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Ectopic expression of *Msx2* in mammalian myotubes recapitulates aspects of amphibian muscle dedifferentiation☆

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ABSTRACT

In contrast to urodele amphibians and teleost fish, mammals lack the regenerative responses to replace large body parts. Amphibian and fish regeneration uses dedifferentiation, i.e., reversal of differentiated state, as a means to produce progenitor cells to eventually replace damaged tissues. Therefore, induced activation of dedifferentiation responses in mammalian tissues holds an immense promise for regenerative medicine. Here we demonstrate that ectopic expression of *Msx2* in cultured mouse myotubes recapitulates several aspects of amphibian muscle dedifferentiation. We found that MSX2, but not MSX1, leads to cellularization of myotubes and downregulates the expression of myotube markers, such as MHC, MRF4 and myogenin. RNA sequencing of myotubes ectopically expressing *Msx2* showed downregulation of over 500 myotube-enriched transcripts and upregulation of over 300 myoblast-enriched transcripts. MSX2 selectively downregulated expression of *Ptgs2* and *Ptger4*, two members of the prostaglandin pathway with important roles in myoblast fusion during muscle differentiation. Ectopic expression of *Msx2*, as well as *Msx1*, induced partial cell cycle re-entry of myotubes by upregulating *CyclinD1* expression but failed to initiate S-phase. Finally, MSX2-induced dedifferentiation in mouse myotubes could be recapitulated by a pharmacological treatment with trichostatin A (TSA), bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 1 (FGF1). Together, these observations indicate that MSX2 is a major driver of dedifferentiation in mammalian muscle cells.

1. Introduction

The induction of cellular plasticity has emerged as a powerful tool for regenerative medicine (Yamanaka and Blau, 2010; Nie et al., 2012; Katsuyama and Paro, 2011). Regeneration seen in a wide range of non-mammalian vertebrates, such as urodele amphibians and teleost fish provides important information about natural mechanisms of using cellular plasticity for renewing large body parts (Brockes and Kumar, 2002; Gemberling et al., 2013). A crucial question for the field of regenerative medicine is whether urodele regeneration can be recapitulated in mammalian cells.

Studies on amphibian or fish regeneration identified reversal and plasticity of the differentiated state as a major mechanism to produce stem cell-like cells during regeneration (Jopling et al., 2010; Echeverri et al., 2001; Kragl et al., 2009). This process, classically called dedifferentiation, reprograms differentiated cells at the wound site back to a progenitor stage, which can then proliferate and replace the missing tissue with appropriate patterning (Stoick-Cooper et al., 2007). Dedifferentiation has been best characterized in salamander muscle cells. It has been shown that upon signals produced in the regenerating tissue, multinucleated postmitotic differentiated myotubes can reverse their differentiated state and give rise to proliferating progenitors (Lo et al., 1993; Kumar et al., 2000; Sandoval-Guzmán et al., 2014). To date, there is not any convincing evidence demonstrating dedifferentiation occurring physiologically in mammalian muscles. Therefore, an intriguing question is whether this process could be engineered into mammalian muscle.

Mammalian muscle differentiation is governed by the expression of a series of master regulatory transcription factors, collectively called

Abbreviations: msx2, msh (muscle segment homeodomain) homeobox homolog 2; msx1, msh homeobox homolog 1; MHC, myosin heavy chain; Mrf4, muscle regulatory factor 4; Ptgs2, prostaglandin-endoperoxide synthase 2; Ptger4, prostaglandin E receptor 4; TSA, trichostatin A; BMP4, bone morphogenetic protein 4; FGF1, fibroblast growth factor 1; TBF, trichostatin–BMP4–FGF1.

☆ Data availability for RNA-seq files: NIH Gene Expression Omnibus (GEO): GSE65619 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=sbibegcmzjudhep&acc=GSE65619).

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myogenic regulatory factors (MRFs) (Bentzinger et al., 2012; Molkentin and Olson, 1996). Proliferating myoblasts start expressing MRFs, i.e. MyoD, myogenin, MRF4 and MYF5, in an orderly fashion, withdraw from cell cycle and fuse to each other to form multinucleated postmitotic myotubes (Perry and Rudnick, 2000). Differentiated myotubes express structural proteins such as MHC and exhibit muscle-specific structural properties (Bentzinger et al., 2012). Due to their multinucleated morphology and their postmitotic differentiated states, myotubes have to reverse several processes to dedifferentiate. The steps that myotubes have to go through for dedifferentiation are: 1) phenotypic dedifferentiation (loss of differentiated muscle-specific markers) 2) cellularization (fragmentation of multinucleated myotubes into mononucleated cells) and 3) cell cycle re-entry (Duckmanton et al., 2005). Previous studies in newts and mammals have identified several cell cycle regulators or transcription factors regulating muscle dedifferentiation (Echeverri and Tanaka, 2002; Tanaka et al., 1999; McGann et al., 2001; Odelberg et al., 2000; Kumar et al., 2004; Pajcini et al., 2010; Tanaka et al., 1997; Lööf et al., 2007). Among these factors, the homeodomain-containing transcription factor, MSX1, has been attributed a master regulatory role in amphibian myotube dedifferentiation (Duckmanton et al., 2005; Kumar et al., 2004). Two studies also showed that MSX1 leads to dedifferentiation of mammalian myotubes (Odelberg et al., 2000; Yang et al., 2014). However, expression of the MSX1 homolog, MSX2, during amphibian regeneration precedes MSX1 expression and MSX2 is highly expressed at the early regenerates (Echeverri and Tanaka, 2002; Carlson et al., 1998). Despite this temporal importance in animals, the potential role of MSX2 in dedifferentiation was previously not addressed in salamander or mammalian muscle cells.

We sought to test the potential role of MSX2 during muscle cell dedifferentiation by ectopically expressing it in cultured mouse myotubes. We found that ectopic expression of *Msx2* leads to dedifferentiation with partial cell cycle re-entry in postmitotic multinucleated mammalian muscle cells and this response can be recapitulated by a pharmacological treatment with trichostatin a (TSA), bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 1 (FGF1).

2. Materials and methods

2.1. Antibodies and materials

Anti-myogenin antibody was obtained from BD Pharmingen (Franklin Lakes, New Jersey, USA). Anti-MHC antibody was obtained from DSHB. Anti-CyclinD1 antibody was obtained from Abcam. Cy3-conjugated goat anti-mouse antibody was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Anti-α-tubulin, heparin, TSA and AraC were obtained from Sigma (Rowville, Victoria, Australia). BMP4 and FGF1 were obtained from R&D systems (Minneapolis, MN, USA). Selective soluble adenylyl cyclase inhibitor, KH7, was obtained from Tocris Bioscience (Bristol, UK). *Msx1* (Addgene plasmid #21024) and *Msx2* (Addgene plasmid #21025) constructs were obtained from Addgene depository (Stelnicki et al., 1997).

2.2. Mammalian cell culture

Mouse pmi28 myoblasts (Kaufmann et al., 1999) were cultured in DMEM supplemented with 20% fetal bovine serum and 1% L-glutamine and cultured at 37 °C in 5% CO_2. Myotubes were obtained by switching the confluent myoblast cultures to a medium with DMEM supplemented with 10% horse serum and 1% L-glutamine at 37 °C in 5% CO_2 for three days. To induce *Msx1*, *Msx2* or *GFP* alone, myotube cultures were supplemented with 2 μg/ml doxycycline in growth medium that was prepared with doxycycline-free fetal bovine serum (Clontech, Mountain View, CA, USA). For TSA, BMP4, FGF1 and heparin treatments, the medium was aspirated and fresh differentiation medium with or without the factors was added on the myotube cultures.

2.3. Lentivirus production and transduction

Msx1, *Msx2* and *GFP* constructs were subcloned into pLVX-TRE3G lentiviral vector (Clontech, Mountain View, CA, USA). Lentiviruses were packaged in Lenti-X 293T cells (Clontech, Mountain View, CA, USA). For the expression of doxycycline-dependent transactivator, lentiviruses containing pLVX-Tet3G vector were produced. Pmi28 myoblasts were then transduced with two lentiviral stocks simultaneously in a ratio of 1:1. Double-transduced myoblasts were selected under the pressure of Puromycin (for pLVX-TRE3G) and G418 (for pLVX-Tet3G), expanded and frozen.

2.4. Myotube isolation

3-day-old heterogeneous myotube cultures of pmi28 line were gently washed with PBS and incubated in a pre-warmed StemPro Accutase dissociation reagent (Invitrogen, Carlsbad, CA, USA) for 10 °min at 37 °C. The cell suspension was filtered with a 40 mm nylon filter mesh (BD Falcon, Franklin Lakes, New Jersey, USA). The filter was washed with a differentiation medium several times to wash off the mononucleated cells. Myotubes that are trapped on the mesh were retrieved and recultured on gelatin-coated culture dishes in a differentiation medium containing 25 mM AraC. After three days, the medium was replaced with fresh differentiation medium to get rid of AraC before the treatments were initiated.

2.5. Western blots

Treated pmi28 myotube cultures were washed with PBS and lysed in a SDS-RIPA buffer supplemented with protease inhibitors (cOmplete Proteinase Inhibitor, Roche, Basel, Switzerland) and benzonase (Novagen, Darmstadt, Germany). Lysates were cleared by a centrifugation at 13 k rpm for 10 min. Total protein was measured by BCA (Pierce, Waltham, MA, USA). Equal amounts of total protein were separated on 5%–15% gradient SDS-PAGE gels, transferred to nitrocellulose membranes and immunoblotted for anti-myogenin (1:100), anti-MHC (1:200) and anti-α-tubulin (1:5000).

2.6. Live cell imaging

Isolated transgenic pmi28 myotubes were treated with 2 μg/ml doxycycline in growth medium. Over the course of next four days, phase contrast images were acquired from live myotube cultures using a 5× phase-contrast objective, an AxioCamHRm camera (resolution of 1388 × 1040 pixels) with a Zeiss AxioObserver Z.1 and the AxioVision software. Individual pictures were saved as compressed, but lossless PNGs. Cells were observed using self-programmed software. Quantification of cleavage was done by analyzing time-course image series for each condition and manually assigning cleavage events to individual myotubes.

2.7. RNA extraction, reverse transcription and quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), DNase-treated with TURBO™ DNase (Ambion, Life Technologies, Waltham, MA, USA) and reverse-transcribed into first strand cDNA (Thermo Scientific, Waltham, MA, USA). The qRT-PCR reactions were run in LightCycler 96 (Roche Applied Science, Basel, Switzerland). Relative quantification was performed. Expression of each gene was normalized to 18S rRNA expression. The primer sequences used in qRT-PCR reactions were as follows: 5′- AGGAGTGGGCCTGCGGCTTA -3′ and 5′- AACGGCCATGCACCACCACC -3′ for mouse 18S rRNA; 5′- GAGTTCTCCAGCTCGCTCAG -3′ and 5′- CTTCAGCTTCTCCAGCTCCG-3′ for mouse *Msx1*; 5′- AAAGGCGGTGACTTGTTTTCG -3′ and 5′- CCTTGATCCTGCGCTCCTCT-3′ for mouse *Msx2*; 5′- TGAAGAGCCGAGAGGTTCAC -