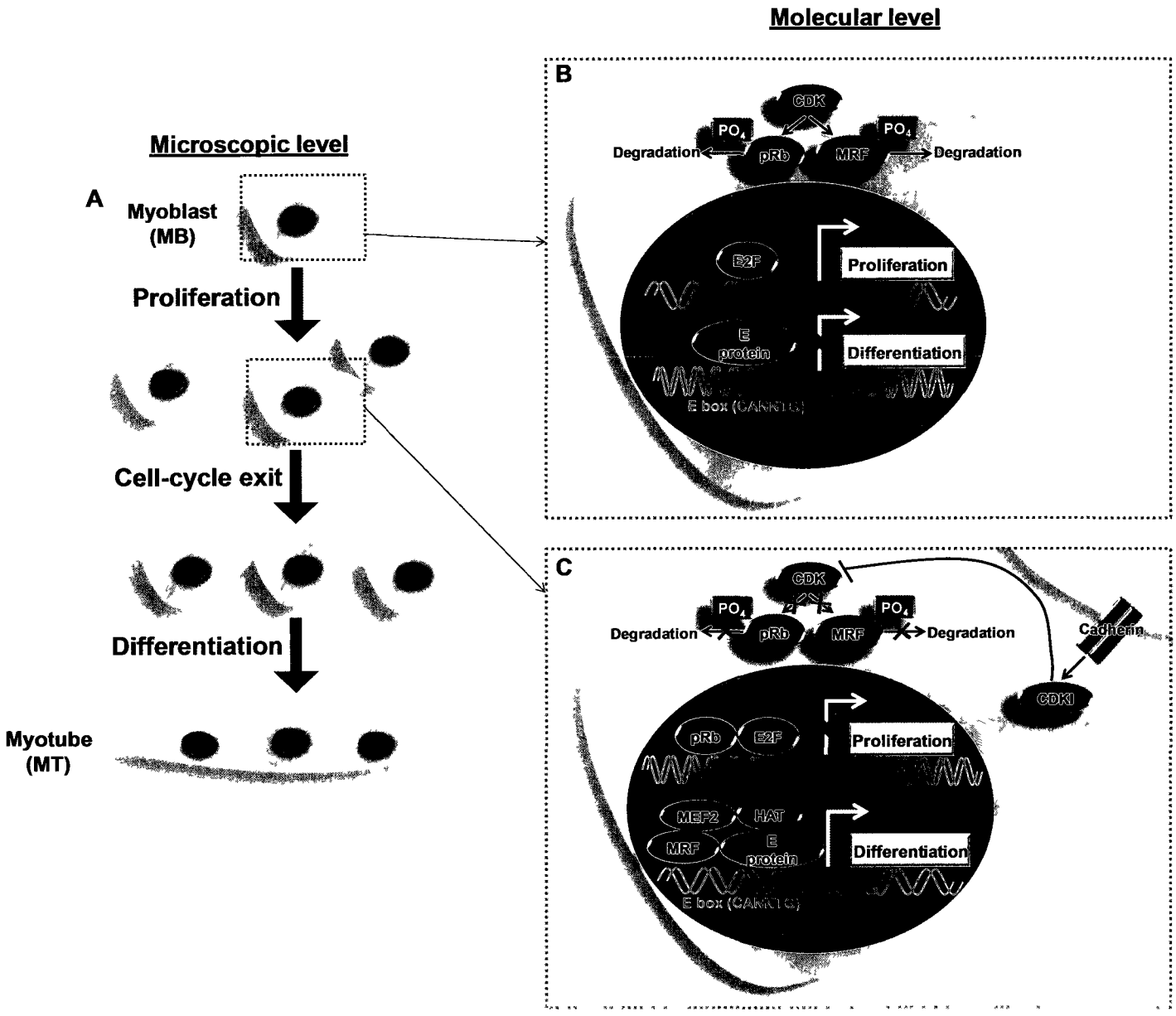


Figure 4. Skeletal myogenesis at the microscopic- and molecular-level. (A) During myogenesis, mononucleated myoblasts (MB) proliferate, followed by cell-cycle exit, and fusion to form multinucleated myotube (MT); **(B)** during proliferation, active cyclin-dependent kinase (CDK) triggers MB proliferation by phosphorylating and subjecting retinoblastoma protein (pRb) to degradation, in which E2F transcription factor is free

from the inhibitory effect of pRb and elicits the proliferation of MBs. Simultaneously, CDK sabotages muscle differentiation via phosphorylation-mediated degradation of muscle regulatory factors (MRFs). As a result, E protein by itself cannot drive the differentiation program; **(C)** upon cell-cell contact prevailed in a confluent MB population, cadherin is activated, by which CDK inhibitor (CDKI) is induced. This in turn inhibits CDK from phosphorylating its downstream substrates: pRb and MRF. Hence, both pRb and MRF are exempted from degradation, in which pRb complexes with the E2F transcription factor and inhibits it from activating the proliferation-associated events, hence the cells are subjected to the cell-cycle exit; whereas MRF dimerizes with the E protein, along with the co-activator myocyte enhancer factor-2 (MEF2), and the chromatin-remodeling molecule histone acetyltransferase (HAT), to synergistically evoke the muscle differentiation program. Phosphate groups are indicated as "PO_4".

At the molecular level, this hierarchical process is orchestrated by two groups of transcriptional regulators. The first group are the muscle regulatory factors (MRFs), which comprise Myf5 (Braun, T. *et al.*, 1989; Tajbakhsh, S. *et al.*, 1996a), MyoD (Davis, R.L. *et al.*, 1987; Choi, J. *et al.*, 1990), myogenin (Edmondson, D.G. and Olson, E.N., 1989; Wright, W.E. *et al.*, 1989; Hasty, P. *et al.*, 1993; Nabeshima, Y. *et al.*, 1993), and MRF4 (Rhodes, S.J. and Konieczny, S.F., 1989). These MRFs belong to the basic helix-loop-helix (bHLH) family, which heterodimerize with another bHLH member called the

E proteins (E12, E47, HEB). The dimer thus formed binds to the consensus DNA sequence (CANNTG) named the E box, which is present in the promoters of most muscle-specific genes (Lassar, A.B. *et al.*, 1991). The second group of factors are the myocyte enhancer factor-2s (MEF2s), which belong to the MADS box family comprised of four isoforms MEF2A, MEF2B, MEF2C, and MEF2D. These isoforms homo- or hetero-dimerize with each other and bind to the consensus AT-rich sequence ((C/T)TA(A/T)$_4$TA(G/A)), which is also found in the promoters of many muscle-specific genes (Gossett, L.A. *et al.*, 1989; Pollock, R. and Treisman, R., 1991; Yu, Y.T. *et al.*, 1992; Olson, E.N. *et al.*, 1995; Brand, N.J., 1997; Black, B.L. and Olson, E.N., 1998; Naya, F.J. and Olson, E., 1999). MEF2 functions as a co-activator of MRFs to synergize in the muscle differentiation program (Molkentin, J.D. *et al.*, 1995; Molkentin, J.D. and Olson, E.N., 1996).

One key feature of skeletal myogenesis is the mutually exclusive occurrence of cell proliferation and differentiation; the tipping point between the two is governed by a master regulator: the retinoblastoma protein (pRb) (Benedict, W.F. *et al.*, 1983; Cavenee, W.K. *et al.*, 1985; Friend, S.H. *et al.*, 1986; Lee, W.H. *et al.*, 1987; Huang, H.J. *et al.*, 1988).

During proliferation, cyclin/cyclin-dependent kinases (CDKs), such as cyclin A/CDK2, cyclin D/CDK4, cyclin D/CDK6, and cyclin E/CDK2, are active. These kinases phosphorylate pRb, sequestering it inactive (Buchkovich, K. *et al.*, 1989; Chen, P.L. *et al.*, 1989; DeCaprio, J.A. *et al.*, 1989; Kato, J. *et al.*, 1993; Obeyesekere, M.N. *et al.*, 1995; Harbour, J.W. *et al.*, 1999). As a consequence, pRb is unable to bind to the E2F transcription factor complex and inhibit its activation of downstream proliferation-associated cellular events, including chromosome segregation, mitotic spindle formation, and chromatin

remodeling (Ren, B. *et al.*, 2002). Simultaneously, MBs are detained from differentiation by repressing the MRFs in various ways: firstly, E2F transcription factor not only regulates genes essential for cell proliferation, but also functions as a transcriptional repressor of MyoD and myogenin (Wang, J. *et al.*, 1996); secondly, analogous to pRb, MyoD is also phosphorylated by CDK and subjected to degradation (Song, A. *et al.*, 1998; Kitzmann, M. *et al.*, 1999; Kitzmann, M. and Fernandez, A., 2001). As a result, MB population propagates sporadically while its differentiation program is prohibited **(Figure 4B)**. This initial repression of muscle differentiation is essential for ensuring a sufficiently large number of muscle precursor cells are attained prior to differentiation to populate the vast amount of skeletal musculature in the metazoan species.

After iterative cycles of proliferation, the MB population becomes confluent and cell-cell contact prevails. It is evident that cell-cell contact or the 'community effect' is essential to trigger the cell-cycle exit, and subsequently terminal muscle differentiation (Gurdon, J.B., 1988; Gurdon, J.B. *et al.*, 1993; Cossu, G. *et al.*, 1995). The switch between cell-cell contact and cell-cycle exit is governed by transmembrane proteins, such as cadherins (Hatta, K. *et al.*, 1987; Knudsen, K.A. *et al.*, 1990; Holt, C.E. *et al.*, 1994; Zeschnigk, M. *et al.*, 1995; George-Weinstein, M. *et al.*, 1997; Goichberg, P. and Geiger, B., 1998). In a highly-confluent MB pool cell-cell contact provokes the activation of cadherins. This in turn induces CDK inhibitors (CDKIs), such as p21 and p57. As the name suggests, CDKI inhibits CDK from phosphorylating its respective substrates, i.e., pRb and MyoD (Gavard, J. *et al.*, 2004; Messina, G. *et al.*, 2005). The corollary of that is two-fold: (1) non-phosphorylated pRb is exempted from degradation, binds and inhibits E2F from activating proliferation events, thus cell cycle-exit is elicited (Nevins, J.R., 1992;

Flemington, E.K. *et al.*, 1993; Schneider, J.W. *et al.*, 1994; Harbour, J.W. and Dean, D.C., 2000; Frolov, M.V. and Dyson, N.J., 2004); (2) in similar fashion, non-phosphorylated MyoD is spared from degradation (Holt, C.E. *et al.*, 1994; George-Weinstein, M. *et al.*, 1997; Reynaud, E.G. *et al.*, 2000; Tintignac, L.A. *et al.*, 2004), and subsequently dimerizes with the E protein to set the differentiation program in motion **(Figure 4C)**. In addition, MyoD induces CDKI (Guo, K. *et al.*, 1995; Halevy, O. *et al.*, 1995) and pRb (Martelli, F. *et al.*, 1994). These positive feedback loops are pivotal to reinforce the irreversible or terminal nature of myogenesis, in which MBs withdrawn from the cell cycle are not allowed to re-enter the proliferation cycle, but terminally differentiate into MTs.

Chromatin remodeling and myogenesis

The genetic code deoxyribonucleic acid (DNA) is packaged into a compact structure called chromatin by wrapping approximately every 200 base pairs (bp) of DNA around an octamer of histone proteins which corresponds to the basic repeating unit named nucleosome (Kornberg, R.D., 1974; Kornberg, R.D. and Thomas, J.O., 1974; Olins, A.L. and Olins, D.E., 1974; Luger, K. *et al.*, 1997; Kornberg, R.D. and Lorch, Y., 1999). Further coiling and looping confers an additional level of chromatin packaging and results in a highly condensed structure called chromosome **(Figure 5)**.

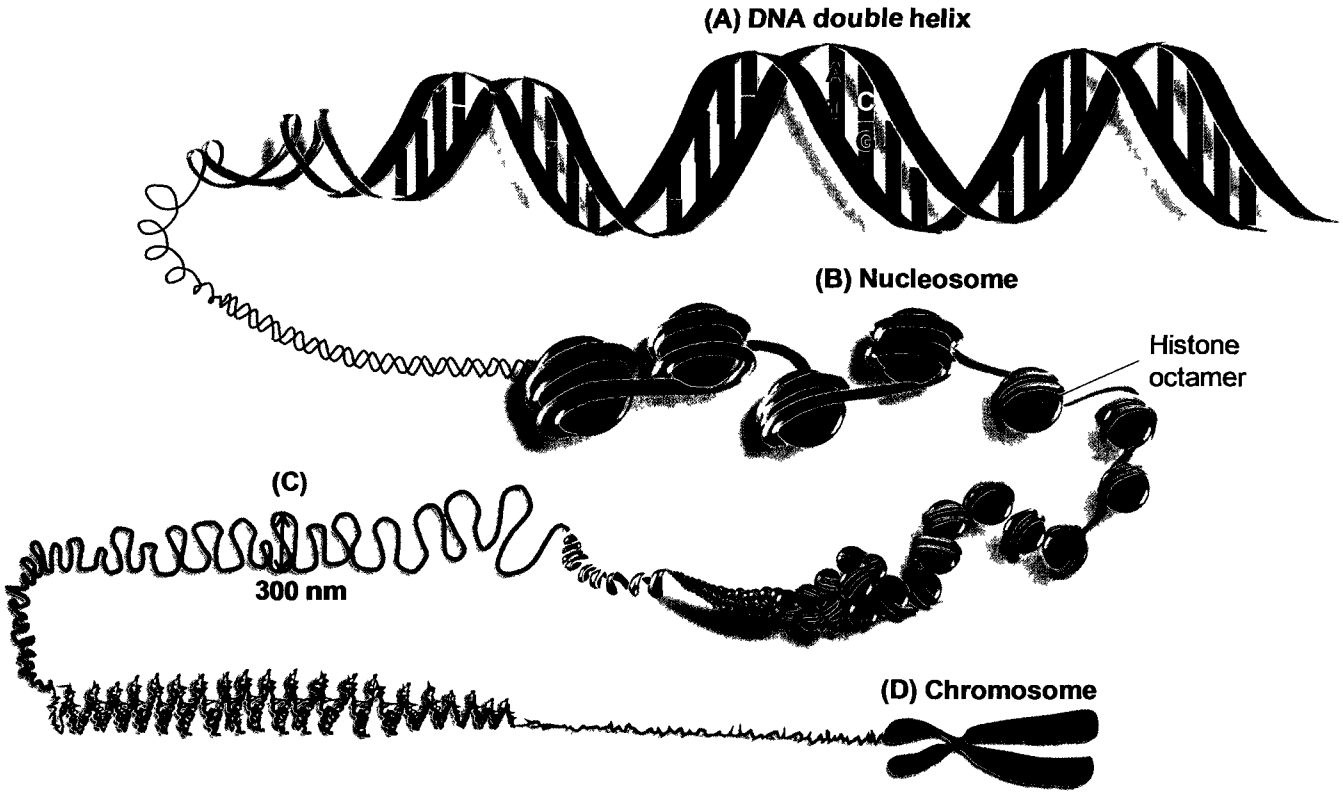


Figure 5. Hierarchical packaging of chromatin. (A) DNA is a double-stranded helical structure with complementary base-pairing between adenine (A) and thymine (T); cytosine (C) and guanine (G); **(B)** the first level of chromatin packaging is accomplished by wrapping ~200 bp of DNA around an octamer of histone proteins, which corresponds to the basic repeating unit of the chromatin called nucleosome; **(C)** further coiling and looping condenses the chromatin into a 300 nm-wide fiber; **(D)** with additional level of coiling and looping, the chromatin is compressed into an extensively compact structure called chromosome.

This highly condensed organization of chromatin impedes RNA polymerase II (pol II) from accessing the DNA to elicit transcription, and thus the subsequent gene expression (Orphanides, G. *et al.*, 1996; Struhl, K., 1996; Nikolov, D.B. and Burley, S.K., 1997; Kornberg, R.D. and Lorch, Y. 1999; Venters, B.J. and Pugh, B.F., 2009a; Venters, B.J. and Pugh, B.F., 2009b). To initiate gene expression, the chromatin has to be remodelled from a compact structure (i.e., transcriptionally repressive) to a relaxed conformation (i.e., transcriptionally active). The conformational changes of the chromatin are modulated by the chromatin-remodeling complexes, which are recruited by the cognate transcription factors (Latchman, D.S., 1997; Kornberg, R.D. and Lorch, Y., 1999; Lorch, Y. *et al.*, 1999; Strahl, B.D. and Allis, C.D., 2000; Jenuwein, T. and Allis, C.D., 2001; Narlikar, G.J. *et al.*, 2002). There are two major classes of chromatin-remodeling complexes. The first class is ATP-dependent complexes, such as SWI/SNF, which utilize ATP as the energy source to expose DNA sequences by sliding the nucleosome positions (de la Serna, I.L. *et al.*, 2001; Sif, S., 2004; de la Serna, I.L. *et al.*, 2005). The second class is histone acetyltransferases (HATs), such as cAMP response element-binding (CREB)-binding protein (CBP)/p300, which neutralizes the positively charged amine groups of the histones by acetylation, and attenuates their binding affinities to the DNA. As a consequence, a loosened potentially transcriptionally active conformation of the chromatin is induced. By contrast, histone deacetylases (HDACs) erase the the effect of HATs by deacetylation to restore the compact transcriptionally inactive conformation of the chromatin. As such, HATs and HDACs are generally perceived as positive and negative regulators of myogenesis, respectively (McKinsey, T.A. *et al.*, 2001; Polesskaya, A. *et al.*, 2001).

During muscle proliferation and differentiation, HATs and HDACs are differentially recruited to a subset of genes to orchestrate the two mutually exclusive events. During proliferation, HDAC physically interacts with MyoD (Puri, P.L. *et al.*, 2001) to diminish its transcriptional activity on the target genes, such as CDKI (Mal, A. *et al.*, 2001), in which CDK remains active to subject both pRb and MyoD to degradation. As a result, E2F transcription factor triggers cell proliferation in the absence of pRb, while the absence of MyoD sabotages the differentiation program. In addition, HDAC also associates with the myogenic co-activator MEF2 and debilitates its pro-myogenic activity (Miska, E.A. *et al.*, 1999; Lu, J. *et al.*, 2000; Dressel, U. *et al.*, 2001; Chan, J.K. *et al.*, 2003; Haberland, M. *et al.*, 2007; McGee, S.L., 2007). Upon differentiation, HDAC sequentially dissociates from MyoD and associates with pRb-E2F complex to trigger the cell-cycle exit and differentiation (Puri, P.L. *et al.*, 2001). Furthermore, the transcriptional activator HATs are recruited to MyoD (Eckner, R. *et al.*, 1996; Yuan, W. *et al.*, 1996; Gerber, A.N. *et al.*, 1997; Sartorelli, V. *et al.*, 1999; Polesskaya, A. *et al.*, 2000; Polesskaya, A. and Harel-Bellan, A., 2001; Polesskaya, A. *et al.*, 2001a; Dilworth, F.J. *et al.*, 2004), and MEF2 (Sartorelli, V. *et al.*, 1997; Ma, K. *et al.*, 2005), to induce CDKI (Puri, P.L. *et al.*, 1997a; Puri, P.L. *et al.*, 1997b) and pRb (Magenta, A. *et al.*, 2003), in an effort to elicit muscle differentiation.

Signaling pathways and myogenesis

The differential recruitment of the chromatin-remodeling complexes during myogenesis is responsive to the extracellular environment or stimuli, which are conveyed

by the cellular regulatory circuits or signaling pathways (Whiteside, S.T. and Goodbourn, S., 1993; Brivanlou, A.H. and Darnell, J.E., Jr., 2002). There are three major signaling pathways involved in myogenesis: (1) extracellular signal-regulated kinases signaling; (2) p38 signaling; (3) phosphatidylinositol-3-kinase-Akt signaling. These pathways also communicate or crosstalk with each other to fine-tune the temporal gene expression during muscle differentiation.

Extracellular signal-regulated kinases (ERK) signaling

ERK signaling belongs to the classical mitogen-activated protein kinase (MAPK) cascade, which involves a series of phosphorylation events of protein kinases, in a hierarchical order of MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and MAPK. For ERK activation, receptor phosphorylation triggers Raf, MEK1/2, and ERK1/2, which ultimately triggers the nuclear transcriptional events (reviewed in Davis, R.J., 1993; Cobb, M.H. and Goldsmith, E.J., 1995; Cobb, M.H., 1999; Chang, L. and Karin, M., 2001) (**Figure 6**).

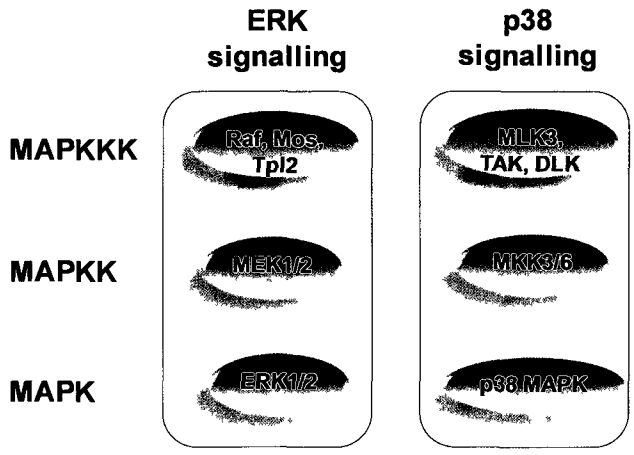


Figure 6. ERK- and p38-signaling cascade.

In myogenesis, the decision between proliferation and differentiation is affected by the amount of ERK activity (Bennett, A.M. and Tonks, N.K., 1997). Under growth conditions, activated ERK signaling promotes the proliferation of MBs (Dee, K. *et al.*, 2002), by inducing the cell-cycle regulator cyclin D1 (Bennett, A.M. and Tonks, N.K., 1997). Simultaneously, ERK signaling hinders muscle differentiation (Olson, E.N. *et al.*, 1987; Payne, P.A. *et al.*, 1987; Dorman, C.M. and Johnson, S.E., 1999; Wu, Z. *et al.*, 2000; Jo, C. *et al.*, 2005), by decreasing the expression of MyoD (Lassar, A.B. *et al.*, 1989), and its transcriptional activity (Perry, R.L. *et al.*, 2001; Tortorella, L.L. *et al.*, 2001); sabotaging the nuclear accumulation of MEF2 (Winter, B. and Arnold, H.H., 2000); and recruiting the transcriptional repressor HDAC to the nucleus to repress muscle differentiation (Zhou, X. *et al.*, 2000). Reciprocally, inhibition of ERK signaling allows

cell-cycle exit (Peeper, D.S. *et al.*, 1997), and leads to precocious muscle differentiation (Yokoyama, T. *et al.*, 2007).

p38 signaling

Another MAPK cascade, p38 MAPK, as opposed to ERK, has to be pro-myogenic (Cuenda, A. and Cohen, P., 1999), in which activated p38 signaling promotes MyoD/E protein heterodimerization (Lluis, F. *et al.*, 2005); enhances the transcriptional activity of both MyoD (Zetser, A. *et al.*, 1999) and MEF2 (Ornatsky, O.I. *et al.*, 1999; Yang, S.H. *et al.*, 1999; Zhao, M. *et al.*, 1999; Wu, Z. *et al.*, 2000; De Angelis, L. *et al.*, 2005); facilitates the subsequent binding of MyoD and MEF2 to the promoters of the target genes (Penn, B.H. *et al.*, 2004); and recruites SWI/SNF to form stable transcriptional complexes to provoke muscle gene expression (Simone, C. *et al.*, 2004).

Phosphatidylinositol-3-kinase (PI3K)-Akt signaling

Analogous to p38, PI3K-Akt signaling is pro-myogenic, in which Akt, also known as protein kinase B, is a downstream effector of PI3K (Datta, K. *et al.*, 1996). Activated PI3K pathway initiates cell-cycle exit by inducing the CDKI p21 (Fujio, Y. *et al.*, 1999; Lawlor, M.A. and Rotwein, P., 2000a). In addition, it promotes muscle differentiation by elevating the expression of both myogenin (Calera, M.R. and Pilch, P.F., 1998) and MyoD (Jiang, B.H. *et al.*, 1998; Jiang, B.H. *et al.*, 1999); and promoting the transcriptional activity of MEF2 (Tamir, Y. and Bengal, E., 2000).

Crosstalk between signaling pathways

Taken together, p38- and PI3K-pathway are pro-myogenic (i.e., promote differentiation); while ERK signaling is mitogenic (i.e., enhances proliferation). These pathways do not function independently, but interact or crosstalk with each other to deliberately orchestrate the muscle differentiation program (Weng, G. *et al.*, 1999; Friedman, A. and Perrimon, N., 2007; Jothi, R. *et al.*, 2009; Bhardwaj, N. *et al.*, 2010; Levy, E.D. *et al.*, 2010). Under growth condition, ERK signaling is active to induce the cell-cycle regulator cyclin D1 to promote proliferation (Bennett, A.M. and Tonks, N.K., 1997); and simultaneously inhibits p38 signaling from initiating the muscle differentiation (Khurana, A. and Dey, C.S., 2002; Shields, J.M. *et al.*, 2002). Upon differentiation, ERK signaling is not active which allows cell-cycle exit, and de-repression of p38 signaling which elicits muscle differentiation. Reciprocally, both p38- (Chen, G. *et al.*, 2000) and PI3K- (Rommel, C. *et al.*, 1999) pathways inhibit the activity of ERK signaling, and obstruct the muscle cells from re-entering the proliferation cycle, and subject them to terminal differentiation. In addition, both pro-myogenic p38- and PI3K-signaling cooperates and synergizes the activity of each other to provoke myogenesis (Li, Y. *et al.*, 2000), in which p38 induces the expression of Akt, the downstream effector of PI3K to promote the transcriptional activity of MEF2 (Cabane, C. *et al.*, 2004); whereas PI3K enhances the activity of p38 signaling by triggering its upstream activator MKK6 (Gonzalez, I. *et al.*, 2004).

From intra- to extra-cellular perspective of myogenesis

Irrespective of well-documented intracellular factors involved in myogenesis, the key step in developing a more comprehensive picture of the regulation of muscle development is to investigate the extracellular factors that prime these downstream intracellular events. This, in turn, may provide valuable insight into the acute cellular response as a result of extrinsic cues in normal muscle development and regeneration. Intriguingly, the effects exerted by the 'conditioned' media (CM) on the development of muscle cells have been documented some time ago (Bischoff, R., 1986a; Bischoff, R., 1990), illustrating the phenomena that myogenic cells modify their own extracellular milieu by secreting factors that exert autocrine and paracrine effects on the differentiation program. Furthermore, skeletal muscle has been recognized as the largest endocrine organ in humans for secreting extracellular factors, the myokines, which orchestrate cellular processes in an autocrine or paracrine fashion, i.e., factors secreted from the cells regulate their own cellular programs or the surrounding cells, respectively (Engler, D., 2007; Pedersen, B.K. and Febbraio, M.A., 2008). There are two types of secreted proteins based on their secretion mechanisms: (1) classical secreted proteins; (2) non-classical secreted proteins. Classical secreted proteins contain endoplasmic reticulum (ER)-targeting sequence, a 16-30 residues long sequence which starts with 1-2 basic amino acid(s), followed by a stretch of 6-12 hydrophobic residues. This signal peptide guides the protein to the ER by interacting with acidic phospholipid head groups and ER receptors associated with the ER membrane. After which, this signal peptide is cleaved by signal peptidase in the ER lumen, and the remaining protein is then packaged into a secretory vesicle and transported through the Golgi apparatus towards the plasma

membrane. The fusion between the secretory vesicle and plasma membrane leads to protein secretion via exocytosis. In non-classical secreted proteins, four mechanisms are involved: (1) a plasma membrane transporter, which directly translocates proteins, such as fibroblast growth factor (FGF) from cytoplasmic to extracellular space; (2) membrane flip-flop, in which protein is first packaged into a secretory vesicle and then transported towards the plasma membrane. According to the membrane flip-flop model, these proteins are flipped from the inner to outer leaflet of the plasma membrane; (3) endosomal recycling, proteins such as interleukin (IL) are packaged into vesicles which are then fused with the plasma membrane and released into the extracellular milieu; (4) membrane blebbing, proteins such as galectin accumulate beneath the plasma membrane and form membrane bound vesicles called exosomes, which then pinch off into the extracellular space (Lodish, H. et al., 2000). To date, a number of extracellular growth factors have been implicated in playing a role in myogenesis. For instance, members of FGF (Spizz, G. *et al.*, 1986; Gossett, L.A. *et al.*, 1989; Li, L. *et al.*, 1992; Hardy, S. *et al.*, 1993; Gerber, A.N. *et al.*, 1997); the insulin-like growth factor-1 (IGF1) (Coleman, M.E. *et al.*, 1995; Coolican, S.A. *et al.*, 1997; Musaro, A. and Rosenthal, N., 1999; Wang, Z.M. *et al.*, 1999; Lu, J. *et al.*, 2000; Xu, Q. and Wu, Z., 2000); and transforming growth factor (TGF) families (Gossett, L.A. *et al.*, 1989; Brennan, T.J. *et al.*, 1991; Martin, J.F. *et al.*, 1992; Gerber, A.N. *et al.*, 1997; De Angelis, L. *et al.*, 1998). Apart from the well-known myokines, there were individual studies investigating other myokines, such as plasminogen activator (Festoff, B.W. *et al.*, 1982); collagenase (Beach, R.L. *et al.*, 1985); decorin (Brandan, E. *et al.*, 1991); glial growth factor (Florini, J.R. *et al.*, 1996a); neurocrescin (Nishimune, H. *et al.*, 1997); meltrin alpha (Gilpin, B.J. *et al.*, 1998);

musculin (Nishizawa, H. *et al.*, 2004; Engler, D., 2007); IL-1 beta (Tateno, K. *et al.*, 2006); IL-7 (Haugen, F. *et al.*, 2010); ADAMTS-like 2 (Koo, B.H. *et al.*, 2007); follistatin-like 1 (Ouchi, N. *et al.*, 2008); and secreted protein acidic and rich in cysteine (SPARC) (Bassuk, J.A. *et al.*, 1993; Cho, W.J. *et al.*, 2000; Jorgensen, L.H. *et al.*, 2009). To make progress on the characterization of the 'secretome' (i.e., profile of secreted factors) in an unbiased manner, we initiated a mass spectrometry-based proteomics strategy to identify secreted proteins during skeletal myogenesis.

Instrumentation of mass spectrometry (MS)

The mass spectrometer is a sophisticated instrument for the accurate mass determination of molecules, small and large, and has been recognized as the most versatile and comprehensive tool in the proteomics field for large-scale characterization of proteins (James, P., 1997; Aebersold, R. and Mann, M., 2003; Yates, J.R. *et al.*, 2009). Two key processes that take place in the mass spectrometer are: (1) generation of gas-phase analyte ion by ionization processes, such as matrix-assisted laser desorption/ionization, electrospray ionization; (2) determination of the mass-to-charge ratio (*m/z*) of the gas-phase analyte ion with the mass analyzer, such as time-of-flight, quadrupole, and linear ion trap.

Ionization methods

Matrix-assisted laser desorption/ionization (MALDI)

MALDI is one of the two major ionization techniques for biological molecules (Karas, M. and Hillenkamp, F., 1988). A schematic diagram of the MALDI source is depicted in **Figure 7**.

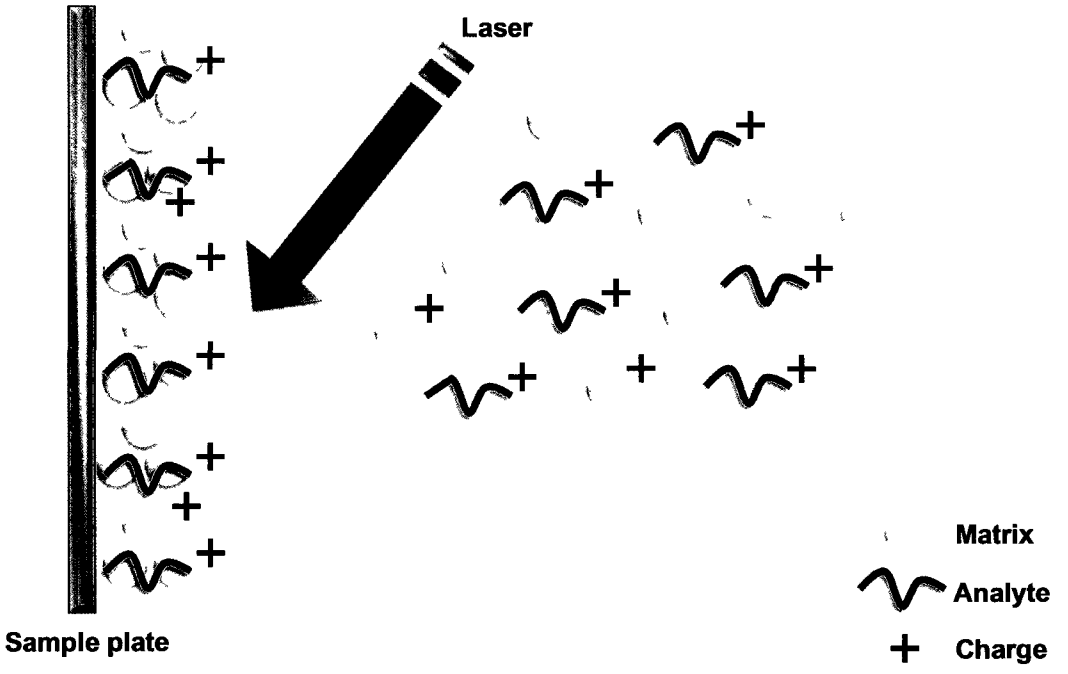


Figure 7. Schematic diagram of a MALDI source.

The analyte is dispersed in a 'matrix' which is typically an organic acid that contains an UV chromophore. The sample is prepared by first dissolving the analyte in a solution of the matrix and deposited onto the sample plate. As the solvent evaporates, analyte molecules co-crystallize with the matrix on the sample plate. Under irradiation by pulses of the nitrogen laser, the matrix absorbs the UV energy. What follows is still incompletely understood. The consensus is that the UV energy induces transfer of the analyte into the gas phase. Some of the molecules may desorb as ions; however, for biomolecules the most likely routes are UV dispersion followed by proton transfer from the matrix molecules in the gas phase immediate to the solid sample (Hillenkamp, F. *et al.*, 1991; Beavis, R.C. and Chait, B.T., 1996; Karas, M. *et al.*, 2000; Karas, M. and Kruger, R., 2003). MALDI is versatile in that it is fairly tolerant towards salts and detergents, which are commonly found in biological samples. In addition, it is compatible with gel-based separation: gel bands or spots can be excised; the proteins are then trypsinized and extracted for MALDI MS.

Electrospray ionization (ESI)

ESI was perfected by Fenn and co-workers (Whitehouse, C.M. *et al.*, 1985; Fenn, J. B. *et al.*, 1989) for the analyses of biomolecules. A schematic diagram of the ESI process is shown in **Figure 8**. In ESI, the sample is an aqueous based solution containing the analytes, which is infused through a capillary. A high voltage is applied to the capillary to electrically disperse the emerging droplets.

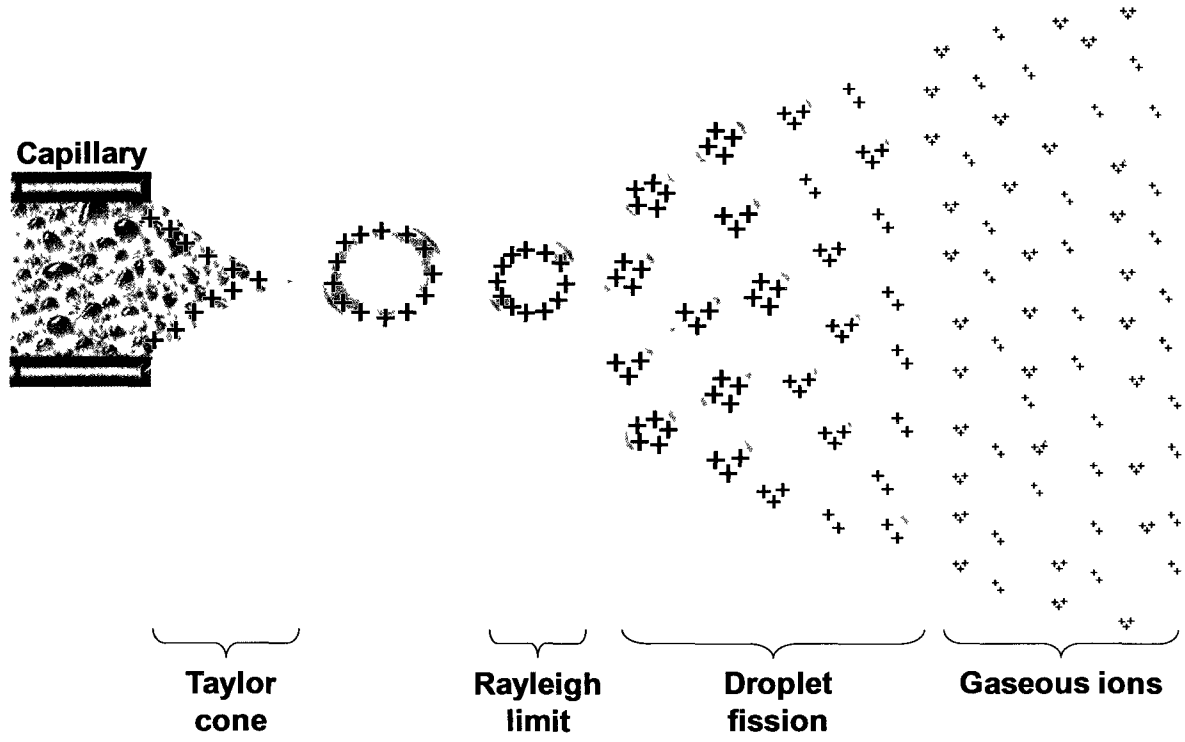


Figure 8. Schematic diagram of ESI.

The high voltage applied to the capillary sets up a strong electric field at the capillary tip. As a result, the emerging droplet is highly charged (Taylor, G.I. and McEwan, A.D., 1965). The Coulombic repulsion among the charges tends to disperse the droplet; these forces are counterbalanced by the surface tension of the droplet. As the droplets are small, they have high evaporation rates. Droplet evaporation results in higher charge density on the surface, to the point that the resulting Coulombic repulsion overcomes surface tension (reaching the Rayleigh limit (Rayleigh, L., 1882)), and the

droplet disintegrates. This fission process tends to be uneven with the consequence that a larger offspring droplet carries the bulk of the liquid, but a disproportionately lower fraction of the charge, and a smaller offspring droplet that carries a disproportionately larger fraction of the charge. The cycle of droplet evaporation and uneven Rayleigh fission proceeds iteratively until protonated analytes result in the gas phase. Ionization is thought to occur by two mechanisms: (1) the ion evaporation model (IEM) proposed by Thomson and Iribarne (Thomson, B.A. and Iribarne, J.V., 1979), in which ions eventually desorb directly into the gas phase from very small droplets after repeated cycles of evaporation and fission; (2) the charge residue model (CRM) suggested by Dole and co-workers, in which gas-phase ions are produced after all solvent molecules eventually evaporate after iterative cycles of solvent evaporation, the remains or the residuals give rise to the gaseous ions (Dole, M. *et al.*, 1968). The current consensus is that IEM is operative for small ions, while CRM is applicable to large, biological ions.

Mass analyzers

Time-of-flight (TOF)

TOF mass spectrometry separates ions of different *m/z* according to their differences in flight time when given the same kinetic energy (Wiley, W.C. and McLaren, I.H., 1955). Key components of a TOF mass spectrometer include: (1) the extraction region, where ions are accelerated by an electric field to a given kinetic energy; (2) the drift region, where ions are separated according to their flight times in the absence of an electric field **(Figure 9)**.

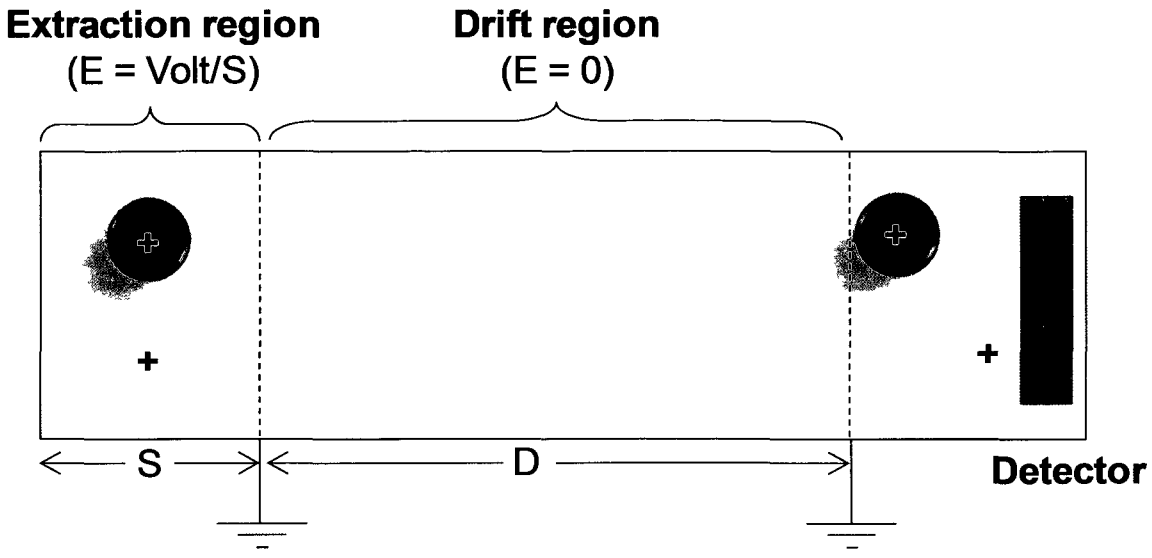


Figure 9. Principle of TOF.

Gaseous ions (pictorially depicted as grey and yellow) enter the extraction region where they are accelerated by the electric field *E*. The potential energy that these ions experience are: *Eez*, where e is the electronic charge and *z* is the numerical charge. The ions gain kinetic energy equal to the loss of potential energy:

$$Eez = \frac{1}{2}mv^2 \qquad \text{(Equation 1)}$$

where m and v are the mass and the velocity of the ions, respectively. The ions are then injected into the flight tube where they are separated by their times of flight.

Since $v = \frac{D}{t}$

where D is the flight length and t is the flight time. Substituting these parameters into Equation 1:

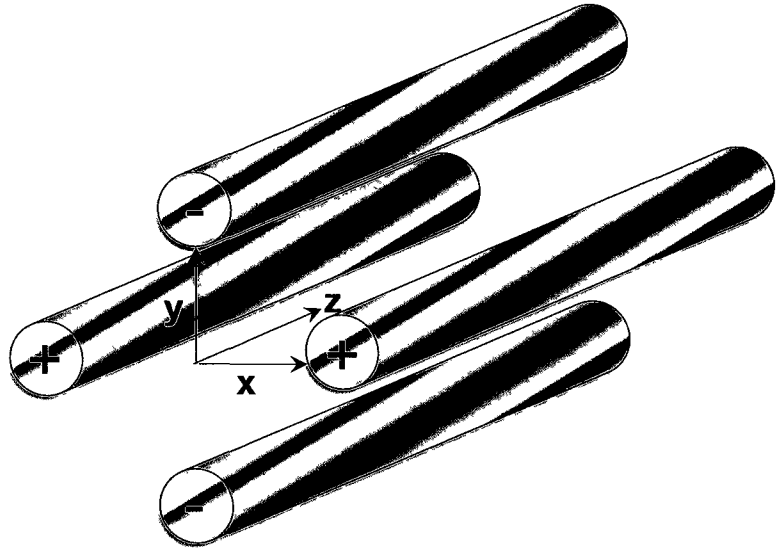
$$\frac{m}{z} = 2eE\left(\frac{t}{D}\right)^2 \qquad \text{(Equation 2)}$$

According to equation 2, m/z is directly proportional to t^2. By measuring the flight time t of an ion, its m/z can be deduced. In practice, the sequential arrival of ions per injection (pulse) is recorded in a TOF mass spectrum. Many spectra are typically accumulated per analysis for improved signal over noise (Cotter, R.J. 1989; Chevrier, M.R. and Cotter, R.J., 1991; Cotter, R.J., 1992; Guilhaus, M. *et al.*, 2000).

Quadrupole

A quadrupole mass analyzer is composed of four parallel cylindrical electrodes, arranged in two opposing pairs. Each electrode pair is electrically biased with both direct current (DC) and radio frequency (RF) voltage components. The DC voltages of the two electrode pairs are oppositely charged (**Figure 10A**), whereas the RF voltages are 180° out-of-phase to each other (**Figure 10B**).

(A)



(B)

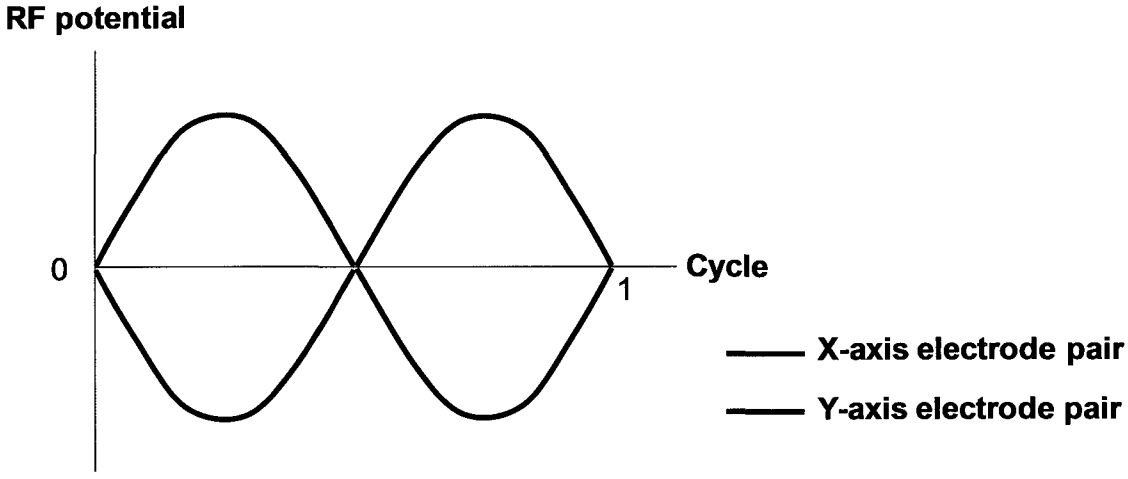


Figure 10. The quadrupole mass analyzer.

The quadrupole mass analyser separates ions of different *m/z* values by means of application of a time-dependent combination of DC and RF voltages. The operating voltages of both DC and RF affect the oscillating behaviour of the ions. Depending on the choice of DC and RF biases, two general operating modes of the quadrupole are in common use: (1) high pass mass filter, in which ions above a certain *m/z* cut-off are allowed to transverse the length of the quadrupole (and be transmitted); (2) selective mass filter, which permits ions within a narrow range of *m/z* to be transmitted.

In the high pass mass filter mode, only RF potential is applied (DC = 0). When the RF potential is positive, the protonated ions encounter repulsion between the electrode pair and are driven into the central channel of the quadrupole **(Figure 10A)**. Conversely, when the RF potential is negative, the ions are attracted to the electrode pairs. The resultant tracjectory is a series of oscillations as ions traverse the length of the quadrupole. Within a given MS experiment, whether or not the ions will collide with the electrode and discharge depends on their *m/z* values. Ions with higher *m/z* values are less sensitive to the alternating phases of the RF voltages; as a result, they traverse the quadrupole and are recorded by the detector. Ions with lower *m/z* values are more perturbed by the RF voltage and they eventually hit the quadrupole rods or escape through the space between the rods. The high pass mass filter mode is used in quadrupoles that function as an ion guide or as a collision cell in tandem mass spectrometry.

In the selective mass filter mode, both DC and RF voltages are applied. When the DC component is added, the resulting electric field oscillations destabilize the trajectories

of some ions causing them to either collide with the rods or be ejected radially. The motion of an ion in a quadrupole mass analyzer is described according to its *m/z* by a second-order differential equation known as the Mathieu equation (Mathieu, E.M., 1868; March, R.E., 1998). This equation effectively demonstrates that the oscillating DC and RF electric fields generated by the oppositely charged pairs of rods have both stabilizing and destabilizing influence on the ions. A light, positively charged ion, for example, is too highly influenced by the oscillating negative rod set to maintain a steady trajectory, whereas a heavy, positively charged ion is insufficiently swayed by the restorative forces of the positive rod set to allow it to fare any better. At a particular DC-to-RF ratio, however, a certain range of masses of ions will establish a steady trajectory and will pass safely through the quadrupole mass filter. This 'window of transmission' can be tuned by adjusting the DC and RF voltages while maintaining this ratio. As such, the quadrupole mass 'filter' can be used to selectively transmit ions of a particular mass, or to allow sequential transmission of a range or spectrum of masses in a controlled fashion. In this way, a quadrupole mass filter is a very effective ion manipulation tool and can be used to transmit an entire population of ions (see above); to selectively transmit ions of a specific *m/z* over a period of time, thereby separating them from all others present; or to rapidly scan through a desired *m/z* range and allow for the generation of a mass spectrum upon ion detection. When the quadrupole is being used in the resolving mode, it is transmitting some ions at all times, but is never transmitting all the ions that enter its front end. In fact, since the quadrupole selectively transmits a specific *m/z* value by destabilizing the trajectory of all others, the transmission efficiency and subsequent instrument sensitivity

relate reciprocally to the resolving power of the instrument. In simpler terms, better quadrupole resolution equals poorer sensitivity and vice versa.

Tandem mass spectrometry

In tandem mass spectrometry (MS/MS), two stages of mass analyses are implemented in a single experiment. Ion selection is performed in the first stage mass analyzer, followed by fragmentation of the selected (precursor) ion, and finally mass analysis of the fragment (product) ions is performed on the second stage mass analyzer. In general, MS/MS can be implemented as: (1) tandem-in-space, in which more than one mass analyzer are physically arranged in tandem to perform different stages of mass analyses, e.g., quadrupole/time-of-flight (QqTOF); (2) tandem-in-time, in which one mass analyzer is employed to conduct all stages of mass analyses; the stages are separated temporally, e.g., ion traps.

Quadrupole/time-of-flight (QqTOF)

In a QqTOF hybrid tandem mass spectrometer, two different types of mass analyzers are employed: the quadrupole mass analyzer, Q1, and the time-of-flight mass analyzer, TOF. In addition, two high pass mass filters, q0 and q2, are also employed; the former functions as an ion guide to focus the ions into Q1, while the latter functions either as an ion guide or a collision cell depending on the operation mode. In QqTOF, there are two major operation modes: (1) MS mode, and (2) MS/MS mode.

In MS mode **(Figure 11A)**, Q1 operates as a high pass mass filter to allow all the ions above a certain m/z cut-off (pictorially as red, yellow, and green) to tranverse; while q2 works as an ion guide to transmit and focus the ions prior to mass analysis with TOF.

In MS/MS mode **(Figure 11B)**, Q1 operates as a mass filter to select the precursor ion of interest (red), and subject it to q2 which now functions as a collision cell filled with an inert collision gas, nitrogen. The collision between an ion and the neutral gas converts some of the ion's kinetic energy into internal energy. The internal energy is redistributed rapidly within the ion. When the redistributed energy exceeds the dissociation threshold of a given bond, dissociation ensues. Typically this collision-induced dissociation (CID) results in a fragment (product) ion and a neutral. In CIDs, the weakest bonds tend to break first (Chernushevich, I.V. et al., 2001). An examination of the MS/MS spectrum reveals the connectivity within the precursor ion. Accumulation of knowledge through the study of a large number of MS/MS spectra allows the formulation of fragmentation rules and fragmentation chemistry. For protonated peptides, the fragmentation chemistry is relatively well understood to the extent that the primary structure (the sequence of the amino acid residues) can often be deduced from the MS/MS spectrum. In practice, the identification of the peptide is typically performed by matching the experimental MS/MS spectrum with predicted spectra generated from known protein sequences in a library (see later).

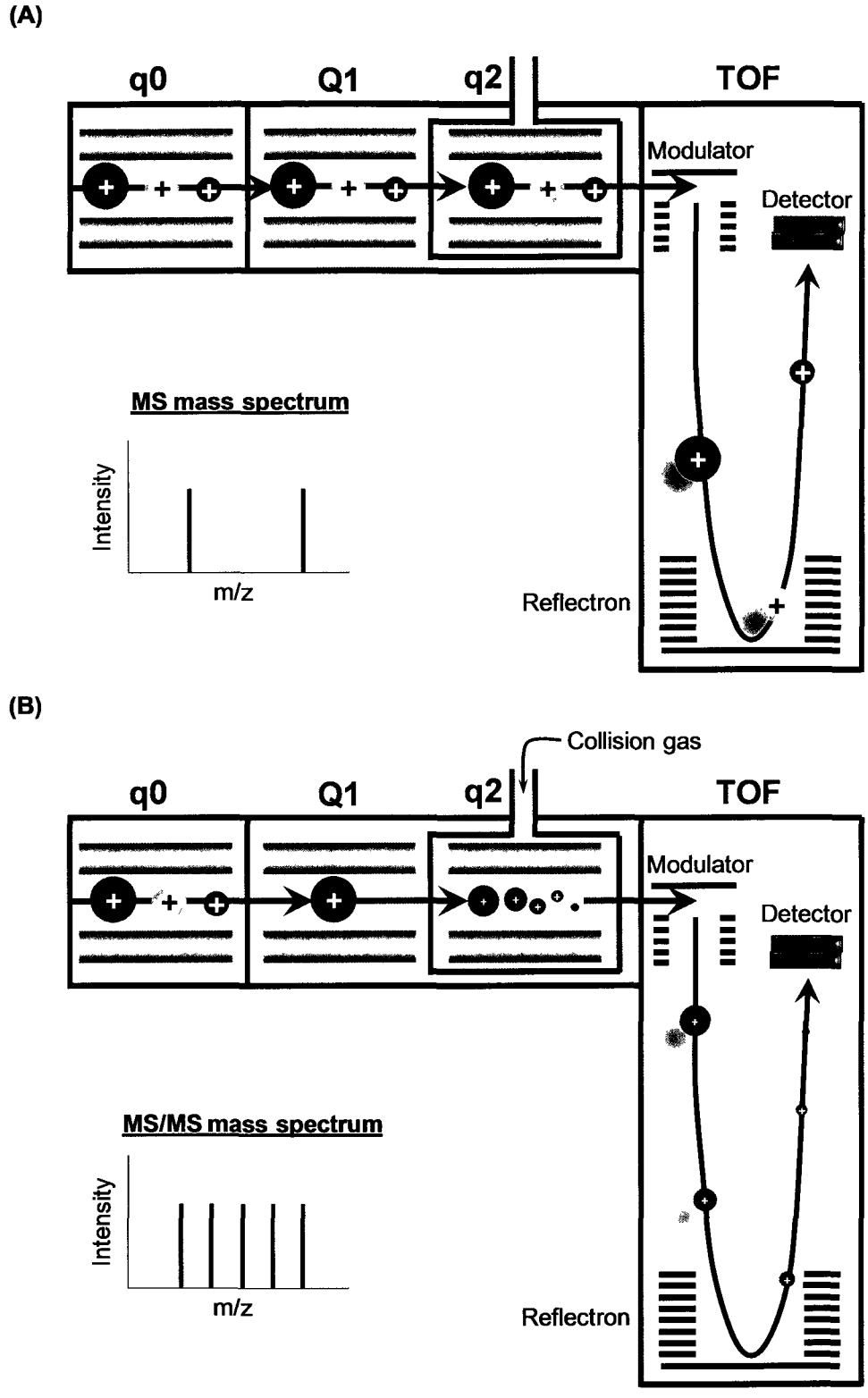


Figure 11. Schematic diagram of the QqTOF hybrid tandem mass spectrometer: (A) MS mode and **(B)** in MS/MS mode.

Applications of MS

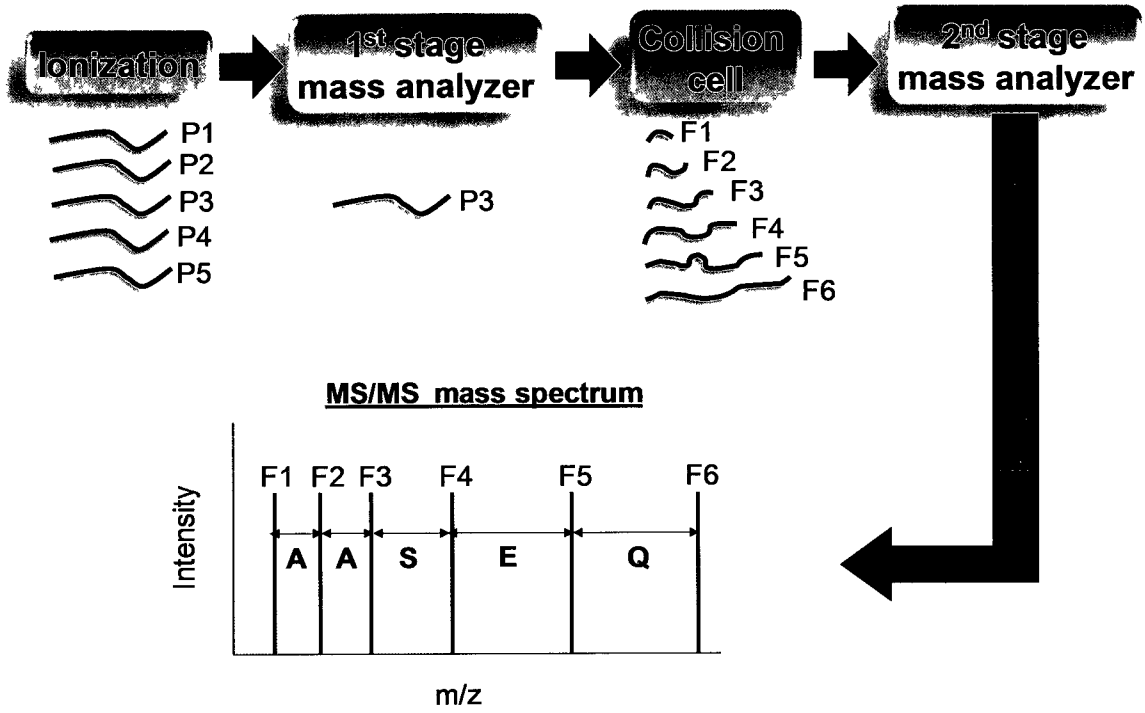
MS has become a valuable tool in proteomics for identifying and quantifying proteins, often in a high-throughput manner (James, P., 1997; Aebersold, R. and Mann, M., 2003).

Protein identification (ID)

MS-based protein ID was initially conducted with peptide mass fingerprinting (PMF), in which the target protein is cleaved or digested enzymatically, typically trypsin that offers high cleavage specificity at the C-terminus of lysine and arginine (Olsen, J.V. *et al.*, 2004). The resulting digested peptides serve as the unique fingerprint of that particular protein, whose identity can be retrieved by matching the peptide masses against a protein database (Henzel, W.J. *et al.*, 1993; James, P. *et al.*, 1993; Mann, M. *et al.*, 1993; Pappin, D.J. *et al.*, 1993; Yates, J.R., III *et al.*, 1993). This method is not applicable to a protein mixture, in which the resulting peptide mixture of the proteins complicates the subsequent interpretation. In this regard, MS/MS has emerged to be the method of choice for protein ID as it can lead to confident peptide and protein identification based even on a single tryptic peptide. Proteins are first enzymatically cleaved with trypsin, the tryptic peptides generated are subject to the first stage mass analyzer for precursor ion selection (i.e., the peptide of interest). This is followed by CID in the collision cell (Shukla, A.K. and Futrell, J.H., 2000, **Figure 12A**). The most common cleavages are those of the peptide bonds, thereby generating b and/or y ions, in which the charge

resides on the N- or C-terminal side of the cleaved peptide, respectively (Roepstorff, P. and Fohlman, J., 1984; Hunt, D.F. *et al.*, 1986; Biemann, K., 1988; **Figure 12B)**.

(A)



(B)

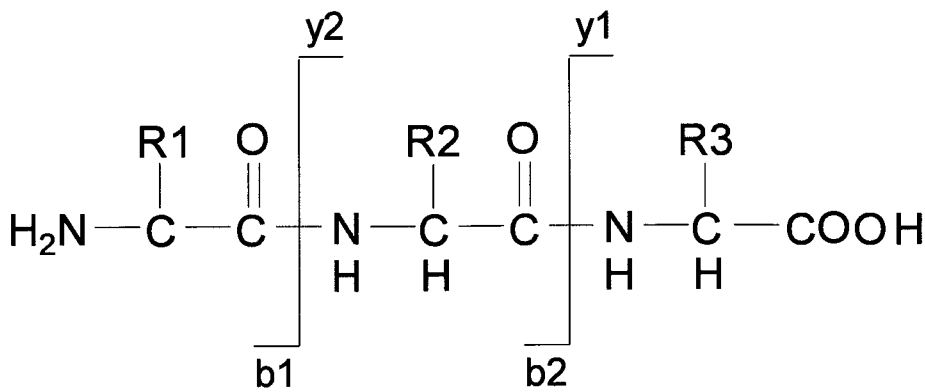


Figure 12. Protein ID by MS/MS. (A) The protein of interest is first digested into peptides (P1-P5) with trypsin. This is followed by selection of the target precursor ion (e.g., P3) by the first stage mass analyzer; the precursor ion is then fragmented in the collision cell via CID. The resulting fragment ions (F1-F6) are then analyzed by the second stage mass analyzer, and recorded in the MS/MS mass spectrum, which reveals the amino acid sequence of the respective parent ion; **(B)** According to the nomenclature proposed by Roepstorff and Fohlman (1984), after the cleavage of the peptide bond (shaded in yellow), the fragment ion as resulted with the charge retained on the N- and C-terminus is designated as b- and y-ion, respectively. The mass difference between the consecutive b- or y-ions corresponds to the mass of a particular amino acid residue. By extension, the amino acid residue sequence of the selected peptide can be retrieved from the b- or y-ion series. R1, R2 and R3 represent the side chains of the residues.

Protein quantification (Quan)

A preponderance of biological processes is regulated by a cohort of proteins, whose expression levels alter according to different physiological states, such as differentiation versus proliferation. Having a high throughput tool to snapshot these alternations advances our capability to understand the protein functions during myogenesis. Nonetheless, MS is not quantitative *per se* due to the variability in sample preparation, processing and instrument acquisition. To circumvent this, stable isotope

labelling is employed in which protein samples from different cell states are labelled with different versions of the stable isotope, for instance proliferation- and differentiation-protein sample is labelled with the light $^{12}C_6$- and heavy $^{13}C_6$-isotope, respectively. There are two crucial properties of the stable isotopes which confer their success in MS-based quantitative study: firstly, the stable isotopes are different in terms of mass based on the number of neutrons they contain, for instance $^{13}C_6$ is 6 amu 'heavier' than $^{12}C_6$. This allows the heavy and light labelled protein samples to combine, and be processed together in order to eliminate the variability in sample processing, such as protein fractionation and trypsin digestion, while the tryptic peptides generated from the two can be distinguished in the mass spectrum by the signature mass difference. Secondly, the stable isotopes are chemically identical which means both light- and heavy-labelled tryptic peptide possess equal ionization efficiency, and the ratio of their peak intensities revealed in the mass spectrum corresponds to the relative expression level of the respective protein **(Figure 13)**.

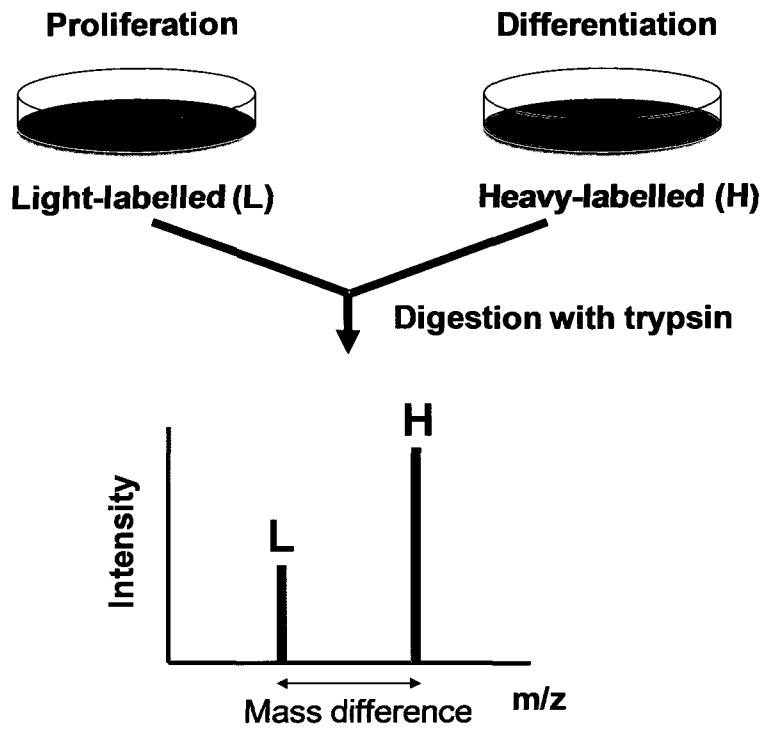


Figure 13. Determination of relative protein expression level with stable isotope labelling. To compare the protein expression level between different cell states, for instance differentiation versus proliferation, protein samples from the two are labelled with heavy- and light-stable isotope, respectively. The labelled protein samples are then combined, digested with trypsin, and acquired in the same mass spectrum, in which the labelled tryptic peptides appear as peptide pair with a signature mass difference in between, and the ratio of their peak intensities corresponds to the relative protein expression level.

There are two general ways to introduce the stable isotopes into the samples: (1) chemical labelling, such as isotope-coded affinity tags (ICATs) and isobaric tag for relative and absolute quantitation (iTRAQ); (2) metabolic labelling, such as stable isotope labelling with amino acids in cell culture (SILAC) (Ong, S.E. and Mann, M., 2005).

The ICAT reagent comprises three components: (1) the reactive iodoacetamide group which targets at the sulfhydryl group of cysteine; (2) the linker region which carries either light- or heavy-stable isotope; (3) the biotin group which allows subsequent isolation and recovery of the labelled peptides. Protein samples are chemically tagged with the labelling reagent via the reactive iodoacetamide group, after which the labelled proteins are enzymatically digested, enriched using the avidin column, and eluted for LC-MS analysis (Gygi, S.P. *et al.*, 1999) **(Figure 14)**.

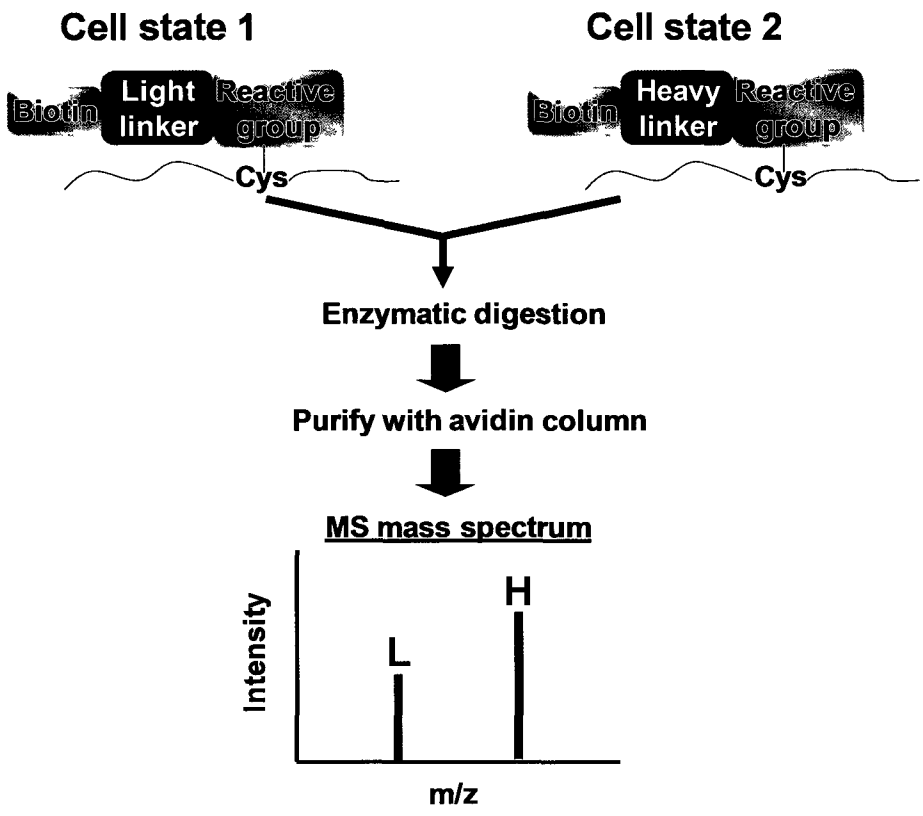


Figure 14. Schematic diagram of ICAT analysis.

Analogous to ICAT, the iTRAQ reagent is also composed of three components: (1) the reactive group which targets the primary amine group, including the N-terminus of the peptide and the side chain of the lysine residue; (2) the reporter group which carries either the light- or heavy-isotopes; (3) the balance group which balances out the mass difference between the light- and heavy-reporter group. The last feature makes the intact light- and heavy-iTRAQ reagent isobaric, and so are the labelled peptides. As a

result, the light- and heavy-labelled peptides are unresolved chromatographically and superimpose as a single LC-MS peak of the same *m/z* value. CID of this peak produces fragment ions including the reporter ions whose intensity ratio gives the relative abundance of the cell states that their respective reagents label, and larger fragement ions from which the peptide can be identified via a database search (Ross, P.L. *et al.*, 2004) **(Figure 15)**.

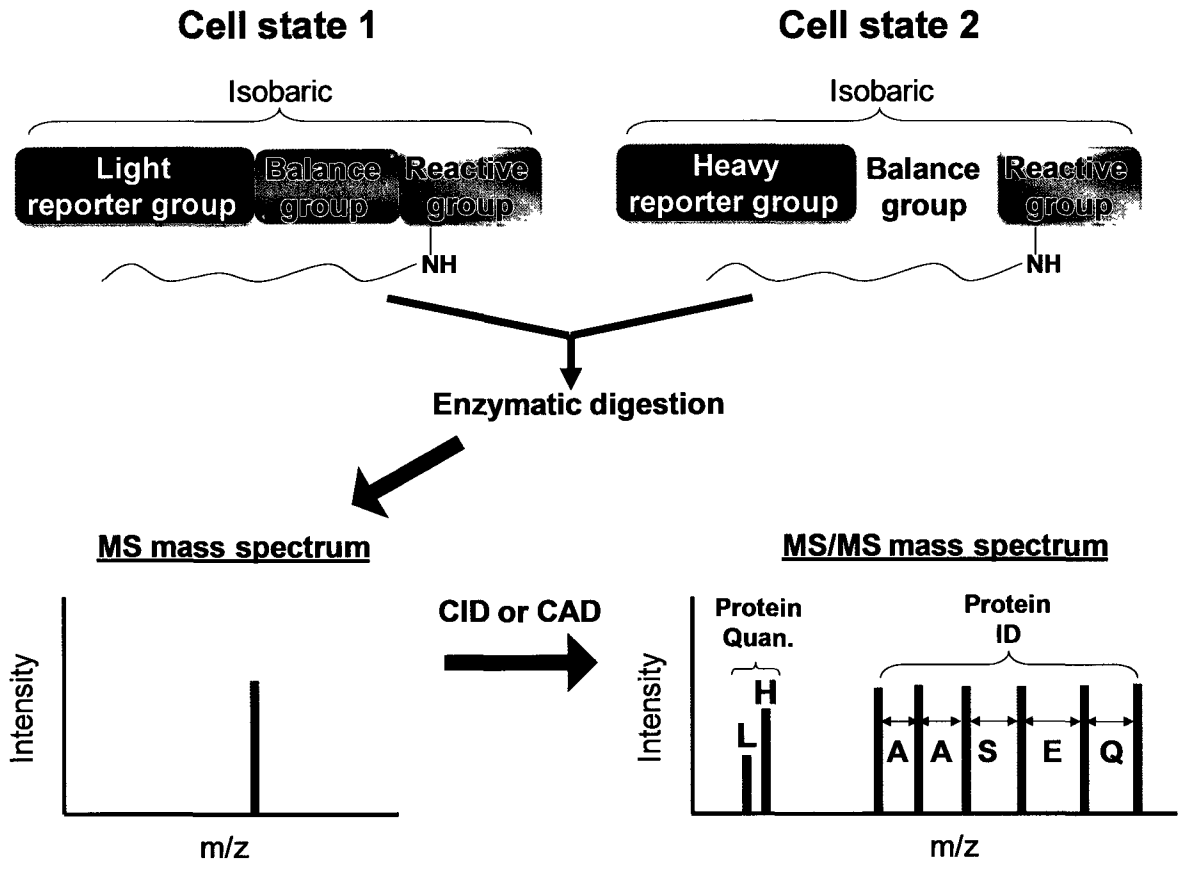


Figure 15. Principle of iTRAQ analysis.

In SILAC, light- and heavy-labelled amino acids, such as $^{12}C_6$- versus $^{13}C_6$-lysine, are incorporated into the cells via metabolism. Cells of different physiological states are propagated in the culture media which contain either light- or heavy-labelled amino acids. After several cell doublings, the isotope-labelled amino acids are incorporated into the cellular proteome, in which the labelled protein samples are combined, processed and analyzed with MS. The labelled peptides appear as two peaks in the MS mass spectrum, and their relative peak intensities corresponds to the relative protein expression level (Ong, S.E. *et al.*, 2002) **(Figure 16)**.

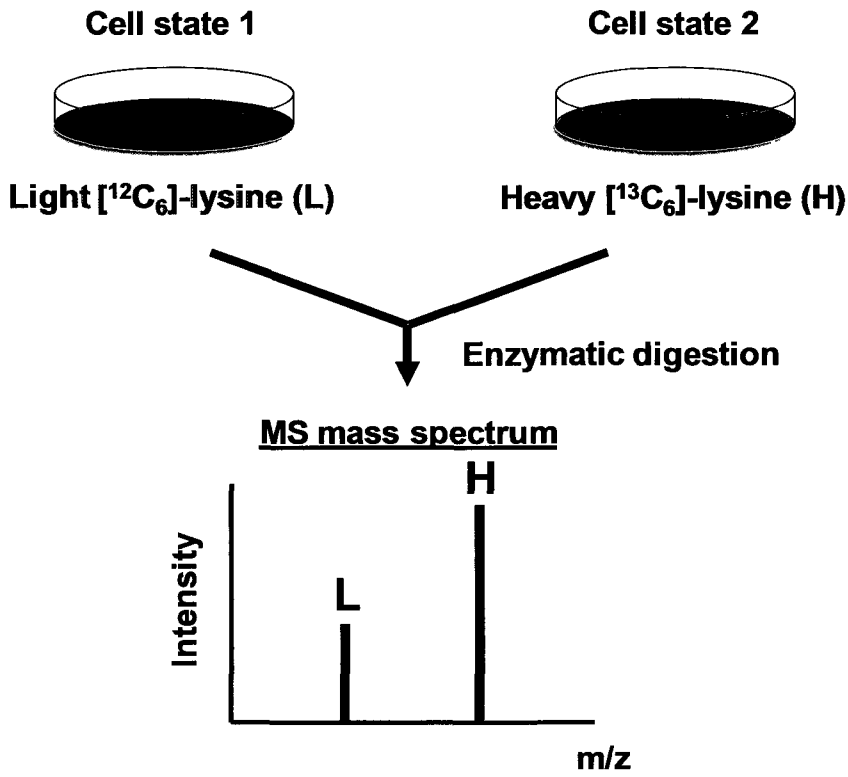


Figure 16. Schematic diagram of SILAC analysis.

Sample fractionation

Biological samples are complex, and they require fractionation before MS analysis to reduce the sample complexity prior to analysis, which in turn enhances the chance to identify crucial, albeit low abundance, regulatory proteins. In our work, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS PAGE), and reverse phase liquid chromatography (RPLC), were employed for sample fractionation.

In 1D-SDS PAGE, the protein sample is first mixed with the sample buffer containing two key ingredients: (1) sodium dodecyl sulphate (SDS), which uniformly coats the protein with negative charges; (2) β-mercaptoethanol which linearizes the protein by reducing or cleaving the disulfide bond, in order to eliminate the impact of protein conformation on migration. During separation, negatively charged proteins migrate towards the positive electrode through the uniformly sized pores of the polyacrylamide gel. Interaction with the pores restricts or slows down the migration of larger proteins. As a result, proteins are fractionated according to their size, which is proportional to molecular weights (Shapiro, A.L. *et al.*, 1967; Weber, K. and Osborn, M., 1969) **(Figure 17)**.

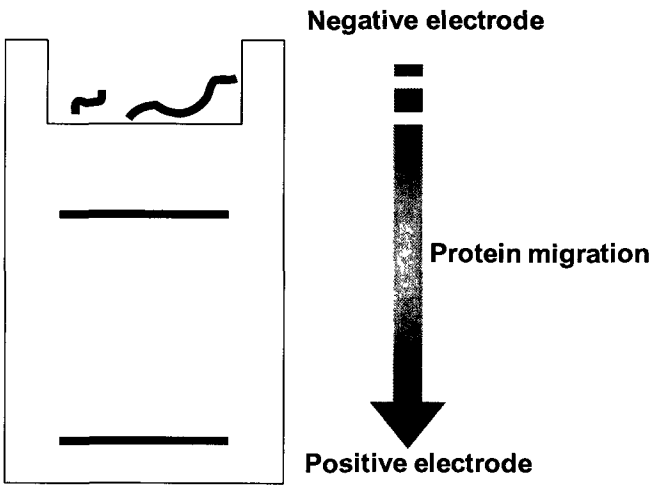


Figure 17. Protein separation with 1D-SDS PAGE. Green and red line represents smaller and larger protein, respectively. Both proteins migrate towards the positive electrode, but with different migration rates, in which the smaller the protein, the faster the migration, vice versa.

In RPLC, proteins are separated based on their hydrophobicity which affects their differential partition between the non-polar stationary phase and the polar mobile phase. As depicted in **Figure 18**, the stationary phase is composed of silica beads conjugated with hydrophobic C18 hydrocarbon chains which interact with the proteins (pictorially depicted by red and green) via hydrophobic interactions. By increasing the percentage of the polar mobile phase, such as acetonitrile or methanol, the hydrophobic interactions between the two are sequentially competed out, according to the protein hydrophobicity, i.e., the greater the protein hydrophobicity, the higher the percentage of the polar mobile

phase is required to elute the protein (Bushey, M.M. and Jorgenson, J.W., 1990; Fournier, M.L. *et al.*, 2007).

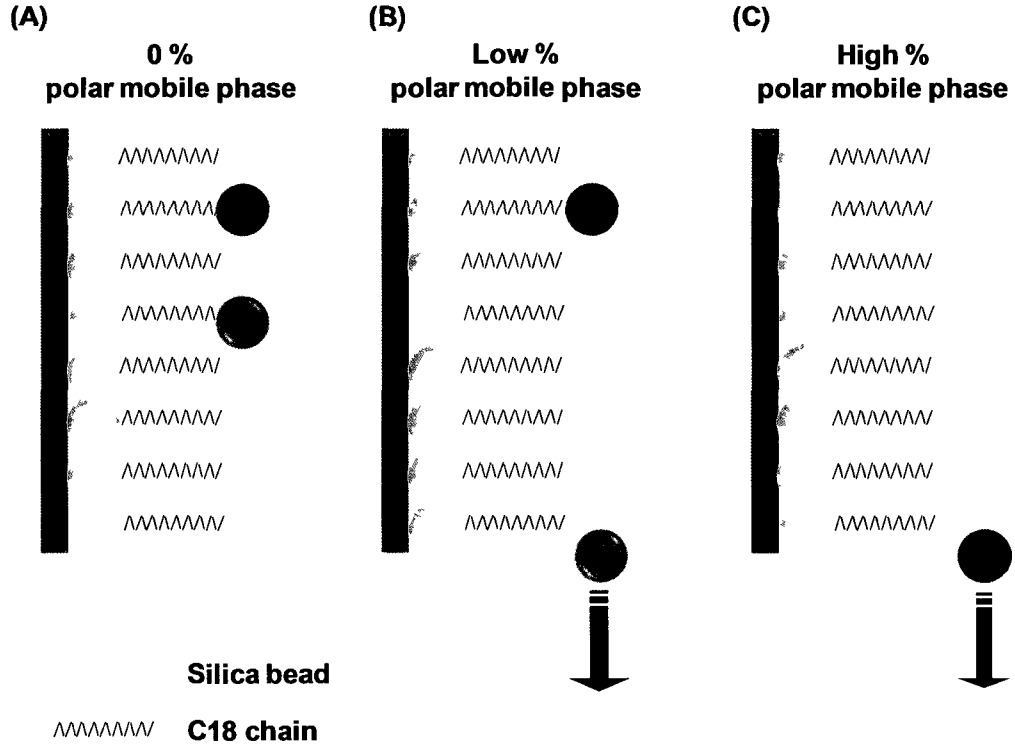


Figure 18. Schematic diagram of RPLC. Red and green ball represents the protein with higher and lower hydrophobicity, respectively.

Chapter 2:

Statement of purpose

The program of muscle differentiation is orchestrated by a hierarchy of well-characterized transcription factors, which are responsive to extracellular cues. Skeletal muscle has been shown to secrete extracellular factors or myokines to modulate myogenesis in an autocrine or paracrine fashion. In an attempt to characterize the secretome (i.e., the profile of secreted factors), we initiated a novel discovery based study using an MS-based proteomic approach to identify and quantify the secretome of mammalian skeletal muscle cells during differentiation.

Identification of the secretome during skeletal myogenesis (Chapter 3)

In our first attempt to systematically characterize the secretome during myogenesis, we employed serum-free culture condition in tandem with MALDI-QqTOF to identify secreted proteins from the mouse skeletal muscle cell line C2C12. This work has subsequently been published in Chan, C.Y. *et al.*, 2007.

Quantification of the secretome during skeletal myogenesis (Chapter 4)

To further substantiate the initial discovery study, next we employed SILAC in tandem with high throughput online RPLC-MS/MS to identify differentially expressed secreted proteins during myogenesis. This work has recently been published in Chan, C.Y. *et al.*, 2011a.

Chapter 3:

Identification of the secretome during skeletal myogenesis

Rationale

The manuscript illustrates the first discovery study adapting an MS-based proteomic approach to identify secreted proteins during muscle differentiation. All of the work was conducted in the labs of John C. McDermott and K.W. Michael Siu. I was responsible for optimizing the serum-free culture conditions, preparing samples, devising the non-redundant data acquisition scheme, acquiring and analyzing the data generated with MALDI-QqTOF hybrid mass spectrometer.

This manuscript was written by me with editing by John McDermott and Michael Siu.

Journal of Proteome Research, **6(2)**, 698-710, 2007.

Identification of secreted proteins during skeletal muscle development

C.Y. X'avia Chan[1, 2], John C. McDermott[1, 2 *], and K.W. Michael Siu[1, 2, 3 *]

[1]*Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3*

[2]*Centre for Research in Mass Spectrometry, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3*

[3]*Department of Chemistry, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3*

* To whom correspondence should be addressed.

John C. McDermott

247 Farquharson Building

York University

4700 Keele Street

Ontario, Canada

M3K 1P3

Phone: 416-736-2100 x30389

K.W. Michael Siu

242 Chemistry Building

York University

4700 Keele Street

Ontario, Canada

M3J 1P3

Phone: 416-650-8021

Received for review September 3, 2006. Accepted January 11, 2007.

Abstract

The differentiation program of skeletal muscle cells is exquisitely sensitive to secreted proteins. We developed a strategy to maximize the discovery of secreted proteins, using mass spectrometry-based proteomics, from cultured muscle cells, C2C12, grown in a serum-free medium. This strategy led to the identification of 80 non-redundant proteins, of which 27 were secreted proteins that were identified with minimum of two tryptic peptides. A number of the identified secreted proteins are involved in extracellular matrix remodeling, cellular proliferation, migration and signaling. These findings open a new avenue in studying skeletal muscle differentiation in the aspect of extracellular milieu.

Introduction

The program of muscle cell differentiation has proven to be a regulatory paradigm for understanding principles of cellular differentiation. At the molecular level this process is regulated by two families of transcriptional regulators, the muscle regulatory factor (MRF) family, comprised of MyoD, Myf5, myogenin and MRF4 (Ott, M.O. *et al.*, 1991; Hasty, P. *et al.*, 1993; Rudnicki, M.A. *et al.,* 1993; Tajbakhsh, S. *et al.,* 1996b; Kablar, B. *et al.*, 1997; Kassar-Duchossoy, L. *et al.*, 2004) and the myocyte enhancer factor-2 (MEF2A-D) transcriptional regulatory proteins, which function as obligatory partners of the MRFs in the differentiation of cultured myogenic cells (Cox, D.M. *et al.*, 2000; McKinsey, T.A. *et al.*, 2002). One key aspect of the control of myogenic transcription factor activity is an acute responsiveness to cellular signaling pathways; an example is the control exerted over these factors by growth-factor-activated signaling pathways (Florini, J.R. *et al.*, 1991; Florini, J.R. *et al.*, 1996b). Understanding the link between myogenic transcription factors and signals secreted from cells within their 'community' is regarded as a fundamental question in developmental biology.

A thorough understanding of the mechanisms involved in extracellular regulation is a prerequisite to understanding the control exerted by intracellular effectors in the differentiation program. To date, a number of extracellular growth factors have been found to play a functional role as myogenic regulators (Bass, J. *et al.*, 1999). For example, members of the insulin-like growth factor (IGF) 1 and transforming growth factor (TGF) families were observed to have potent, but opposing, effects on myogenesis (Florini, J.R. *et al.*, 1996b; Bass, J. *et al.*, 1999). In addition, dramatic effects exerted by

the 'conditioned' media (CM) on muscle cells were documented, indicating that myogenic cells modify their own extracellular milieu by secreting factors that exert autocrine and paracrine effects on the differentiation programme (Bischoff, R., 1986b; Bischoff, R., 1990). Indeed, it has been suggested that therapeutic use of these secreted factors may constitute the basis for a cell-based treatment for Duchenne Muscular Dystrophy (DMD) by activating skeletal muscle stem cells (Seale, P. and Rudnicki, M.A., 2000). Unfortunately, this optimism is not justified by the scarcity of knowledge on these secreted factors (Currie, P.D. and Ingham, P.W., 1998; Huh, M.S. *et al.*, 2005). Characterization of the full spectrum of myogenic secreted factors, and subsequent analysis of their functions, are a critical first step in better understanding the regulation of myogenesis and muscle pathology.

Here we report the results of a proteomic study on identifying secreted proteins central to skeletal muscle differentiation; this study uses the mouse skeletal muscle cell line C2C12 as a model system and matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry (MS) and tandem mass spectrometry (MS/MS) for analysis. The C2C12 muscle cell line, initially developed by Yaffe *et al.* has been a cornerstone of work concerning the differentiation of muscle cells since its introduction in the 1960's (Yaffe, D. and Feldman, M., 1965; Yaffe, D., 1968). These cells proliferate under high mitogen conditions and differentiate on exposure to low mitogen containing media. The C2C12 culture model has led to many significant advances in our understanding of muscle differentiation, including MRFs and MEF2 transcriptional regulatory proteins in this process (Ott, M.O. *et al.*, 1991; Hasty, P. *et al.*, 1993; Rudnicki, M.A. *et al.*, 1993; Tajbakhsh, S. *et al.*, 1996b; Kablar, B. *et al.*, 1997; McKinsey, T.A. *et al.*, 2002; Kassar-

Duchossoy, L. *et al.*, 2004). MS-based protein identification methodologies via tryptic peptide mapping, or MS/MS analysis, in combination with searching against a protein database are now established practices that have led to mapping of proteomes (Aebersold, R. and Goodlett, D.R., 2001; Aebersold, R. and Mann, M., 2003). There have been a number of reported studies on identifying secreted proteins and mapping of secretomes from various cells and cell lines (Lim, J.W. and Bodnar, A., 2002; Dupont, A. *et al.*, 2004; Tjalsma, H. *et al.*, 2004; ; Wang, P. *et al.*, 2004; Chen, X. *et al.*, 2005; Dupont, A. *et al.*, 2005; Trost, M. *et al.*, 2005; Volmer, M.W. *et al.*, 2005; Voigt, B. *et al.*, 2006); however, none have involved skeletal muscle cells. As far as we know, this is the first report on secreted proteins in myogenesis.

Materials and methods

A general sample workflow is shown in **Figure 19**:

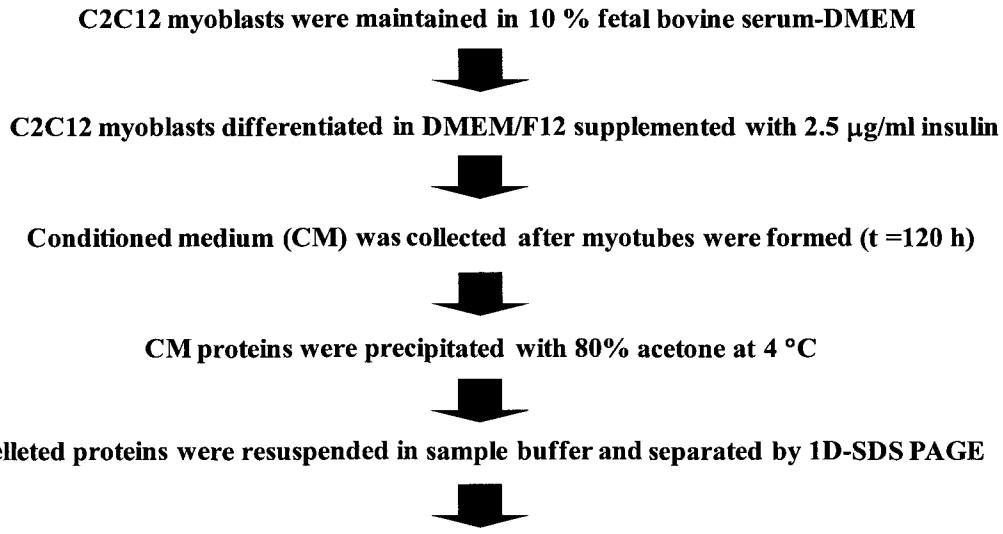


Figure 19. General workflow of preparation of conditioned media and subsequent protein identification with mass spectrometry.

Cell culture

Mitogenic murine C2C12 myoblasts (American Type Culture Collection, ATCC) were seeded on 10-cm gelatin-coated (Sigma) culture plates (Fisher scientific), containing 10 mL of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10 % FBS (Hyclone), 2 mM L-glutamate (Invitrogen), 50 units/mL penicillin-streptomycin (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). Confluent

(90 %) myoblasts were committed to differentiating into multinucleated myotubes by switching them into the differentiation medium comprising 5 mL of Dulbecco's MEM supplemented with 2 mM L-glutamate, 50 units/mL penicillin-streptomycin, 1 mM sodium pyruvate, and 5 % horse serum (Atlanta).

For serum-free inoculation, confluent (90 %) myoblasts were briefly washed with versene (Bioshop) and digested with 1 mL of 0.125 % trypsin (Gibco) for 1 min. The trypsinization process was ceased by addition of 5 mL of the serum-free differentiation medium, comprising 1:1 Dulbecco's MEM:Ham's Nutrient Mixture F-12 (Gibco) supplemented with 2 mM L-glutamine, 50 units/mL penicillin-streptomycin, 1 mM sodium pyruvate, and 2.5 μg/mL bovine insulin (Sigma). The cells were pelleted by centrifugation for 10 min at 153 ×g. The pellet was resuspended in 5 mL of the serum-free differentiation medium and pelleted by centrifugation. This washing step was repeated. The resulting pellet was resuspended in 5 mL of the serum-free differentiation medium and inoculated directly onto a 10-cm gelatin-coated culture plate. The CM was collected after 24, 48, 72, 96, 120 h of incubation. After the collection of CM at each specific time point, the cells remained on the culture dish were washed extensively with 10 mL of phosphate-buffered saline (Gibco). This washing step was repeated four more times, followed by replenishment of the serum-free differentiation medium.

Protein fractionation by 1D-SDS PAGE

The CM collected were purified by two-step centrifugation at 4 °C (98 ×g) for 5 min, followed by 13,000 ×g for 30 min to remove cells and debris. The clarified medium

was then syringe-filtered (0.45 μm pore size, Millex-HV) to remove remaining particles. The proteins dissolved in the CM were precipitated by addition of acetone (Caledon) at -20 °C to a final concentration of 80 %; precipitation was allowed to proceed overnight. The proteins were then pelleted by centrifugation at 4 °C (15,000 ×g for 1 h). After removal of acetone, the proteins were allowed to air dry. The dried proteins were dissolved in a minimum volume of SDS-gel loading buffer, containing 50 mM Tris-HCl (Bioshop), 2 % SDS (Bioshop), 25 % glycerol (BDH), and 2.93 % β-mercaptoethanol (Bioshop). The protein concentration was determined by Bradford assay (Bio-Rad). Equal amounts of protein samples (125-μg) were loaded onto a 12 % 1D-SDS PAGE (20 cm × 20 cm, Bio-Rad). The resolved protein bands were visualized by staining with Coomassie blue (ICN Biomedicals, Inc.)

In-gel digestion with trypsin

The SDS-PAGE separation revealed 52 bands per lane at the time point of 120 h at which myotubes were clearly evident (**Figures 21**). These bands were excised and each was subjected to individual in-gel reduction, alkylation, and trypsin digestion. Briefly, proteins in the gel slice were reduced with 30 μL of 50 mM ammonium bicarbonate (Sigma) /10 mM DTT (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 the dark. Enzymatic digestion was then performed overnight

with sequencing-grade trypsin (Promega) at 37 °C.

Protein identification with MS

Each tryptic peptide mixture thus generated was concentrated and desalted with ZipTip^{TM} (Millipore), and subsequently eluted with 1.5 μL of 10 mg/mL α-cyano-4-hydroxycinnamic 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 MS and MS/MS spectra generated were searched against, respectively, the SWISS-PROT and NCBInr databases using the Mascot software (Matrix Science). The search parameters were: taxonomy, *Mus musculus*; allowed modifications, carboxyamidomethylation of cysteine and oxidation of methionine; missed cleavages allowed, one; peptide and MS/MS tolerance, ±50 ppm and ±100 ppm, respectively, with a peptide charge of 1+. Secreted proteins identified had to have a minimum of two ≥ 10-residue peptides identified with high confidence and had to have been verified by manual inspection for having a consecutive series of matched and abundant *y* or *b* ions (≥ 3 residues). The vast majority of proteins were identified with three or four peptides.

Non-redundant data acquisition

To maximize the number of secreted proteins identified per MALDI spot, a non-redundant data acquisition strategy **(Figure 20)** was implemented in the selection of precursor ions (protonated tryptic peptides) for MS/MS.

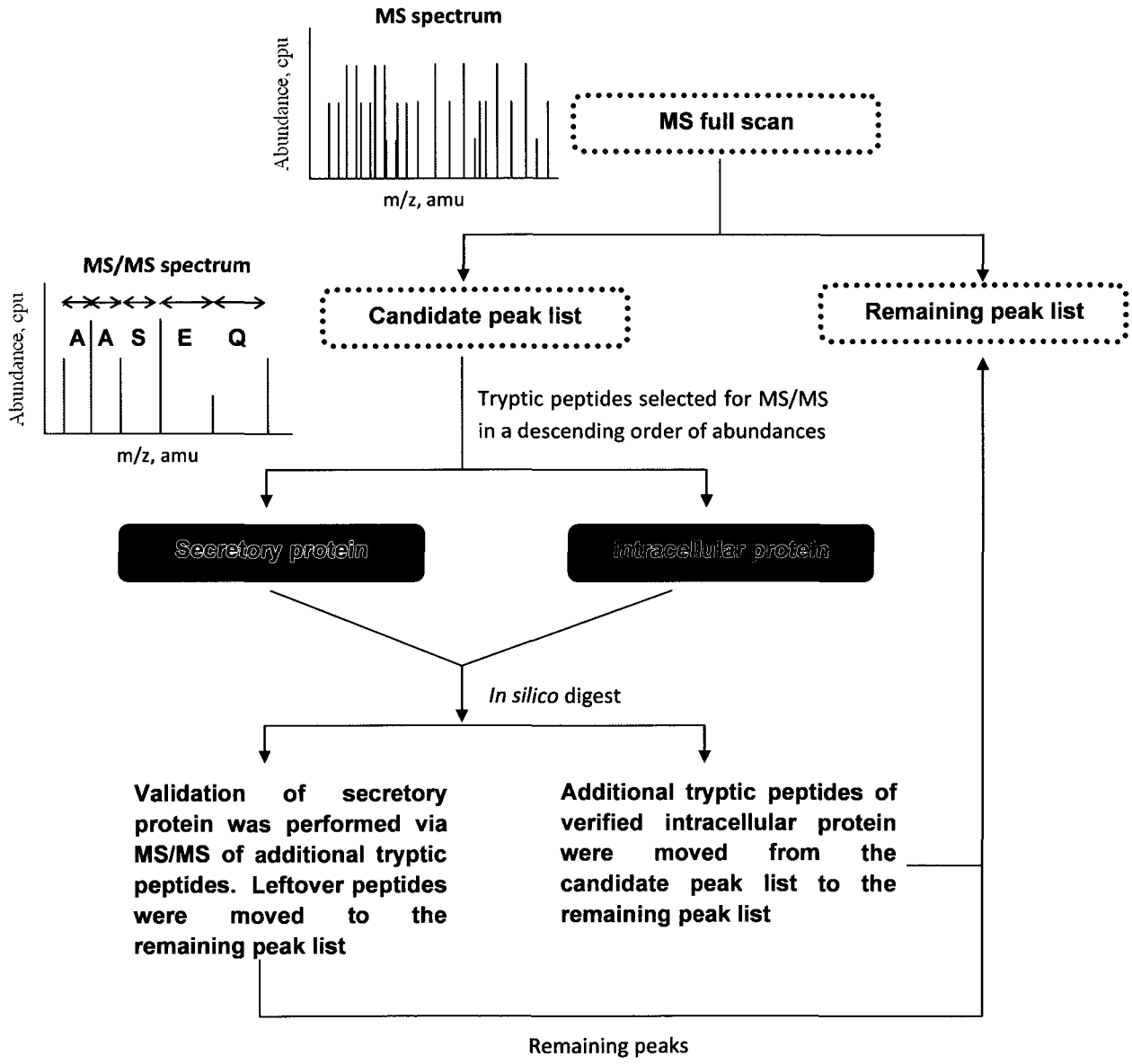


Figure 20. Non-redundant data acquisition for maximizing the discovery of secretory proteins.

All peaks in a given MALDI-TOF mass spectrum exceeding a user-defined threshold (typically S/N ≥ 2) were employed in a first-round of identification via tryptic peptide mass fingerprinting. The tryptic peptides of the candidate protein thus identified were noted and entered into a 'candidate peak list' earmarked for subsequent MS/MS analyses. The remaining peaks within the range of m/z 900-2000 were entered into a 'remaining peak list'. MS/MS was then performed first on the ions in the candidate peak list in a descending order of abundance. Proteins thus confirmed were classified as intracellular or secreted. All possible tryptic peptides of a verified intracellular protein were immediately transferred from the candidate peak list to the remaining peak list. By contrast, once a secreted protein was verified, additional validation was immediately sought via sequencing of a second and sometimes third tryptic peptide. As before, the remaining tryptic peptides of a secreted protein thus validated were also transformed to the remaining peak list. Once all the ions in the candidate peak list were analyzed, MS/MS was then performed on the ions in the remaining peak list, again in a descending order of abundance. These analyses were performed until all peptides within a given sample spot were completely consumed.

Myosin heavy chain expression with immunocytochemistry

Myosin heavy chain subunits are encoded by distinct members of a multigene family expressed at different stages of muscle development and are the archetypal marker proteins to indicate cellular differentiation of muscle. In order to visualize myosin heavy chain expression in cultured muscle cells we used the MF20 monoclonal antibody

(Bader, D. *et al.*, 1982) which recognizes all sarcomeric myosins. Briefly, after fixation (6 mins 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 anti mouse secondary antibody at a dilution of 1:1000 (Bio-Rad). Positively stained cells were visualized using a colorimetric substrate (diaminobenzidine substrate; Sigma) which results in a visually brown stain of the positive cells.

Results

Cell Culture and Preparation of Secreted Proteins

A key determinant of our selection of the mouse C2C12 myoblast as the skeletal muscle model was due to its discernible phenotypic changes during myogenesis **(Figure 21)**.

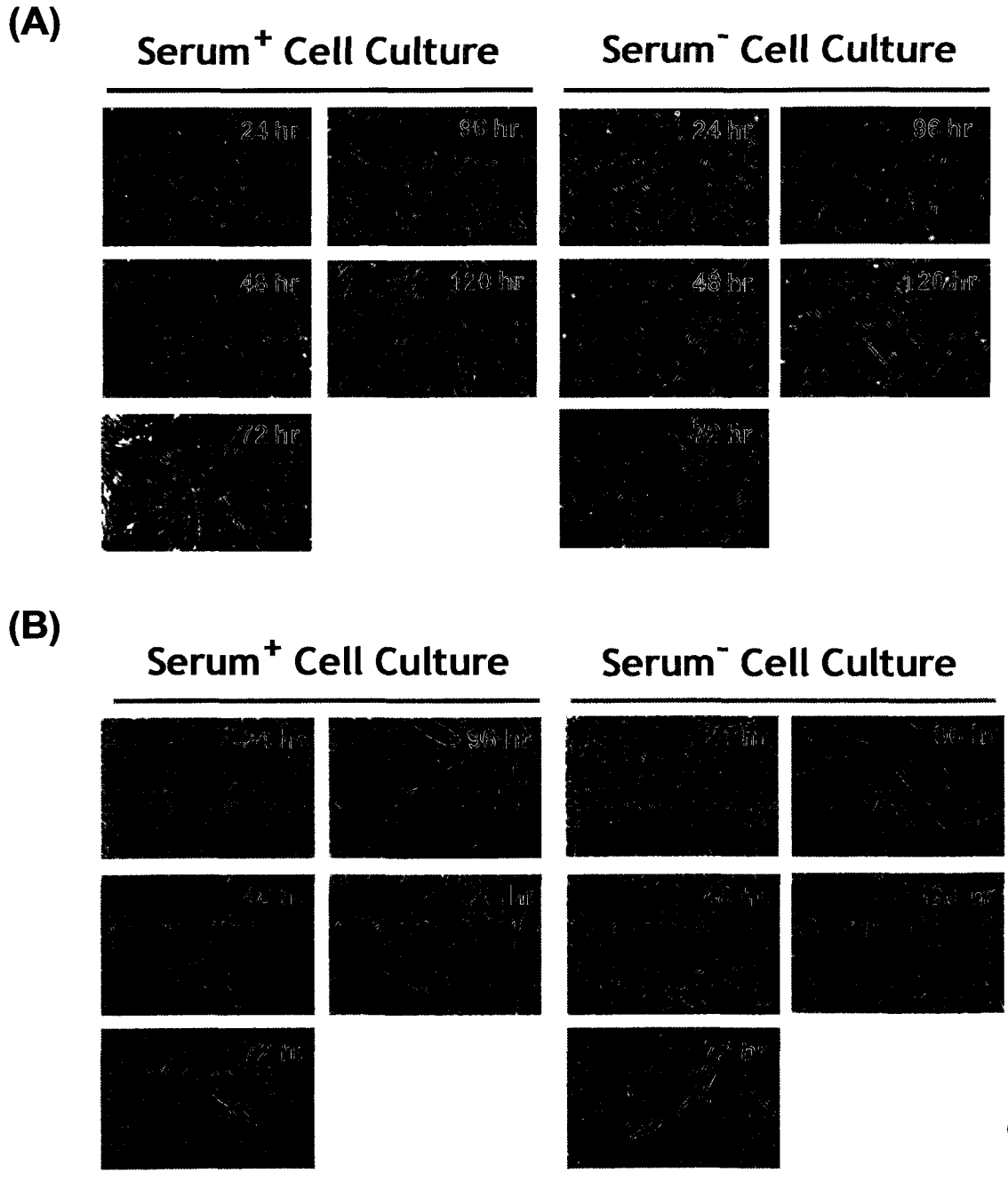


Figure 21. Phenotypic changes of C2C12 cells during myogenesis. **(A)** Light microscopic images of C2C12 myoblasts during myogenesis in serum containing- versus serum free-culture system (left and right panel, respectively). The images were taken at various time points (24, 48, 72, 96 and 120 h) following switching to, respectively, 5 % horse serum or DMEM/F12 supplemented with 2.5 μg/ml insulin. **(B)** Parallel set of samples as (A), but with myosin heavy chain staining via immunocytochemistry. Positively stained cells were visualized using a colorimetric substrate (diaminobenzidine substrate) in which brown staining indicates myosin positive cells.

The choice of 120 h for CM collection was based on a compromise between prominent myotube formation (as revealed by prominent fusion of multinucleated myotubes) and cell viability. Avoidance of fetal bovine serum (FBS), a common medium in cell culturing (Goto, S. *et al.*, 1999), was a key step, as it is a rich source of proteins thus making differentiation between C2C12 proteins and FBS proteins difficult. We found that C2C12 cells grow reasonably well in the serum-free culture medium DMEM/F12 supplemented by 2.5 μg/ml of bovine insulin **(Figure 21)**, which had been reported to be a potent mitogen, survival and differentiation factor for muscle cells (Haba Gde, L. *et al.*, 1966; Mandel, J.L. and Pearson, M.L., 1974; Smith, C.W. *et al.*, 1999; Conejo, R. *et al.*, 2001). Comparable morphological changes to the C2C12 myoblasts were apparent during myogenesis in serum-containing and the serum-free media: both cell culture systems showed the same onset time for differentiation (48 h) and maturation

time for terminal differentiation (120 h), as indicated by, respectively, the partial alignment of myoblasts and fusion of myotubes **(Figure 21A)**. Insulin (supplied exogenously) and Insulin like growth factor (secreted endogenously) act through the IGF-1 receptor in cultured muscle cells. IGF-1 is a critical paracrine factor in differentiating muscle cells *in vitro* and *in vivo*. Thus, the addition of insulin to serum free media is required for cell survival and maintenance but is also something that the cells are normally exposed to *in vivo*. In normal culture media (both growth and differentiation conditions), Insulin and IGF-1 are critical components of the media and *in vivo*, the cells are exposed to high circulating levels of Insulin (especially in the embryonic and fetal phases of development).

The CM collected at 120 h was precipitated with 80 % acetone, followed by dissolution in the SDS-gel loading buffer and one dimensional-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D-SDS PAGE) separation. The results of experiments in which we have analyzed the expression of a muscle marker gene (myosin heavy chain) by immunocytochemistry **(Figure 21B)**, demonstrate that a developmental progression in myosin gene expression, which occurs with high frequency and under relatively simple conditions ($serum^-$) can be achieved compared to more usual complex culture conditions ($serum^+$), thus making this model culture system more amenable to proteomic analysis. The data in **Figure 21B** indicate that the initial appearance of myosin positive cells (as indicated by the brown staining) occurs in both the $serum^+$ and $serum^-$ conditions at 72 h. As the differentiation proceeds at 96 and 120 h, the caliber and number of multinucleated, elongated myotubes that are positively stained for myosin in

the serum$^+$ and serum$^-$ culture conditions are qualitatively equivalent indicating the efficacy of these conditions for studying muscle differentiation.

Mass Spectrometric Analysis of Secreted Proteins

The SDS-PAGE-separated proteins were visualized as discrete bands by Coomassie blue staining **(Figure 22)**.

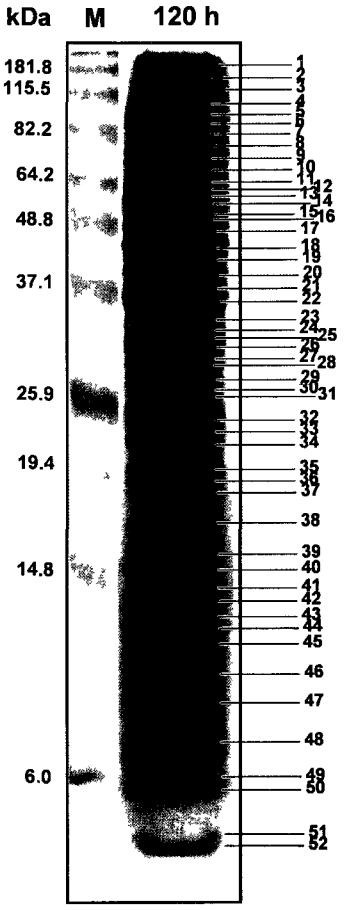
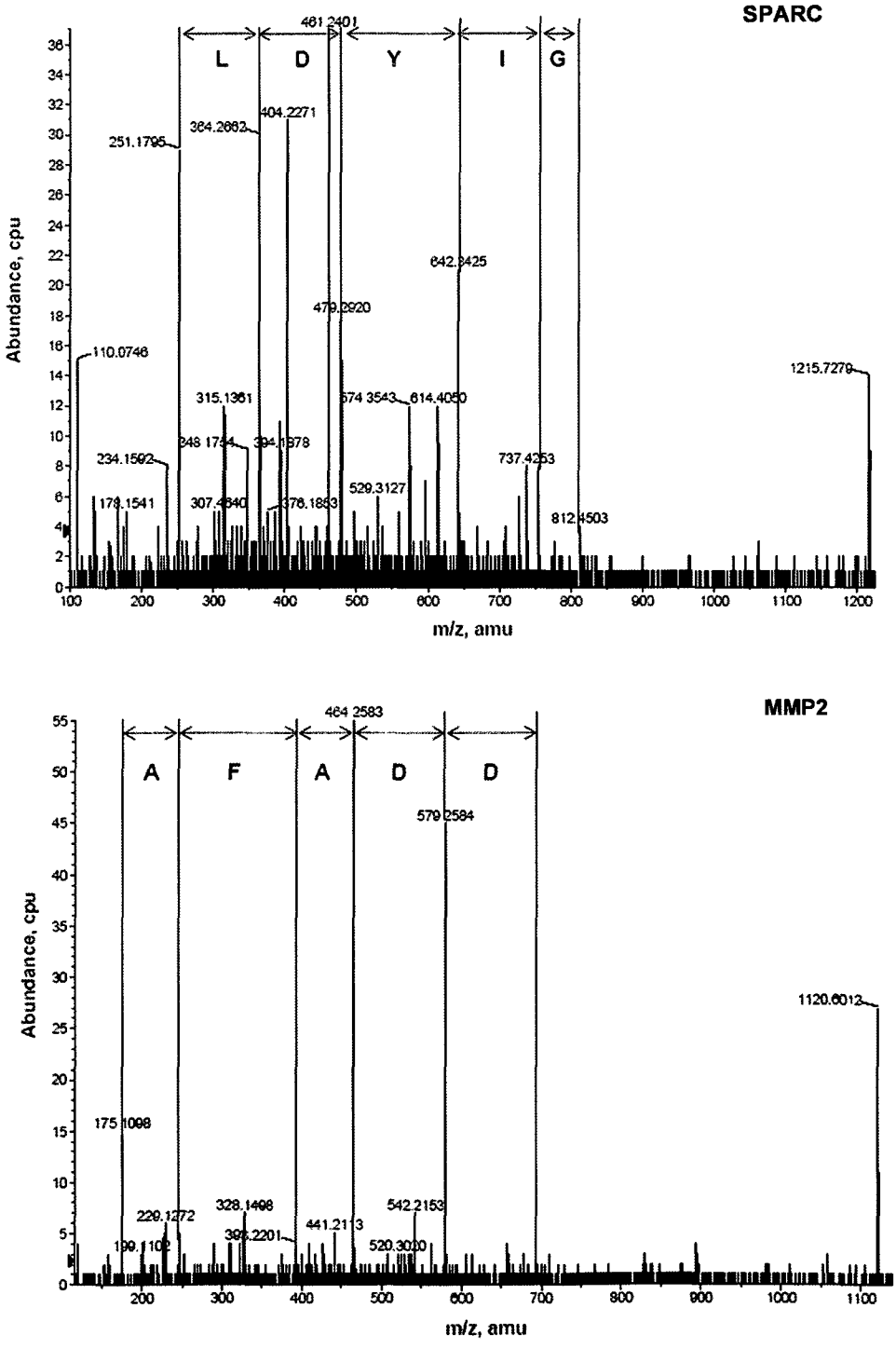


Figure 22. 1D-SDS PAGE separation of conditioned media collected at 120 h. Fifty two bands were revealed after Coomassie blue staining. Excised bands were trypsinized and analyzed using MALDI MS and MS/MS.

After in-gel tryptic digestion, the peptides were extracted for mass spectrometric analysis. We opted to use MALDI MS and MS/MS for this study because of its ruggedness in comparison with online electrospray MS/MS. The representative MS/MS spectra of three secreted proteins, secreted protein rich in cysteine protein (SPARC), matrix metalloproteinase 2 (MMP2) and cystatin C were revealed in **Figure 23**.



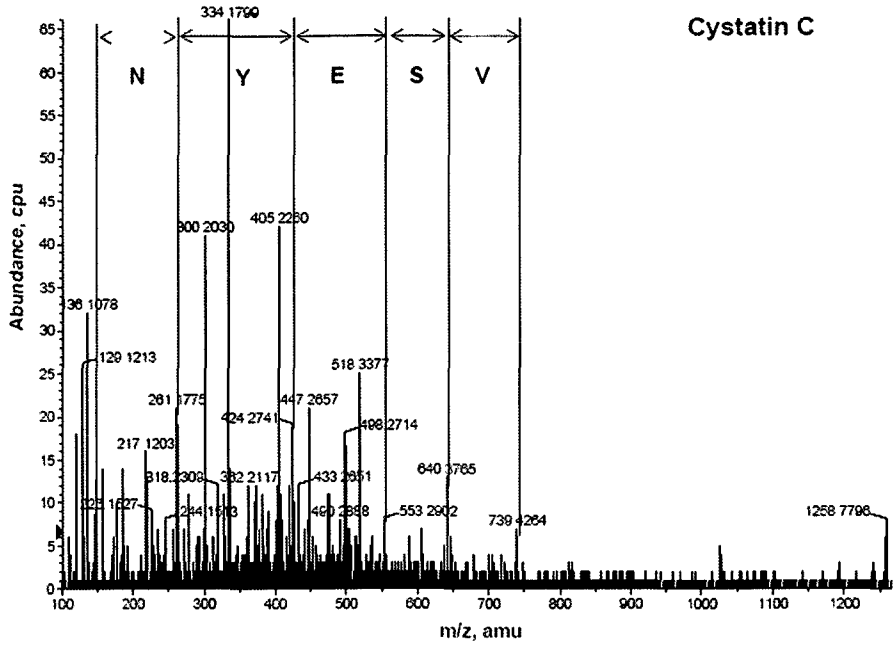


Figure 23. MS/MS spectra of tryptic peptides that contribute toward identification of three secretory proteins: SPARC, MMP2 and cystatin C.

In total, we have identified 80 proteins in the collected CM; 27 have been classified as secreted (Caroni, P. *et al.*, 1991; Bernocco, S. *et al.*, 2003; Tombran-Tink, J. and Barnstable, C.J., 2003; John, C.D. *et al.*, 2004; Ochieng, J. *et al.*, 2004; Yamaji, R. *et al.*, 2005) **(Table 1)** and 53 as intracellular proteins based on their currently known functions (Balvay, L. and Fiszman, M.Y., 1994; ; Sciote, J.J. and Morris, T.J., 2000; Merrit, T.J. and Quattro, M., 2003; Liu, Y. *et al.*, 2006) **(Table 2)**. Two proteins, e.g. fibronectin and procollagen, appeared in more than one region of the 1D-SDS gel, indicating the presence of degradation products and/or different forms of the proteins. Of

the 27 secreted proteins, the vast majority were identified with three or more peptides, thus making these highly confident identifications.

Table 1. Summary of secreted proteins.

protein name	Accession #	Band #	Peptide ions matched (Th)	Amino acid sequence of peptide matched	b & y ions matched	Functions
Fibronectin (Fn1)	gi|29835174	1	1401.67	HYQINQQWER	b2-7; y1, 4-5	extracellular matrix protein
		2	1373.68	TFYQIGDSWEK	b2-3; y2-6	
		6	1373.69	TFYQIGDSWEK	b2-3; y5-10	
			1690.87	HALQSASAGSGSFTDVR	b2-6; y1,2	
		8	1690.91	HALQSASAGSGSFTDVR	b2-6; y1,2	
		30	1401.70	HYQINQQWER	b2-5, 7; y1,2,5	
		31	1401.70	HYQINQQWER	b2-6;	

					y1, 5,6	
Fibronectin	gi|1181242	1	1593.82	VTDATETTITISWR	b6-7; y1-6, 8,11	extracellular matrix protein
			1717.92	GVTYNIIVEALQNQR	b3-6; y1-4,6	
			1906.09	VTWAPPPSIELTNLLVR	b3; y1,3-5,7	
			1958.02	N TFAEITGLSPGVTYLFK	b2-6; y2-5,8,9	
		2	1169.69	TVLVTWTPPR	b2-5; y1,3,4	
			1906.12	VTWA PPPSIELTNLLVR	b3,9; y1,3-7	
		3	1169.69	TVLVTWTPPR	b2-4; y1,3,4	
			1937.03	EESPPLIGQQATVSDIPR	y2,3,5,6	
		5	1616.86	V EVLPVSLPGEHGQR	b2,3; y1,4,7	

		8	1329.74	IHLYTLNDNAR	b2,3,5; y1-4	
Nidogen	gi|128200	3	1230.65	VLFDTGLVNPR	b2,6; y1,2,4,6,7	extracellular matrix protein
			1324.65	GNLYWTDWNR	b2-5; y1,3,4	
			1621.87	VVYWTDISEPSIGR	b2,3; y1,2,5-8	
		6	1230.71	VLFDTGLVNPR	b6; y1-4,6,7	
Biglycan	gi|348962	3	1312.77	IQAIELEDLLR	b2-5; y1-4,6	extracellular matrix protein
		4	1312.79	IQAIELEDLLR	b2-5; y1,3,4,6	
			2027.19	NHLVEIPPNLPSSLVELR	b2-6,8; y1,2,5,6,8,9,12,13	
		5	2027.13	NHLVEIPPNLPSSLVELR	b2-6,8; y6,8,12	

			2286.15	EISPDTTLLDLQNNDISELR	y1,2,4-7,10	
		6	2027.17	NHLVEIPPNLPSSLVELR	b2-6,8,9; y1,2,5,6,8,9	
		7	2027.17	NHLVEIPPNLPSSLVELR	b2-6,8; y1,2,4,5,8	
Procollagen α 2 (I)	gi|6680980	4	1421.78	GLPGEFGLPGPAGPR	y1,3,5,7,10	extracellular matrix protein
		29	1971.04	AVLLQGSNDVELVAEGNSR	b3; y1,2,4,6-8,10	
Moesin	gi|199765	9	1182.66	APDFVFYAPR	b3-5; y1-5,7	extracellular matrix protein
			1233.67	IQVWHEEHR	b2,3,5,6; y1-3	
			1677.91	TQEQLASEMAELTAR	b2,4-6; y1,2,4,6,7	
Procollagen α 1 (III)	gi|33859526	28	1344.65	KHWWTDSGAEK	b1-4; y1-8	extracellular matrix protein
			1433.69	FTYTVLEDGCTK	b2,4; y2-7	

			1497.72	NSIAYMDQASGNVK	b3-6; y2,4-7	
Procollagen α 1 (I)	gi|424104	28	1118.56	NWYISPNPK	b2,3; y2-6	extracellular matrix protein
			1187.65	SLSQQIENIR	b2-5; y1-4	
			1455.82	ALLLQGSNEIELR	b2-4; y1,2,4,5	
Heparan sulfate proteoglycan (HSPG2)	gi|62948096	36	1615.91	T STADGLLLWQGVVR	y1,4,6,10	extracellular matrix protein
			1644.87	SLPEVPETIEFEVR	y1,2,4-7,9,10	
			1666.82	LVSEDPINDGEWHR	y1,3,5,6,9	
			1674.93	DIIYIGGAPDVATLTR	b2-4; y1,5,6,8	
Matrix Metalloproteinase-2 (MMP-2)	gi|6678902	10	1223.68	WDKNQITYR	b4, y1-3,7	Metalloproteinase, regulation of cell migration
			1374.79	QDIVFDGIAQIR	b2,3; y1,3,6,10	
			2108.07	IIGYTPDLDPETVDDAFAR	y1,4-6,10	

Procollagen C-proteinase enhancer protein (PCEP)	gi|6679221	17	1098.61	GFLLWYSGR	b2-5; y1,3,4	collagenase enhancer
			1657.85	YDALEVFAGSGTSGQR	b2-5; y1-4,6,11	
Pigment epithelium-derived factor (PEDF)	gi|1747298	18	1278.67	DTDTGALLFIGR	b6-8; y1,3,6,9,11	Retinal epithelial secreted protein, member of inhibitory Serpin (serine protease inhibitors) superfamily, regulation of cell migration
			1559.77	LAAAVSNFGYDLYR	b2-5; y1-4	
Secreted protein rich in cysteine protein (SPARC)	gi|56800418	21	1106.64	NVLVTLYER	b2-5; y1,4	basement membrane protein, anti-proliferation and counteradhesion effect
			1215.65	LHLDYIGPCK	b2-7; y2-6	
			1575.88	RLEAGDHPVELLAR	b6; y1,4,7,8,11,13	
		22	1106.61	NVLVTLYER	b2,3,5; y1	
			1215.65	KLHLDYIGPCK	b2-5;	

					y3-6	
			1425.73	APLIPMEHCTTR	b2-4; y1-5	
		23	1419.77	LEAGDHPVELLAR	b2,6,7,9; y1,3-5,7,8	
			1923.98	YIAPCLDSELTEFPLR	y1,3-5,6,9	
		24	1924.02	YIAPCLDSELTEFPLR	y1,4,6,7,9	
		25	1923.9	LDYIGPCKYI APCLDSELTE FPLR	y1,3-5,7,9	
		26	1419.76	LEAGDHPVELLAR	b6-8; y1,4,7-8	
		27	1419.77	LEAGDHPVELLAR	b2,6,8; y1,4,7,8	
Serine Protease Inhibitors (Serpin), clade E	gi|6679373	19	1286.67	TPFLEASTHQR	b2-5; y1-6	inhibitors of serine protease, regulate cell migration
			1463.77	NEISTADAIFVQR	b2,3,8; y1-4,6	
Annexin A1	gi|6754570	23	1262.63	TPAQFDADELR	y1-3,5,6	calcium-binidng protein,

			1724.88	FLENQEQEYVQAVK	b2-9; y1-9	regulation of cell migration, anti-inflammatory effect
			1746.94	GLGTDEDTLIEILTTR	b7,8,10; y1,3-6,8,9,11	
		29	1262.63	TPAQFDADELR	b1-3,5,6	
			1746.93	GLGTDEDTLIEILTTR	b7,8; y1,3-7,9,11	
Galectin 3	gi|33859580	32	1273.61	GNDVAFHFNPR	b7-9; y1,2,4-8	galactose-binding protein, regulation of cell proliferation and migration
			1469.75	QSAFPFESGKPFK	b1-5; y1-6,9	
			1649.83	VAVNDAHLLQYNHR	b5,7-9; y1,2,4,5,9	
Vimentin	gi|55408	38	1115.58	VELQELNDR	b2-5; y1,2,4,5,7	cytoskeletal protein, macrophage secreted protein involved in immune response
			1254.59	LGDLYEEEMR	b3; y1-4,7	
			1296.63	EEMLQREEAESTLQSFR	b2-4;	

					y1-4,7	
			1533.88	KVESLQEEIAFLK	b3,5-10; y5,6	
Cyclophilin CyP-S1	gi|53035	39	1286.61	DFMIQGGDFTR	b2-4; y1,3,6,10	chaperone, protein folding
			1364.72	TVDNFVALATGEK	b2,5; y3-7	
Serpin	gi|200966	19	1306.69	DNQSGSLLFIGR	b3,6,7; y1-6,11	*inhibitor of serine* protease, regulation of cell migration
			1659.83	DNQSGSLLFIGR	b3,6,7; y1-6	
Lectin, galactose binding, soluble 1 (galectin-1)	gi|12805209	46	1187.52	EDGTWGTEHR	b5,9; y1-6,8,9	galactose-binidng protein, regulation of cell proliferation and migration
			1486.78	DSNNLCLHFNPR	b4-6,8; y1-6	
Annexin A2	gi|6996913	24	1111.59	QDIAFAYQR	b1-5; y1-4,7	calcium-binding protein, regulation of cell migration, anti-inflammatory effect
		25	1980.84	AEDGSVIDYELIDQDAR	y1-4,9	

			2064.98	RAEDGSVIDYELIDQDAR	b4,9; y2-4,9	
Tumor protein NM 23	gi|387496	41	1344.81	TFIAIKPDGVQR	b2-5; y1,4,6,7	blocking differentiation of leukemic cells
			1785.98	VMLGETNPADSKPGTIR	b2; y1-3,5,6,7,10	
		42	1051.59	GDFCIQVGR	b3-5; y1-5,7	
		43	1344.84	TFIAIKPDGVQR	b2,3; y1,4,6	
Cystatin C	gi|31981822	46	1256.70	ALDFAVSEYNK	b3-5; y2-6	cysteine protease (e.g. Cathepsin) inhibitors
			1598.82	ALCSFQIYSVPWK	b3,4,6,7; y2-4	
			1843.90	MLGAPEEADANEEGVRR	b9; y1,2,4,5,8	
Glyceraldehyde-3-phosphate dehydrogenase	gi|41054968	23	1779.84	LISWYDNEYGYSNR	y1,2,5,6,8	inhibit cell spreading without influencing cell growth
		24	1779.85	LISWYDNEYGYSNR	b2,3,6;	

(GAPDH)					y1-8	
Ubiquitin	gi|38373984	49	1039.56	EGIPPDQQR	y1-3,6,8	protein degradation
			1523.81	I QDKEGIPPDQQR	y1-3,5,6,8,10	
			1787.95	TITLEVEPSDTIENVK	b2-7; y3-6,9	
		50	1039.53	EGIPPDQQR	b2-4; y1-6,8	
			1787.96	TITLEVEPSDTIENVK	b2-7; y3-7,9,10	
Insulin-like growth factor binding protein-5 (IGBF 5)	gi|437125	27	1255.64	FVGGAENTAHPR	b8,10; y1-4,6	growth factor, regulation of cell proliferation
			1642.89	QDEEKPLHALLHGR	b1,3,7; y1-7,9,10,12	
		28	1255.63	FVGGAENTAHPR	y1,2,4-6	
		39	1642.89	QDEEKPLHALLHGR	y1,4-7,9,10,12	
		44	1219.66	HMEASLQEFK	b2-4,6; y2,4	
			1255.71	FVGGAENTAHPR	y1-6	

Insulin-like growth factor binding protein-2 (IGBF 2)	gi|550379	27	1378.67	QEGEACGVYIPR	b2,5; y1-4,6,8	growth factor, regulation of cell proliferation

Table 2. Summary of intracellular proteins.

Protein name	Accession #	Band #	Peptides ions matched (Th)	Amino acid sequence of peptide matched	# of residues matched
Pristanoyl-CoA oxidase	gi|12055059	1	1759.92	W LVCYLLQESHRR	13
Vinculin	gi|309533	4	1175.64	MSAEINEIIR	10
Elongation factor 2	gi|33859482	6	1138.55	YEWDVAEAR	10
			1274.73	EDLYLKPIQR	9
Elongation factor α-1	gi|13278382	19	1404.74	YYVTIIDAPGHR	12
HSP 84	gi|123681	7	1194.68	IDILPNPQER	10
HSP 1 α	gi|6754254	7	1264.69	RAPFDLFENR	10
HSP 70	gi|29748016	9	1430.77	TWNDPSVQQDIK	12
			1460.84	SDIDEIVLVGGSTR	12
			1512.84	AKFEELNMDLFR	14
HS 70 protein	gi|1661134	10	1253.62	FEELNADLFR	10
			1745.83	NQTAEKEEFEHQQK	14
Heat shock protein HSP 27	gi|424145	34	1149.61	LFDQAFGVPR	10
Chaperone TCP-1	gi|695625	13	1150.6	FAEAFEAIPR	10

		14	1307.63	HFSGLEEAVYR	11
Calreticulin	gi|50568	15	1451.65	EQFLDGDAWTNR	12
			1784.85	IKD PDAAKPEDWDER	15
α- tubulin	gi|202210	17	1701.96	AVFVDLEPTVIDEVR	15
α-tropomyosin	gi|439846	29	1243.67	IQLVEEELDR	11
			1284.77	KLVIIEGDLER	10
Beta-tropomyosin	gi|50190	26	1243.66	IQLVEEELDR	10
			1243.67	IQLVEEELDR	10
Tropomyosin 3	gi|62027399	29	1642.83	IQVLQQQADDAEER	14
Actin related protein 2/3 complex	gi|23621467	29	1343.73	DNTINLIHTFR	11
			1450.78	ASHTAPQVLFSHR	13
Beta-actin FE-3	gi|13516473	36	1790.9	SYELPDGQVITIGNER	16
α-actin	gi|49870	48	1198.71	AVFPSIVGRPR	11
Myosin light chain	gi|71051212	38	1260.62	GNFNYIEFTR	10
			1415.66	FTDEEVDELYR	13
Desmin	gi|8250510	39	1673.89	FLEQQNAALAAEVNR	15
Kinesin	gi|41327746	25	1296.42	DESGCEEEEGR	11

Cofilin 1	gi|6680924	40	1519.73	HELQANCYEEVK	12
			1790.87	HELQANCYEEVKDR	14
Transgelin	gi|6755714	41	1221.67	QMEQVAQFLA	10
Zinc finger	gi|63635047	42	1032.68	RYRPIAT	8
Cysteine-rich protein 1	gi|39795495	48	913.44	EVYFAER	7
			1111.55	DWHRPCLK	8
			1316.6	CDKEVYFAER	10
Ribosomal protein S21	gi|21536222	48	1584.73	DHASIQMNVAEVDR	14
Ribosomal protein S19	gi|12963511	50	1134.58	DVNQQEFVR	9
Nuclear ribonucleprotein A/B	gi|6754222	23	1499.72	EVYQQQQYGSGGR	13
Tyr 3-monooxygenase	gi|30583161	31	1189.72	DSTLIMQLLR	10
			1256.67	YLAEFATGNDR	11
			1820.08	AASDIAMTELPPTHPIR	17
		33	1819.94	AASDIAMTELPPTHPIR	17
Tyr 3-monooxygenase	gi|29748002	32	1330.69	FLIPNASQPESK	12
Phosphoglycerate mutase	gi|12963669	32	1312.61	HGESAWNLENR	11
			2115.12	NLKPIKPMQFLGDEETVR	18

Phosphoglycerate kinase	gi|202423	20	1634.88	LGDVYVNDAFGTAH R	15
			1769.09	ALESPERPFLAILGGAK	17
Testis-specific phosphoglycerate kinase	gi|200326	28	1634.80	LGDVYVNDAFGTAHR	15
Proteasome (prosome, macropain) subunit, alpha type 6	gi|6755198	33	1156.62	HITIFSPEGR	10
GDP(Guanosine DiP) *dissociation inhibitor*	gi|40254781	18	1351.68	DLGTDSQIFISR	12
Rho GDP dissociation inhibitor (GDI) α	gi|55742827	33	1601.78	IDKTDYMVGSYGPR	15
			1650.92	VAVSADPNVPNV IVTR	16
			1783.82	AEEYEFLTPMEEAPK	14
Triosephosphate isomerase	gi|54855	34	954.49	FFVGGNWK	8
			1466.74	TATPQQAQEVHEK	13
Glutathione S-transferase	gi|2624496	35	1351.74	PPYTIVYFPVR	16
			1854.94	FEDGDLTLYQSNAILR	11
Peroxiredoxin 1	gi|56103807	36	1006.55	IGYPAPNFK	9
			1196.65	LVQAFQFTDK	10
Peroxiredoxin 4	gi|7948999	37	1225.69	QITLNDLPVGR	11
Keratin complex 2	gi|46275805	52	1179.63	YEELQVTAVK	10

α-Enolase	gi|12963491	18	1439.74	YITPDQLADLYK	12
Aldolase 1, A isoform	gi|6671539	22	1342.74	ADDGRPFPQVIK	12
Malate dehydrogenase	gi|319837	26	1393.72	FVEGLPINDFSR	12
Lactate dehydrogenase	gi|6754524	26	1055.63	DQLIVNLLK	9
			1118.59	SADTLWGIQK	10
Pyruvate Kinase M	gi|551295	13	1665.86	FDEILEASDGI MVAR	15
		14	1019.5	GDYPLEAVR	18
			1586.77	DAVLNAWAEDVDLR	22
			1764.96	KGVNLPGAAVDLPAVSEK	14
			2145.11	LAPITSDPTEAAAVGAVEAS FK	9
		15	1586.8	DAVLN AWAEDVDLR	14
Dstn	gi|71059761	41	1307.68	YALYDASFETK	11
			1542.75	HEYQAVGPEDLNR	13
Prolyl 4-hydroxylase, β polypeptide	gi|62533147	15	1780.83	VDATEESDLAQQYGVR	15
			1833.9	ILFIFIDSDHTDNQR	16
Hexosaminidase beta	gi_226165	12	1224.66	LQPALWPFPR	10
Lysyl oxidase	gi|6754568	35	1051.55	EPPAAPGAWR	10

			1447.71	HWFQAGFSPSGAR	13
Cyclophilin C complexed w/ Cyclosporin A	gi|1000036	38	1357.65	DFMIQGGDFTAR	12
Cyclophilin A	gi|38049599	42	1154.66	FEDENFILK	9
Peptidylprolyl isomerase A	gi|71051228	42	1278.68	EGMNIVEAMER	11
		43	1055.61	VSFELFADK	12
			1379.83	VSFELFADKVP	9

Non-redundant Data Acquisition

In addition to secreted proteins, intracellular proteins might also exist in CM which could arise as a result of cell stress, by which they were released to the culture media subsequent to apoptosis and/or as a consequence of washing. In order to maximize the number of secreted proteins identified, we implemented the strategy of non-redundant data acquisition **(Figure 20)** in which the detail of it was described in the Materials and Methods session. The general rationale of this approach is to categorize the tryptic peptides into intracellular and extracellular proteins with the help a protein database. The result of this could in return facilitate our focus on secreted protein identification.

In the secreted protein identification list as shown in **Table 1**, the number of distinct MS/MS analyses (accumulated spectra) per sample varied from a low of 7 to a high of 33 (due to different quantities of proteins available in the bands), with an average of 19 analyses per sample band. With the implementation of a non-redundant data acquisition approach, these numbers dropped enormously in intracellular protein list with an average of <3 analyses per protein of a range from 1 to 3 **(Table 2)**. This supports the potent effectiveness of the nonredundant approach in maximizing secreted protein identification.

Discussion

Proteins secreted into the extracellular milieu by skeletal muscle cells, sometimes referred to collectively as the 'secretome', are potent mediators of cell survival, proliferation, differentiation, and fusion. To begin unraveling the complex biology underlying the role played by secreted proteins in myogenesis, the first necessary step is to characterize the proteins involved. A critical initial requirement prior to this protein identification is the development of a method for preparing secretome samples from cultured muscle cells without extensive contamination by intracellular or culture media derived proteins. In this study, we have developed an effective method for myogenic secretome isolation and characterization using mass spectrometry. A number of the secreted proteins thus identified have previously been reported as proteins secreted from muscle (Bernocco, S. *et al.*, 2003; Tombran-Tink, J. and Barnstable, C.J., 2003) indicating the validity of the approach taken. More importantly, some identified proteins appear to function as key secreted regulators of the muscle growth and differentiation program. It is not possible at this stage to classify unequivocally all of the remainder as secreted proteins, as the possibility exists that these could arise as a result of cell stress and were released to the culture media subsequent to apoptosis and/or as a consequence of washing. Validation experiments, including localization with tagged proteins, will need to be performed in the future.

We initially assigned the secreted proteins to several functional groups, in order to putatively link proteins together in known pathways and to identify possible roles in muscle differentiation. A number of the proteins segregate into the 'extracellular matrix' (ECM) class of proteins, including fibronectin, nidogen, biglycan, various isoforms of

procollagen, moesin and heparan sulfate proteoglycan. Evidence indicates that the ECM not only acts as a structural support for maintaining the cell architecture, but also plays a pivotal role in both morphogenesis and tissue regeneration via ECM remodeling. This process, in turn, is regulated by ECM-associated proteases (John, C.D. *et al.*, 2004; Ochieng, J. *et al.*, 2004). In particular, the ECM proteins belonging to MMPs have been implicated in muscle differentiation (Caroni, P. *et al.*, 1991). Several of these proteases, including MMP2 and procollagen C-proteinase enhancer protein (PCEP), were found in this study. MMP2 exerts a direct degradation effect on the ECM by cleaving the major ECM components, such as type IV collagen (El-Mallakh, R.S. *et al.*, 1992; Balvay, L. and Fiszman, M.Y., 1994; Kraft, T. *et al.*, 2000; Sciote, J.J. and Morris, T.J., 2000; Merrit, T.J. and Quattro, M., 2003; Yamaji, R. *et al.*, 2005; Liu, Y. *et al.*, 2006). PCEP triggers the degradation of ECM indirectly by promoting the activity of the ECM-degrading enzyme, procollagen C-proteinase (Werb, Z., 1997; Cho, W.J. *et al.*, 2000; Goetsch, S.C. *et al.*, 2003; Lluri, G. and Jaworski, D.M., 2005).

A number of proteins identified belong to the class of proteins involved in cell migration. During skeletal muscle development, myoblasts migrate across the basal lamina; this process stops once the differentiation program commences (Egeblad, M. and Werb, Z., 2002). Several secreted proteins that have been identified in this study, including serine protease inhibitors (serpin) (Kherif, S. *et al.*, 1999; El Fahime, E. *et al.*, 2000; Balcerzak, D. *et al.*, 2001; Oh, J. *et al.*, 2004), pigment epithelium-derived factor (PEDF) (Couch, C.B. and Strittmatter, W.J., 1983; Reponen, P. *et al.*, 1992; Carmeli, E. *et al.*, 2004), annexin-A1 and -A2 (Scott, I.C. *et al.*, 1999; Steiglitz, B.M. *et al.*, 2002; Bernocco, S. *et al.*, 2003), and galectin-1 and -3 (Low, D.A. *et al.*, 1981; Hughes, S.M.

and Blau, H.M., 1990; Carrell, R.W. *et al.*, 1991; Verdière-Sahuquè, M. *et al.*, 1996; Mbebi, C. *et al.*, 1999; Simonovic, M. *et al.*, 2001; Meyer, C. *et al.*, 2002; Ricard-Blum, S. *et al.*, 2002), are speculated to be involved in the regulation of myogenesis consistent with their capability in regulating cell migration.

An intriguing link between the MMP2, secreted protein rich in cysteine (SPARC), and cystatin C proteins was identified by combining our protein identification data (representative MS/MS data shown in **Figure 23**) with published literature concerning the functional properties of these proteins. This putative network of extracellular proteins is based on the following observations: MMP2 has been implicated in myoblast migration (Sciote, J.J. and Morris, T.J., 2000), myotube formation (Yamaji, R. *et al.*, 2005), and regulation of satellite cells (El-Mallakh, R.S. *et al.*, 1992); SPARC functions as a potent anti-proliferative protein and is highly expressed during the differentiation of myoblasts (Tombran-Tink, J. and Barnstable, C.J., 2003). Both SPARC (Tombran-Tink, J. and Barnstable, C.J., 2003; Rescher, U. and Gerke, V., 2004) and MMP2 (Barondes, S.H. *et al.*, 1994; Perillo, N.L. *et al.*, 1998; Alldridge, L.C. and Bryant, C.E., 2003; John, C.D. *et al.*, 2004) are common targets of the TGFβ signaling pathway in muscle cells. Moreover, MMP2 directly cleaves and activates the SPARC protein (Yang, R.Y. and Liu, F.T., 2003). In addition, cystatin C, a cysteine protease inhibitor (Krzeslak, A. and Lipinska, A. *et al.*, 2004), is TGFβ responsive (Ochieng, J. *et al.*, 2004; Califice, S. *et al.*, 2004; Sato, S. and Nieminen, J., 2004) and is directly associated with MMP2 regulation (Schiemann, B.J. *et al.*, 2003; Francki, A. *et al.*, 2004). Thus we propose a model for this proteomic model in which reciprocal feedback loops between the respective proteins will contribute to a functional role in the myogenic differentiation program (**Figure 24**).

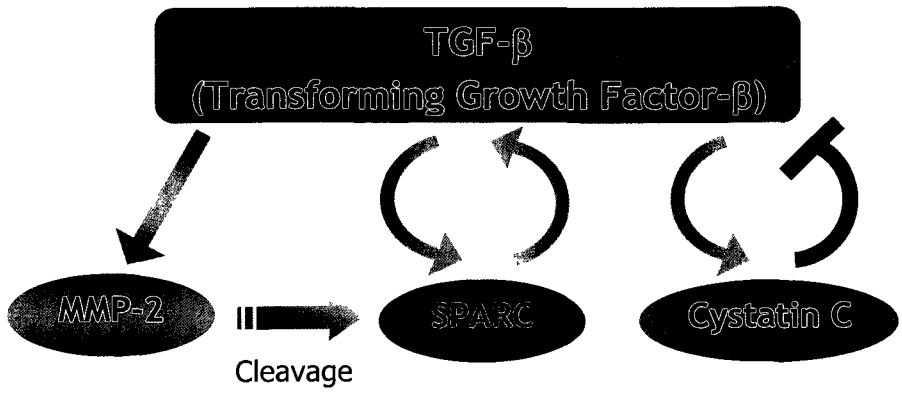


Figure 24. Proposed model of secretory proteins associated with TGFβ. Positive interactions are indicated by arrowheads, inhibitory interactions are indicated by right angled lines. Direction of arrows and angled lines indicates the directionality of signaling. Bi-directional lines indicate a proposed feedback loop. In this hypothesis, TGFβ induces MMP2 by increasing its transcription and/or prolonging the stability of MMP2 transcripts. A positive feedback loop is proposed between SPARC and TGFβ: TGFβ elicits SPARC expression at the transcriptional level; SPARC interacts with TGFβ receptor and thus facilitates TGFβ signaling. A negative feedback loop is proposed between cystatin C and TGFβ: TGFβ upregulates cystatin C; however, cystatin C deactivates TGFβ signaling by antagonizing cognate receptors of the TGFβ pathway. MMP2 is proposed to generate a cleaved form of SPARC, the physiological role of which is currently unknown.

Concluding remarks

In summary, a strategy was developed for the isolation and identification of secreted proteins during the differentiation of cultured muscle cells. A number of secreted proteins belonging to several functional groups were identified that hold considerable potential as key regulators of the myogenic differentiation program. These studies provide a testable framework for identifying mechanistic links within the network of secreted proteins responsible for the differentiation of myogenic cells. The identification and characterization of secreted proteins may facilitate the development of small molecule-based therapeutic strategies for *in vivo* and *in vitro* manipulation of muscle differentiation and growth in a variety of congenital and acquired myopathies.

Acknowledgements

This work was made possible by grants from CIHR and the Heart and Stroke Foundation of Canada

Chapter 4:

Quantification of the secretome during skeletal myogenesis

Rationale

To further our understanding of the role of the secretome during skeletal myogenesis, we implemented SILAC in tandem with RPLC-MS/MS to identify differentially expressed secreted factors during muscle differentiation.

This manuscript represents work done in the labs of K.W. Michael Siu, John C. McDermott and Kenneth R. Evans at Ontario Cancer Biomarker Network (OCBN). I was responsible for the cell culture, SILAC labelling, Western blot analyses, immunocytochemistry, and data acquisition with LTQ LIT mass spectrometry with Jian Chen, Dharsee Moyez, and Peihong Zhu. Data acquisition with QSTAR Pulsar mass spectrometry was performed by Olena Masui in collaboration with myself. Statistical analyses were done by Olga Krakovska, Shaun Ghanny and Sebastien Voisin. Plasmid construction, Reverse transcription-polymerase chain reaction (RT-PCR) and reporter assays were conducted by Vladimir Belozerov. I was primarily responsible for the interpretation of mass spectra and results, and their implications. This manuscript was written by me with editing by John McDermott and Michael Siu.

Molecular and Cellular Proteomics, **10(5)**, M110.004804. Epub 2011 Feb 22

Identification of differentially regulated secretome components during skeletal myogenesis

C.Y. X'avia Chan[1,2], Olena Masui[2,3], Olga Krakovska[2,3], Vladimir E. Belozerov[2,3], Sebastien Voisin[2,3], Shaun Ghanny[1,2], Jian Chen[4], Dharsee Moyez[4], Peihong Zhu[4], Kenneth R. Evans[4], John C. McDermott[1,2,5,6], and K.W. Michael Siu[1,2,3]

Author affiliation:

[1]Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[2]Centre for Research in Mass Spectrometry (CRMS), York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[3]Department of Chemistry, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[4]Ontario Cancer Biomarker Network (OCBN), MaRS Centre, South Tower, Suite 200, 101 College street, Toronto, Ontario, M5G 1L7

[5]Muscle Health Research Centre (MHRC), York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[6]Centre for Research in Biomolecular Interactions (CRBI) York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

To whom correspondence should be addressed:

John C. McDermott

247 Farquharson Building, York University, 4700 Keele Street, Ontario, Canada, M3K 1P3

Phone: 416-736-2100 (×30389)

Email: jmcderm@yorku.ca

K.W. Michael Siu

242 Chemistry Building, York University, 4700 Keele Street, Ontario, Canada, M3J 1P3

Phone: 416-650-8021

Email: kwmsiu@yorku.ca

Fax: 416-736-5752

Received for review September 11, 2010. Accepted February 22, 2011.

This research was originally published in Molecular and Cellular Proteomics. C.Y. X'avia Chan, Olena Masui, Olga Krakovska, Vladimir E. Belozerov, Sebastien Voisin, Shaun Ghanny, Jian Chen, Dharsee Moyez, Peihong Zhu, Kenneth R. Evans, John C. McDermott, and K.W. Michael Siu. Identification of differentially regulated secretome components during skeletal myogenesis. Molecular and Cellular Proteomics. 2011; 10: M110.004804. Epub 2011 Feb 22 © the American Society for Biochemistry and Molecular Biology.

Abstract

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 (Chan, C.Y. *et al.*, 2007). 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 (SILAC) 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 a number of 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 (Fstl-1), osteoglycin (OGN), spondin-2 and cytokine-induced apoptosis inhibitor-1 (CIAPIN-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.

Introduction

Development, growth and maintenance of skeletal musculature are a critical feature of all metazoan species (Ordahl, C.P. and Le Douarin, N.M., 1992; Buffinger, N. and Stockdale, F.E., 1994; Christ, B. and Ordahl, C.P., 1995; Cossu, G. *et al.*, 1996a; Cossu, G. *et al.*, 1996b; Tajbakhsh, S. and Cossu, G., 1997; Venters, S.J. *et al.*, 1999; Cossu, G. *et al.*, 2000). 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 (Youn, B.W. and Malacinski, G.M., 1981; Ordahl, C.P. and Le Douarin, N.M., 1992; Pourquie, O. *et al.*, 1993; Christ, B. and Ordahl, C.P., 1995; Kato, N. and Aoyama, H., 1998). 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), 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) (Capers, C.R., 1960; Konigsberg, I.R. *et al.*, 1960; Stockdale, F.E. and Holtzer, H., 1961; Stockdale, F. *et al.*, 1964). At the molecular level, each of these steps is regulated by the interplay of intracellular signal transducers and nuclear transcription factors. In particular, the muscle regulatory factor (MRF) family, MyoD (Davis, R.L. *et al.*, 1987; Choi, J. *et al.*, 1990), Myf5 (Braun, T. *et al.*, 1989; Tajbakhsh, S. *et al.*, 1996b), myogenin (Knoepfler, P.S. *et al.*, 1999; Berkes, C.A. *et al.*, 2004; de la Serna, I.L. *et al.*, 2005) and MRF4 (Rhodes, S.J. and Konieczny, S.F., 1989) are essential for myoblast

lineage commitment (Rudnicki, M.A. *et al.*, 1992; Rudnicki, M.A. *et al.*, 1993; Braun, T. and Arnold, H.H., 1996), 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 (Edmondson, D.G. and Olson, E.N., 1989; Rhodes, S.J. and Konieczny, S.F., 1989; Wright, W.E. *et al.*, 1989). 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 (Lassar, A.B. *et al.*, 1991).

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 remodeling (Buchkovich, K. *et al.*, 1989; Chen, P.L. *et al.*, 1989; DeCaprio, J.A. *et al.*, 1989; Kato, J. *et al.*, 1993; Obeyesekere, M.N. *et al.*, 1995; Harbour, J.W. *et al.*, 1999; Ren, B. *et al.*, 2002). This allows MBs to proliferate. In addition, CDKs can also phosphorylate MRFs and promote their degradation, thereby further suppressing the differentiation program (Song, A. *et al.*, 1998; Kitzmann, M. *et al.*, 1999; Floyd, Z.E. *et al.*, 2001). 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 confluency *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 (Hatta, K. *et al.*, 1987; Knudsen, K.A. *et al.*, 1990; Holt, C.E. *et al.*, 1994; Zeschnigk, M. *et al.*, 1995; George-Weinstein, M. *et al.*, 1997; Goichberg, P. and Geiger, B., 1998). 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 (Gavard, J. *et al.*, 2004; Messina, G. *et al.*, 2005). 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 pre-requisite for differentiation to occur (Nadal-Ginard, B., 1978).

While 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 (Bischoff, R., 1986a; Bischoff, R., 1990). Intriguingly, while 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 (Engler, D.,

2007; Pedersen, B.K. and Febbraio, M.A., 2008). Apart from the well-known myokines, such as members of the insulin-like growth factor-1 (IGF-1) (Florini, J.R. *et al.*, 1993; Liu, J.P. *et al.*, 1993; Powell-Braxton, L. *et al.*, 1993; Coleman, M.E. *et al.*, 1995; Barton-Davis, E.R. *et al.*, 1998; Lawlor, M.A. *et al.*, 2000; Lawlor, M.A. and Rotwein, P., 2000a; Lawlor, M.A. and Rotwein, P., 2000b; Barton, E.R. *et al.*, 2002; Musaro, A. *et al.*, 2004) and transforming growth factor (TGF) families (Florini, J.R. *et al.*, 1986; Massague, J. *et al.*, 1986; Olson, E.N. *et al.*, 1986; Brennan, T.J. *et al.*, 1991; Martin, J.F. *et al.*, 1992; De Angelis, L. *et al.*, 1998; Liu, D. *et al.*, 2001; Liu, D. *et al.*, 2004; Cohn, R.D. *et al.*, 2007), there are studies investigating other myokines, such as plasminogen activator (Festoff, B.W. *et al.*, 1982), collagenase (Beach, R.L. *et al.*, 1985), decorin (Brandan, E. *et al.*, 1991), glial growth factor (Florini, J.R. *et al.*, 1996a), neurocrescin (Nishimune, H. *et al.*, 1997), meltrin alpha (Gilpin, B.J. *et al.*, 1998), musculin (Engler, D., 2007; Nishizawa, H. *et al.*, 2004), IL-1 beta (Tateno, K. *et al.*, 2006), IL-7 (Haugen, F. *et al.*, 2010), ADAMTS-like 2 (Koo, B.H. *et al.*, 2007), Fstl-1 (Ouchi, N. *et al.*, 2008), and secreted protein acidic and rich in cysteine (SPARC) (Bassuk, J.A. *et al.*, 1993; Cho, W.J. *et al.*, 2000; Jorgensen, L.H. *et al.*, 2009). 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 a 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 (Chan, C.Y. *et al.*, 2007), we have implemented a quantitative proteomics approach involving SILAC (Ong, S.E. *et al.*, 2002) to compare the secretomes of MBs and MTs derived from mouse skeletal muscle

cells (C2C12) that were metabolically labeled with light [$^{12}C_6$]-lysine (L) and heavy [$^{13}C_6$]-lysine (H), respectively.

Materials and methods

Cell culture and SILAC labelling

The workflow of cell culture and SILAC labeling is depicted in **Figure 25**.

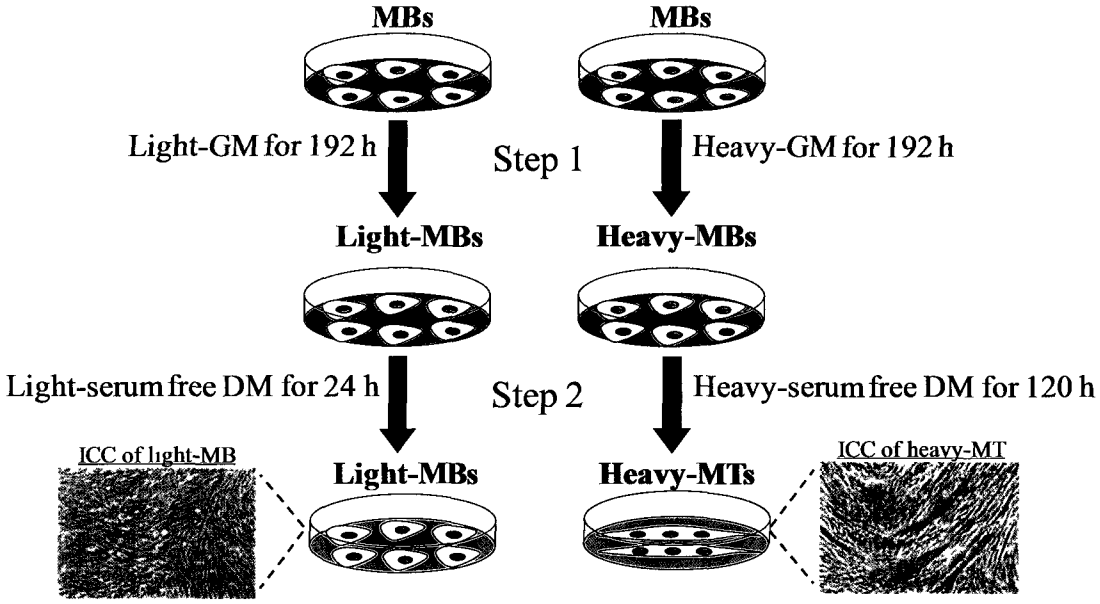


Figure 25. Workflow of cell culture and SILAC labelling. MBs were cultured in either light- or heavy-labeled growth medium (GM) for 192 h (Step 1). Light- and heavy-labeled MBs were then subjected to serum-free differentiation medium (DM) for 24 h and 120 h, respectively (Step 2). This produced light-labeled MBs and heavy-labeled MTs, the latter of which prominently expressed myosin heavy chain (MyHC), a muscle differentiation marker visualized as a brown stain by immunocytochemistry (ICC).

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 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 % CO_2. 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, supplemented with either 0.7 mM [$^{12}C_6$]- or [$^{13}C_6$]-lysine (Invitrogen), respectively **(Step 1 of Figure 25)**.

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) and segregated in 1 mL of 0.125 % trypsin (Gibco) for 1 min. Trypsinization was terminated by the addition of 5 mL of serum-free DM, comprising SILAC Dulbecco's MEM: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 (Chan, C.Y. *et al.*, 2007). This medium allows differentiation of the cells in a serum protein-free environment which has proven critical for secretome analysis (Haba Gde, L. *et al.*, 1966; Mandel, J.L. and Pearson, M.L., 1974; Smith, C.W. *et al.*, 1999; Conejo, R.

et al., 2001; Li, H. *et al.*, 2007; Polacek, M. *et al.*, 2010; Zhang, Y. *et al.*, 2010; Zhong, J. *et al.*, 2010). In addition, supplementation of the media as we have identified above is critical as culture of cells in non-supplemented 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×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 [$^{12}C_6$]- or [$^{13}C_6$]-lysine) in which light- and heavy-labeled MBs were allowed to inoculate for 24 h and 120 h, respectively **(Step 2 of Figure 25)**. During the differentiation of heavy-labeled MBs, cells were washed with 10 mL of phosphate-buffered saline (PBS) (Gibco) 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 **(Figure 25)**.

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 **(Step 1 of Figure 25)**.

Cells were washed with PBS twice and scraped from culture plates in 1 mL of PBS, followed by centrifugation at 57×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-HCl (Bioshop), 150 mM NaCl (Bioshop), 0.5 % NP40 (BioRad), 2 mM EDTA (Bioshop), 100 mM NaF (Sigma), 10 mM Na_2HPO_4 (Bioshop), 1 mM Na_3VO_4 (Sigma), 1 mM PMSF (Bioshop), 1 µg/mL leupeptin (Bioshop), 1 µg/mL aprotinin (Bioshop) and 1 µg/mL pepstatin A (Bioshop). 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×g for 5 min at 4 °C. The supernatant was collected.

Preparation of conditioned media (CM)

To examine the expression pattern of the secretome 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 (**Step 2 of Figure 25**). The CM collected were purified by two-step centrifugation at 98 ×g for 5 min at 4 °C, followed by 12,000 ×g for 30 min. The clarified media were then syringe-filtered (0.45-µm pore size, Millex-HV) to remove remaining particles. Proteins in the CM were precipitated by 80 % acetone (v/v) (Caledon) at -20 °C for 24 h. The proteins were then pelleted by centrifugation at 4 °C (15,344×g for 1 h) and allowed to air dry. The dried proteins were then dissolved in a minimal volume of sodium dodecyl sulphate (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 density (OD) at 595 nm using a spectrophotometer (Beckman). Protein concentrations in the samples were determined by comparing their OD with those in the BSA standard curve.

One dimensional (1D)-SDS PAGE

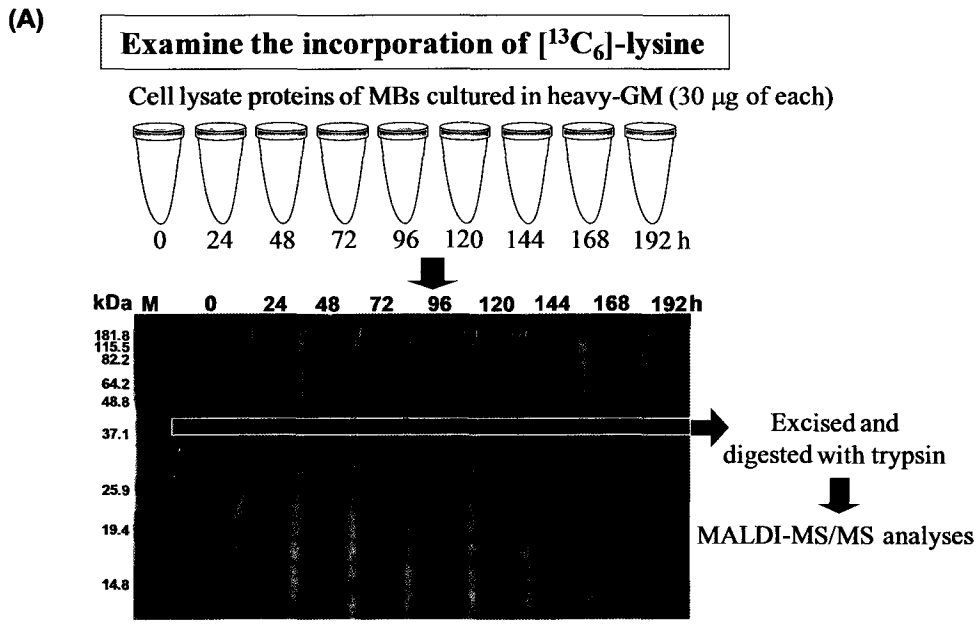
All 1D-SDS PAGE were performed on 12 %-polyacrylamide gels (10 cm × 10 cm, Bio-Rad) using Coomassie blue (ICN Biomedicals, Inc.) to visualize the resolved protein bands.

For evaluating the incorporation of [$^{13}C_6$]-lysine, 30 μg of cell lysate protein derived from MBs culturing in heavy-labeled GM from 0 to 192 h **(Step 1 of Figure 25)** 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 **(Figure 26A)**.

For determining the thresholds for differential expression, cell lysates from MBs cultured in light- and heavy-labeled GM for 192 h **(Step 1 of Figure 25)** 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 **(Figure 26B)**.

For examining the relative expression of the secretome at the MT versus MB stage, CM collected from light-labeled MBs and heavy-labeled MTs **(Step 2 of Figure 25)** 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 **(Figure 26C)**.



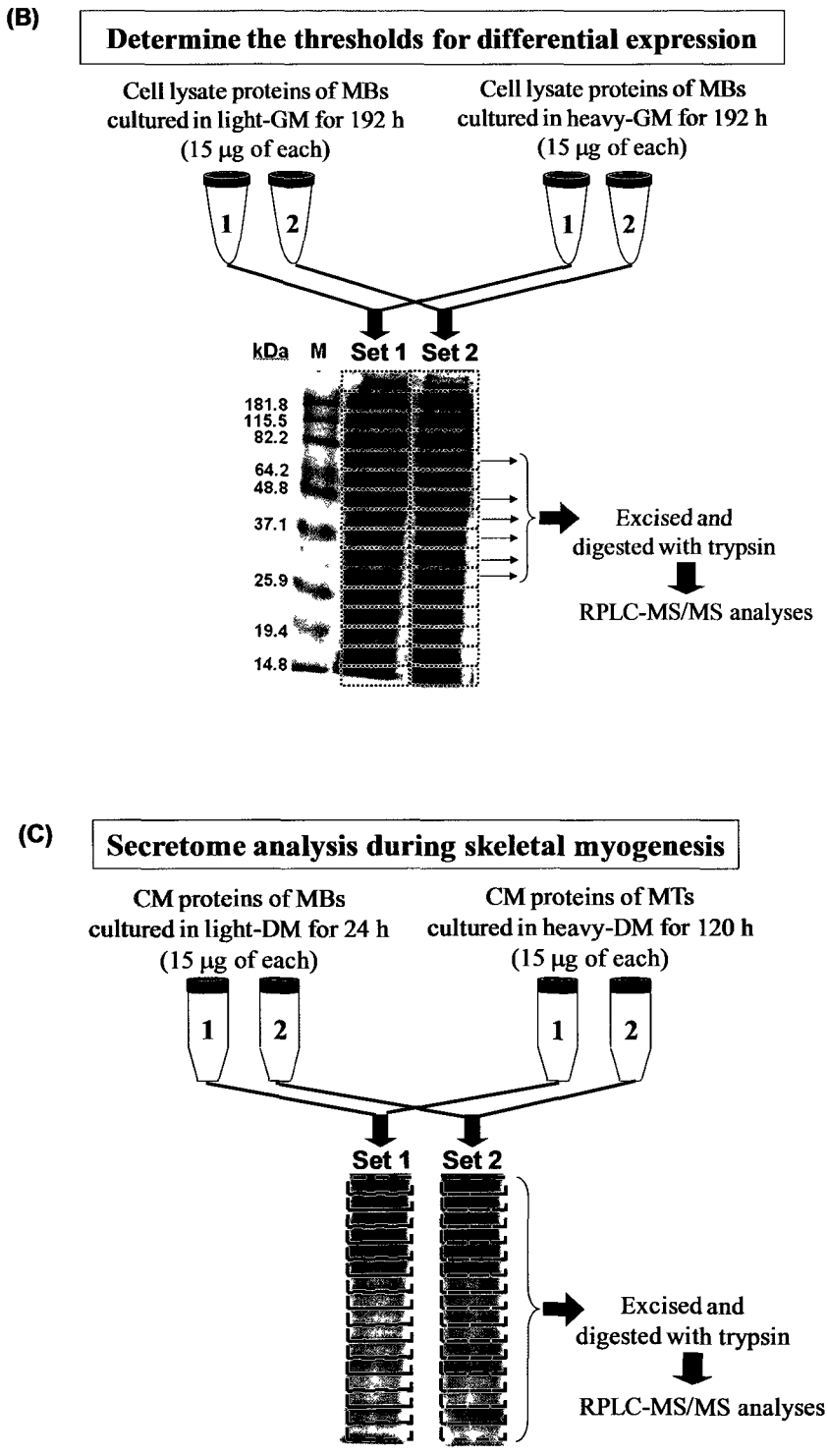


Figure 26. 1D-SDS PAGE. (A) for examining the incorporation of [$^{13}C_6$]-lysine, **(B)** for determining the thresholds for differential expression, and **(C)** for secretome analysis during skeletal myogenesis. M shows the protein ladder.

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 (DTT) (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-hydroxycinnamic acid (Sigma) in 60 % acetonitrile (ACN) (Sigma) and 0.3 % trifluoroacetic acid (TFA) (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 NCBInr databases (released on February 25, 2008 with a total of 137762 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, ICPL: 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 % ACN. The peptide solution was brought to dryness by speedvac (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, while those from set 2 to an LC Packings RPLC-QSTAR Pulsar hybrid QqTOF (Applied Biosystems/MDS SCIEX) instrument.

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.) 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 multi-segment 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 LIT tandem mass spectrometer in data-dependent acquisition (DDA) 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, NCBInr; species, *Mus musculus*; enzyme, trypsin; fixed modifications, cysteine carboxyamidomethylation; variable modifications, lysine $^{13}C_6$ and methionine oxidation; missed cleavages, 0; peptide charge, 2+ and 3+; peptide and fragment tolerance, 10 Da and 0.8 Da, respectively; and instrument type, ESI-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) at 25 μL/min for 4 min. This was followed by peptide separation on a 15-cm long Integrafrit 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 multi-segment 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 DDA 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 (Shilov, I.V. *et al.*, 2007) for analysis with the following search parameters: sample type, SILAC (Lys+6); cysteine alkylation, iodoacetamide; digestion, trypsin; instrument, QSTAR ESI; species, *Mus musculus*; quantitate, checked; ID focus, biological modifications; database, NCBInr; search effort, thorough ID; detected protein threshold [unused ProtScore (Conf)], >1.3 (95 %). The false discovery rate (FDR) was determined by searching mass spectra against a reversed decoy database in which proteins with local FDR <5 % were regarded as significant hits.

To ensure high-confidence identifications, proteins identified with a minimum of two distinct peptides (i.e., different amino acid sequences) in which one of them being unique (i.e., the peptide was only present in that particular protein) were reported. To determine the relative expression of a protein, full MS scans within the full width at half maximum of the chromatographic peak (i.e., ≥ 50 % of the chromatographic peak intensity) were averaged to generate an average full MS scan. From the peaks, the ratio of heavy- to light-labeled monoisotopic peak height of the unique peptide contributed to the peptide ratio, and the peptide ratios derived from the same protein were averaged and reported as the relative protein expression level in MT versus MB.

Statistical analysis

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 **(Step 1 of Figure 25)**. Since 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 $\min_i(p_i)$ and the maximum ratio by $\max_i(p_i)$, in which p_i 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. $\min_i(\log(p_i)) \approx -\max_i(\log(p_i))$. In linear space, we can express this equation as $\max_i(p_i) \approx \frac{1}{\min_i(p_i)}$. 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., $\max_i(p_i) >> \frac{1}{\min_i(p_i)}$, we then use the maximum of detected protein ratios to reconstruct the theoretical distribution range as $\left[\frac{1}{\max_i(p_i)}, \max_i(p_i)\right]$. Analogously, if the minimum of the determined protein ratios is less than one over the maximum, i.e., $\min_i(p_i) << \frac{1}{\max_i(p_i)}$, we reconstruct the theoretical protein range as $\left[\min_i(p_i), \frac{1}{\min_i(p_i)}\right]$. 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 $\min(\min_{i}(p_i), \frac{1}{\max_{i}(p_i)})$ and $\max(\max_{i}(p_i), \frac{1}{\min_{i}(p_i)})$, respectively.

Assignment of classical and non-classical secreted proteins

Proteins identified were parsed with two open-source algorithms, SignalP 3.0 (Bendtsen, J.D. *et al.*, 2004a) and SecretomeP 2.0 (Bendtsen, J.D. *et al.*, 2004b) for secreted protein prediction. Proteins with SP probability ≥0.5 were assigned as classical secreted proteins (C); those with SP probability <0.5 and NN-score ≥0.5 were regarded as non-classical 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 (Haematologic Technologies Inc.), α-actin (Santa Cruz Biotechnology Inc.), OGN (R&D), BSA (Sigma), and cleaved caspase-3 (Cell signaling technology). 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 1D-SDS PAGE, followed by an overnight electrophoretic transfer to Immobilon-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.) 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 fluorescence-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 (ICC)

MyHC subunits are encoded by distinct members of a multigene family expressed at different stages of muscle development and are the archetypal 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 (Bader, D. *et al.*, 1982) 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 (HRP)-conjugated goat anti-mouse 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'TAGGATCCATGGAAAACGTGAGTCTTG3' and

5'ATGCGGCCGCTTAGACGCAGTTATCTGG3' (BamHI and NotI sites are underlined). Spondin-2 cDNA was inserted into BamHI and NotI digested pcDNA3 (Invitrogen), and the resulting construct was verified by sequencing. Three pre-designed MISSION siRNAs (Sigma) with the highest possible ranking were used against each target gene. Some of the primers for semi-quantitative 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 pre-designed 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 approximately 80-90% confluence, cells were co-transfected with an MCK promoter-driven luciferase reporter plasmid (Donoviel, D.B. *et al.*, 1996), a CMV-β-galactosidase control plasmid, and 50 nM siRNA using lipofectamine reagent (Invitrogen) in serum-free D-MEM. 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). By 2.5 days of differentiation period, cells were rinsed twice with ice-cold PBS, and lysed with 20 mM Tris at pH 7.6 with 0.1% Triton X-100. Luciferase- and β-galactosidase-activity were determined as previously described (Kollias, H.D. *et al.*, 2006; Miyake, T. *et al.*, 2010). In brief, relative luciferase unit (RLU) 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 these 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 HRP-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 wash. The cells were then developed with AEC Chromogen kit as the chromogenic substrate (Sigma). 0 nM OGN and 8 nM bone morphogenetic 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.

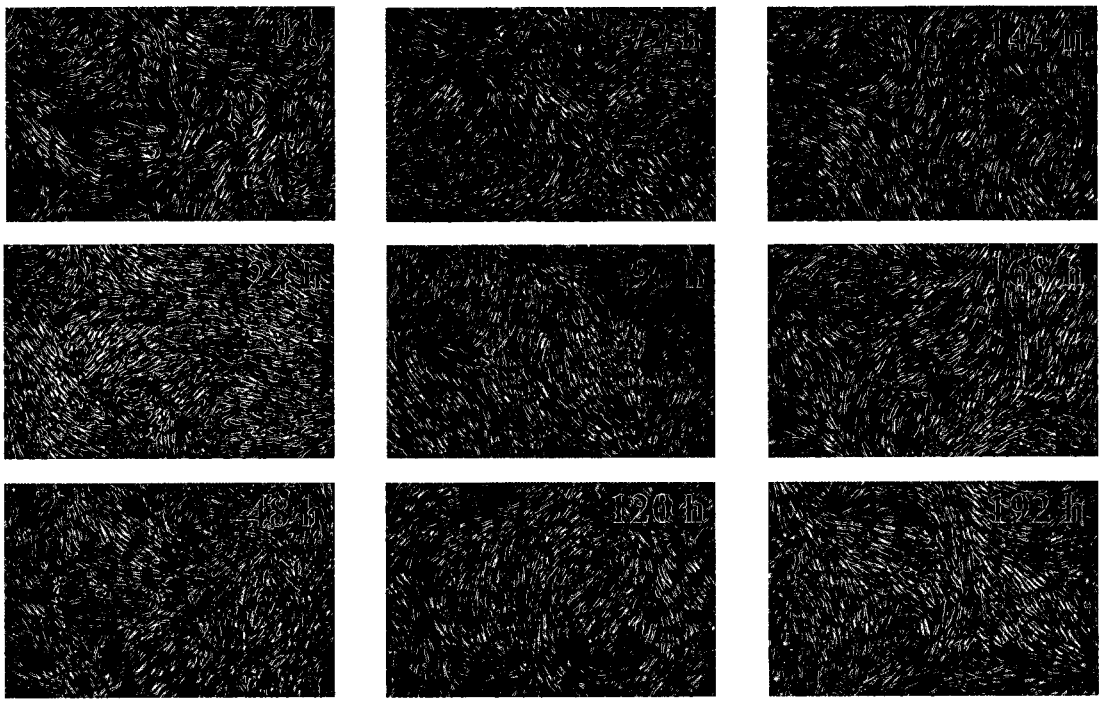
Results

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 (Ohno, T., 1979; Ganassin, R.C. and Bols, N.C., 1992; Ong, S.E. *et al.*, 2002). The morphologies of MBs were monitored throughout the course of SILAC labeling. MBs were observed to maintain a typical mononucleated polygonal shape **(Figure 27A)** 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 **Figure 27B**, 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 due to the incorporation of [$^{13}C_6$]-lysine, as verified by MS/MS analyses in which both peaks at m/z 1954.1 and 1960.2, corresponding to non-labeled and labeled version of the tryptic peptide, respectively, were monitored **(Figure 27C)**. This gave us a preliminary idea on the minimal labeling time, which was 120 h according to **Figure 27B**. To be more stringent, cells labeled for 192 h were used. The completeness 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.

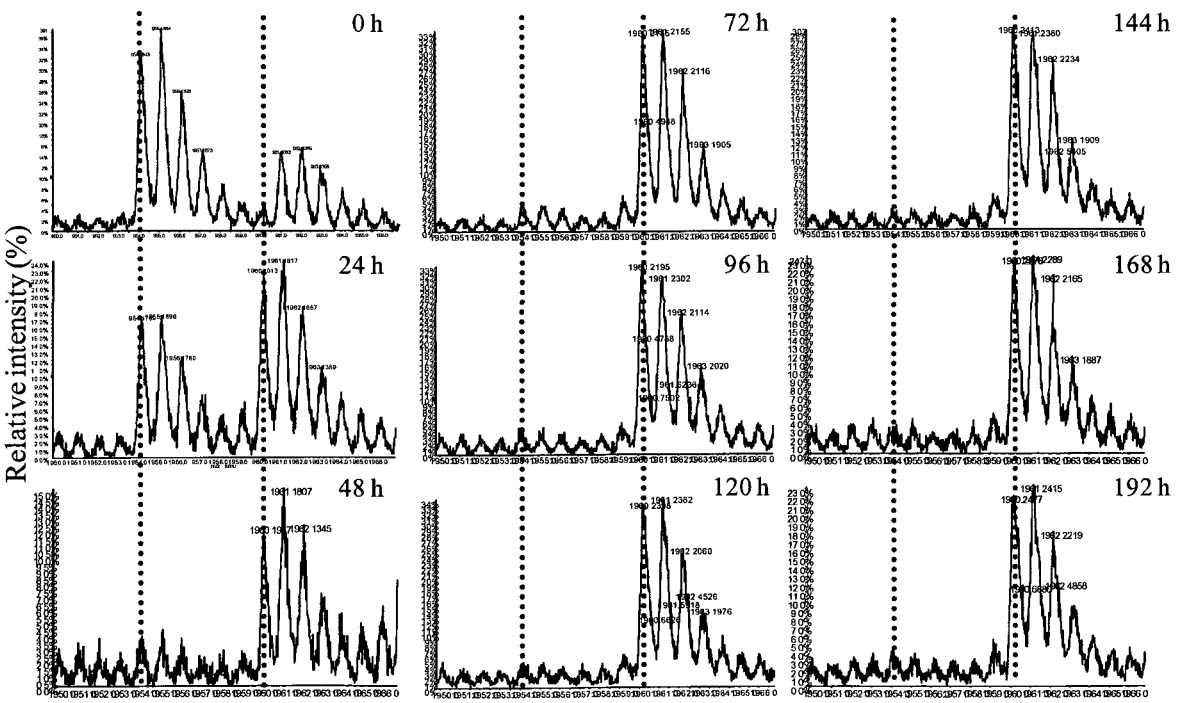
(A)

MBs cultured in heavy-GM



(B)

MS mass spectra



(C)

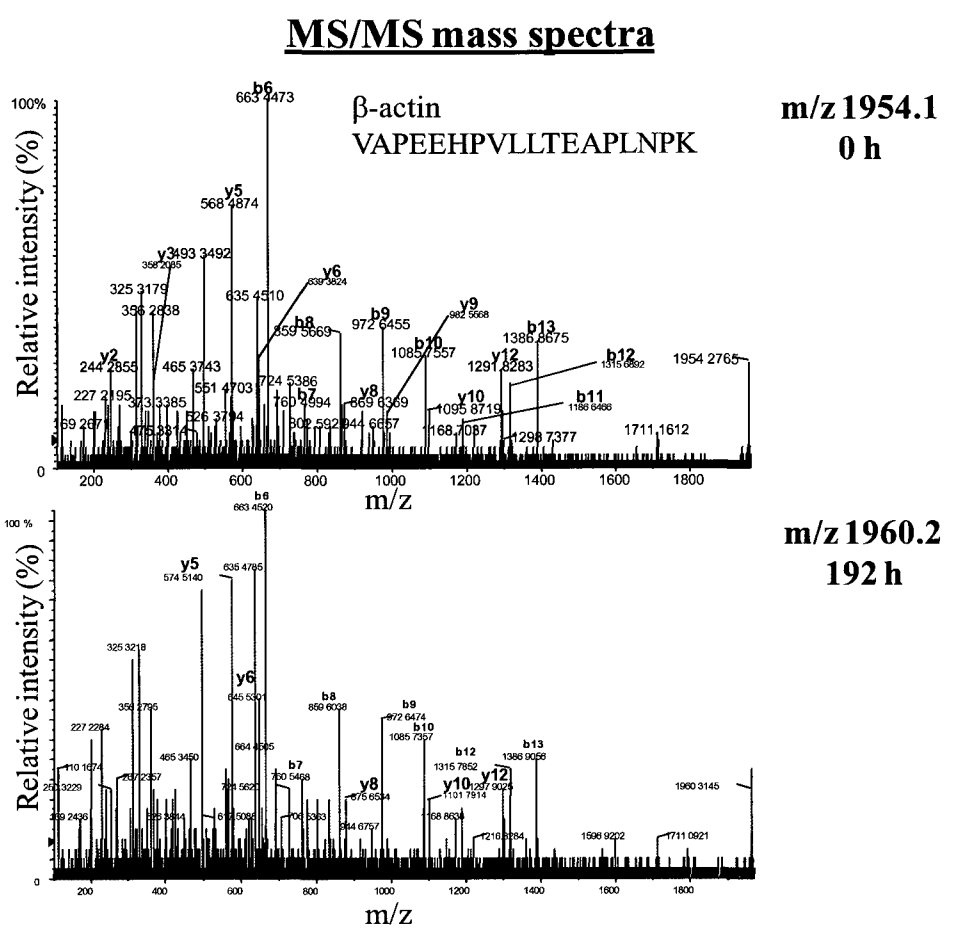


Figure 27. Optimization of SILAC experiments: (A) Phase-contrast photomicrographs of MBs cultured in heavy-labeled GM from 0 to 192 h. **(B)** Incorporation of heavy-labeled lysine into MBs. At 0 h, a non-labeled tryptic peptide at m/z 1954.1 (green dashed line) was present in the MS scan. This was followed 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 (VAPEEHPVLLTEAPLNPK) of β-actin. Light- and heavy-labeled y ions were labeled in green and red, respectively.

For complete labeling, protein ratios derived from such a mix should be close to 1. According to **Figure 28A and 28B**, 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)**.

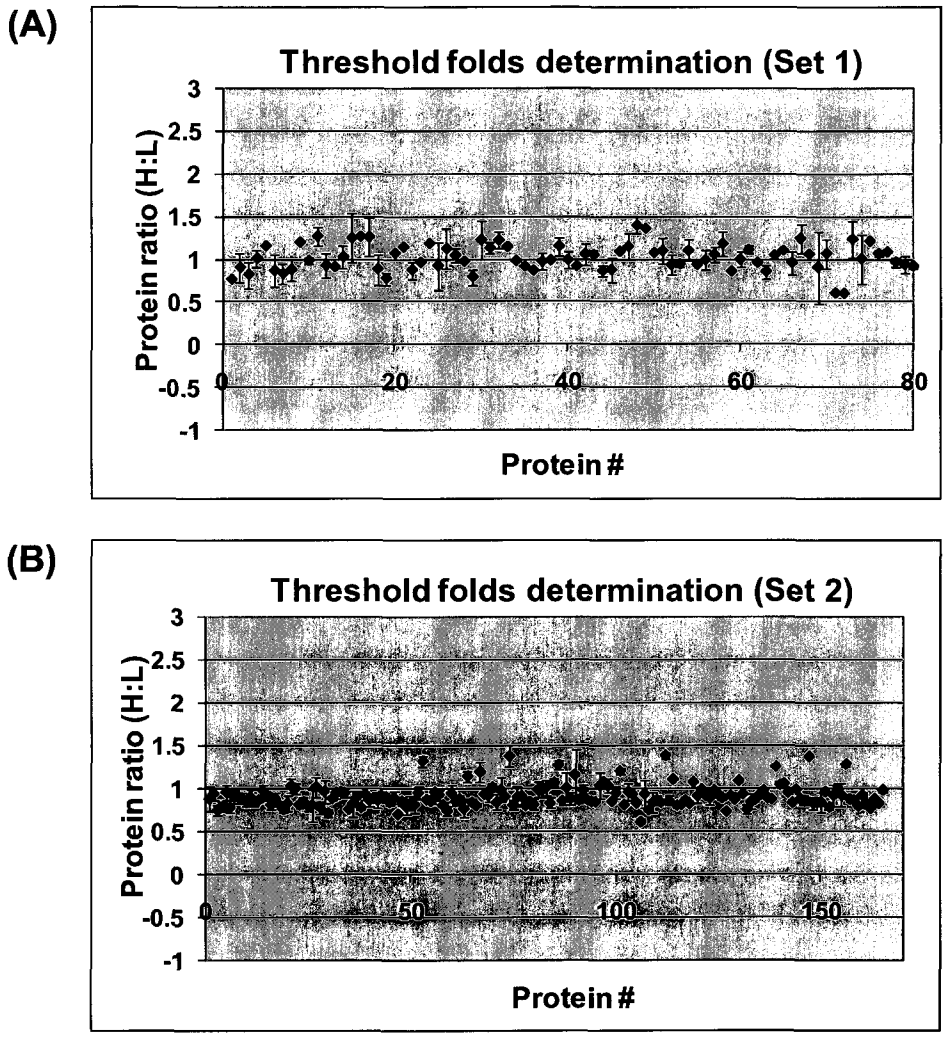


Figure 28. Determination of the thresholds for differential expression. Tryptic peptides from sets 1 and 2 (Fig. 26B) were analyzed with **(A)** Agilent 1100 RPLC-LTQ LIT tandem mass spectrometer, and **(B)** LC Packings RPLC-QSTAR Pulsar hybrid

QqTOF tandem mass spectrometer. Peptide ratios were given by the ratio of heavy- to light-labeled monoisotopic peak intensities of unique peptides. Protein ratios were determined by averaging the peptide ratios for a given protein. Error bars represented the SD of peptide ratios.

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 **(Figure 25)**. 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 non-differentiated 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 dataset 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 (**Figure 26B**). 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 **Figure 28**, the experimental protein ratios from sets 1 and 2 were tightly centralized around 1. After statistical analysis, protein ratios (H:L) $\geq$1.68 and $\leq$0.60 were categorized as up- and down-regulated in MT versus MB respectively, in set 1; whereas in set 2, protein ratios $\geq$1.58 and $\leq$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 favour 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 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 (**Figure 26C**). 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 SignalP 3.0 and SecretomeP 2.0 **(Supplemental data S1)**. The relative expression levels of these secreted proteins were determined manually. 34 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 3, Supplemental data S2)**.

Table 3. Secreted proteins quantified. Down- and up-regulated expression levels (H:L) were shaded in green and red respectively, whereas those in grey represented non-differential expression. Classical- and non-classical 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 (%)
			Protein ratio (H:L)	SD of protein ratio	Protein ratio (H:L)	SD of protein ratio					
gi|6754976	Peroxiredoxin 1	N	0.46	0.05	0.37	0.04	26	202	14	12	80.9
gi|437125	Insulin-like growth factor binding protein 5	C	0.52	0.09	0.36	0.03	34	275	12	11	58.9
gi|6996913, gi|63594732	Annexin A2	N	0.24	0.06	0.23	0.03	8	69	12	10	40.7
gi|6680924, gi|94406453	Cofilin 1, non-muscle	N	0.41	0.07	0.35	0.03	28	227	8	5	56.0
gi|6755040	Profilin 1	N	0.51	0.06	0.35	0.02	43	242	9	8	90.7
gi|62201487, gi|6679937, gi|50233866, gi|47607490	Glyceraldehyde -3-phosphate dehydrogenase	N	0.60	0.05	0.42	0.03	17	104	11	10	55.3

gi|6678077, gi|94390366	Secreted acidic cysteine rich glycoprotein	C	1.34	0.21	0.90	0.07	27	292	11	10	48.3
gi|126116574	NAC alpha domain containing	C	9999	0	NA	NA	4	82	6	6	3.5
gi|18314528	Ciapin1 protein	N	9999	0	NA	NA	7	23	4	4	23.4
gi|585122	Fibulin-2 precursor	C	1.25	0	NA	NA	1	8	4	4	3.8
gi|24586721, gi|31980922	Eukaryotic translation elongation factor 1 beta 2	C	0.82	0.06	NA	NA	2	20	5	4	39.7
gi|7304963	Chloride intracellular channel 4	N	0.67	0.03	NA	NA	3	50	8	7	37.5
gi|74222953	Phosphatidylethanolamine binding protein 1	N	0.60	0.07	NA	NA	3	21	6	5	50.3
gi|309315, gi|7305155	Hypoxanthine-guanine phosphoribosyltransferase	N	0.59	0.06	NA	NA	3	10	3	2	15.6

gi|148675904	Malate dehydrogenase 1, NAD (soluble), isoform CRA_c	N	0.58	0.08	NA	NA	3	34	8	7	27.6
gi|33468857	Histidine triad nucleotide binding protein 1	N	0.57	0	NA	NA	1	7	2	2	16.7
gi|12846252	Peroxiredoxin 2	N	0.57	0.05	NA	NA	6	43	9	7	70.2
gi|4759158	Small nuclear ribonucleoprote in polypeptide D2	N	0.56	0	NA	NA	1	31	4	4	40.7
gi|6754524	Lactate dehydrogenase A	N	0.50	0	NA	NA	1	126	15	4	60.5
gi|3914434, gi|6755204	Proteasome subunit beta type-5 precursor	C	0.5	0.09	NA	NA	5	35	10	9	55.5
gi|148708561	AP-1, beta 1 subunit, isoform CRA_a	N	0.48	0.15	NA	NA	2	5	3	3	4.0
gi|226471	Cu/Zn superoxide	N	0.47	0.04	NA	NA	3	29	4	3	32.7

	dismutase										
gi|143811353	Sterile alpha motif domain-containing protein 3	N	0.4	0	NA	NA	1	13	4	4	7.1
gi|2498391, gi|31560699	Follistatin-like 1	C	0.36	0	NA	NA	1	17	5	4	18.3
gi|30519911, gi|94365804	Transgelin 2	N	0.35	0.03	NA	NA	3	106	11	8	68.3
gi|6679166	Osteoglycin	C	0.16	0	NA	NA	1	35	8	7	38.3
gi|148706007	Ubiquitin-activating enzyme E1-like 2, isoform CRA_b	N	0	0	NA	NA	1	44	6	6	11.0
gi|3914439	Proteasome subunit beta type-4	N	0	0	NA	NA	2	12	5	5	26.1
gi|18252782	Serine (or cysteine) proteinase inhibitor, clade C, member 1	C	0	0	NA	NA	3	12	5	5	18.3
gi|1167907,	Alpha-1(XVIII)	C	0	0	NA	NA	4	65	11	3	11.2

gi|511298	collagen										
gi|50190	Beta-*tropomyosin*	N	0	0	NA	NA	7	13	2	2	11.6
gi|13435747	Rho GDP dissociation inhibitor (GDI) alpha	N	0	0	NA	NA	1	49	7	6	49.0
gi|12836985, gi|10946870, gi|94372279	Aldo-keto reductase family 1, member A4	N	0	0	NA	NA	1	11	7	5	37.3
gi|74183811	Annexin A1	N	0	0	NA	NA	2	2	3	2	8.7

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 (**Figure 29A-D**). 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 **Figure 29E**). In contrast, Western blotting does not (cannot) make this distinction and yields a protein ratio of ~1.

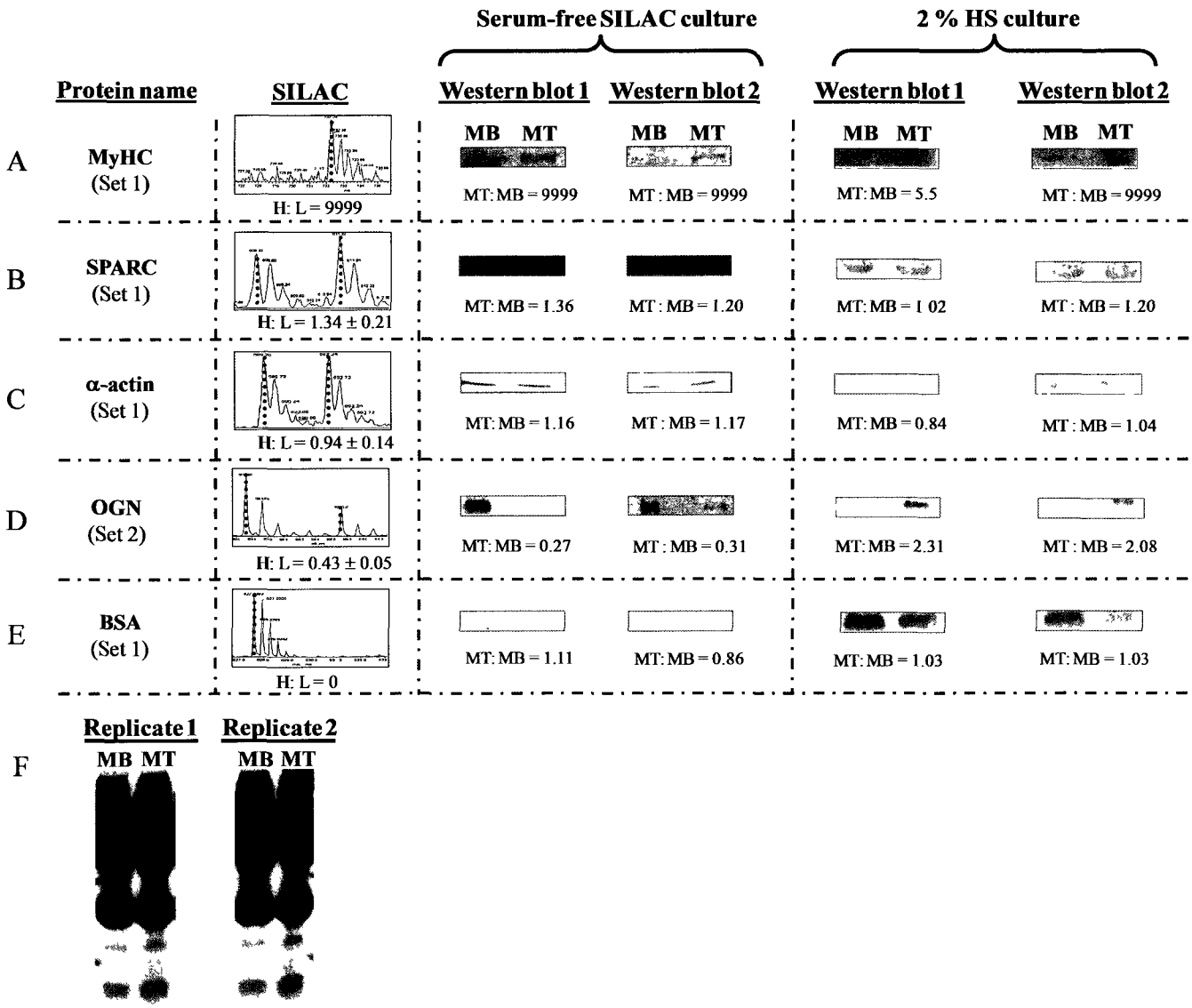


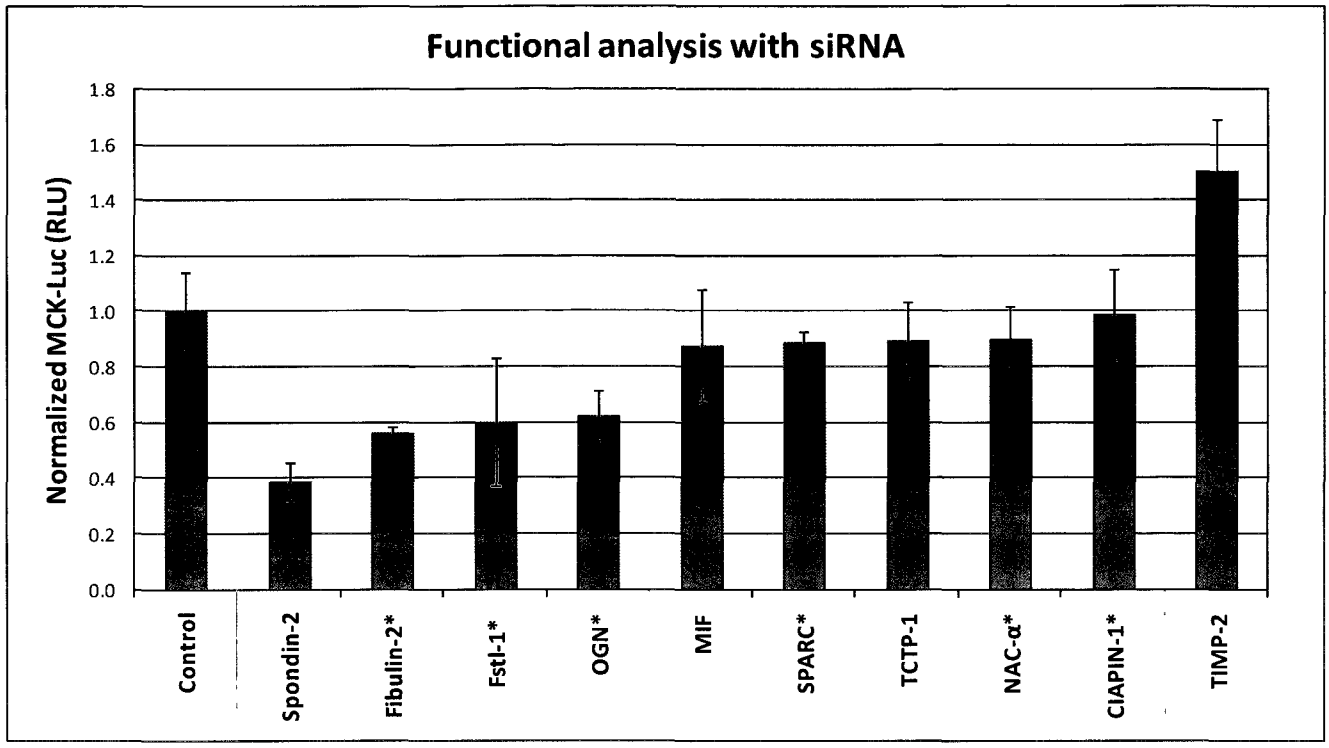
Figure 29. Comparison of SILAC and Western blot analyses. In SILAC, samples from set-1 and -2 were analyzed with Agilent 1100 RPLC-LTQ LIT tandem mass spectrometer and LC Packings RPLC-QSTAR Pulsar hybrid QqTOF tandem mass spectrometer respectively. Peptide ratios were determined by the ratio of heavy- versus light-labeled monoisotopic peak intensity, as indicated by red- and green-dashed lines in the representative MS mass spectra. Average and SD of which contributed to the corresponding protein ratios (H:L) and ±error respectively as revealed beneath each MS

mass spectrum. For verification, the same set of samples were subjected to Western blot analyses twice as shown in the column 'Western blot-1' and 'Western-blot-2' under the 'serum-free SILAC culture'. Protein ratios were deduced by the ratio of fluorescence intensity associated with the protein band in MT- versus MB-lane (MT:MB) as revealed beneath each blot. Protein ratio of '9999' and '0' represented the protein was exclusively expressed in MT and MB respectively. To verify the protein expression levels in the classical culture conditions, i.e., 2 % horse serum (HS), replicate sets of conditioned media derived from the 2% HS culture condition were subjected to Western blot analyses, and the results were tabulated under the column '2 % HS culture'. Coomassie blue staining of the 1D SDS-PAGE was used as the loading control.

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, since 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 **(Figure 29)**. 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. While 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 since siRNA mediated knock down of OGN resulted in a modest decrease in myoblast differentiation as indicated by our MCK-Luc reporter gene system **(Figure 30)**. 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.

(A)



(B)

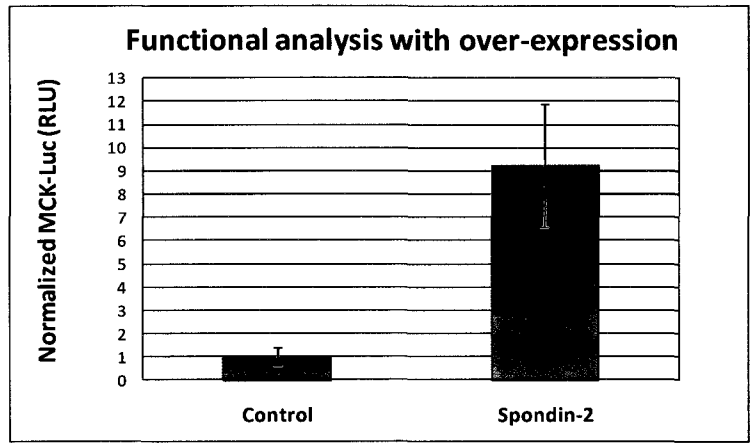


Figure 30. Functional analysis of identified secretome components using a myogenic differentiation marker MCK promoter-driven luciferase reporter system: (A) Loss of function analyses using siRNA: knock down of individual gene in MBs, such as spondin-2, fibulin-2, Fstl-1, and OGN, attenuated MCK-luciferase activity. Conversely, knock down of TIMP-2 increased MCK-luciferase activity. Knock down of MIF, SPARC, TCTP-1, NAC-α, and CIAPIN-1 did not result in significant changes in MCK-

luciferase activity. Proteins successfully quantified with SILAC were marked with asterisks. **(B)** Gain of function analyses using over-expression: up-regulation of spondin-2 increased MCK-luciferase activity significantly.

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 ten proteins: spondin-2, fibulin-2, Fstl-1, OGN, macrophage migration inhibitory factor (MIF), SPARC, translationally controlled tumour protein-1 (TCTP-1), NAC alpha domain containing-protein (NAC-α), CIAPIN-1, and tissue inhibitor of metalloproteinase-2 (TIMP-2) were selected for testing in a functional assay using a myogenic differentiation marker MCK promoter-driven luciferase reporter gene system **(Figure 30A)**. Six of these proteins had been successfully quantified with SILAC. C2C12 MBs were co-transfected 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 knock-down 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 knock-down was confirmed by semi-quantitative RT-PCR **(Supplemental data S4B)**. Knock down of a number of genes, spondin-2, Fstl-1, fibulin-2, and OGN, significantly attenuated MCK-luciferase levels, suggesting a positive role for these

proteins in myogenesis, whereas knock down 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-α, and CIAPIN-1, no significant alteration of the MCK-luciferase activity was discernible **(Figure 30A)**.

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 **(Figure 30A)**. To further address the role of spondin-2 in myogenesis, we carried out gain of function analysis by over-expressing 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 **(Figure 30B)**. We suggest that adapting an siRNA-mediated loss of function analysis in tandem with a gain of function examination by over-expressing candidate proteins are useful to initially assess the role of identified components in the biological process under study, in this case, myogenesis. Of course, the output measurement (in this case, an MCK luciferase reporter gene assay) is only one criterion for assessing the effect of secretome factors on the differentiation program.

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 six-fold **(Table 3)**; 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 (Madisen, L. *et al.*, 1990). Thus, we speculated that OGN might be a repressor of myogenesis in multipotent mesenchymal cells, while promoting the osteogenic-lineage. We had previously reported that C2C12 cells are multipotent and can progress down the osteogenic lineage when exposed to appropriate extracellular factors such as BMP-2 (Aziz, A. *et al.*, 2009). To test this, C2C12 cells were treated with purified OGN at various concentrations (0.02, 0.2, 2, and 10 nM). As shown in **Figure 31A and 31B**, 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 **(Figure 31C)**. 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 **(Figure 31C)**.

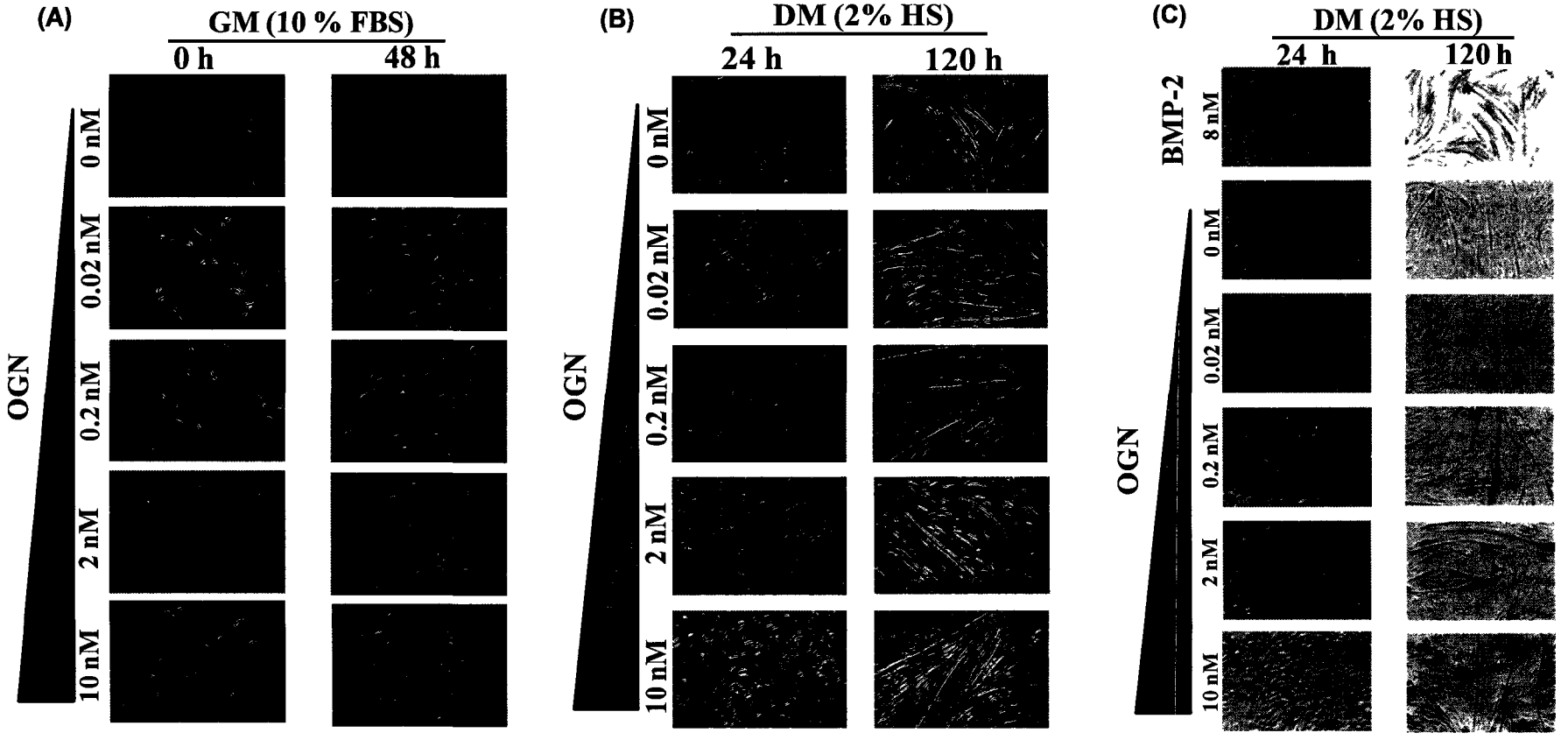


Figure 31. Light microscopic images of C2C12 cells cultured in various OGN-supplemented media: (A) growth media (GM), **(B)** differentiation media (DM), and **(C)** DM subjected to MyHC-ALP double-staining. BMP-2 (8 nM) and OGN (0 nM) were employed as positive controls for ALP (purple) and MyHC (brown) expression, respectively.

Thus to fully assess the role of each identified secretome factor, a number of 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 pre-requisite 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 a number of 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 (Fernando, P. *et al.*, 2002; Larsen, B.D. *et al.*, 2010). 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 **Figure 32**, 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 since one might have predicted larger differences which 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 differentiation program.

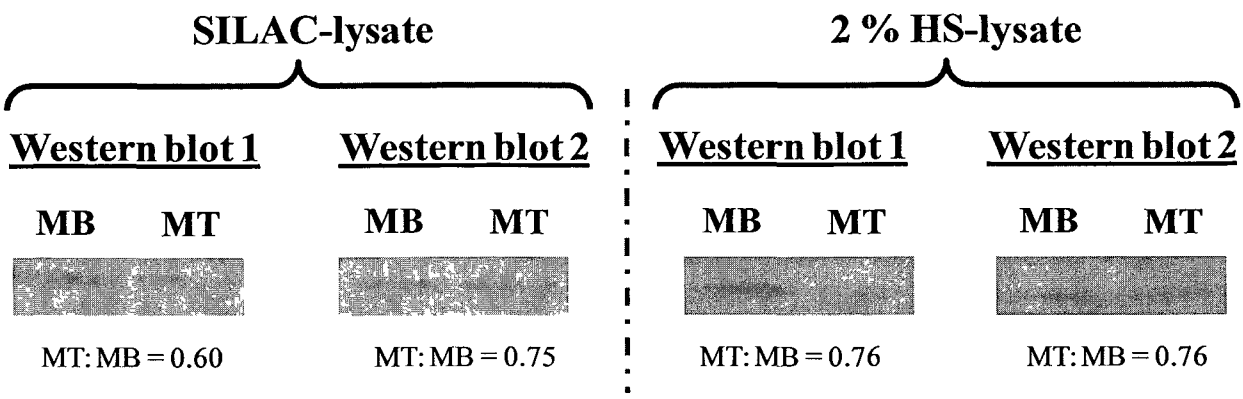


Figure 32. Comparison of apoptosis between SILAC and 2 % horse serum (HS) culture condition. Cleaved (activated) caspase-3 was implemented as the apoptotic indicator in both myoblast (MB) and myotube (MT) state.

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 (Cusella-De Angelis, M.G. *et al.*, 1994; McPherron, A.C. *et al.*, 1997; Liu, D. *et al.*, 2001; Stewart, J.D. *et al.*, 2003; Gosselin, L.E. *et al.*, 2004; Droguett,

R. *et al.*, 2006; Suryawan, A. *et al.*, 2006; Cohn, R.D. *et al.*, 2007; Schabort, E.J. *et al.*, 2009; Droguett, R. *et al.*, 2010). 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 (Shibanuma, M. *et al.*, 1993). During development, Fstl-1 is first expressed in the mesenchymal component (Adams, D. *et al.*, 2007), and subsequently regionalized in the dorsomedial compartment of the somite (Amthor, H. *et al.*, 1996). 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 (Johnson, S.E. and Allen, R.E., 1990; Amthor, H. *et al.*, 1996; McPherron, A.C. *et al.*, 1997; Liu, D. *et al.*, 2001; Kamanga-Sollo, E. *et al.*, 2003; Cohn, R.D. *et al.*, 2007; Schabort, E.J. *et al.*, 2009). 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 agreement with this, knock down of Fstl-1 decreased the transcriptional activity of the MCK differentiation marker by 1.7-fold **(Figure 30)**. 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 (Oshima, Y. *et al.*, 2008). As Fstl-1 was more abundant in MB **(Table 3)**, 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 (Ouchi, N. *et al.*, 2008),

ovarian and endometrial cancer cells (Chan, Q.K. *et al.*, 2009). Hence, it would be of interest to examine whether Fst-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 (Madisen, L. *et al.*, 1990; Funderburgh, J.L. *et al.*, 1997). 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 (Yamamoto, N. *et al.*, 1997; Nishimura, R. *et al.*, 1998; Partridge, K. *et al.*, 2002; Vinals, F. *et al.*, 2002; Yeh, L.C. *et al.*, 2002; Kaihara, S. *et al.*, 2003; Otsuka, E. *et al.*, 2003; Susperregui, A.R. *et al.*, 2008). 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 favours osteoblast, rather than myoblast, commitment in multipotent mesenchymal cells such as C2C12 and C3H10T1/2 cell lines (Aziz, A. *et al.*, 2009). To test this hypothesis, C2C12 cells were treated with purified OGN at various concentrations. As shown in **Figure 31A and 31B**, 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 **(Figure 31C).** Much to our surprise, knock down of OGN decreased MCK transcriptional activity by 1.6-fold **(Figure 30A)**. 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 (Mu, Z.M. *et al.*, 2002) and c-Abl (Wen, S.T. and Van Etten, R.A., 1997). 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 (Chang, J.W. *et al.*, 2006; Kim, J.H. *et al.*, 2007; Kim, J.H. *et al.*, 2008). In addition, it was found to be secreted from A549 cells (Chang, J.W. *et al.*, 2006). 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 since 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 IL-3-independent cell line, which conferred resistance to factor-deprived apoptosis (Shibayama, H. *et al.*, 2004). According to **Table 3**, CIAPIN-1 was

solely expressed in MT. Knock down of CIAPIN-1, however, did not significantly alter the transcriptional activity of MCK **(Figure 30A)**. 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 post-differentiation role in maintaining a subset of quiescent muscle stem cells, the satellite cells, which reside along the muscle fibres beneath the basal lamina (Mauro, A., 1961; Armand, O. *et al.*, 1983; Partridge, T.A. *et al.*, 1989). This satellite cell population is crucial for post-natal muscle regeneration (reviewed in Cossu, G. and Biressi, S., 2005; Dhawan, J. and Rando, T.A., 2005; Machida, S. and Narusawa, M., 2006; Le Grand, F. and Rudnicki, M.A., 2007; Boonen, K.J. and Post, M.J., 2008; Kuang, S. and Rudnicki, M.A., 2008; Rhoads, R.P. *et al.*, 2009). CIAPIN-1 has been shown to inhibit renal carcinoma cell proliferation by arresting cancer cells in the G1/S phase of the cell cycle (Li, X. *et al.*, 2007a; Li, X. *et al.*, 2007b; He, L. *et al.*, 2009). This suggests that the anti-proliferative 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 (DMD) and cachexia.

In addition to differentially expressed proteins, some proteins whose abundances do not change substantially upon myogensis 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 (Pan, T.C. *et al.*, 1993). Fibulin-2 is expressed in heart, placenta and ovarian tissues (Zhang, R.Z. *et al.*, 1994), and has been shown to link the cells to the basement

membrane in epithelial (Utani, A. *et al.*, 1997) and haematopoietic cells (Gu, Y.C. *et al.*, 2000). This linkage contributes to cell migration in epicardial cells during coronary vasculogenesis and angiogenesis (Tsuda, T. *et al.*, 2001), and in smooth muscle cells (SMCs) during atherosclerotic lesions (Strom, A. *et al.*, 2006). In addition, fibulin-2 was persistently expressed in myocardial cells (Tsuda, T. *et al.*, 2001) and the adult heart valves to maintain tensile strength of the cardiac valves (Zhang, H.Y. *et al.*, 1995). This persistent expression was also observed in our skeletal muscle model, in which fibulin-2 was constitutively expressed in both MB and MT (**Table 3**). Taking into account of the fibulin-2 knock-down data, in which MCK transcriptional activity was decreased by 1.8-fold (**Figure 30A**), we hypothesize that fibulin-2 may be a house-keeping protein entailed in cell-cell interaction, cell migration, and extracellular matrix (ECM) remodeling, 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, while that of spondin-2 inhibited MCK transcription by 2.6-fold (**Figure 30A**).

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 (Carmeli, E. *et al.*, 2004). In addition, MMPs can degrade all ECM constituents to facilitate cell migration and tissue remodeling (El Fahime, E. *et al.*, 2000). MMP activity is inhibited by interaction with TIMPs in a 1:1 stoichiometric ratio (Nuttall, R.K. *et al.*, 2004). This ratio is critical in modulating muscle cell migration and differentiation, and in maintaining homeostasis of the muscle fibres (Lewis, M.P. *et al.*, 2000). Intriguingly, over-expression of TIMP-2 decreased myogenin

expression, which implies that TIMP-2 may function as a negative regulator of myogenesis (Tomczak, K.K. *et al.*, 2004). This is in accordance with our observation that knock down of TIMP-2 rescues MCK transcription (**Figure 30A**). Interestingly, there are multiple E-box consensus sequences in the mouse TIMP-2 promoter (Lluri, G. *et al.*, 2008), 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 (**Figure 30A**). Conversely, MCK transcriptional activity was enhanced by 9.2-fold with exogenous over-expression of spondin-2 (**Figure 30B**). 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 homologue of spondin-2) promotes adhesion and outgrowth of hippocampal embryonic neurons (Klar, A. *et al.*, 1992; Feinstein, Y. *et al.*, 1999). In addition, spondin-2 has also been implicated in innate immunity, in which it bound to bacteria as an opsonin for subsequent macrophage phagocytosis (He, Y. W. *et al.*, 2004; Jia, W. *et al.*, 2005). In the context of tumour growth, spondin-2 exerts different effects depending on the cell type: in ovarian cancer, spondin-2 was over-expressed and deployed as a new serum diagnostic biomarker (Simon, I. *et al.*, 2007); analogously, in prostate cancer, spondin-2 was found to be over-expressed in > 80 % of the prostate cancers metastatic to bone or the lymph nodes, as well as in locally recurrent tumours in androgen-unresponsive patients. Prominent anti-tumour effects were achieved with a single administration of radiolabeled spondin-2-antibodies to animals (Parry, R. *et al.*, 2005). In contrast, over-expression of spondin-2 was shown to moderate the invasiveness of hepatocellular carcinoma by attenuating cell

migration (Liao, C.H. *et al.*, 2010). 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.* (Henningsen, J. *et al.*, 2010) 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 acknowledged that at this stage, our study is predominantly discovery in nature and requires many subsequent mechanistic studies to address the roles of the identified differentially expressed secretome components. These ongoing mechanistic studies will ultimately determine the biological function and relevance of the identified secretome components during myogenesis.

Concluding remarks

In conclusion, SILAC in conjunction with high throughput online RPLC-MS/MS analyses were successfully implemented to quantitatively profile secretome components during skeletal muscle differentiation. Some of the proteins identified, including Fstl-1, CIAPIN-1, OGN and fibulin-2, may be novel regulators of various stages of the myogenic program **(Figure 33)**. Gain and loss of function studies to determine the individual and combinatorial role(s) of these factors will elucidate a broader understanding of myogenesis, and possibly uncover generic principles determining how the extracellular milieu contributes toward the fate of pluripotent mesenchymal cells. Understanding the role of these secreted factors will ultimately inform diagnostic and therapeutic approaches to our understanding of muscle ontogeny, physiology, and pathology.

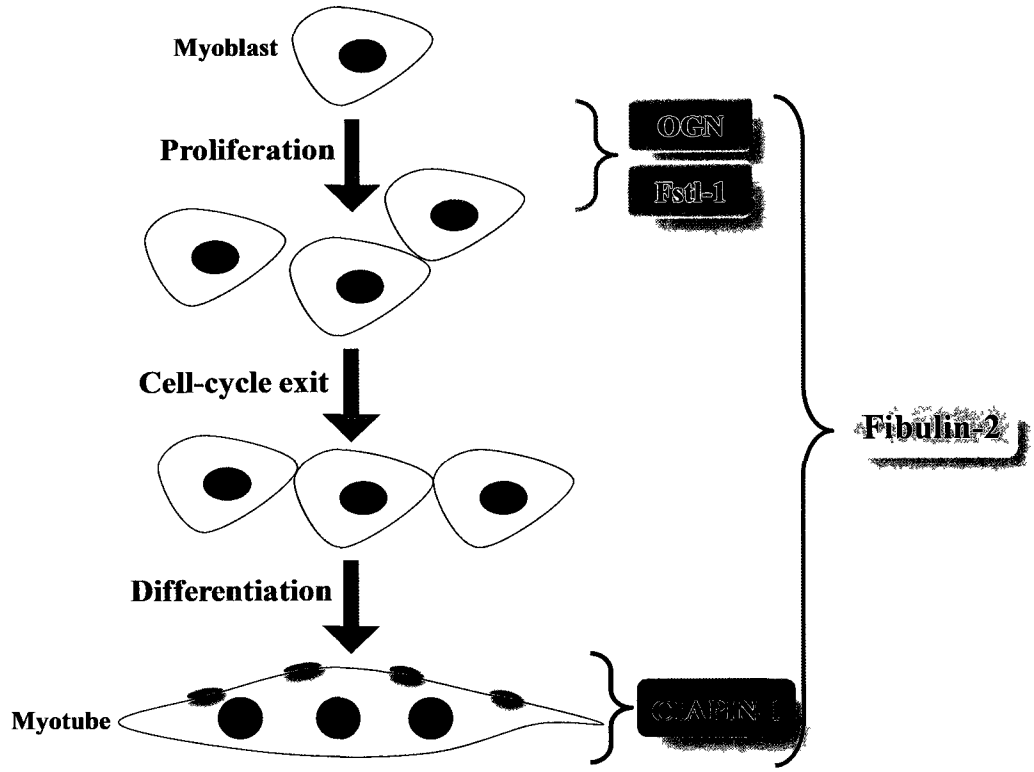


Figure 33. Plausible roles of OGN, Fstl-1, CIAPIN-1, and fibulin-2 in various stages of myogenesis. Up- and down-regulated proteins are shaded in red and green, respectively; non-differentially expressed proteins in grey. Satellite cells are depicted as purple ovals residing along the myotube.

Acknowledgements

The work was made possible by support from the Natural Sciences and Engineering Research Council of Canada (NSERC) and from the Canadian Institutes of Health Research (CIHR) to JCM and KWMS. The authors are indebted to Professor Michael Scheid (York University) for access to the Odyssey Infrared Imaging System Platform.

Summary

The studies reported here represent a combination of biological and mass spectrometry-based proteomic approaches to investigate the secretome during skeletal myogenesis. An optimized serum-free culture condition was implemented, which facilitates normal muscle growth and differentiation, while minimizing protein interference derived from serum-containing culture medium, such as albumin, in an effort to enhance the discovery of secreted proteins. With the implementation of MALDI-QqTOF, 27 secreted proteins were identified, in which we were able to identify previously uncharacterized secreted proteins, and the findings of which represented the first proof-of-principle study to identify the secretome during skeletal muscle differentiation using an MS-based approach.

In addition, we reasoned that during myogenesis, a cluster of proteins may alter in their expression levels and if we could establish an unbiased high throughput tool to profile such alterations occurring in the secretome, this would further advance our understanding and insight into the regulation of muscle differentiation by the extracellular milieu. To substantiate this, we adapted SILAC in conjunction with two parallel RPLC-MS/MS, in an attempt to identify differentially expressed secretome components in the myotube versus myoblast stage. 34 secreted proteins were quantified, 30 of which were shown to be differentially expressed during muscle development. Classical biological techniques such as Western blot analyses, over-expression, and si-RNA were also employed for verification and validation, which furthered the characterization of differentially expressed secretome components.

There are several directions that could be further pursued based on the secretome analysis carried out to date. Firstly, in addition to MCK-luciferase reporter gene assay, other muscle differentiation readouts such as Western blot analyses against a battery of MRFs and structural protein MyHC, electric excitability and contractibility of skeletal muscle, should be put in place, so to provide a more comprehensive examination of effect of the secreted protein(s) we identified on myogenic progression. In addition, supplementing cells with purified form of the proteins of interest or the cognate neutralizing antibodies could further verify their extracellular roles in myogenesis. In terms of SILAC, labelled arginine, for instance ($^{13}C_6$, $^{15}N_4$Arg) could be adapted in conjunction with $^{13}C_6$ labelled lysine, in order to increase the coverage of quantified proteins. This in turn would advance the likelihood of identifying more novel secreted proteins.

In the longer term, the characterization of the secretome could help in identifying factors that may influence cellular programming. For instance, a very prominent field at the moment is the establishment and programming of induced pluripotent stem cells (iPS). Our knowledge of extracellular factors that regulate the phenotype of muscle cells could be used in programming these cells or establishing conditions in which their lineage conversion is optimized. This could be a very productive area for future studies.

In conclusion, the unbiased identification and quantification approach I have developed during my doctoral work offers several major advantages over conventional approaches of protein analysis such as Western blotting which is a fairly labour intensive approach that requires high quality antibodies for each protein to be detected. The approach I have worked on does not require any a priori knowledge of the proteins that

will be detected, nor does it require any specialized protein specific reagents. This could therefore advance the pace of protein analysis and also uncover previously unrecognized proteins that are involved in the secretome.

Apart from characterizing the secretome during differentiation, this approach should be freely transferable to many other cellular contexts such as the changes in gene expression that occur when stem cells commit to a particular cell lineage or when cells are experimentally reverted back or re-programmed to a stem cell like state (IPS). Thus, I envision that the procedural analysis I have worked on and future modifications of it in the application to a variety of cell biological contexts should prove fruitful in contributing to the global understanding of the proteome that will be at the forefront of modern biology for the foreseeable future.

Appendices:

A: Secretome analysis of skeletal myogenesis using SILAC and shotgun proteomics

International Journal of Proteomics (In press)

Secretome analysis of skeletal myogenesis using SILAC and shotgun proteomics

C.Y. X'avia Chan[1,2], John C. McDermott[1,2,4,5], and K.W. Michael Siu[1,2,3]

Author affiliation:

[1]Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[2]Centre for Research in Mass Spectrometry (CRMS), York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[3]Department of Chemistry, York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[4]Muscle Health Research Centre (MHRC), York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

[5]Centre for Research in Biomolecular Interactions (CRBI) York University, 4700 Keele Street, Toronto, Ontario, Canada M3J 1P3

To whom correspondence should be addressed:

John C. McDermott

247 Farquharson Building, York University, 4700 Keele Street, Ontario, Canada, M3K 1P3

Phone: 416-736-2100 (×30389)

Email: jmcderm@yorku.ca

K.W. Michael Siu

242 Chemistry Building, York University, 4700 Keele Street, Ontario, Canada, M3J 1P3

Phone: 416-650-8021

Email: kwmsiu@yorku.ca

Fax: 416-736-5752

Received for review November 15, 2010. Accepted January 26, 2011.

Abbreviations

1D-SDS PAGE	One-dimensional gel electrophoresis
ASK	Apoptosis signal-regulating kinase
bHLH	Basic helix-loop-helix
CDKIs	CDK inhibitors
CDKs	Cyclin-dependent kinases
CDO	CAM-related/down-regulated by oncogenes
CIAPIN1	Cytokine-induced apoptosis inhibitor 1
CM	Conditioned media
DML	Dorsal medial lip
ECM	Extracellular matrix
HATs	Histone acetyltransferases
HDACs	Histone deacetyltransferases
ICAT	Isotope-coded affinity tag
IGF1	Insulin-like growth factor-1
iTRAQ	Isobaric tag for relative and absolute quantitation
MEF2	Myocyte enhancer factor 2

MRFs	Myogenic regulatory factors
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation factor
OGN	Osteoglycin
PCAF	p300/ CBP-associated factor
pRb	Retinoblastoma protein
Prx1	Peroxiredoxin 1
ROS	Reactive oxygen species
RPLC-MS/MS	Reversed phase liquid chromatography tandem mass spectrometry
SILAC	Stable isotope labelling by amino acids in cell culture
SLRP	Small leucine-rich repeat proteoglycan
SPARC	Secreted protein acidic and rich in cysteine
TGF	Transforming growth factor
VLL	Ventral lateral lip

Abstract

Myogenesis, the formation of skeletal muscle, is a multi-step event that commences with myoblast proliferation, followed by cell-cycle arrest, and finally the formation of multinucleated myotubes via fusion of mononucleated myoblasts. Each step is orchestrated by well-documented intracellular factors, such as cytoplasmic signaling molecules and nuclear transcription factors. Regardless, a key step in getting a more comprehensive understanding of the regulation of myogenesis is to explore the extracellular factors that are capable of eliciting the downstream intracellular factors. This could further provide valuable insight into the acute cellular response to extrinsic cues in maintaining normal muscle development. In this review, we survey the intracellular factors responsible for the cascades of events during myogenesis: myoblast proliferation, cell cycle arrest of myoblasts, and differentiation of myoblasts into myotubes. This focus on extracellular perspective of muscle development illustrates our mass spectrometry-based proteomic approaches to identify differentially expressed secreted factors during skeletal myogenesis.

1. Introduction

Myogenesis, the formation of skeletal muscle, has been recognized as a hierarchical cellular event, commencing with myogenic lineage specification and followed by iterative proliferation of the muscle precursor cells called myoblasts in which cell-cell contact is initiated. This triggers withdrawal of myoblasts from the proliferation cycle (i.e., cell-cycle arrest) and in turn switches on the differentiation program in which mononucleated myoblasts are fused to each other and give rise to multinucleated myotubes (i.e., building blocks for contractile muscle fibres in the mature animal). Each step is orchestrated by groups of intracellular factors, such as cytoplasmic signaling molecules and nuclear transcription factors, which are described in further detail below.

1.1 Myogenic lineage specification

Skeletal muscle originates from the paraxial mesoderm, epithelialization and segmentation of which gives rise to the somites in a cranio-caudal manner (i.e., somites are generated and specified from head to tail) **(Figure 1)**. Various compartments of the somite are committed to distinct cell lineages: myotome (muscle), dermatome (skin) and sclerotome (bone and cartilage), according to their relative orientations to the surrounding tissue, such as ectoderm, neural tube, notochord and lateral mesoderm [1]. The ventral medial portion of the somite is specified as the sclerotome; whereas the double-layered structure remaining is called the dermomyotome which gives rise to dermatome and myotome. The latter is sub-divided into two compartments: dorsal medial lip (DML) and ventral lateral lip (VLL). The former compartment gives rise to the epaxial myotome that

becomes the back muscle, whereas the latter gives the hypaxial myotome that generates the muscles of the body wall, limbs, and tongue [2-5].

1.2 Myoblast proliferation with simultaneous repression of muscle differentiation

After the primary wave of myoblasts is generated from the somite, they enter the cell cycle and undergo iterative propagation to expand the cell population, eventually cell-cell contact occurs. This step has been shown to be essential to withdraw the myoblasts from the proliferation cycle and initiate the differentiation program **(Figure 2A)** [6-8]. Thus, the proliferation and differentiation of myoblasts are mutually exclusive events; the tipping point between the two is governed by a master regulator: the retinoblastoma protein (pRb) [9-11].

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, holding it inactive [12-17]. As a result, pRb is unable to bind to the E2F transcription factor complex and inhibit its activation of downstream proliferation-associated cellular events, including chromosome segregation, mitotic spindle formation, and chromatin remodeling [18] **(Figure 2B)**.

Notably, the differentiation of these myoblasts is critically dependent upon a family of myogenic transcription factors: the myogenic regulatory factors (MRFs), including myogenic differentiation factor (MyoD) [19, 20] and myogenic factor 5 (Myf5) [21, 22]. The MRFs confer on the myoblasts a potent ability to differentiate. By contrast, mitogenic myoblasts may be prohibited from differentiation by myogenic repressors,

including Id [23, 24], twist [25-27], MyoR [28, 29], Mist 1 [30], and I-mf [31]. In the absence of myogenic repressors, MRFs, which are members of the class II basic helix-loop-helix (bHLH) superfamily, can dimerize with members of the class I bHLH family, the E proteins. The E protein: MRF heterodimer recognizes and binds to the consensus DNA sequence (CANNTG) named the E-box, which lies upstream of most muscle-specific genes, e.g., the myosin heavy chain, muscle creatine kinase [32]. Conversely, in the presence of myogenic repressors, the dimerization between MRF and the E protein inside the nucleus is negated, either by (1) competitive binding to MRFs or the E proteins by means of Id, twist, MyoR, and Mist 1, or (2) sequestering MRFs in the cytoplasm by means of I-mf. Additional control can come via other interactions, including those of pRb and CDKs which can also phosphorylate MRFs and subject them to degradation [33-35] **(Figure 2B)**. The initial repression of muscle differentiation is essential for ensuring a sufficiently large number of myoblasts are attained prior to differentiation to populate the vast amount of skeletal musculature in the metazoan species.

1.3 Cell-cycle arrest of myoblasts with simultaneous activation of muscle differentiation

Under growth conditions, myoblasts proliferate until they reach confluency and cell-cell contact provokes growth arrest. The switch between cell-cell contact and cell-cycle arrest is mediated by transmembrane proteins, such as m-cadherin [36-41]. Upon cell-cell contact, m-cadherin is activated and induces CDK inhibitors (CDKIs), e.g., p21 and p57 [42, 43]. As the name suggests, CDKIs inhibit CDK from phosphorylating its

respective substrates, such as pRb and MRF [44, 45]. As a result, both pRb and MyoD are spared from degradation. The corollary to that is two-fold: (1) non-phosphorylated pRb can bind and inhibit E2F from activating the downstream proliferation events, by which cell-cycle arrest of myoblasts is achieved [46, 47]; and (2) non-phosphorylated MyoD can dimerize with the E protein and cooperatively binds to the E box to activate the expression of muscle-specific genes, thus triggering the differentiation program. Furthermore, with the recruitment of myogenic co-activators, such as myocyte enhancer factor 2 (MEF2) [48-51], as well as the chromatin remodeling factors, the histone acetyltransferases (HATs), for example p300 and p300/ CBP-associated factor (PCAF) [52-61], the differentiation program is initiated **(Figure 2C)**. In addition, activated cadherin interacts and triggers a cell adhesion molecule of the Ig superfamily called CAM-related/down-regulated by oncogenes (CDO) [62, 63]. The CDO complex promotes myogenesis by activating the p38 MAPK signaling pathway [64-67], which is a well-known pro-myogenic signal acting at various steps [68-70]. p38 such as enhances the activity of MyoD [71], and its co-activator MEF2 [72], favouring MyoD/E protein heterodimerization by phosphorylating E protein [73], recruiting SWI-SNF chromatin-remodeling complex to the promoter of muscle-specific genes to enhance accessibility to transcriptional regulators required for subsequent gene expression [74]. Intriguingly, CDO is a target of MyoD, establishing a positive feedback loop which reinforces the muscle differentiation program [63, 75].

1.4 From intra- to extra-cellular perspective of myogenesis

Irrespective of well-documented intracellular factors involved in myogenesis, the key step in developing a more comprehensive picture of the regulation of muscle development is to investigate the extracellular factors that prime these downstream intracellular events. This, in turn, may provide valuable insight into the acute cellular response as a result of extrinsic cues in normal muscle development and regeneration. Intriguingly, the effects exerted by the 'conditioned' media (CM) on the development of muscle cells have been documented some time ago [76, 77], illustrating the phenomena that myogenic cells modify their own extracellular milieu by secreting factors that exert autocrine and paracrine effects on the differentiation program. Furthermore, the skeletal muscle has been recognized as the largest endocrine organ in humans for secreting extracellular factors, the myokines that orchestrate muscle development in an autocrine fashion [78, 79]. Apart from the well-known myokines, such as members of the insulin-like growth factor-1 (IGF1) [80-89] and transforming growth factor (TGF) families [90-98], which have potent, but opposing effects on myogenesis, there were individual studies investigating other myokines, such as plasminogen activator [99]; collagenase [100]; decorin [101]; glial growth factor [102]; neurocrescin [103]; meltrin alpha [104]; musculin [78, 105]; IL-1 beta [106]; IL-7 [107]; ADAMTS-like 2 [108]; follistatin-like 1 [109]; and secreted protein acidic and rich in cysteine (SPARC) [110-112]. To make progress on the characterization of the 'secretome' in an unbiased manner, we implemented an initial mass spectrometry-based proteomics study to identify secreted proteins in the mouse skeletal muscle cell line C2C12 [113]. Furthermore, a more quantitative approach using stable-isotope labelling by amino acids in cell culture

(SILAC) in conjunction with online reversed phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS), has now been implemented to identify differentially expressed secreted proteins during myogenesis.

2. Workflow of SILAC quantification

In differential proteomics, stable isotope-labelling, e.g., ^{2}H versus ^{1}H, ^{13}C versus ^{12}C, and ^{15}N versus ^{14}N, is employed to introduce a signature mass difference between the samples of interest (e.g., treatment versus control). After enzymatic protein digestion, the ratios of the labelled peptide peak intensities reveal the relative protein expression. There are two general ways to introduce the stable isotope label into the sample: (1) chemical labelling, typically achieved via the isotope-coded affinity tag (ICAT) or the isobaric tag for relative and absolute quantitation (iTRAQ); and (2) metabolic labelling, conveniently performed via SILAC. ICAT targets the sulfhydryl group on the cysteine residue [114], whereas iTRAQ modifies the amino group on the N-terminus and the lysine residue [115]. For SILAC, stable isotope-labelled amino acids are metabolically incorporated into the living cells as they grow. Irrespective of the labelling methodology, the tagged samples are then combined and processed as one in subsequent treatment, separation, and analysis. This minimizes the impact of non-quantitative recovery of the proteins and peptides in these steps on the accuracy of the quantification [116, 117].

In recent years, SILAC has been widely applied to various biological models and cell types, including immune B cells [118], fibroblasts [119], neuronal cells [120], blood cells [121], lung cells [122], chondrocytes [123], prostate cancer [124], ovarian cancer

[125], liver cancer [126, 127], breast cancer [128, 129], esophageal cancer [130, 131], and embryonic stem cells [132-134]. In addition, it has also been successfully implemented in tissues [135, 136], and living organisms [121, 137-139].

We employed SILAC labelling in an attempt to identify differentially expressed secreted factors at the myotube- versus myoblast-stages (i.e., differentiation versus proliferation) in C2C12 cells. As illustrated in **Figure 3**, CM proteins derived from [$^{12}C_6$]-lysine labelled myoblasts (light) and [$^{13}C_6$]-lysine labelled myotubes (heavy) were mixed in equal amounts and subjected to one-dimensional gel electrophoresis (1D-SDS PAGE), followed by trypsin digestion. The resulting tryptic peptides were analyzed by online RPLC-MS/MS. The ratio of the heavy- versus light-labelled peptide peak intensities in the MS mass spectrum mirrored the relative expression level of that particular protein during myogenesis.

3. Implications of the secreted proteins identified in myogenesis

As previously discussed, myogenesis is a multi-step process, beginning with myogenic lineage specification, followed by cell proliferation, cell cycle arrest and ultimately the differentiation of myoblasts into myotubes. We postulated that each of these steps is regulated by secreted factor(s). According to our preliminary data, novel secreted proteins, such as osteoglycin (OGN), peroxiredoxin 1 (Prx1), and cytokine-induced apoptosis inhibitor 1 (CIAPIN1), were identified as differentially expressed proteins. Their respective role(s) in myogenesis were proposed as follows.

3.1 OGN

OGN is also known as mimecan which belongs to the small leucine-rich repeat proteoglycan (SLRP) family of proteins [140-146]. This protein was found to be essential in maintaining the integrity of the extracellular matrix (ECM) of the cornea [147, 148] and the vascular smooth muscle [149, 150] by inhibiting the ECM-cleaving enzyme gelatinase [151]. This anti-ECM cleaving property contributed to OGN's tumour suppressor role in hepatocarcinoma cells by attenuating tumour cell migration [152]. Given OGN's differential expression in myogenesis, we hypothesized that OGN might play an inhibitory role by hindering myoblast migration and the subsequent cell-cell contact. As result, cell-cycle arrest is inhibited and hence the muscle differentiation program is sabotaged. Interestingly, the E box has been identified in the promoter region of OGN [153]. This projects a compelling regulation mechanism of OGN during myogenesis in which binding of the MRF and E protein heterodimer to the E box may function as a docking site to recruit a chromatin remodeling molecule, such as histone deacetyltransferases (HDACs); as a consequence, the transcription and subsequent expression of OGN decreases.

Furthermore, OGN may also play a role in myogenic lineage commitment, where the protein was initially identified as a bone-inductive factor [154-158]. Intriguingly, we have demonstrated the possibility that C2C12 myoblasts could be re-committed to the osteoblast lineage by over-expressing a bone-inductive gene called menin1 [159]. With this taken into account, it is tempting for us to speculate a plausible link between OGN and menin1 in which down-regulation of OGN may be essential in directing the myoblasts to myogenic lineage.

3.2 Prx1

Prx1, also known as Pag [160] and MSP23 [161], belongs to the antioxidant protein family for cellular defence against reactive oxygen species (ROS) [162]. Prx1 was revealed to be up-regulated in various cancer types, such as oral cancer [163], lung cancer [164-171], pancreatic cancer [172], and esophageal cancer [173]. Expression level of Prx1 was shown to positively correlate with cancer progression; knocking down Prx1 not only attenuated malignancy, but also sensitized the cancer cells to chemotherapy and improved survival [174-176]. Given the role of Prx1 as a pro-survival factor by blocking apoptosis signal-regulating kinase (ASK)-induced cell death [177-179], we hypothesized that Prx1 might function as a mitogen that promotes the proliferation of myoblasts. As proliferation and differentiation are mutually exclusive events, the down-regulation of Prx1 (unpublished data) may be essential for the withdrawal of myoblasts from the proliferation cycle and subsequent differentiation.

3.3 CIAPIN1

CIAPIN1 has been characterized as an anti-proliferation molecule in cell division and angiogenesis [180-182]. CIAPIN1 was shown to be a suppressor of various cancers, for instance gastric cancer [183], renal carcinoma [184], esophageal cancer [185], and colorectal cancer [186]. The anti-proliferation effect of CIAPIN1 was found to be mediated by up-regulating CDKI, which in turn allows pRb to inhibit the E2F transcription factor from activating downstream proliferation events; as a result, cell-cycle arrest prevails [184, 187]. We postulated that CIAPIN1 may function as a positive

regulator of myogenesis, in which the up-regulation of CIAPIN1 (unpublished data) may be essential in triggering cell-cycle arrest of myoblast for subsequent differentiation to take place.

4. Conclusion

We have demonstrated the fidelity of applying SILAC to identify secreted factors during skeletal myogenesis in an unbiased proteomics approach. OGN, Prx1 and CIAPIN1 were identified as novel differentially expressed extracellular factors that are proposed to play a role in the myogenic program (**Figure 4**). Based on the findings of this 'discovery' approach, gain and loss of function studies are now in progress to further dissect these proteins' individual and combinatorial roles in myogenesis. The identification of secretome factors that regulate myogenesis will enhance our knowledge of extracellular regulation of differentiation as well as identify biomarkers of potential therapeutic value in muscle regeneration and stem cell programming.

Acknowledgement

The work was made possible by support from the Natural Sciences and Engineering Research Council (NSERC) of Canada and from the Canadian Institutes of Health Research (CIHR) to JCM and KWMS.

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Figure Legends

Figure 1. Myogenic lineage specification. Dorsal medial lip and ventral lateral lip were denoted as DML and VLL, respectively.

Figure 2. Skeletal muscle differentiation at the microscopic- and molecular-level. (A) During myogenesis, mononucleated myoblast proliferates, followed by cell-cycle exit, and fusion to form multinucleated myotube; **(B)** during proliferation, at the molecular level, active CDK could trigger myoblast proliferation by phosphorylating and subjecting pRb to degradation, in which E2F transcription factor is free from the inhibitory effect of pRb and elicits the proliferation of myoblasts. Simultaneously, CDK can also block myoblasts from differentiation via the phosphorylation-induced degradation of MRF. As a consequence, E protein by itself cannot drive the differentiation program; **(C)** upon cell-cell contact, m-cadherin is activated, by which CDKI is induced. This in turn inhibits CDK from phosphorylating its downstream substrates: pRb and MRF. Hence, both pRb and MRF are exempted from degradation, in which the former can withdraw the myoblasts from the cell cycle by inhibiting E2F transcription factor from activating the proliferation-associated events; whereas the latter complexes with E protein, myogenic co-activator MEF2, and the chromatin remodeling molecule HATs, in an effort to evoke the differentiation program of myoblasts synergistically. Phosphate groups were indicated as "PO_4".

Figure 3. The workflow of using SILAC to identify differentially expressed secreted factors during skeletal myogenesis.

Figure 4. Overview of the implications of OGN, Prx1, and CIAPIN1 in myogenesis.

Figure 1

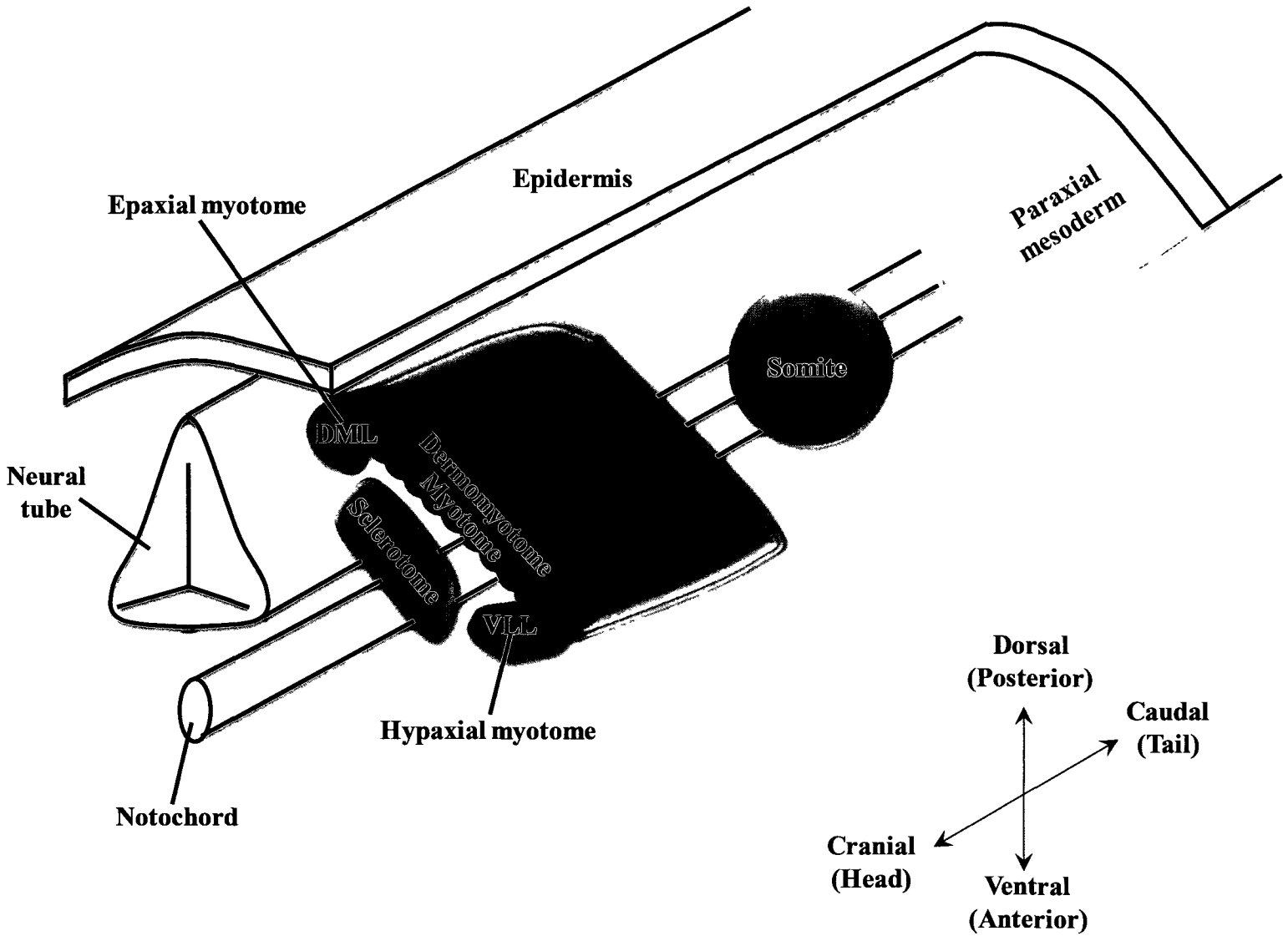

Epaxial myotome
Epidermis
Paraxial mesoderm
Sonite
DML
Dermomyotome
Myotome
Sclerotome
VLL
Neural tube
Hypaxial myotome
Notochord
Dorsal (Posterior)
Caudal (Tail)
Cranial (Head)
Ventral (Anterior)

Figure 2

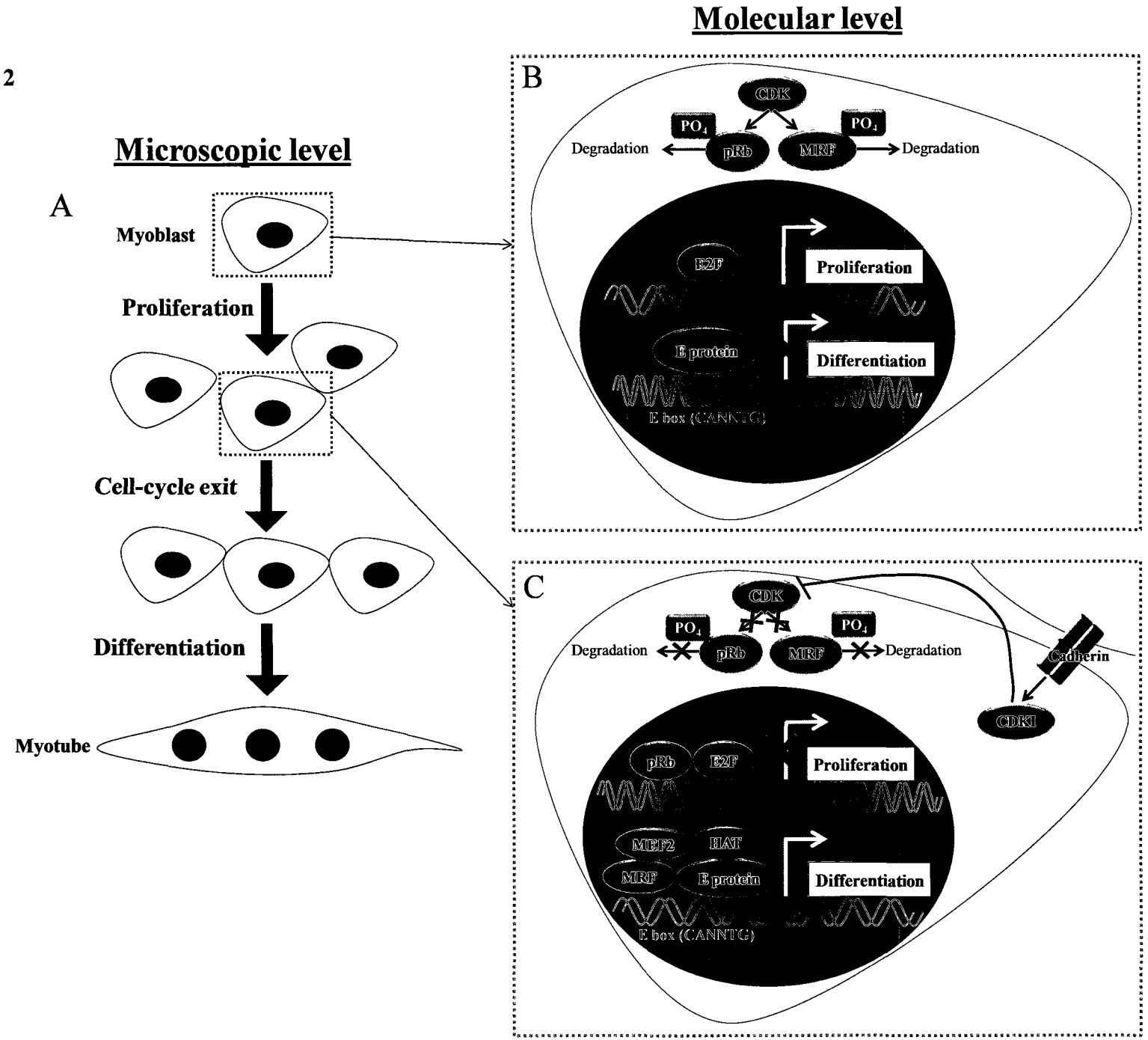

Microscopic level
A
Myoblast
Proliferation
Cell-cycle exit
Differentiation
Myotube
Molecular level
B
CDK
PO4
PO4
Degradation
pRb
MRF
Degradation
E2F
Proliferation
E protein
Differentiation
E box (CANNTG)
C
CDK
PO4
PO4
Degradation
pRb
MRF
Degradation
Cadherin
CDKI
pRb
E2F
Proliferation
MEF2
HAT
MRF
E protein
Differentiation
E box (CANNTG)

Figure 3

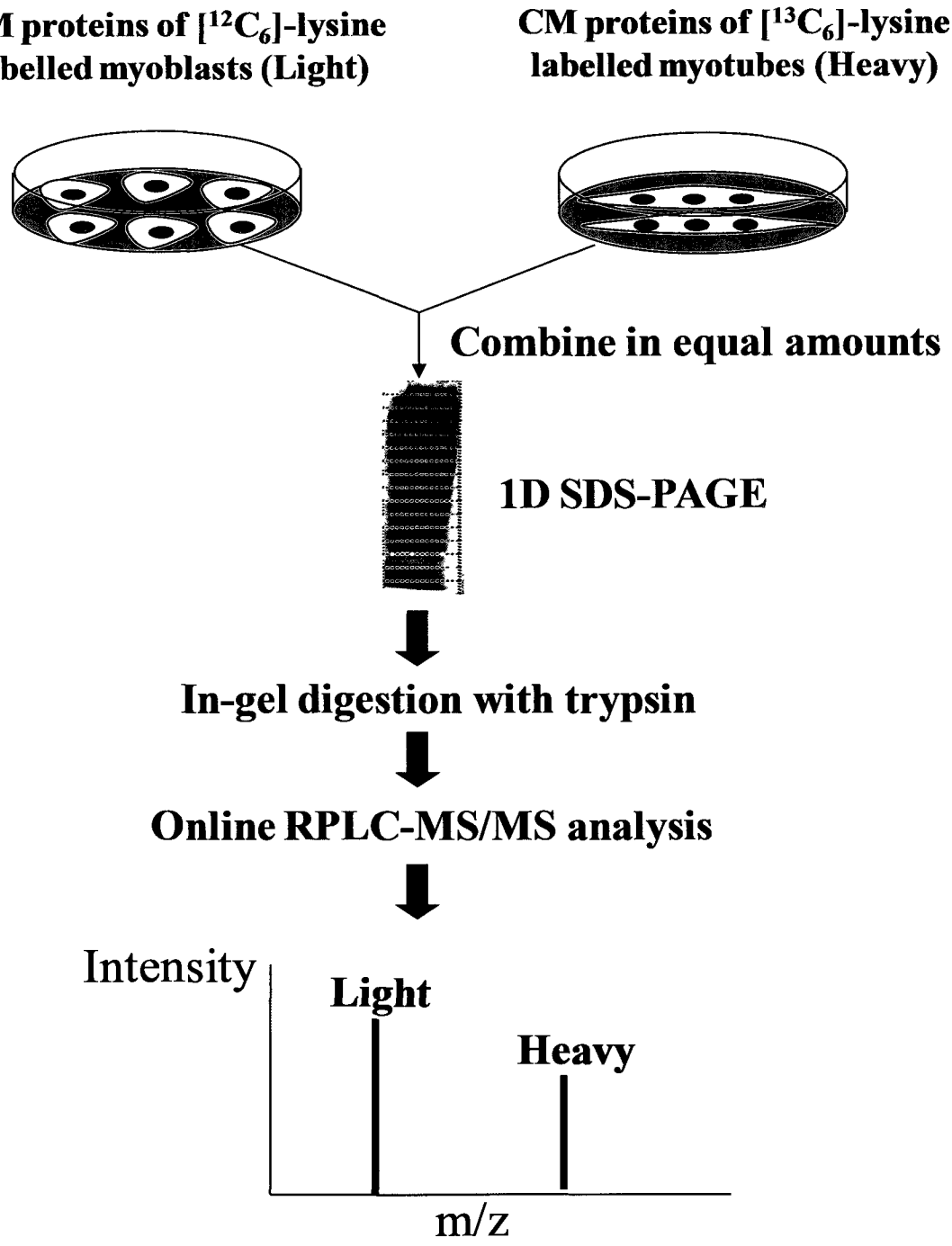
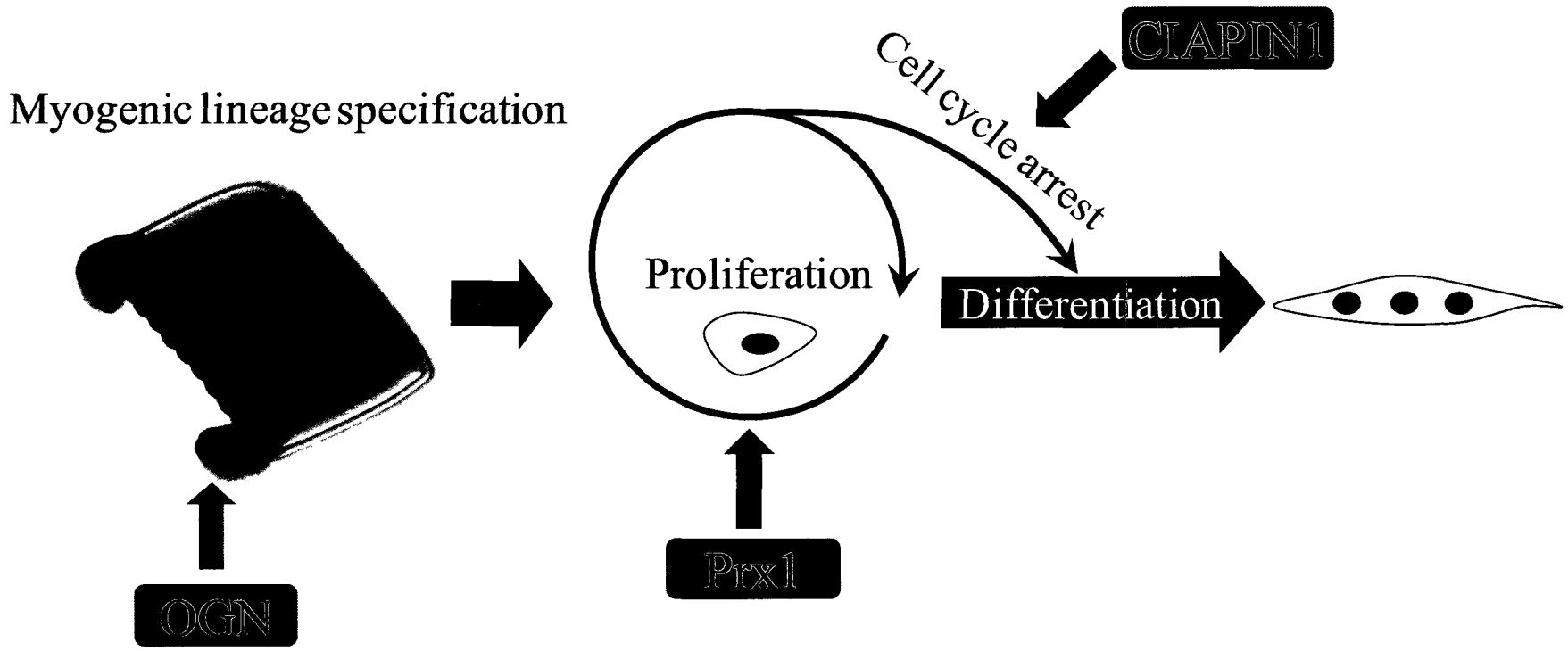

CM proteins of [12C6]-lysine labelled myoblasts (Light)
CM proteins of [13C6]-lysine labelled myotubes (Heavy)
Combine in equal amounts
1D SDS-PAGE
In-gel digestion with trypsin
Online RPLC-MS/MS analysis
Intensity
Light
Heavy
m/z

Figure 4


CIAPIN1
Cell cycle arrest
Myogenic lineage specification
Proliferation
Differentiation
Prx1
OGN

B: Supplemental data

Supplemental data S1

Supplemental S1: Total proteins identified. Proteins identified were parsed to two open-source algorithms SignalP and SecretomeP for secreted proteins prediction in which proteins with SP probability score ≥ 0.5 were assigned as classical-secreted proteins (C); whereas those with SP probability < 0.5 and NN-score ≥ 0.5 were grouped as non-classical secreted proteins (N). The rest with both SP probability and NN-score < 0.5 were categorized as hypothetical intracellulr proteins (I). Proteins identified with a minimal of two distinct peptides (i.e., different amino acid sequence) and one of them being unique (i.e., peptide is only found in that particular protein) were reported.

gi #	Protein name	SignalP	SecretomeP	Protein group	Total # of Peptides ID	# of distinct Peptides ID	# unique Peptides ID	Protein coverage (%)
		SP probability	NN score					
gi|148678464	Dynein, axonemal, heavy chain 9	0	0.405	I	35	21	21	7.7
gi|148676427	Procollagen, type V, alpha 1	1	0.051	C	62	23	15	22.8
gi|70794816, gi|12963491	Enolase 1	0	0.439	I	212	20	8	69.6
gi|124378033	Nucleoporin 214	0	0.196	I	20	8	6	5.7
gi|126116574	NAC alpha domain containing	0.757	0.323	C	82	6	6	3.5
gi|148706007	Ubiquitin-activating enzyme E1-like 2, isoform CRA_b	0.098	0.545	N	44	6	6	11.0
gi|73921193	Myosin heavy chain 9	0	0.09	I	76	12	6	8.0

gi|6753912	Ferritin heavy chain 1	0	0.581	N	19	5	5	26.9
gi|3914439	Proteasome subunit beta type-4	0.002	0.587	N	12	5	5	26.1
gi|18252782	Serine (or cysteine) proteinase inhibitor, clade C, member 1	0.996	0.557	C	12	5	5	18.3
gi|23956222	ARP3 actin-related protein 3	0.013	0.442	I	9	5	5	18.9
gi|58037237	Coiled-coil domain containing 57	0	0.204	I	14	5	5	4.8
gi|40254507	Phosphoglycolate phosphatase	0	0.496	I	11	5	5	23.1
gi|20138800, gi|160333280	Intersectin-1 (EH and SH3 domains protein 1)	0.015	0.138	I	16	9	5	7.1
gi|12850643	Hydroxysteroid (17-beta) dehydrogenase 10	0.98	0.684	C	15	5	4	29.9
gi|116283387	Nefl protein	0	0.093	I	18	5	4	12.0
gi|19527064	ATPase, H+ transporting, lysosomal V1 subunit B1	0.001	0.601	N	12	4	4	11.1
gi|18314528	Ciapin1 protein	0.008	0.668	N	23	4	4	23.4

gi|148693430	Decapping enzyme, scavenger	0	0.58	N	7	4	4	16.3
gi|12832182	Delta-aminolevulinic acid dehydratase	0.057	0.529	N	5	4	4	18.2
gi|585122	Fibulin-2 precursor	1	0.477	C	8	4	4	3.8
gi|2253159	Peripherin	0.007	0.582	N	13	4	4	11.3
gi|4759158	Small nuclear ribonucleoprotein polypeptide D2	0	0.914	N	31	4	4	40.7
gi|143811353	Sterile alpha motif domain-containing protein 3	0	0.594	N	13	4	4	7.1
gi|404057	Adenylosuccinate synthetase	0.002	0.452	I	4	4	4	14.3
gi|22122515	AHA1, activator of HSP 90kDa ATPase	0	0.294	I	4	4	4	13.0
gi|21730459	Chain A, RNA-Binding Protein (Tb-Rbp)	0.012	0.28	I	14	4	4	29.4
gi|2970691	Thioredoxin-related protein	0	0.456	I	7	4	4	23.2
gi|26342124	Neurofilament 3, medium	0	0.071	I	8	5	4	8.6

gi|226471	Cu/Zn superoxide dismutase	0.002	0.708	N	29	4	3	32.7
gi|148684009	Procollagen, type I, alpha 1, isoform CRA_a	0.996	0.205	C	4	4	3	4.8
gi|110625681	Serologically defined colon cancer antigen 10	0	0.543	N	75	4	3	16.2
gi|47059073	Thrombospondin 1	0.971	0.428	C	8	4	3	3.8
gi|223115	Dehydrogenase C4,lactate	0	0.452	I	5	4	3	18.5
gi|161016799	Annexin A4	0	0.551	N	13	3	3	11.0
gi|148708561	AP-1, beta 1 subunit, isoform CRA_a	0.001	0.518	N	5	3	3	4.0
gi|74180373	Archain 1	0.622	0.257	C	61	3	3	6.1
gi|467315	Immunoglobulin light chain VJ region	0.177	0.574	N	5	3	3	50.9
gi|29244192	Leucine-rich repeat-containing 8	0.002	0.82	N	7	3	3	5.1
gi|21313588	Small glutamine-rich tetratricopeptide repeat containing protein	0.108	0.588	N	4	3	3	11.7
gi|31324022	Stanniocalcin 1	0.999	0.903	C	20	3	3	19.5

gi|6755863	Tumor rejection antigen gp96	1	0.481	C	3	3	3	4.5
gi|40807354	A kinase (PRKA) anchor protein 10	0	0.349	I	75	3	3	5.7
gi|112363072	actin related protein 2/3 complex, subunit 2	0	0.36	I	3	3	3	10.3
gi|74184317	CAP, adenylate cyclase-associated protein 1, isoform CRA_b	0	0.353	I	6	3	3	8.0
gi|10946972	Epsilon subunit of coatomer protein complex	0.001	0.349	I	15	3	2	16.6
gi|21703726, gi|29366820, gi|94394670, gi|94394670	Isopentenyl-diphosphate delta isomerase isoform 1	0	0.479	I	14	3	3	18.5
gi|45544618	*Methylthioadenosine* phosphorylase	0.055	0.449	I	7	3	3	18.4
gi|12846524	Obg-like ATPase 1 isoform a	0	0.444	I	6	3	3	8.6
gi|148707143	Prefoldin 2, isoform CRA_b	0	0.167	I	8	3	3	31.1
gi|1730067	Pyruvate kinase isozymes R/L (L-PK)	0	0.425	I	5	3	3	7.1

gi|28972365	Rho-related BTB domain containing 2	0.002	0.247	I	17	3	3	8.4
gi|23956082	Ribosomal protein L5	0	0.488	I	16	3	3	14.5
gi|74187271	Ribosomal protein S6 kinase, polypeptide 1 isoform 1	0.001	0.489	I	45	3	3	11.4
gi|4501885	Beta actin	0	0.498	I	325	16	2	67.7
gi|1167907, gi|511298	Alpha-1(XVIII) collagen	1	0.163	C	65	11	3	11.2
gi|148681536	zyxin, isoform CRA_b	0.273	0.462	I	11	6	1	14.4
gi|74204932	Proteasome beta 6 subunit precursor	0.085	0.496	I	19	5	2	27.8
gi|1000033	Chain A, Cyclophilin C Complexed With Cyclosporin A	0	0.342	I	11	4	3	30.8
gi|110591195	Chain A, Glutathione S-Transferase, Mu7 (Gstm7)	0.016	0.435	I	9	4	1	19.9
gi|148665650	Proteasome alpha 3 subunit	0	0.326	I	10	4	2	18.0
gi|74209434	YTH domain family 3 isoform 1	0	0.456	I	16	4	2	10.4

gi|7710086, gi|21313162, gi|37718983, gi|94385160, gi|38372905, gi|94387350, gi|27734154, gi|6679587, gi|21311975, gi|31559981	RAB10, member RAS oncogene family	0.218	0.548	N	22	6	3	19.0
gi|6678131	Spermidine synthase	0.001	0.537	N	10	3	2	16.9
gi|4507791	Ubiquitin-conjugating enzyme E2M	0	0.74	N	7	3	2	16.4
gi|148667170	Heterogeneous nuclear ribonucleoprotein F	0	0.26	I	14	3	2	11.8
gi|33667042	Heterogeneous nuclear ribonucleoprotein L	0	0.484	I	24	3	1	9.2
gi|74181503	Syndecan binding protein isoform 1	0.001	0.273	I	3	3	3	19.7
gi|26325678	A disintegrin and metalloproteinase domain 6-like	0.998	0.606	C	36	2	2	3.1
gi|6680770	Bcl2-associated X protein	0	0.741	N	2	2	2	16.7

gi|50190	Beta-tropomyosin	0	0.555	N	13	2	2	11.6
gi|549047	CD81 antigen	0.093	0.714	N	5	2	2	13.1
gi|55741460	DJ-1 protein	0.02	0.537	N	20	2	2	21.2
gi|33468857	Histidine triad nucleotide binding protein 1	0	0.795	N	7	2	2	16.7
gi|148690423	Hydroxyacyl glutathione hydrolase	0.96	0.793	C	16	2	2	7.4
gi|12842364	Inosine triphosphatase	0.002	0.505	N	8	2	2	10.1
gi|6680387	Insulin-like growth factor binding protein 6	1	0.68	C	9	2	2	8.8
gi|12833728	Microtubule-associated protein 1 light chain 3 beta	0	0.58	N	4	2	2	16.8
gi|9910548	SH3-binding domain glutamic acid-rich protein like	0.003	0.733	N	4	2	2	20.2
gi|14625464	Stathmin	0	0.733	N	8	2	2	12.8
gi|18700032	Acidic nuclear phosphoprotein 32 family, member B	0	0.101	I	6	2	2	11.8
gi|151180645	Aldolase A retroprotein 1	0.056	0.437	I	3	2	2	6.0

gi|12840311	Glyoxalase domain containing 4	0	0.396	I	6	2	2	9.3
gi|51304	Histone cluster 1, H2ae	0	0.465	I	10	2	2	20.7
gi|74220666	Lamin A isoform C	0.003	0.297	I	2	2	2	5.6
gi|6679337	Phosphatidylinositol transfer protein, alpha	0	0.489	I	3	2	2	12.2
gi|13654249	Phosphoglycerate kinase 2	0	0.367	I	3	2	2	6.2
gi|13385434	Phosphoribosylaminoimidazole carboxylase	0	0.485	I	3	2	2	6.1
gi|12842359	RAN binding protein 1	0	0.183	I	6	2	2	15.8
gi|14589953	RNA polymerase II, polypeptide H	0	0.484	I	7	2	2	17.3
gi|2144100	Set beta isoform	0	0.142	I	11	2	2	11.6
gi|14523046	Intermediate filament protein nestin	0	0.345	I	68	15	2	12.2
gi|157787199	Alpha-tropomyosin	0	0.624	N	12	6	1	22.2
gi|2690302	Aspartate aminotransferase precursor	0.996	0.51	C	8	6	1	15.1
gi|51593432	Ptms protein	0	0.56	N	9	5	1	29.9

gi|148669988	Glutathione S-transferase mu 2	0.011	0.376	I	13	4	2	21.0
gi|13435924	Aldolase 3, C isoform	0.001	0.374	I	5	3	2	15.2
gi|6681273	Eukaryotic translation elongation factor 1 alpha 2	0.003	0.251	I	5	3	1	13.8
gi|508270	A+U-rich RNA-binding protein	0.798	0.66	C	6	2	1	16.0
gi|13626116	Angiopoietin-like 2	0.998	0.568	C	3	2	1	6.3
gi|5815459	Insulin-like growth factor binding protein 5 protease	1	0.754	C	7	2	1	5.4
gi|148694194	Tropomyosin 1, alpha, isoform CRA_b	0	0.525	N	30	2	1	6.7
gi|7106546	14-3-3 protein sigma	0	0.481	I	4	2	2	12.1
gi|202229	Beta-tubulin	0	0.498	I	2	2	1	6.9
gi|148685497	Fructose-bisphosphate aldolase A	0.014	0.379	I	5	2	1	3.3
gi|15029724	Fus protein	0	0.492	I	8	2	1	13.6
gi|200022	Neurofilament protein	0.458	0.037	I	42	2	1	3.5
gi|31982186	Malate dehydrogenase 2, NAD	0.336	0.449	I	75	13	12	49.7

gi|6754976	Peroxiredoxin 1	0	0.542	N	202	14	12	80.9
gi|437125	Insulin-like growth factor binding protein 5	1	0.922	C	275	12	11	58.9
gi|33563250	Desmin	0.022	0.684	N	55	12	11	28.4
gi|6996913, gi|63594732	Annexin A2	0.001	0.739	N	69	12	10	40.7
gi|6647554, gi|58037546	Isocitrate dehydrogenase 1	0	0.563	N	42	11	10	39.6
gi|62201487, gi|6679937, gi|50233866, gi|47607490	Glyceraldehyde-3-phosphate dehydrogenase	0.002	0.541	N	104	11	10	55.3
gi|6678077, gi|94390366	SPARC precursor	0	0.646	N	292	11	10	48.3
gi|3914434, gi|6755204	Proteasome subunit, beta type 5	0.013	0.753	N	38	10	9	55.5
gi|6755040	Profilin 1	0.001	0.56	N	242	9	8	90.7
gi|12844989, gi|94380051, gi|94379874, gi|94370545, gi|94369219, gi|94369185	Phosphoglycerate mutase 1 (isozyme B)	0	0.408	I	108	11	7	67.7
gi|30519911, gi|94365804	Transgelin 2	0.406	0.727	N	106	11	8	68.3
gi|12846252, gi|31560539	Peroxiredoxin 2	0	0.494	I	59	9	7	70.2

gi|5803225, gi|31981925	Tyrosine 3-/ Tryptophan 5- monooxygenase activation protein, epsilon polypeptide	0	0.33	I	238	9	8	44.3
gi|125490382, gi|6679221	Procollagen C-Proteinase enhancer protein	1	0.616	C	35	8	7	20.3
gi|6678097	Serine (or Cysteine) proteinase inhibitor, clade B, member 6A	0.002	0.451	I	28	8	7	31.0
gi|6679166	Osteoglycin	0.997	0.567	C	35	8	7	38.3
gi|7304963	Chloride intracellular channel 4	0	0.563	N	50	8	7	37.5
gi|148675904, gi|31982178	Malate dehydrogenase 1, NAD	0.248	0.428	I	124	8	7	27.6
gi|6679439	Peptidylprolyl isomerase A	0	0.421	I	258	8	7	64.0
gi|497775, gi|6679745	Fascin 1	0.003	0.322	I	13	7	6	20.3
gi|21312564, gi|94421109	Calponin 3, acidic	0	0.663	N	28	7	6	29.7
gi|13435747, gi|31982030	RHO GDP Dissociation inhibitor (GDI), alpha	0	0.427	I	139	7	6	49.0

Supplemental data S2

Supplemental S2: Secreted proteins quantified. Down- and up-regulated expression levels were shaded in green and red respectively, whereas those in grey represented non-differential expression.

gi #	Protein name	SignalP	Secretome P	Protein group	Set 1		Set 2		Total # unique peptides quantified	Total # Peptides identified	Total # distinct peptides identified	# unique Peptides ID	Protein coverage (%)
		SP probability	NN score		Protein ratio (H:L)	SD of protein ratio	Protein ratio (H:L)	SD of protein ratio					
gi|6754976	Peroxiredoxin 1	0	0.542	N	0.46	0.05	0.37	0.04	26	202	14	12	80.9
gi|437125	Insulin-like growth factor binding protein 5	1	0.931	C	0.52	0.09	0.36	0.03	34	275	12	11	58.9
gi|6996913, gi|63594732	Annexin A2	0.001	0.739	N	0.24	0.06	0.23	0.03	8	69	12	10	40.7
gi|6680924, gi|94406453	Cofilin 1, non-muscle	0	0.629	N	0.41	0.07	0.35	0.03	28	227	8	5	56.0
gi|6755040	Profilin 1	0.001	0.56	N	0.51	0.06	0.35	0.02	43	242	9	8	90.7
gi|62201487, gi|6679937, gi|50233866, gi|47607490	Glyceraldehyde-3-phosphate dehydrogenase	0.001	0.541	N	0.60	0.05	0.42	0.03	17	104	11	10	55.3
gi|6678077, gi|94390366	Secreted acidic cysteine rich glycoprote	1	0.921	C	1.34	0.21	0.90	0.07	27	292	11	10	48.3

gi|74186081, gi|70778915	Moesin	0	0.558	N	141	7	6	16.6
gi|148690909, gi|94396246, gi|94395498, gi|94395328, gi|6753914	Ferritin light chain 1	0	0.5	N	71	10	6	76.0
gi|6671672	Capping potein muscle Z-line, alpha 2	0	0.454	I	30	8	5	42.3
gi|47894398	Tropomyosin 4	0	0.485	I	78	8	5	40.7
gi|6680924, gi|94406453	Cofilin 1, non-muscle	0	0.629	N	227	8	5	56.0
gi|12836985, gi|10946870, gi|94372279	Aldo-keto reductase family 1, member A4	0.016	0.458	I	16	7	5	37.3
gi|14250422, gi|82896232, gi|63518159, gi|94372948, gi|94372849, gi|94374014, gi|94374016	6-phosphogluconate dehydrogenase, decarboxylating isoform 6	0.119	0.436	I	31	7	5	21.9
gi|6755202	Proteasone beta 3 subunit	0	0.401	I	37	7	5	40.5
gi|33563282	Proteasome subunit, alpha type 1	0	0.345	I	11	6	5	30.0
gi|19882207	Farnesyl diphosphate synthetase	0	0.468	I	16	6	5	24.4

gi|7106389	Proteasone subunit, alpha type 7	0	0.236	I	17	6	5	31.9
gi|74222953, gi|84794552	Phosphatidylethanolamine binding protein 1	0.001	0.496	I	46	6	5	50.3
gi|6754524	Lactate dehydrogenase 1, A chain	0.001	0.568	N	126	15	4	60.5
gi|148676868, gi|6756041	Tyrosine 3-/ Tryptophan 5- monooxygenase activation protein, zeta polypeptide	0	0.244	I	223	11	4	52.7
gi|4504445, gi|94404313, gi|85060507, gi|94404317, gi|94404315, gi|6754220	Heterogeneous nuclear ribonucleoprotein A1	0	0.111	I	86	9	5	37.8
gi|6679108, gi|94389192, gi|94388148, gi|94388033, gi|94386888, gi|94385999, gi|94385641	Nucleophosmin	0	0.469	I	12	7	5	33.2
gi|15617203, gi|148694736	Chloride intracellular channel 1	0	0.406	I	44	7	5	38.2

gi|130488506, gi|94373764, gi|94410172, gi|6753868	Four and a half LIM domains protein 3	0	0.533	N	26	6	4	25.3
gi|293689, gi|6754556	Lamin B1	0.007	0.389	I	21	6	5	11.2
gi|2498391, gi|31560699	Follistatin-like 1	1	0.507	C	17	5	4	18.3
gi|58037267, gi|82941744	Protein disulfide isomerase-associated 6 isoform 2	0.966	0.63	C	12	5	4	18.2
gi|6753060	Annexin A5	0.001	0.463	I	14	5	4	21.3
gi|6753284, gi|94385006	Caspase 3	0	0.459	I	14	5	4	24.5
gi|6754090	Glutathione S-transferase omega 1	0	0.744	N	18	5	4	29.6
gi|24586721, gi|31980922	Eukarotic translation elongation factor 1 beta 2	0	0.655	N	20	5	4	39.7
gi|202054, gi|31543867	Tissue inhibitor of Metalloproteinase 2	1	0.866	C	21	5	4	34.1
gi|13374567	Carbonic anhydrase 13	0	0.42	I	21	5	4	26.0
gi|9790219, gi|94368815	Destrin	0.096	0.686	N	46	5	4	38.2
gi|34328108	Procollagen, type I, alpha 1	0.996	0.174	C	29	15	3	18.0

gi|74144541, gi|6680980	Procollagen, type I, alpha 2	0.986	0.207	C	24	14	3	16.4
gi|148680653, gi|6679651	Enolase 3, beta muscle	0	0.282	I	57	8	3	27.3
gi|74198639, gi|94371270, gi|94369912, gi|94391781, gi|94390243, gi|6677769	60 S ribosomal protien L12	0.002	0.868	N	57	7	3	61.2
gi|27754065	Pyrophosphatase	0	0.41	I	11	5	4	19.4
gi|457880, gi|31982260	Insulin-like growth factor binding protein 2	1	0.805	C	22	5	3	22.0
gi|74193793, gi|6679497	Proteasome subunit, alpha type 2	0.187	0.399	I	12	4	3	21.5
gi|7106387, gi|94388799, gi|94389513, gi|94389511, gi|94388526, gi|94388524	Proteasome subunit, alpha type 5	0	0.48	I	14	4	3	18.7
gi|148684442, gi|6755372	ribosomal protein S3	0	0.489	I	18	4	3	18.7
gi|6753086	Apurinic/Apyrimidinic endonuclease 1	0	0.663	N	11	4	3	17.0
gi|5542285, gi|6754696	Macrophage migration inhibitory factor	0.003	0.707	N	23	4	3	44.7

gi|27501448	Integrin beta 4 binding protein	0.001	0.556	N	17	4	3	29.4
gi|9910832, gi|31981327	Proteasome subunit, beta type 2	0	0.439	I	14	4	3	17.4
gi|6755198	Proteasome subunit, alpha type 6	0	0.429	I	33	4	3	20.3
gi|148703873, gi|6678437	Tumor protein, translationally-controlled 1	0	0.527	N	45	4	3	35.6
gi|227293, gi|6681079	Cathepsin B preprotein	1	0.852	C	26	4	3	15.3
gi|12835914, gi|149271040	Lamin A isoform A	0.001	0.067	I	206	25	2	42.7
gi|74191337, gi|6753304	Serine (or Cysteine) proteinase inhibitor, clade H, member 1	1	0.859	C	24	10	2	32.4
gi|56699438, gi|54287684	Eukaryotic translation elongation factor 1 delta isoform A	0.005	0.471	I	47	10	2	18.9
gi|123227997, gi|11875203	Tropomyosin 2, beta	0	0.518	N	89	9	2	36.3
gi|6679078, gi|94377018, gi|94375800, gi|82900057	Nucleoside-diphosphate kinase 2	0	0.307	I	152	8	2	61.8

gi|6756037	Tyrosine 3-/ Tryptophan 5- monooxygenase activation protein, eta polypeptide	0	0.349	I	23	6	4	32.5
gi|6680229, gi|94385061, gi|94418431	High mobility group box 2	0	0.275	I	14	5	3	27.1
gi|6754222	Heterogeneous nuclear ribonucleoprotein A/B	0	0.559	N	38	5	2	23.2
gi|13384778	6-phosphogluconolactonase	0.841	0.725	C	13	4	2	26.1
gi|148706176, gi|6753364, gi|94381643, gi|94379626, gi|82903467	Cell division cycle 42	0.006	0.747	N	4	3	2	27.6
gi|74183811, gi|6754570	Annexin A1	0	0.47	I	165	3	2	8.7
gi|6679803	FK506 binding protein 1A	0	0.383	I	24	3	2	37.0
gi|9910218	Fibroblast growth factor 21	0.994	0.928	C	6	3	2	21.4
gi|309315, gi|7305155	Hypoxnthine guanine phosphoribosyl transferase 1	0	0.766	N	10	3	2	15.6

gi|950002, gi|94373495, gi|6752954	actin, gamma cytoplasmic 1	0.071	0.569	N	296	3	2	7.1
gi|4886998, gi|6753558	Cathepsin L preprotein	1	0.686	C	22	3	2	10.8
gi|6671539	Aldolase 1, A isoform	0	0.357	I	212	16	1	72.0
gi|118091, gi|71774133	Peptidylprolyl isomerase B	0.438	0.864	N	21	6	3	32.7
gi|33859640	Transaldolase 1	0	0.417	I	15	5	1	16.6
gi|6755252	Purine rich element binding protein N	0	0.178	I	11	5	1	23.1
gi|50363232	Nestin	0	0.345	I	7	4	1	2.5
gi|31981611, gi|148705465	Spondin 2, extracellular matrix protein	0.999	0.669	C	21	4	2	22.1
gi|51704904, gi|94364890, gi|94364892, gi|82878765, gi|82795789, gi|27754099, gi|82795783	Elongation factor 1-gamma	0.062	0.41	I	4	3	1	10.2
gi|28916703, gi|6755114	Peroxiredoxin 5 precursor	0.347	0.682	N	10	3	2	14.3
gi|6754994	Poly(RC) binding protein 1	0.002	0.549	N	11	3	1	13.8
gi|113205063, gi|6755795	Tissue inhibitor of Metalloproteinase 1	1	0.881	C	14	3	2	19.5

gi|6753618	D-dopachrome tauromerase	0	0.38	I	5	2	1	18.6
gi|4506697, gi|13385652	Ribosomal protine S20	0	0.769	N	7	2	1	17.6
gi|74227478, gi|51873060	Eukaryotic translation elongation factor, alpha 1	0.003	0.155	I	36	2	1	7.6
gi|192912, gi|31981822	Cystatin C	1	0.945	C	49	2	1	17.9
gi|54855, gi|6678413	Triosephosphate isomerase 1	0.083	0.397	I	88	2	1	9.6
gi|134152436, gi|31982755	Vimentin	0.003	0.728	N	206	2	1	10.6
gi|7242171	Proliferating cell nuclear antigen	0.001	0.562	N	8	2	1	8.8
gi|6671746	Cofilin 2, muscle	0	0.683	N	22	2	1	13.9
gi|148665968, gi|45597447, gi|94378683, gi|94377409, gi|82901591	Superoxide dismutase 1, soluble	0.003	0.76	N	32	2	1	16.7
gi|74181120, gi|94373894	Heparan sulfate proteoglycan core protein precursor (HSPG)	1	0.509	C	47	2	1	0.8
gi|148703432, gi|42741690, gi|31543918	Ubiquitin-conjugating enzyme E2 variant 1	0	0.799	N	6	2	1	19.0

gi|78000203, gi|31560030	Tropomyosin 1, alpha	0	0.638	N	29	2	1	9.3
gi|4502203, gi|6680718, gi|6680716, gi|6671571	ADP-Riboxylation factor 3	0	0.439	I	26	2	1	11.0
gi|148690465, gi|6678481	Ubiquitin-conjugating enzyme E2L3	0	0.582	N	18	4	2	15.6

Supplemental data S2

Supplemental S2: Secreted proteins quantified. Down- and up-regulated expression levels were shaded in green and red respectively, whereas those in grey represented non-differential expression.

gi #	Protein name	SignalP	Secretome P	Protein group	Set 1		Set 2		Total # unique peptides quantified	Total # Peptides identified	Total # distinct peptides identified	# unique Peptides ID	Protein coverage (%)
		SP probability	NN score		Protein ratio (H:L)	SD of protein ratio	Protein ratio (H:L)	SD of protein ratio					
gi|6754976	Peroxiredoxin 1	0	0.542	N	0.46	0.05	0.37	0.04	26	202	14	12	80.9
gi|437125	Insulin-like growth factor binding protein 5	1	0.931	C	0.52	0.09	0.36	0.03	34	275	12	11	58.9
gi|6996913, gi|63594732	Annexin A2	0.001	0.739	N	0.24	0.06	0.23	0.03	8	69	12	10	40.7
gi|6680924, gi|94406453	Cofilin 1, non-muscle	0	0.629	N	0.41	0.07	0.35	0.03	28	227	8	5	56.0
gi|6755040	Profilin 1	0.001	0.56	N	0.51	0.06	0.35	0.02	43	242	9	8	90.7
gi|62201487, gi|6679937, gi|50233866, gi|47607490	Glyceraldehyde-3-phosphate dehydrogenase	0.001	0.541	N	0.60	0.05	0.42	0.03	17	104	11	10	55.3
gi|6678077, gi|94390366	Secreted acidic cysteine rich glycoprote	1	0.921	C	1.34	0.21	0.90	0.07	27	292	11	10	48.3

	in[a]											
gi|126116574	NAC alpha domain containing[a]	0.757	0.323	C	9999	0	NA	NA	4	82	6	6	3.5
gi|18314528	Ciapin1 protein[a]	0.008	0.668	N	9999	0	NA	NA	7	23	4	4	23.4
gi|585122	Fibulin-2 precursor[a]	1	0.477	C	1.25	0	NA	NA	1	8	4	4	3.8
gi|24586721, gi|31980922	Eukaryotic translation elongation factor 1 beta 2	0.981	0.667	C	0.82	0.06	NA	NA	2	20	5	4	39.7
gi|7304963	Chloride intracellular channel 4	0	0.563	N	0.67	0.03	NA	NA	3	50	8	7	37.5
gi|74222953	Phosphatidylethanolamine binding protein 1	0.001	0.767	N	0.6	0.07	NA	NA	3	21	6	5	50.3
gi|309315, gi|7305155	Hypoxanthine-guanine phosphoribosyltransferase	0	0.766	N	0.59	0.06	NA	NA	3	10	3	2	15.6

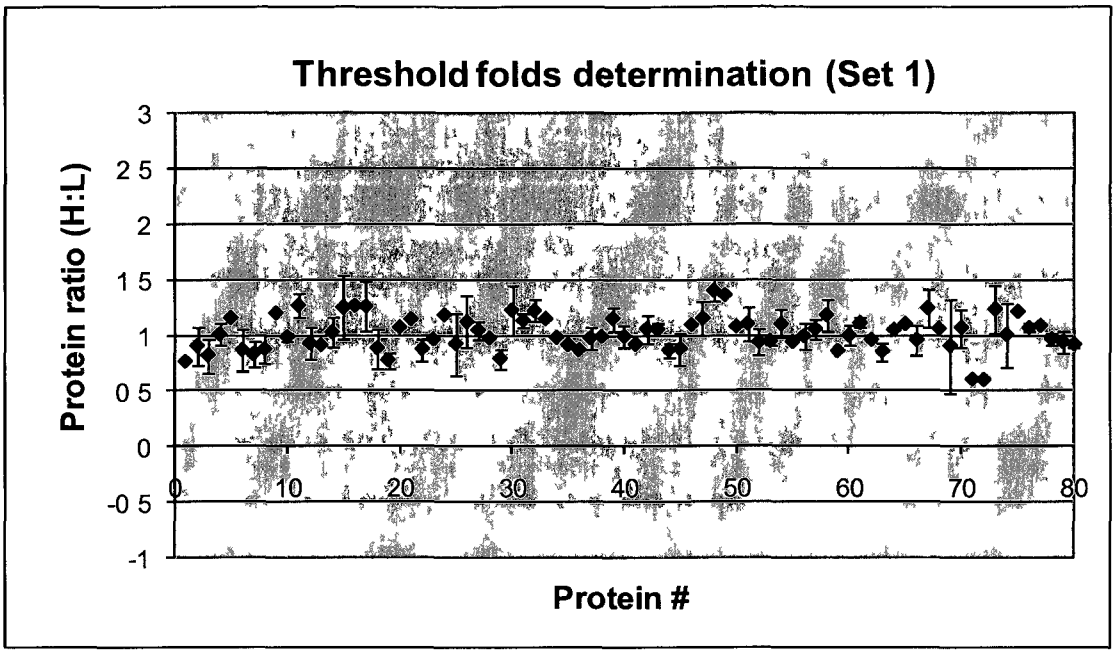
gi|148675904	Malate dehydrogenase 1, NAD (soluble), isoform CRA_c	0.004	0.516	N	0.58	0.08	NA	NA	3	34	8	7	27.6
gi|33468857	Histidine triad nucleotide binding protein 1	0	0.795	N	0.57	0	NA	NA	1	7	2	2	16.7
gi|12846252	Peroxiredoxin 2	0	0.562	N	0.57	0.05	NA	NA	6	43	9	7	70.2
gi|4759158	Small nuclear ribonucleoprotein polypeptide D2	0	0.914	N	0.56	0	NA	NA	1	31	4	4	40.7
gi|6754524	Lactate dehydrogenase A	0.001	0.568	N	0.50	0	NA	NA	1	126	15	4	60.5
gi|3914434, gi|6755204	Proteasome subunit beta type-5 precursor	0.53	0.411	C	0.5	0.09	NA	NA	5	35	10	9	55.5
gi|148708561	AP-1, beta 1 subunit, isoform CRA_a	0.001	0.518	N	0.48	0.15	NA	NA	2	5	3	3	4.0

gi|226471	Cu/Zn superoxide dismutase	0.002	0.708	N	0.47	0.04	NA	NA	3	29	4	3	32.7
gi|143811353	Sterile alpha motif domain-containing protein 3	0	0.594	N	0.4	0	NA	NA	1	13	4	4	7.1
gi|2498391, gi|31560699	Follistatin-like 1[a]	1	0.507	C	0.36	0	NA	NA	1	17	5	4	18.3
gi|30519911, gi|94365804	Transgelin 2	0	0.79	N	0.35	0.03	NA	NA	3	106	11	8	68.3
gi|6679166	Osteoglycin[a,b]	0.997	0.567	C	0.16	0	NA	NA	1	35	8	7	38.3
gi|148706007	Ubiquitin-activating enzyme E1-like 2, isoform CRA_b	0.098	0.545	N	0	0	NA	NA	1	44	6	6	11.0
gi|3914439	Proteasome subunit beta type-4	0.002	0.587	N	0	0	NA	NA	2	12	5	5	26.1
gi|18252782	Serine (or cysteine) proteinase inhibitor, clade C, member 1	0.996	0.557	C	0	0	NA	NA	3	12	5	5	18.3
gi|1167907, gi|511298	Alpha-1(XVIII)	1	0.163	C	0	0	NA	NA	4	65	11	3	11.2

	collagen												
gi|50190	Beta-tropomyosin	0	0.555	N	0	0	NA	NA	7	13	2	2	11.6
gi|13435747	Rho GDP dissociation inhibitor (GDI) alpha	0	0.755	N	0	0	NA	NA	1	49	7	6	49.0
gi|12836985, gi|10946870, gi|94372279	Aldo-keto reductase family 1, member A4	0	0.547	N	0	0	NA	NA	1	11	7	5	37.3
gi|74183811	Annexin A1	0	0.589	N	0	0	NA	NA	2	2	3	2	8.7

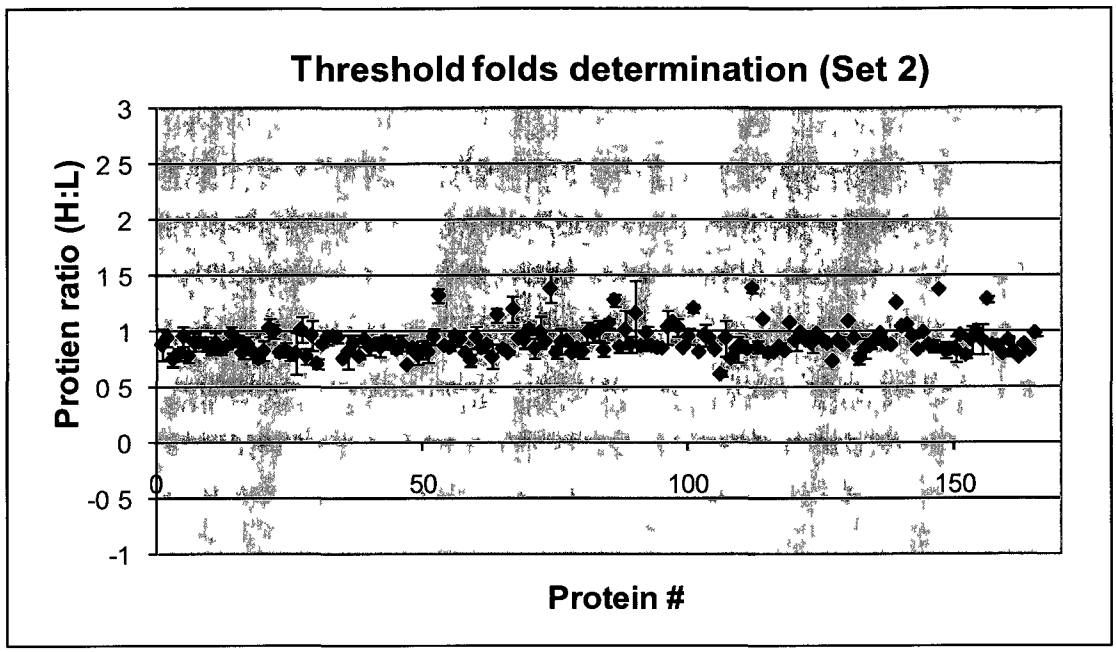
a: functional study using siRNA was performed
b: functional study using purified protein was conducted

Supplemental data S3A



# of protein ratios	80	
# of peptide ratios	229	
Average CV of peptide ratios from the same protein	8 4	%
Median CV of peptide ratios from the same protein	8 1	%
Average protein ratios	1 02	
SD of protein ratios	0 156558	
CV of protein ratios	15.4	%

Supplemental data S3B



# of protein ratios	165	
# of peptide ratios	858	
Average CV of peptide ratios from the same protein	5.6	%
Median CV of peptide ratios from the same protein	4.9	%
Average protein ratios	0.91	
SD of protein ratios	0.1	
CV of protein ratios	14.1	%

Supplemental data S4A

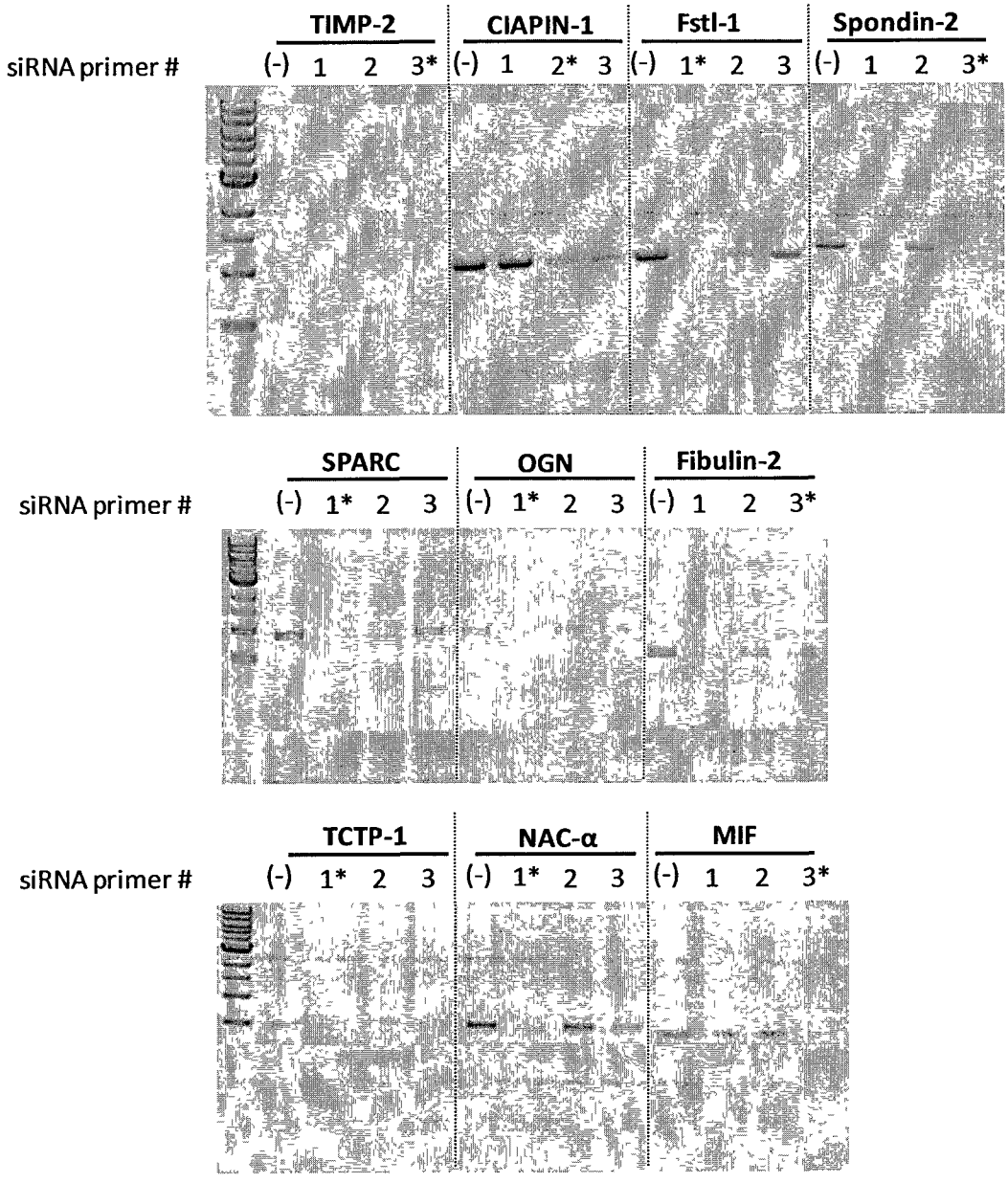
siRNAs (Sigma) and primers used for functional studies of secreted proteins. Three pre-designed MISSION® siRNAs with highest possible ranking were used against each target gene. Some of the primers for semi-quantitative RT-PCR include restriction sites for cDNA cloning.

Gene	MISSION® siRNA ID	Primers for RT-PCR
Spondin-2	SASI_Mm01_00020011 SASI_Mm01_00020012 SASI_Mm01_00020016	5' TAGGATCCATGGAAAACGTGAGTCTTG 3' 5' ATGCGGCCGCTTAGACGCAGTTATCTGG 3'
Fstl-1	SASI_Mm01_00107056 SASI_Mm01_00107058 SASI_Mm01_00107059	5' TCAAGCTTATGTGGAAACGATGGCTG 3' 5' ATGCGGCCGCTTAGATCTCTTTGGTGTTC 3'
Fibulin-2	SASI_Mm02_00298371 SASI_Mm02_00298372 SASI_Mm02_00298373	5' GCTGAACTGCCCAATAGC 3' 5' CGTCCACGTCTGTGCATTC 3'
OGN	SASI_Mm01_00105762 SASI_Mm02_00314650 SASI_Mm01_00105763	5' TAGGATCCATGGAGACTGTGCACTCTA 3' 5' ATGCGGCCGCTTAGAAGTATGACCCTATG 3'
SPARC	SASI_Mm01_00101826 SASI_Mm01_00101828 SASI_Mm01_00101829	5' TAGGATCCATGAGGGCCTGGATCTTC 3' 5' ATGCGGCCGCTTAGATCACCAGATCCTTG 3'
MIF	SASI_Mm01_00059557 SASI_Mm01_00059553 SASI_Mm02_00319369	5' TAGGATCCATGCCTATGTTCATCGTG 3' 5' ATGCGGCCGCTCAAGCGAAGGTGGAACC 3'
TCTP	SASI_Mm01_00277140 SASI_Mm01_00277141 SASI_Mm01_00277142	5' TAGGATCCATGATCATCTACCGGGAC 3'

		5′ ATGCGGCCGCTTAACATTTCTCCATCTC 3′
NACα domain containing protein	SASI_Mm02_00298803 SASI_Mm02_00298804 SASI_Mm02_00298805	5′ AGTAGGGCTACATTCCCAG 3′ 5′ CTGAGGTGGGAACTGTACTT 3′
CIAPIN-1	SASI_Mm02_00339401 SASI_Mm02_00339402 SASI_Mm02_00339403	5′ TAGAATTCATGGAGGAGTTTGGGATC 3′ 5′ ATGCGGCCGCCTAGGCATCCTGGAGATTG 3′
TIMP-2	SASI_Mm01_00154973 SASI_Mm01_00154974 SASI_Mm01_00154975	5′ TAGGATCCATGGGCGCCGCGGCCCG 3′ 5′ ATGCGGCCGCTTACGGGTCCTCGATGTC 3′

Supplemental data S4B

Determine the efficiency of siRNA knock-down with semi-quantitative RT-PCR. Three pairs of siRNA primers were tested to knock down each gene. Primers marked with asterisks were the ones with the highest efficiency to knock down the mRNA of the respective gene, as revealed by the band intensities of the RT-PCR products (i.e., the ones with the lowest band intensities in the agarose gels). These potent siRNA primers were used for subsequent functional analysis using MCK-driven luciferase reporter assay. The scrambled siRNAs denoted as '(-)' were used as negative controls.



C: Materials and methods

Cell freezing

1.) Trypsinized 10-cm plates of cells with 3 ml of 0.125 % trypsin.
2.) Add 10 ml of 10 % FBS in DMEM to the cell suspension
3.) Spin down the cell suspension at 1000 rpm for 5 min
4.) Resuspend the cell pellet with 10 ml of 10 % DMSO in 10 % FBS-DMEM which was filtered with 0.2 μm syringe filter
5.) Aliquot the cell suspension into 1 ml-vial
6.) Store them in - 80°C

Thawing cells

1.) Thaw the frozen cells in 37-40 °C water bath
2.) Add 5 ml of 10 % FBS-DMEM to the cells
3.) Centrifuge at 1000 rpm for 10 min
4.) Resuspend the cell pellet and inoculate onto the 10-cm tissue culture plate with 10 ml of 10 % FBS-DMEM

Passaging cells

1.) Aspirate the old culture medium
2.) Rinse the cells with 10 ml of Versene (x 2)
3.) Trypsinize the cells with 1 ml of 0.125 % trypsin and incubate at 37 °C for 1 min
4.) Add 9 ml of 10 % FBS-DMEM to the trypsinized cells
5.) For 1:10 split ratio, 1 ml of the trypsinized cell suspension was diluted in 9 ml of 10% FBS-DMEM and inoculated onto a new 10-cm culture plate.

Preparation of cell lysates

1.) Wash the cells with cold PBS (×2)
2.) Scrap the cells off the plate with 1 ml of PBS and transfer to the eppendorf tube
3.) Spin the cells at 2000 rpm for 3 min at 4 °C

4.) Resuspend the cell pellet with 200 µl of NP-40 buffer (50 mM of Tris-HCl at pH8, 150 mM of NaCl, 0.5 % of NP40, 2mM of EDTA, 100 mM of NaF, 10 mM of sodium pyrophosphate, 1 mM of Na_3VO_4, 1 mM of PMSF, 1 µg/ml of leupeptin, 1 µg/ml of aprotinin, 5 µg/µl of pepstatin A).

5.) Vortex the suspension for 30 sec and stay on ice for 10 min (×3)

6.) Spin down pellet at maximum speed for 5 min at 4°C

7.) Transfer supernatant to new eppendorf

In-gel digestion of proteins for MS analysis

1.) Rinse the gel slices with 50 µl of 50 mM ammonium bicarbonate and discard the solution

2.) Shrink the gel slices with 50 µl of 50 % acetonitrile in 25 mM ammonium bicarbonate at room temperature for 15 min

3.) Short spin and discard the solution

4.) Add 30 µl of 50 mM ammonium bicarbonate in 10 mM DTT to the gel slices and incubate at 56 °C for 15 min, followed by cooling down at room temperature for 15 min

5.) Short spin and discard the solution

6.) Add 30 µl of 100 mM Iodoacetamide in 50 mM of ammonium bicarbonate and stay in dark for 15 min

7.) Short spin and discard the solution

8.) Add 50 µl of 50 mM ammonium bicarbonate to the gel slices and stay for 10 min

9.) Short spin and discard the solution

10.) Shrink the gel with 50 µl of 50% acetonitrile in 25mM of ammonium bicarbonate and stay for 15 min

11.) Short spin and discard the solution

12.) Add 30 µl of sequencing-grade trypsin to the gel slices and stay on ice for 15 min

13.) Add 10 µl of 25 mM ammonium bicarbonate to the gel slices and incubate at 37 °C for overnight

14.) Short spin and transfer solution to an eppendorf tube

15.) Add 50 µl of 25 mM ammonium bicarbonate to the gel slices and stay at room temperature for 20 min
16.) Short spin and pool the solution to the eppendorf tube
17.) Add 50 µl of 5% formic acid in 50% acetonitrile to the gel slices for 20 min
18.) Short spin and pool the solution to the eppendorf tube

Western blot analyses

1.) Soak the nitrocellulose membrane in 100 % methanol for 1 min
2.) Rinse the nitrocellulose membrane with H_2O (x3)
3.) Soak Whatman papers, nitrocellulose membranes and the sponges in transfer buffer tank which contains transfer buffer (0.2 M of glycine and 25 mM of Tris)
4.) Sandwich the SDS gel with nitrocellular membrane, whatman paperes and sponges and assemble the sandwich to the transfer tank
5.) Apply 20 V for overnight
6.) Apply 50 V for 1 h
7.) Block the nitrocellulose membrane with 1:4 diluted Odyssey blocking buffer in PBS for 1 h
8.) Apply primary antibody diluted with Odyssey blocking buffer in 0.1 % Tween and incubate at 4 °C overnight
9.) Wash the membrane with PBS in 0.1 % Tween for 5 min (x4)
10.) Prepare 1: 10,000 diluted 2° FL-antibodies (1:10 dilution, i.e. 1 µl + 9 µl diluted O.block/0.1 % Tween, further diluted to 1:1000, i.e. 2 µl of 10× diluted 2° Ab in 2 ml diluted O.block/ 0.1 % Tween) in dark
11.) Apply secondary antibody diluted with Odyssey blocking buffer in 0.1 % Tween at a dilution ratio of 1:10,000 and incubate at room temperature for 1 h in dark
12.) Wash the membrane with PBS in 0.1 % Tween in dark for 5 min (x4)
13.) Rinse the membrane in PBS
14.) Develop the membrane using LI-COR Odyssey system

Immunocytochemistry

1.) Aspirate the culture media and rinse the cells with 10 ml of PBS (×3)
2.) Fix the cells with 6 ml of 90 % methanol at room temperature for 6 min
3.) Rinse the cells with 10 ml of PBS (×3)
4.) Incubate the cells with 6 ml of MF-20 primary antibody at room temperature for 2 h
5.) Rinse the cells with 10 ml of PBS for 5 min (×3)
6.) Incubate the cells with 6 ml of secondary antibody at room temperature for 1 h
7.) Rinse the cells with 10 ml of PBS for 5 min (×3)
8.) Incubate the cells with 10 ml of 3,3'-diaminobenzidine (DAB) reagent at room temperature for 30 min
9.) Rinse the cells with 10 ml of PBS (×3), followed by 10 ml of H_2O
10.) Counterstain plate with 10 ml of hematoxylin for 2 min
11.) Rinse the cells with H_2O (×3)

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