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A global downregulation of microRNAs occurs in human quiescent satellite cells during myogenesis

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ABSTRACT

During myogenesis, human satellite cells differentiate and form multinucleated myotubes, while a fraction of the human satellite cells enter quiescence. These quiescent satellite cells are able to activate, proliferate and contribute to muscle regeneration. Post-transcriptional regulation of myogenesis occurs through specific myogenic microRNAs, also known as myomiRs. Although many microRNAs are involved in myotube formation, little is known on the involvement of microRNAs in satellite cells entering quiescence. This current study aims to investigate microRNA involvement during differentiation of human satellite cells, specifically proliferating satellite cells entering quiescence.

For this, clonally expanded human satellite cells were differentiated for 5 days, after which myotubes and quiescent satellite cells were separated through FACS sorting. Next, a microRNA microarray comparison of proliferating satellite cells, myotubes and quiescent satellite cells was performed and verified through qRT-PCR.

We show that during human satellite cell differentiation, microRNAs are globally downregulated in quiescent satellite cells compared to proliferating satellite cells, in particular microRNA-106b, microRNA-25, microRNA-29c and microRNA-320c. Furthermore, we show that during myogenesis microRNA-1, microRNA-133, microRNA-206 and microRNA-486 are involved in myotube formation rather than satellite cells entering quiescence. Finally, we show an overall decrease in total mRNA in quiescent satellite cells, and an indication that RNaseL regulation plays a role in promoting and maintaining quiescence. Given the importance of quiescent satellite cells in skeletal muscle development and regenerative medicine, it is imperative to distinguish between myotubes and quiescent satellite cells when investigating skeletal muscle development, especially in microRNA studies, since we show that microRNAs are globally downregulated in quiescent human satellite cells.

1. Introduction

Muscle tissue has its own endogenous repair and maintenance system which is based on myogenic progenitor cells, *i.e.* satellite cells. *In vivo*, satellite cells are activated upon tissue damage, they proliferate and differentiate, fuse with existing myofibers and thereby contribute to the regeneration of damaged muscle (Buckingham and Montarras, 2008; Le and Rudnicki, 2007; Sacco et al., 2008; Ten Broek et al., 2010; Zammit and Beauchamp, 2001). During differentiation *in vitro*, part of the human satellite cells form multinucleated myotubes, and the other part enters quiescence (Fukada et al., 2007,2011). While myotubes are terminally differentiated, these quiescent satellite cells, also called reserve cells (Carnac et al., 2000), are still able to activate, proliferate and differentiate to form myotubes and contribute to muscle regeneration and repair. The ability to adapt to two distinct cell fates, *i.e.* toward myotube formation or quiescence in the same microenvironment is unique for satellite cells (Cosgrove et al., 2009; Relaix and Marcelle, 2009).

The myogenic differentiation process is transcriptionally regulated through factors such as Pax7 and the myogenic regulator factors MyoD and Myogenin. Post-transcriptional regulation further occurs through specific myogenic microRNAs, also known as myomiRs (McCarthy, 2008; Sousa-Victor et al., 2011).

MicroRNAs are small, non-coding RNAs, 20–22 nucleotides in length, involved in post-transcriptional gene regulation through inhibition of protein translation or enhancing messenger RNA degradation. MyomiRs have an important role in skeletal muscle development and disease (Cheung et al., 2012; Callis et al., 2008; Chen et al., 2009; Crist and Buckingham, 2009,2010; Guller and

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Russell, 2010; Naguibneva et al., 2007). A major effect of microRNAs in myogenesis is that they modulate proliferation as well as differentiation of satellite cells (Ambros, 2001). MicroRNA-133 promotes satellite cell proliferation through repressing the Serum Response Factor (Chen et al., 2006), but is not essential in skeletal muscle development (Deng et al., 2011). MicroRNA-1, microRNA-206 and microRNA-486 are strongly upregulated during satellite cell differentiation and subsequent muscle development. They improve muscle differentiation by inhibiting PAX7 translation such that MYOD is no longer inhibited and myotube formation progresses (Chen et al., 2010; Dey et al., 2011; Hirai et al., 2010; Koning et al., 2011). Finally, other microRNAs that improve myotube formation are microRNA-181 that targets Hox-A11 (Naguibneva et al., 2006), microRNA-24 (Sarkar et al., 2010; Sun et al., 2008) and microRNA-27 that facilitates the start of myotube formation by targeting PAX3 (Crist et al., 2009). Also microRNA-29 improves myotube formation by targeting HDAC4 (Winbanks et al., 2011), thereby preventing muscle degeneration (Wang et al., 2011). Although many microRNAs are involved in myotube formation, little is known on the involvement of microRNAs in satellite cells entering quiescence, which is of importance in skeletal muscle regeneration. Recently, in a hindlimb injury model, microRNA-489 was shown to be involved in maintaining quiescence in mouse satellite cells (Cheung et al., 2012). However, how this relates to human satellite cells is unknown. This current study aims to investigate microRNA involvement during differentiation of human satellite cells through a microRNA microarray of proliferating satellite cells, myotubes and quiescent satellite cells to elevate the knowledge of microRNAs during differentiation of human satellite cells.

2. Materials and methods

2.1. Satellite cell isolation and culture

A muscle biopsy was obtained from a healthy female donor, undergoing blepharoplasty. The age of the donor was 60 years. The study protocol was approved by the institutional medical ethics committee, and the donor gave her informed consent. Satellite cells were isolated with 0.04 mg/ml (0.16Collagenase Wünsch units/ml) Liberase Blendzyme 3 (Roche Applied Science, The Netherlands) as described previously (Koning et al., 2011). Proliferation medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen/Gibco, CA, USA), 20% Fetal Bovine Serum (FBS; Invitrogen/Gibco) and 1% penicillin/streptomycin 50 μg/ml (Sigma-Aldrich, St. Louis, USA). Differentiation medium (DM) contained DMEM, 2% FBS, 1% penicillin/streptomycin, 1% Insulin-Transferrin-Selenium-A (100×; Invitrogen) and 0.4 μg/ml dexamethason (Sigma-Aldrich). The medium was refreshed three times per week. Cells were plated at 5.0×10^3 cells/cm^2 in culture flasks precoated with 1% gelatine/PBS for 30 min. When cells reached 70% confluence, they were enzymatically harvested using accutase (Invitrogen) and passaged. Passage number (P_x) was defined as the xth sequential harvest of a subconfluent cell population. At passage 8, cells were cloned by sorting single cells in 96 wells plates using a MoFlow FACS. The fraction of human satellite cells that was able to clonally expand was $26.3 \pm 4.0\%$. A clone that uniformly expressed the satellite cell marker Pax7 and the myogenic regulator factors MyoD1 and Myogenin was selected for further experiments.

After 5 day differentiation, we sorted mononuclear cells and myotubes by first harvesting the whole cell fraction using accutase (Invitrogen) and then sieving the cell fraction through a 70 μm sieve. Next, we stained the flowthrough with 5 μg/ml Hoechst (Invitrogen) for 30 min at room temperature and finally we performed sorting by FACS to obtain a mononucleated fraction, which represents >90% of the quiescent satellite cells. Proliferating satellite cells were handled similarly, and myotubes were retrieved of the sieve filter.

2.2. Immunofluorescent staining

Cells were cultured on Thermanox® coverslips, Lab-Tek chamber slides or 96 wells plates (all NUNC Brand Products, Roskilde, Denmark) coated with 1% gelatine. At 100% confluence, cells were fixed or cultured for an additional 5 days in DM and subsequently fixed in 2% paraformaldehyde (PFA) at room temperature for 10 min. A permeabilization step was performed with 0.5% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 10 min. Non-specific binding-sites were blocked with 10% goat serum in PBS for 30 min. Cells were incubated with the primary antibody in PBS and 2% serum at room temperature for 60 min or at 4 °C overnight. The primary antibody consisted of either (1) a proliferation marker, rabbit-anti-human Ki67 (1:100; Sanbio, Uden, The Netherlands), (2) a satellite cell marker, mouse-anti-human Pax7 (1:10; Developmental Studies Hybridoma Bank (DSHB), Iowa, USA), (3) a myogenic marker, rabbit-anti-human desmin (1:100; Novus Biological, Littleton, USA), (4) a myogenic transcription factor, mouse-anti-human MyoD (1:100; Dako, Glostrup, Denmark), (5) a myogenic transcription factor, mouse-anti-human myogenin (1:100; DSHB), (6) a sarcomere component, mouse-anti-human myosin (MF20; 1:500; DSHB) and (7) mouse-anti-human RNaseL (1:100; Abcam, Cambridge, UK). After three washes with 0.05% Tween in PBS the cells were incubated with a secondary antibody-cocktail at room temperature for 30 min. The secondary antibody-cocktail consisted of FITC-conjungated goat-anti-rabbit IgG (1:100; Southern Biotech, AL, USA), Alexa Fluor®488 goat-anti-mouse IgM and Alexa Fluor® 555 goat-anti-mouse IgG_1 or IgG_{2b} (all Invitrogen; 1:300 in PBS/DAPI containing 10% normal human serum). Samples were mounted in Citifluor AP1 (Agar Scientific, Essex, UK). Examination was performed by immunofluorescence microscopy using a Leica DMRXA microscope and Leica Software (Leica Microsystems, Wetzlar, Germany), and further quantification was performed by TissueFAXS using a Zeiss AxioObserver.Z1 microscope and TissueQuest Cell Analysis Software (TissueGnostics, Vienna, Austria).

2.3. Gene transcript analysis

Total RNA was isolated from approximately 200,000 cells using the Rneasy Kit (Qiagen Inc., CA, USA), in accordance to the manufacturer's protocol. Briefly, a cell lysate was made and diluted with an equal volume of ethanol (70%). RNA was collected on an RNA binding filter by centrifugation. DNA was removed by incubation with a DNase I solution at 37 °C for 15 min. The RNA-binding filter was washed twice and subsequently the RNA was eluted with 14 μl Elution Buffer. The RNA concentration and purity were determined by spectrophotometry (NanoDrop Technologies, Wilmington, NC). For qRT-PCR analysis, total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas UAB, Lithuania). In summary, 1 μg of total RNA was diluted in a final reaction volume of 20 μl containing random hexamer primer (0.5 μg), RiboLock™ Ribonuclease Inhibitor (20 U), and 1 mM dNTP mix, and incubated at 37 °C for 1 h. The reverse transcription reaction was terminated by heating the mixture to 70 °C for 10 min, after which the samples were placed on ice. Quantitative RT-PCR analysis was performed in a final reaction volume of 10 μl, consisting of SYBR Green Supermix (Bio-Rad, Hercules, USA), 0.5 mM primer-mix (Table 1) and 5 ng cDNA. Reactions were performed at 95 °C for 15 s, 60 °C for 30 s