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Proteomic analysis of secreted proteins from skeletal muscle cells during differentiation

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ABSTRACT

Myokines are muscle-secreted factors to regulate cellular functions. However, it remains elusive what type of myokine is released during muscle differentiation. Here we evaluated the dynamics of myokines. More than 400 proteins were detected in conditioned medium and approximately 8% of them were categorized as myokines. The levels of myokines which promote myotube formation, vascularization or neurogenesis peaked during early differentiation, whereas myokines contributing to repellent activity against nerve cells or suppression of adipogenesis decreased after differentiation. Our findings suggest that muscle cells secrete different types of myokines at different developmental stages to communicate with various types of cells.

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1. Introduction

Skeletal muscles are able to alter their phenotypes, *i.e.*, hypertrophy and atrophy, in response to biological growth and physical exercise. Growth factors are largely involved in these processes. In particular, insulin-like growth factor 1 (IGF-1) increases skeletal muscle mass *in vitro* and *in vivo* through protein synthesis pathways [1], and increases myoblast recruitment to fusion [2–4]. Hepatocyte growth factor (HGF) stimulates activation of satellite cells, which are skeletal muscle stem cells residing in a quiescent state [5]. In contrast to IGF-1 and HGF, myostatin, a transforming growth factor (TGF)-β family member, suppresses satellite cell activation and myoblast proliferation [6]. Loss of myostatin function leads to a significant increase in muscle mass caused by hypertrophy and hyperplasia [7,8]. These growth factors, which are secreted by skeletal muscle and other tissues, directly affect skeletal muscle growth and hypertrophy.

Abbreviations: CM, conditioned medium; DM, differentiation medium; ECM, extracellular matrix; GM, growth medium; iTRAQ®, isobaric tags for relative and absolute quantitation; MS, mass spectrometry.

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Recently, it has been proposed that "myokines" are proteins or peptides released from skeletal muscle cells to modulate the metabolic process in muscle and other tissues [9]. When skeletal muscles undergo physiological exercise, interleukin 6 (IL-6) secreted by skeletal muscle cells increases in serum [10], influencing glucose homeostasis during exercise [11]. In addition to IL-6, IL-8, and other cytokines are also regulated by concentric muscle contraction *in vivo* and electric pulse stimulated cultured myotubes *in vitro* [12,13]. Although physiological function of IL-8 within muscle tissue is poorly understood, it acts as an angiogenic factor and a chemokine in other tissues. This evidence indicates that skeletal muscle tissue functions as an endocrine organ that secretes myokines, like adipose tissue that secretes adipokines.

Mononucleated skeletal muscle cells, *i.e.*, myoblasts, increase their numbers in mitogen-rich growth conditions, but stop proliferating to form multinucleated myotubes in low mitogen conditions. In this way, myoblasts are very sensitive to mitogen, including growth factors and possibly myokines. Mature myotubes also become much larger than mononucleated myoblasts and juvenile myotubes. This increase in myotube or myofiber size occurs even in *in vitro* culture systems, which are independent from the blood vascular system, strongly suggesting that muscle cells secrete proteins and peptides to effectively stimulate their differentiation in an autocrine fashion.

We test the hypothesis that muscle cells secrete different types of myokines at different myogenic growth and differentiation stages. Muscle cell-secreted proteins and peptides collected from four culture stages were comprehensively identified and quantitatively analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and tandem mass spectrometry (MS/MS) with iTRAQ® labeling. The iTRAQ® profile revealed the dynamics of myokines during muscle cell growth and differentiation.

2. Materials and methods

2.1. Cell culture

Animals were cared for according to the procedures outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee of the NARO Institute of Livestock and Grassland Science), and was approved by the committee.

Mouse satellite cells were prepared as previously described with modifications [14]. In brief, nerves, blood vessels, tendons, and fat tissues were removed from hindlimb muscles. Minced muscles were treated with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 40 min at 37 °C. Muscle slurries were filtered through 100 μm and 40 μm nitrex meshes (BD Bioscience). Skeletal muscle cells were cultured on collagen-coated 90 mm dishes (Asahi Techno Glass) in proliferating medium (20% fetal bovine serum [FBS] and 2.5 ng/ml of b-FGF [Life Technologies] in F10 Medium [Life Technologies]) to proliferate the number of cells. These muscle cells were subcultured for further analyses. Cells were shifted from growth medium (GM; 20% FBS in DMEM [Life Technologies]) to differentiation medium (DM; 5% horse serum [HS] in DMEM) to induce muscle differentiation. DM was changed every other day. All media were supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine (Life Technologies). FBS and HS were filtered with a 0.45 μm filter before use to remove debris.

2.2. Medium collection

Conditioned media (CM) were collected from cultured cells in growing conditions and in differentiation conditions at 30, 72, and 120 h after an induction of myogenic differentiation. At each point of collecting CM, cells were rinsed with 37 °C PBS at least 3 times to eliminate serum contamination. Then, cells were rinsed with DMEM that was kept in a cell incubator to adjust pH and temperature (37 °C) at least 1 day before use. Cells were incubated with 5 mL of DMEM per 90 mm dish for 1 h. Morphological changes of cultured cells were not detected during this treatment. The CM were collected, centrifuged, and filtered using 0.2 μm filters (Sartorius) to ensure removal of any debris. The CM were concentrated using spin columns with a cutoff of 3 kDa (Millipore) and stored in a −80 °C freezer. We collected 250–500 mL of conditioned media at each culture stage (G, D30h, D72h and D120h). Namely, 50–100 × 90 mm culture dishes were utilized at each culture stage. Because of low yields of specimens, we performed a single experiment of iTRAQ labeling and MS analysis. Protein concentration was quantified using the Bradford protein assay (Bio-Rad). Equivalent amounts of secreted proteins in CM were subjected to SDS–PAGE and a gel was stained with SYPRO® Ruby (Bio-Rad). The SYPRO® Ruby fluorescent signals were scanned with the Ettan DIGE imaging system (GE Healthcare).

2.3. Immunoblot and antibodies

Cells were harvested with cell scrapers in the presence of homogenizing buffer (0.15 M CsCl, 10 mM Tris/Cl [pH 7.5], 0.1 mM EDTA-Cs [pH 8.0], 1 mM dithiothreitol, 28 μM E64 [Peptide Inst.], 1.5 mM aprotinin [Sigma], 0.1 mM leupeptin [Peptide Inst.], 0.7 mM calpastatin [Takara], 40 μM bestatin [Sigma], and 2 mM phenylmethylsulfonyl fluoride [Sigma]). Whole cell lysates were separated by SDS–PAGE and transferred to Immobilon-P transfer membranes (Millipore) as previously described [15]. After blocking with 0.5% nonfat skim milk in PBS, membranes were incubated with mouse anti-embryonic myosin heavy chain antibody (1:250 dilution; clone#F1.652 developed by Dr. H.M. Blau and obtained from the Developmental Studies Hybridoma Bank; [16]). Subsequently, membranes were incubated with peroxidase-conjugated secondary antibodies (1:10 dilution; Nichirei), and the reacted bands were visualized using a POD immunostaining kit (Wako Pure Chemical Industries).

2.4. MS sample preparation and MS analysis

Twenty micrograms of CM proteins from each time point were dissolved in 20 μL of 0.5 M triethylammonium bicarbonate containing 0.1% Rapigest (Waters). The iTRAQ® labeling method was previously described [15,17]. In brief, each specimen was reduced by TCEP, alkylated by MMTS, and digested by trypsin (Promega) prior to labeling with iTRAQ® 4-plex reagent (ABSciex) according to the manufacturer's

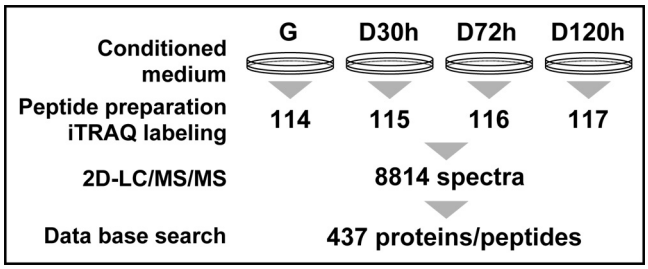

Fig. 1 – A scheme of the experimental design. An experimental overview is shown. Conditioned media (CM) were collected from growth phase (G), and 30 h (D30h), 72 h (D72h), and 120 h (D120h) after muscle differentiation. Concentrated CM were labeled with iTRAQ® following trypsin digestion. Two-dimensional peptide fractionation was performed and each fraction was analyzed with a mass spectrometer. A total of 437 proteins with a Mascot score greater than 45 were identified and quantified from 8814 spectra by a Mascot search engine.

instructions. Samples prepared from growth conditions (G), 30 h DM (D30h), 72 h DM (D72h), and 120 h DM (D120h) were labeled with iTRAQ® 114, 115, 116, and 117, respectively (Fig. 1). iTRAQ® labeled specimens were combined and then multi-step peptide fractionation was performed with a strong cation exchange column (ABSciex). Eight total fractions were eluted with 20, 50, 75, 100, 150, 200, 350, and 500 mM KCl. Every specimen was desalted using a Sep-Pak cartridge (Waters) and concentrated with a vacuum centrifugal concentrator.

Peptide fractionation was further performed on a nano-flow LC system equipped with a MALDI-plate spotter (Chorus). Peptides trapped on a trap column (0.3 mm × 5 mm, 5 μm, 12 nm, L-column ODS; CERI, Tokyo, Japan) were separated onto an analytical column (0.075 mm × 50 mm) packed with Magic C18AQ resin (3 μm, 10 nm particles; Michrom Bioresources); they were eluted with acetonitrile using a gradient of 5–40% solvent B in solvent A for 60 min (solvent A: 1.2% acetonitrile, 0.1% TFA; solvent B: 81.2% acetonitrile, 0.1% TFA), 40–100% solvent B in solvent A for 20 min, and 100% solvent B for 10 min. The column effluent was mixed with the MALDI matrix solution (2 mg/mL α-cyano-4-hydroxycinnamic acid [Shimadzu] dissolved in 70% acetonitrile containing 0.1% TFA) at a rate of 1.8 μL/min at the outlet, and spotted directly onto the ABI 4800 MALDI-plates (ABSciex).

Spotted fractions corresponding to eight gradient segments in SCX chromatography (192 × 8 = 1536 spots) were analyzed with a mass spectrometer (ABSciex 4800 plus MALDI TOF/TOF Analyzer). Proteins were identified using an in-house Mascot search engine (ver. 2.4.0, Matrix Science) and the NCBI database (20,571,509 sequences; 7,061,663,751 residues; Jan. 10, 2013). Mascot search criteria were as follows: Enzyme, "none"; Fixed modifications, "iTRAQ4plex (N-term)" and "iTRAQ4plex (K)"; Variable modifications, "Deamidated (NQ)", "Oxidation (M)", "MMTS (C)" and "iTRAQ4plex (Y)"; Peptide mass tolerance, "±100 ppm"; Fragment mass tolerance, "±0.25 Da". The Mascot search engine detected 437 proteins with a Mascot score above 45 from 8814 MS/MS spectra. Each Mascot score of identified protein in Fig. 4 was greater than 66.

3. Results and discussion

3.1. *Proteins secreted during muscle growth and differentiation*

We hypothesized that muscle cells secrete different molecules at different myogenic developmental stages since proliferating myoblasts and multinucleated myotubes have distinct properties. We comprehensively detected and quantitatively evaluated secreted proteins and peptides in cultured media using a mass spectrometer and iTRAQ® labeling (Fig. 1). We observed morphological alteration of mouse skeletal muscle cells during myogenic differentiation (Fig. 2a). Muscle cells increased in number in growth conditions (G in Fig. 2a), but myoblasts fused to form myotubes following induction of muscle differentiation. Myotubes formed consistently at 72 h after differentiation (D72h) and spontaneous contraction was often observed in myotubes at 120 h after differentiation (D120h). Next, we examined the expression of myosin heavy chain, which is one of the best markers of muscle differentiation. Immunoblot analysis using anti-embryonic myosin heavy chain confirmed higher expression levels of myosin at late stages of development (Fig. 2b). These results demonstrated that muscle cells were successfully differentiated.

3.2. *Comprehensive identification of secreted proteins and peptides by MS/MS analysis*

To avoid contamination, serum-free conditioned media were used in this experiment. These culture media were collected and qualities of each were examined by SDS–PAGE (Fig. 3a). The band patterns for the growth stage were completely different from those for the differentiation stages. Among specimens of muscle differentiation, distinct intensities of bands were observed. These results suggest that muscle cells secreted different types and amounts of proteins and peptides at growth and every differentiation stage.

To comprehensively and quantitatively evaluate what type of proteins and peptides were secreted by skeletal muscle cells during differentiation, those in cultured medium were analyzed with a mass spectrometer after labeling with iTRAQ®. A total of 437 proteins or peptides were identified from 8814 MS/MS spectra using the Mascot search engine. These proteins and peptides were categorized by their original localizations using gene ontology (Fig. 3b). Approximately 8% of identified proteins and peptides were derived from extracellular regions; 76% were of cytoplasmic origin; and 12% were membrane-associated proteins. Unknown proteins and contamination with serum proteins accounted for 1% and 3%, respectively.

Based on gene ontology classification, less than 10% of proteins were defined as secreted proteins. Therefore our data were further evaluated using web-based bioinformatics tools. The SignalP (http://www.cbs.dtu.dk/services/SignalP/) [18] predicted that about 10% of identified proteins contained typical signal peptide sequence (Supplemental Fig. S1). The SecretomeP (http://www.cbs.dtu.dk/services/SecretomeP/) [19] predicted that about 43% of identified proteins were classified as non-classical secreted proteins that can be exported without a classical N-terminal signal peptide (Supplemental

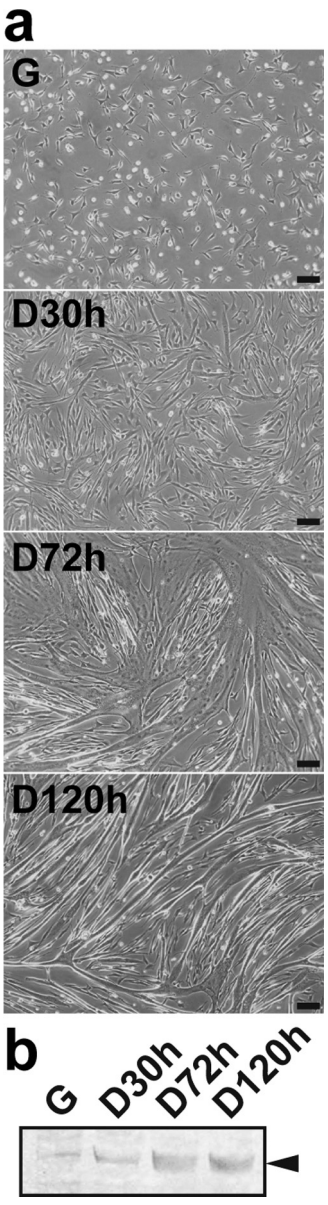

Fig. 2 – Morphological and qualitative alterations of mouse cultured skeletal muscle cells during myogenic differentiation. (a) Light microscopy-based images of muscle cells cultured in growth medium (G), and in differentiation medium at 30 h (D30h), 72 h (D72h), and 120 h (D120h) are shown. Scale bars represent 100 μm. (b) Immunoblot was carried out using anti-embryonic myosin heavy chain antibody. Whole cell lysates prepared from cultured muscle cells in growth medium (G), and in differentiation medium at D30h, D72h, and D120h were subjected to SDS–PAGE. Subsequently, immunoblot was carried out to detect embryonic myosin heavy chain. The black arrowhead indicates bands corresponding to embryonic myosin heavy chain.

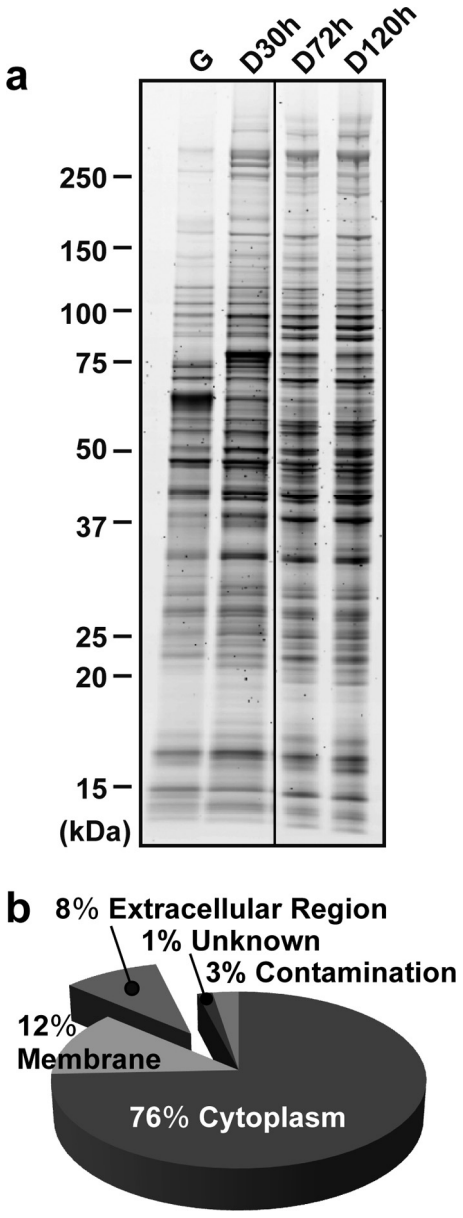

Fig. 3 – Secreted proteins and their subcellular localizations. (a) Equivalent amounts of secreted proteins (1 μg) from each sample (G, D30h, D72h, and D120h) were subjected to SDS–PAGE, and subsequently stained with SYPRO® Ruby. (b) Identified proteins and peptides are categorized by subcellular localizations, which are determined based on the ontology of the National Center for Biotechnology Information. Secreted proteins were involved in the extracellular region. Note that some proteins have multiple cellular localizations.

Fig. S1). Furthermore, proteins in exosomes that are small vesicles to shuttle between cells were verified by the ExoCarta (http://www.exocarta.org/) [20]. Approximately 65% of identified proteins were detected as exosomal proteins (Supplemental Fig. S1).

Proteins and peptides localized to the extracellular region based on gene ontology classification were further classified

Function	GI#	Gene	Name	G/G	D30h/G	D72h/G	D120h/G	SD (D30h/G)	SD (D72h/G)	SD (D120h/G)	Counted peptides
Growth factor activity	gi|148688619	*Prl2c3*	Prolactin-2C3	1.00	0.41	0.69	0.19	1.00	1.00	1.00	1
	gi|6755466	*Sema7a*	Semaphorin-7A	1.00	0.82	0.37	0.20	1.00	1.00	1.00	1
	gi|348567105	*Fstl1*	Follistatin-related protein 1	1.00	0.91	0.64	0.52	1.16	1.15	1.20	3
	gi|148701546	*Sparc*	Secreted acidic cysteine rich glycoprotein	1.00	1.56	0.84	0.58	1.03	1.11	1.15	17
	gi|6678682	*Lgals1*	Galectin-1	1.00	1.18	1.70	1.43	1.06	1.04	1.04	27
	gi|5542285	*Mif*	Macrophage migration inhibitory factor	1.00	1.42	1.89	1.00	1.09	1.01	1.04	2
	gi|148683390	*Hdgf*	Hepatoma-derived growth factor	1.00	1.46	1.52	1.00	1.04	1.24	1.23	7
	gi|9438805	*Psap*	Prosaposin	1.00	1.52	1.74	1.46	1.00	1.00	1.00	1
	gi|6754208	*Hmgb1*	High mobility group protein B1	1.00	1.55	1.78	1.15	1.00	1.00	1.00	1
	gi|148688796	*Lgals3*	Galectin-3	1.00	1.89	3.36	2.29	1.08	1.09	1.11	7
	gi|323668307	*C1qtnf3*	C1q and tumor necrosis factor related protein 3	1.00	3.24	3.11	2.84	1.22	1.12	1.35	2
	gi|437125	*Igfbp5*	Insulin-like growth factor-binding protein 5 precursor	1.00	3.56	3.06	5.32	1.14	1.11	1.08	2
ECM component	gi|351701411	*Bgn*	Biglycan	1.00	1.01	0.72	0.55	1.11	1.44	1.44	3
	gi|344245918	*Svep1*	EGF and pentraxin domain-containing protein 1	1.00	1.03	1.48	1.19	1.00	1.00	1.09	2
	gi|351702680	*Col5a1*	Collagen alpha-1(V)	1.00	1.77	1.08	1.04	1.00	1.00	1.00	1
	gi|149015982	*Fn1*	Fibronectin 1	1.00	2.41	1.82	0.84	1.31	1.31	1.29	12
	gi|47059073	*Thbs1*	Thrombospondin-1 precursor	1.00	2.47	1.93	1.30	1.00	1.00	1.00	1
	gi|7710028	*Gpc1*	Glypican-1	1.00	3.98	2.08	1.38	1.21	1.16	1.21	6
	gi|351705998	*Hspg2*	Heparan sulfate proteoglycan 2	1.00	3.64	2.64	1.67	1.00	1.00	1.00	1
	gi|37589303	*Col1a1*	Collagen alpha-1(I)	1.00	4.85	1.43	2.69	1.27	1.30	1.19	5
	gi|348578396	*Lamc2*	Laminin subunit gamma-2-like	1.00	25.67	11.65	4.91	1.03	1.01	1.19	2
Protease inhibitor	gi|148666388	*Serpinf1*	Serine (or cysteine) peptidase inhibitor, clade F, member 1	1.00	1.01	1.19	0.78	1.09	1.29	1.22	2
	gi|84794552	*Pebp1*	Phosphatidylethanolamine-binding protein 1	1.00	1.54	2.07	1.37	1.15	1.20	1.19	5
	gi|148696604	*Cst3*	Cystatin C	1.00	2.14	2.30	1.98	1.45	1.23	1.09	2
	gi|351698097	*Timp2*	Tissue inhibitor of metalloproteinase 2	1.00	2.65	2.15	1.04	1.29	1.19	1.09	2
	gi|351708313	*Pzp*	Pregnancy zone protein	1.00	15.84	10.88	3.54	1.00	1.00	1.00	1
Other; Unknown	gi|6680836	*Calr*	Calreticulin	1.00	0.84	0.83	0.35	1.08	1.12	1.19	9
	gi|54777	*P4hb*	Prolyl 4-hydroxylase, beta polypeptide	1.00	0.85	0.93	0.47	1.24	1.35	1.69	2
	gi|344248783	*Ppia*	Peptidylprolyl isomerase A	1.00	1.27	1.29	0.83	1.00	1.00	1.00	1
	gi|199821	*Ybx1*	Y box protein 1	1.00	1.49	1.48	0.91	1.10	1.12	1.13	17
	gi|354485301	*LOC100769490*	Peptidyl-prolyl cis-trans isomerase A-like	1.00	1.58	1.53	0.99	1.05	1.07	1.07	12
	gi|45597447	*Sod3*	Superoxide dismutase 3, extracellular	1.00	1.57	1.67	1.16	1.07	1.13	1.16	10
	gi|6678437	*Tpt1*	Tumor protein, translationally-controlled 1	1.00	1.50	2.31	1.33	1.07	1.14	1.24	8
	gi|348554659	*Hsp90aa1*	Heat shock protein 90, alpha (cytosolic), class A member 1	1.00	1.62	3.03	3.30	1.07	1.10	1.11	20
	gi|6671539	*Aldoa*	Aldolase A, fructose-bisphosphate	1.00	2.58	3.97	3.01	1.10	1.13	1.12	47
	gi|6681137	*Dbi*	Diazepam binding inhibitor	1.00	2.65	3.77	2.97	1.07	1.15	1.16	5

Fig. 4 – A list of secreted proteins and peptides categorized by functions. Secreted proteins and peptides from the extracellular region were further classified into four types based on their functions: growth factor activity, extracellular matrix (ECM) component, protease inhibitor, and other (including unknown). iTRAQ® signal intensities of identified proteins and peptides at each point of muscle differentiation were normalized with iTRAQ® signal intensities at growth condition. Proteins and peptides that increased more than 2-fold during muscle differentiation are shown in red rows. Proteins and peptides that decreased less than 2-fold during muscle differentiation are shown in blue rows. Standard geometric deviation (SD) is used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

into four groups by their functions. The number of identified proteins or peptides in the categories of growth factor activity, ECM component, protease inhibitor, and others were 12, 9, 5, and 10, respectively (Fig. 4). We quantified proteins and peptides normalized with iTRAQ® reporter signal intensities for the growth condition. Seventeen proteins increased more than 2-fold during muscle differentiation (red rows in Fig. 4). Four proteins or peptides decreased less than 2-fold during muscle differentiation (blue rows in Fig. 4). iTRAQ® data showed three main patterns of secreted protein or peptide profiles. The first pattern peaks for the growth condition and gradually declines during muscle differentiation. The second pattern is a peak at D30h or D72h. The last pattern is characterized by a peak at late differentiation, D120h. Most of the proteins that localized to the extracellular region are consistent with the second pattern, indicating that myogenic differentiation facilitates myokine release.

3.3. *Identified secreted proteins and peptides: growth factor activity*

Prolactin-2C3, semaphorin-7A (Sema7A), and follistatin-related protein 1 decreased in signal intensity during muscle differentiation. This suggests that these proteins may be necessary during muscle cell proliferation rather than muscle differentiation. In particular, the Sema family functions as chemorepellent against nerve cells during neuromuscular junction formation *in vivo* [21]. Some types of semaphorins are expressed not only in nerve cells, but also skeletal muscle cells [22–24]. Tatsumi et al. [23] propose that satellite cell derived-myoblasts secrete Sema3A, which is triggered by HGF and is highly expressed during the myoblast proliferating stage, to prevent the interaction between motor neuron and immature muscle cells until myotubes or myofibers mature. Our results also showed that proliferating myoblasts secreted semaphorin, and it may be a signal to avoid the interaction between muscle cells and neurons, although the precise functions of Sema7A in developing skeletal muscles are not known.

We detected seven proteins with peaks of growth factor activity at D30h or D72h in our iTRAQ® data (Fig. 4). These proteins are thought to be involved in accelerating muscle differentiation because of the changes in their secreted amounts during muscle differentiation. In fact, at least three of them, galectin-1 (Lgals1), prosaposin (Psap), and high-mobility group protein 1 (Hmgb1), promote muscle differentiation and myofiber formation [25–27]. Intriguingly, Hmgb1 is important in inflammatory response since it is released

from inflammatory cells and is thought to be a lymphokine [28]. However, our results and those of other studies [29] show that Hmgb1 is released from differentiating muscle cells. Thus, it has multiple functions, including as a myokine to enhance myofiber formation and as a lymphokine to promote inflammation.

We captured a characteristic secretion pattern for secreted protein acidic and rich in cysteine (Sparc, also known as osteonectin or BM-40) during muscle differentiation. Sparc increased approximately 1.6-fold at D30h but subsequently decreased (G:D30h:D72h:D120h = 1.00:1.56:0.84:0.58). Our data support previous reports that Sparc is expressed in both myoblasts and myotubes [30,31]. When Sparc expression is knocked down using siRNA, myogenesis was reduced [32]. These results suggest that Sparc secreted by proliferating muscle cells promotes muscle differentiation. It is also associated with adipocyte maturation, obesity, and diabetes [32–34]. Combined, Sparc secreted by muscle cells facilitates muscle development through the suppression of adipogenic differentiation in skeletal muscle tissues consisting of both muscle cells and progenitors of adipocyte [35].

C1q and tumor necrosis factor related protein 3 (C1qtnf3) is an adiponectin paralogous adipokine [36,37]. It is specifically induced during late adipocyte differentiation and plays a role in maintaining adipogenic differentiation [38]. We found that C1qtnf3 was produced by muscle cells, particularly after differentiation, and it is 3-fold more abundant during differentiation than that in the growth stage. Thus, it is possible that muscle cells secrete it during muscle differentiation to prevent the induction of adipogenic differentiation in *in vivo* skeletal muscle development.

Hepatoma-derived growth factor (Hdgf) is a novel angiogenic secreted factor [39]. We found that secretion of Hdgf from muscle cells peaked at D72h. Although its effects on muscle differentiation are not fully understood, Hdgf is secreted from myotubes or myofibers, possibly to promote angiogenesis in skeletal muscle tissues *in vivo*. Hmgb1 also enhances vascularization [29], whereas Sparc suppresses angiogenesis [40]. These functions are reflected in their secretion levels during muscle differentiation, because Hdgf and Hmgb1 peaked at D72h, but Sparc decreased during late muscle differentiation.

3.4. *Identified secreted proteins and peptides: ECM components and Protease inhibitors*

In skeletal muscle tissue, each myofiber is surrounded by the basement membrane. We detected heparan sulfate proteoglycan 2 (Hspg2; also called perlecan) and laminin gamma subunit (Lamc2), both of which are major components of the basement membrane. Secretion of both drastically increased just after induction of muscle differentiation. We also found glypican (Gpc1), which is a glycosylphosphatidylinositol (GPI)-anchored heparan sulfate proteoglycan. Secreted Gpc1 increased 4-fold at D30h compared to its level at the growth condition. This is consistent with the idea that the shedding of Gpc-1 from the satellite cell surface acts as a positive regulator of satellite cell differentiation [41]. Hspg2 not only functions as a component of the basement membrane, but also interacts with superoxide dismutase 3 (Sod3) to prevent cell damage initiated by extracellularly produced reactive oxygen species [42]. We found that secreted levels of Sod3 peaked during early muscle differentiation. These results suggest that Hspg2 and its associated proteins were released from muscle cells synchronously to efficiently construct the extracellular environment.

We also found collagen α subunits of type I and V that make up the endomysium and perimysium in skeletal muscle tissues. Type I collagen interacts with biglycan, a member of the class I family of small leucine-rich proteoglycans, to organize the assembly of collagen fibrils [43,44]. We detected secreted biglycan at the proliferating and early differentiation stages of muscle cell development, consistent with previous reports that mRNA expression of biglycan is initially high and then decreases during skeletal muscle differentiation and maturation [45]. Most other ECM components detected in this study were highly secreted following the induction of muscle differentiation, indicating that muscle cells produce extracellular components to build up ECM architecture along with myotube and myofiber formation.

Sushi, von Willebrand factor type A, EGF, and pentraxin domain containing 1 (Svep1) were found from the early phases of myogenic differentiation through myotube formation. Our quantitative iTRAQ data (G:D30h:D72h:D120h = 1.00:1.03:1.48:1.19) were in agreement with data from a previous study that shows a decline of Svep1 expression in mature myotubes or myofibers [46]. We also detected an increase in Svep1 at D72h, when myogenic cells fuse to form myotubes. Svep1 is thought to be involved in muscle cell fusion through its interaction with α9β1 integrin and Adam 12, because Svep1 is a ligand for α9β1 integrin [47], which interacts with Adam12 during muscle differentiation [48].

Tissue inhibitor of metalloproteinase 2 (Timp2) was also observed, and increased considerably during early muscle differentiation. While matrix metalloproteinase (MMP) is involved in the degradation of the extracellular matrix, Timp2 inhibits the breakdown of ECM structures, which function as scaffolds for myotubes or myofibers. In other words, muscle cells facilitate the production of ECM components to assemble ECM structures by secreting collagens and proteoglycans, and protect ECM components by inhibition of MMP. Furthermore, Timp2 may also affect muscle cell motility, promoting the formation of myotubes or myofibers [49].

Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium-derived factor), member 1 (Serpinf1; also called pigment epithelium-derived factor [PEDF]) is categorized as a protease inhibitor but does not display protease inhibitory activity. It negatively regulates angiogenesis [50]. Our iTRAQ® data showed that Serpinf1 decreases at late differentiation. This pattern of secretion is identical to Sparc, which functions in anti-neovascularization.

3.5. *Positive functions of myofibril components in muscle cell growth*

Unexpectedly, approximately 76% of the identified proteins were derived from cytoplasm by gene ontology classification (Fig. 3). Other groups have also reported cytoplasmic components in secreted fractions by MS/MS analyses [24,51,52]. One of the reasons that high percentage of cytoplasmic proteins were detected might be caused by cell damages including

Table 1 – Relative quantification of immunoglobulin domain containing myofibrillar proteins during muscle differentiation.

GI#	Gene	Name	iTRAQ signal ratio				Counted peptides
			G/G	D30h/G	D72h/G	D120h/G	
77812697	*Ttn*	Titin isoform N2-A	1.00	2.86	6.69	10.98	3
339896547	*Mybpc1*	Myosin binding protein C	1.00	1.36	2.72	5.40	1
109730993	*Mybph*	Myosin binding protein H	1.00	2.56	6.48	5.32	1

apoptotic cell death occurring after the induction of muscle differentiation [53]. The other possibility is that some cytosolic proteins classified by gene ontology may have potential to be localized to extracellular space. Since the SecretomeP and the ExoCarta predicted about 43% and about 65% of proteins that have potential to be exported to extracellular space without classical N-terminal signal peptide (Supplemental Fig. S1). These data suggest that skeletal muscle cells release proteins through not only classical signal peptide pathway but also non classical secretion pathways including exosome.

More interestingly, it has been proposed that titin, myosin binding protein C, and myomesin, which are each immunoglobulin domain containing myofibrillar proteins of skeletal muscles, activate the expression of IGF splicing variants, stimulating myoblast proliferation [54]. We also found fragments of titin, myosin binding protein C and myosin binding protein H, an isoform of myosin binding protein C, in our iTRAQ® data (Table 1). Because the immunoglobulin domain is involved in cell adhesion and binds to cell surface receptors, these fragments of myofibrils may trigger signal transduction of cell proliferation and/or differentiation. The mitogenic activity factor is also found in an extract of crushed adult muscles [55]. Thus, myofibril fractions in the extracellular region might promote muscle cell proliferation and/or differentiation, although it remains controversial how these proteins are exported to the extracellular space.

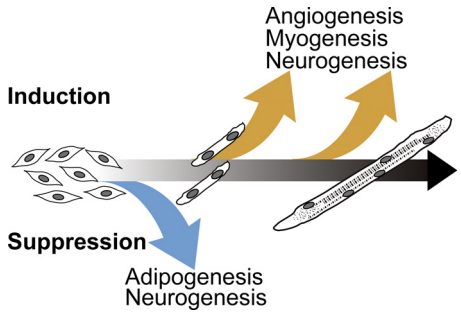

Fig. 5 – A scheme of muscle cell-secreted myokines during muscle differentiation. Skeletal muscle cells secreted different type of myokines at different muscle differentiation stages. Proliferating muscle cells secreted myokines that mainly function as neuronal repellents (Sema7a) and in anti-adipogenic differentiation (Sparc). During early differentiation, muscle cells produced myokines that facilitate myotube/myofiber formation (Sparc, Lgals1, Psap, Hmgb1 and Timp2), vascularization (Hdgf and Hmgb1), and neurogenesis (Hdgf and Psap).

4. Conclusions

We found that cultured skeletal muscle cells produced different types and amounts of myokines during muscle differentiation. During the proliferating stage, muscle cells tend to secrete myokines that suppress neurogenesis and adipogenesis. As muscle differentiation proceeds, muscle cells release myokines that specifically promote myotube formation, vascularization and neurogenesis (Fig. 5). As skeletal muscle tissue consists of not only skeletal muscle cells but also other types of cells, including nerve cells, fat cells, and endothelial cells, the complicated skeletal muscle tissue must be precisely regulated by these types of cells. Our data suggest that skeletal muscle cells take advantage of myokines as signals to organize skeletal muscle tissue even *in vitro*.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.08.001.

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