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In-depth analysis of the secretome identifies three major independent secretory pathways in differentiating human myoblasts

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ABSTRACT

Efficient muscle regeneration requires cross talk between multiple cell types via secreted signaling molecules. However, as yet there has been no comprehensive analysis of this secreted signaling network in order to understand how it regulates myogenesis in humans.

Using integrated proteomic and genomic strategies, we show that human muscle cells release not only soluble secreted proteins through conventional secretory mechanisms but also complex protein and nucleic acid cargos via membrane microvesicle shedding. The soluble secretome of muscle cells contains 253 conventionally secreted signaling proteins, including 43 previously implicated in myogenesis, while others are known to modulate various cell types thus implying a much broader role for myoblasts in muscle remodeling. We also isolated and characterized two types of secreted membrane-derived vesicles: nanovesicles harboring typical exosomal features and larger, morphologically distinct, microvesicles. While they share some common features, their distinct protein and RNA cargos suggest independent functions in myogenesis. We further demonstrate that both types of microvesicles can dock and fuse with adjacent muscle cells but also deliver functional protein cargo.

Abbreviations: CM, Conditioned medium; ECM, Extracellular matrix; MP, Microparticle; MV, Microvesicle; MVE, Multivesicular endosome.

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Thus, the intercellular signaling networks invoked during muscle differentiation and regeneration may employ conventional soluble signaling molecules acting in concert with muscle derived microvesicles delivering their cargos directly into target cells.

1. Introduction

Skeletal muscle has a low basal rate of cellular turnover in adults but retains the capacity to adapt to normal physiological demands and to regenerate in response to injury or disease [1]. Resident myogenic "stem cells" called satellite cells account for nearly all this regenerative potential [2]. Once activated by damage or exercise, these cells proliferate as myoblasts and subsequently differentiate and fuse together to repair muscle fibers. Myogenesis requires coordinated interplay between these satellite cells and other cell types present during various stages of muscle regeneration (e.g. inflammatory, endothelial and muscle-resident stromal cells) [3–5]. There is increasing evidence that skeletal muscle secretes factors that have diverse local and systemic effects, thereby regulating the fate of muscle precursor cells and participating in essential cross-talk with neighboring cells [6,7]. However, knowledge of the intercellular signaling network during human myogenesis remains largely incomplete [8–11], especially regarding the nature and type of communication between cells in a regenerating tissue where inflammation is predominant. In other tissues, cells have been shown to communicate at a distance not only via soluble secreted molecules but also through the release and uptake of microvesicles [12–14]. In the current study we used human muscle cells to model the early stages of regeneration of muscle progenitors and characterized their entire secretome in order to identify the signaling molecules that regulate the activities of the multiple cell types involved in muscle maintenance and repair.

2. Materials and methods

2.1. Human skeletal muscle culture

The satellite cells used in this study originate from primary human cell culture from 1997 [15]. Satellite cells were isolated as previously described in accordance with French legislation on ethical rules [15]. Using the explants method, upon autopsy, neonatal myoblasts were isolated from the quadriceps muscle of a 5-day-old female infant that died due to a developmental heart defect without showing signs of neuromuscular disorder. Cells were expanded in growth medium (Ham's F10, 20% foetal calf serum (FCS) and 50 μg/ml gentamycin (Invitrogen, Paisley, UK) in 5% CO_2, at 37 °C. Their mean population doubling (PDL) was determined at each passage as described previously [16]. In all experiments, myogenicity was greater than 90% as assessed by the expression of desmin localized by immunostaining. Confluent cultures were washed six times in fresh serum-free medium to a serum-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen) which triggered differentiation. These washes were sufficient to reduce contaminating albumin and other elements contained in the fetal calf serum used in proliferation conditions, which were no longer detectable in the culture medium of differentiating cells by SELDI-TOF profiling (data not shown). Cell viability in serum-free medium was >95% as assessed by trypan blue exclusion test (Sigma-Aldrich).The differentiation potential was determined by immunostaining with myosin heavy-chain (MF20) and confirmed by Western Blot analysis of the myogenic factors MyoD and Myogenin.

2.2. Collection of conditioned media and isolation of muscle-derived vesicles

Conditioned media (CM) were collected after 24 h, 48 h, and 72 h incubation in serum free medium. The CM was cleared by centrifugation (10 min at 300*g* followed by 20 min at 2000*g*) to eliminate dead cells e.g. apoptotic cell bodies and cell debris. Cleared CM were subsequently concentrated using an Amicon Ultra-4 5kD cut-off spin Column (Millipore, Watford, UK). For LC-MS/MS analysis and 2D-PAGE, concentrated CM samples from 72 h differentiated cultures were cleaned up for salt and other contaminant by precipitation using acetone/ethanol. Secreted microvesicles (MVs) were recovered from cleared supernatants by differential centrifugation as previously described with slight modifications [17,18]. Briefly, microparticles (MPs) were first sedimented by centrifugation at 20,000*g* for 70 min at 4 °C. The remaining supernatant was further ultracentrifuged at 100,000*g* for 70 min at 4 °C to pellet the exosomes. Finally, purified vesicles were washed twice in phosphate buffered saline (PBS, Invitrogen; 1.06 mM KH_2PO_4, 155.17 mM NaCl and 2.97 mM Na_2HPO_4–$7H_2O$, pH 7.4), and fixed in 2% paraformaldehyde (PAF) prior to electron microscopy or resuspended in PBS. For the density gradient experiment, isolated exosomes were floated in an iodixanol gradient as previously described [19].

2.3. Human multi-analyte profile — Luminex assays

200 μl of unconcentrated CMs from differentiating myoblasts were screened for cytokines and growth factors using Luminex multi-analyte profiling (MAP) technology [20]. The assays (Human MAP® v. 1.6) were performed on 3 independent differentiating cultures by Rules Based Medicine (Houston, TX, USA). Heat-maps for visualization of expression data were produced using FIRe v. 2.2 [21].

2.4. Gel-free nano-flow LC-MS/MS analysis

Precipitated CM and microvesicle pellets (exosomes and MPs) were dissolved in urea-buffer and 30 μg of proteins were reduced, alkylated and subsequently digested with endoproteinase Lys-C (Wako, Neuss, Germany) followed by digestion with trypsin (Promega, Southampton, UK [22]. The resulting peptide mixture was fractionated using an ICAT® Cation Exchange Buffer Pack