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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 -

3′ and 5′- CAGGACAGTGACAAAGAACGTC -3′ for mouse *MHC*; 5′-TGAGGGTGCGGATTTCCTG -3′ and 5′- CACGTTTGCTCCTCCTTCCT -3′ for mouse *Mrf4*; 5′- CTCCTGTGCTGATGATACCG -3′ and 5′- ATTGGCAAAA-CCACACAATG -3′ for mouse Myogenin; 5′- GTGGGTCCACGGAATCTG -3′ and 5′- CACATGTCTGTGCTGGGAAG -3′ for mouse *CyclinD1*; 5′-TTGGGTCACTGGGACTCTGA -3′ and 5′- TTGGAGCCAGAGAAGAACCC-3′ for mouse *CyclinA*; 5′- GTCTTCCAGCCCATTGAACC -3′ and 5′- ACGGAAC-TAAGAGGAGCAGC -3′ for mouse *Ptgs2*; 5′- TCCTCTCTGTTGCGTGTGTC -3′ and 5′- CGTTAAGCAACAGGACATGC -3′ for mouse *Ptger4*; 5′-AACCGCAACTAGAGACCTGC -3′ and 5′- AAACCTGTTGGGGTCAAGGG -3′ for mouse *Pax7*; 5′- AAGACGGTCTCATAGCCCAG -3′ and 5′- TTCCCAG-CACCTAAGGAGTG -3′ for mouse *Fgf4*; 5′- AAGCATTAAGGCCTTGGCAC -3′ and 5′- TGAAGTCCCAAGGCCTCTAG -3′ for mouse *Fgfr4*.

2.8. Transcriptome sequencing and data analysis

RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), DNase-treated with TURBO™ DNase (Ambion, Life Technologies, Waltham, MA, USA), reverse-transcribed and sequenced using Illumina Hi-Seq. Transcripts were aligned with TopHat 2.0 (Kim et al., 2013), and differential expression analysis was performed using the edgeR package (Robinson et al., 2010) in R (R Development Core Team, 2010). A gene was considered differentially expressed if its absolute fold change was greater or equal to 1.5 and its Benjamini–Hochberg-adjusted (Benjamini and Hochberg, 1995) p-value was less than 0.01, such that the false discovery rate (FDR) was controlled at 1%. Gene Ontology (GO) analysis was performed using the DAVID database (Dennis-Jr et al., 2003).

2.9. Immunocytochemistry

Isolated pmi28 myotubes were cultured on gelatin-coated 8-well chamber slides (Nunc Lab-Tek, Waltham, MA, USA). After treatment, myotubes were washed with PBS, fixed in 2% PFA for 15 min, permeabilized with PBS-Triton 0.2% for 10 min and blocked with 1% BSA in PBS-Triton 0.2% for 10 min. Myotubes were incubated with anti-CyclinD1 antibody (1:100) and then with Cy3-conjugated goat anti-mouse IgG (1:1000) in a blocking solution for 1 h at room temperature in a humidified chamber. Cells were stained with DAPI (2 ng/ml in 1% BSA in PBS-Triton 0.2%) and then methanol fixed at −20 °C for 5 min. Fluorescent images were taken using a Nikon fluorescent microscope at 40× magnification.

2.10. EdU staining

Isolated pmi28 myotubes were cultured on gelatin-coated 8-well chamber slides (Nunc Lab-Tek, Waltham, MA, USA). After three days of doxycycline treatment in a growth medium, EdU was added into the doxycycline-containing medium for 24 h. Myotubes were then quickly washed with PBS, fixed in 2% PFA for 15 min, blocked with 3% BSA in PBS and permeabilized with 0.5% Triton for 20 min. Click-iT chemistry was performed on permeabilized myotubes according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Cells were then stained with DAPI. Fluorescent images were taken using a Nikon fluorescent microscope at 40× magnification.

2.11. Statistical analysis

The average values of biological replicates are given as the mean ± SEM. Significance testing for qRT-PCR analyses and the quantification of myotube cellularization was performed with Student's t-test. Significance was defined as $p < 0.05$ with the convention '***' for $p < 0.0001$, '**' for $p < 0.01$, and '*' for $p < 0.05$.

3. Results

3.1. MSX2 downregulates expression of myotube markers

In order to test the potency of MSX2 in dedifferentiation, we generated doxycycline-inducible cell lines expressing *Msx1* or *Msx2* along with *GFP* or *GFP* alone. Fig. 1A shows the ectopic constructs that were packaged into lentiviral vectors and transduced into the myogenic cell line, pmi28 (Kaufmann et al., 1999). pmi28 myoblasts can be cultured until confluence and differentiated into heterogeneous cultures of multinucleated myotubes and undifferentiated mononucleates. When the transgenic myoblast lines were differentiated and induced with 2 μg/ml doxycycline for three days, both *Msx1* and *Msx2* transcript levels increased by 130- and 90-fold, respectively (Fig. S1A). The induction was also confirmed at the protein level for both proteins (Fig. S1B).

One hallmark of dedifferentiation seen in newt muscle cells is the loss of muscle markers, also known as phenotypic dedifferentiation (Duckmanton et al., 2005). Therefore, we asked if MSX1 and MSX2 led to the loss of muscle differentiation markers. Myosin heavy chain (MHC) is a well-characterized late differentiation marker for muscle cells (Bentzinger et al., 2012). Fig. 1B shows that *MHC* transcript levels are decreased by 80% upon *Msx2* expression for three days in heterogeneous myotube cultures. On the other hand, *MHC* transcript levels did not change in *Msx1* and *GFP*-alone expressing cultures, distinguishing the potencies of MSX1 and MSX2 in inducing phenotypic dedifferentiation in mammalian myotubes. We next asked if MSX2 was able to downregulate other prominent myotube markers. MRF4 and myogenin are two myogenic regulatory factors that are crucial for myotube differentiation (Bentzinger et al., 2012). *Mrf4* and *myogenin* transcript levels decreased upon *Msx2* expression by 90% and 30%, respectively (Fig. 1 C–D). *Msx1* expression led to a significant decrease (by 80%) only at *Mrf4* transcript levels (Fig. 1C). The levels of both transcripts did not change in *GFP*-expressing control cultures (Fig. 1 C–D). The expression changes induced by MSX2 were also reflected at the protein levels. MHC and myogenin protein levels were decreased upon three days of *Msx2* expression in myotube cultures (Fig. S2A). In contrast, levels of both proteins did not change in control GFP myotubes (Fig. S2A). To ensure that our *Msx1* construct was functional, we tested its potential for MSX1's previously characterized function to inhibit myoblast differentiation into myotubes (Wang et al., 2011). When overexpressed in myoblasts that are switched to differentiation conditions, both *Msx1* and *Msx2* led to smaller and fewer MHC-positive myotubes compared with their uninduced controls and the GFP control. Therefore, our *Msx1* construct inhibited differentiation as reported previously (Fig. S3). Consistent with the specificity of the responses upon *Msx2* expression, *Mrf4* transcript levels showed a dose–response curve for different levels of MSX2 (Fig. S4 1A–B). We also measured *CyclinD1* transcript levels, a cell cycle regulator whose expression is upregulated by MSX1 and MSX2 (Hu et al., 2001) and saw a similar dose–response curve upon different levels of *Msx2* induction (Fig. S4C), indicating that MSX2 concentration is a key determinant for its downstream effects.

We considered the possibility that the transcript responses seen in heterogeneous cultures were in part due to remaining mononucleated cells. To control for such effects, we isolated myotubes and recultured them in the presence of the anti-metabolic drug arabinofuranosyl cytidine (AraC), which kills the remaining proliferating mononucleated cells by interfering with DNA synthesis (Fig. S2B) (Mastroyiannopoulos et al., 2012). In these isolated, highly pure myotube cultures we measured the transcript levels of *MHC* and *Mrf4* upon MSX2, MSX1 or GFP-alone induction over the course of three days. As shown in Fig. S2 C–D, *MHC* and *Mrf4* transcript levels went down after one day and continued to decrease by >90% after three days of *Msx2* expression. In parallel to what we observed in heterogeneous cultures, *Msx1* expression did not change *MHC* transcript levels and had a more modest effect on *Mrf4* levels compared with that of *Msx2* expression. No change was detected in *GFP*-expressing

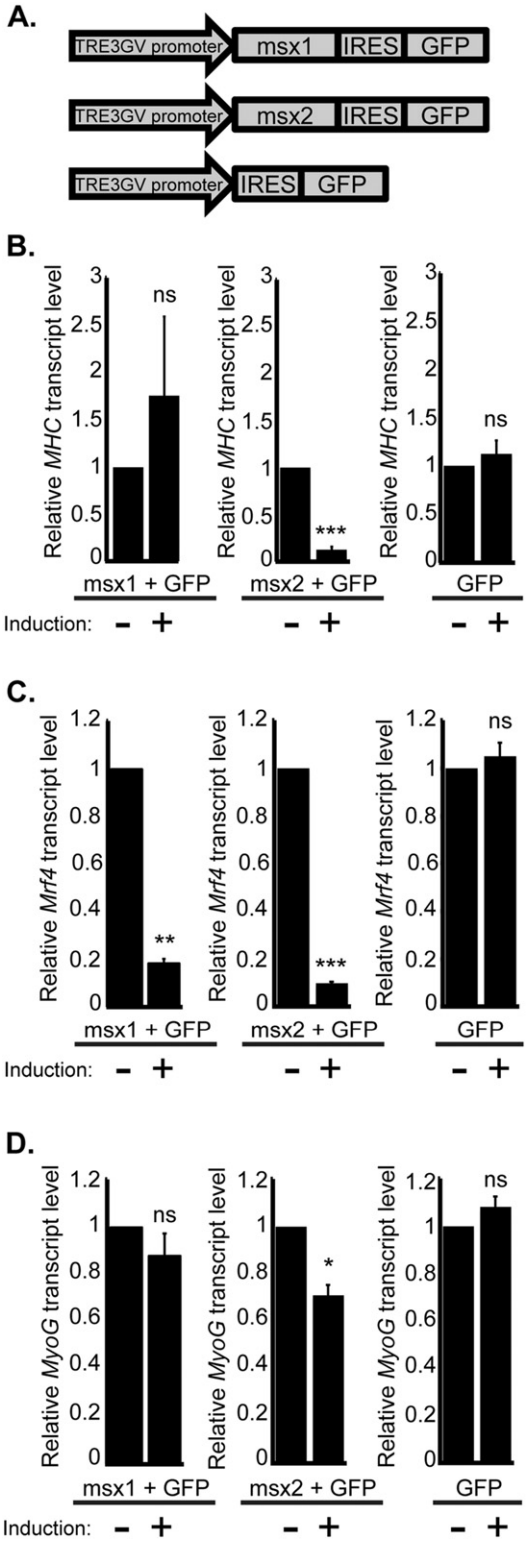

Fig. 1. Ectopic *Msx2* expression downregulates expression of muscle differentiation markers. A. Schematic representation of lentiviral constructs used for the generation of inducible myoblast lines. Doxycycline-inducible promoter is followed by either *Msx1* or *Msx2* or none. *GFP* was cloned in 3′ end of an IRES element. (B-D). *Msx2* downregulates expression of *MHC* (B), *Mrf4* (C) and *myogenin* (D) in myotubes. Heterogeneous myotube cultures were induced with doxycycline for three days. Transcript levels were measured by qRT-PCR. Note that ectopic *Msx1* expression downregulates only *Mrf4* expression. Data are representative of three independent experiments and are represented as means ± SEM (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$; ns, not significant versus untreated conditions).

control myotubes for any of these transcripts at any time point (Fig. S2 C–D). Taken together, MSX2 downregulates transcript and protein levels of myotube markers.

3.2. MSX2 reprograms myotube nuclei

To extensively characterize transcriptome-wide gene expression upon ectopic *Msx2* expression in myotubes, we performed RNA sequencing (RNA-seq) on uninduced and induced isolated myotubes carrying *Msx2* and *GFP* or *GFP* alone expression cassettes. As a reference for the undifferentiated state, we also sequenced the transcriptomes of uninduced myoblasts carrying these two transgenic constructs. To minimize the potential cell-to-cell variation in response to long-term *Msx2* expression, we focused on a relatively early time point and induced the isolated myotube cultures for one day. First, we identified 4964 upregulated and 4464 downregulated transcripts in myotubes compared with myoblasts (uninduced GFP control cells; ≥1.5-fold change; FDR corrected p-values < 0.01) (Fig. 2 A–B). Upon ectopic *Msx2* expression in myotubes, 923 transcripts were downregulated, whereas 1283 transcripts were upregulated (Fig. 2 A–B). Based on the potential role of MSX2 in dedifferentiation, we reasoned that the transcripts that are normally upregulated in myotubes in comparison with myoblasts should be downregulated upon *Msx2* expression. In accord with this idea, 575 myotube-enriched transcripts were downregulated after one day of *Msx2* expression (Fig. 2A). Similarly, 331 myoblast-enriched transcripts were upregulated upon *Msx2* expression (Fig. 2B). A heat map generated for these latter 906 genes in total shows that there are clear differences between expression profiles of myoblasts and myotubes independent of their transgenic background. Induction of *Msx2* expression in myotubes reverses myotube expression profile towards the myoblast profile (Fig. 2C). A principal component analysis (PCA) done for the same transcript set across samples also confirmed this result (Fig. S5 A–B). It demonstrated a clear separation between myoblast and myotube samples and showed that the expression profiles of *Msx2*-expressing myotubes are more similar to those of the myoblast samples. We then performed gene ontology (GO) enrichment analysis for the transcripts regulated by *Msx2* expression in myotubes. As suggested by the phenotypic dedifferentiation induced by *Msx2* expression, downregulated transcripts were enriched in GO terms related to muscle tissue development and differentiated muscle structures (Table S1). On the other hand, upregulated transcripts were enriched in GO terms related to signaling, extracellular matrix and transcription (Table S2).

We next validated selected transcript hits from the RNA-seq analysis by qRT-PCR. As shown in Fig. 2D, transcript levels of myotube marker *Mrf4* decrease by ~80% upon MSX2 induction, whereas they remain unchanged by the expression of GFP alone. Furthermore, transcript levels of the myoblast marker *Pax7* increase by ~3.5-fold upon MSX2 induction, as opposed to no change by GFP alone. We also validated several other transcript responses, which were shown to have a role in regeneration blastema in salamanders and zebrafish or in muscle differentiation pathways (Ferretti et al., 2001; Poss et al., 2000). Two Fgf pathway members, FGF4 and FGFR4, which are upregulated in regeneration blastema in animals were also upregulated upon *Msx2* expression by 11- and 3-fold, respectively. The prostaglandin pathway has a prominent role in myoblast fusion (Horsley and Pavlath, 2003; Zalin, 1977). Prostaglandin E (PGE) has been shown to positively regulate myoblast fusion (Entwistle et al., 1986; David and Higginbotham, 1981; Bondesen et al., 2006). Two members of the prostaglandin pathway, namely *Ptgs2* and *Ptger4*, were downregulated in the RNA-seq analysis. *Ptgs2* encodes for COX2 enzyme, which is the most critical enzyme in the prostaglandin pathway (Bondesen et al., 2006). *Ptger4*, on the other hand, is a PGE receptor (Yokoyama et al., 2013). We validated the transcript decrease for *Ptger4* by qRT-PCR and showed that there was a non-significant trend towards a decrease for *Ptgs2* transcripts after one day of msx2 induction (Fig. 2 H-J). Given the role of prostaglandin pathway in myoblast fusion, we reasoned that this pathway might need to be

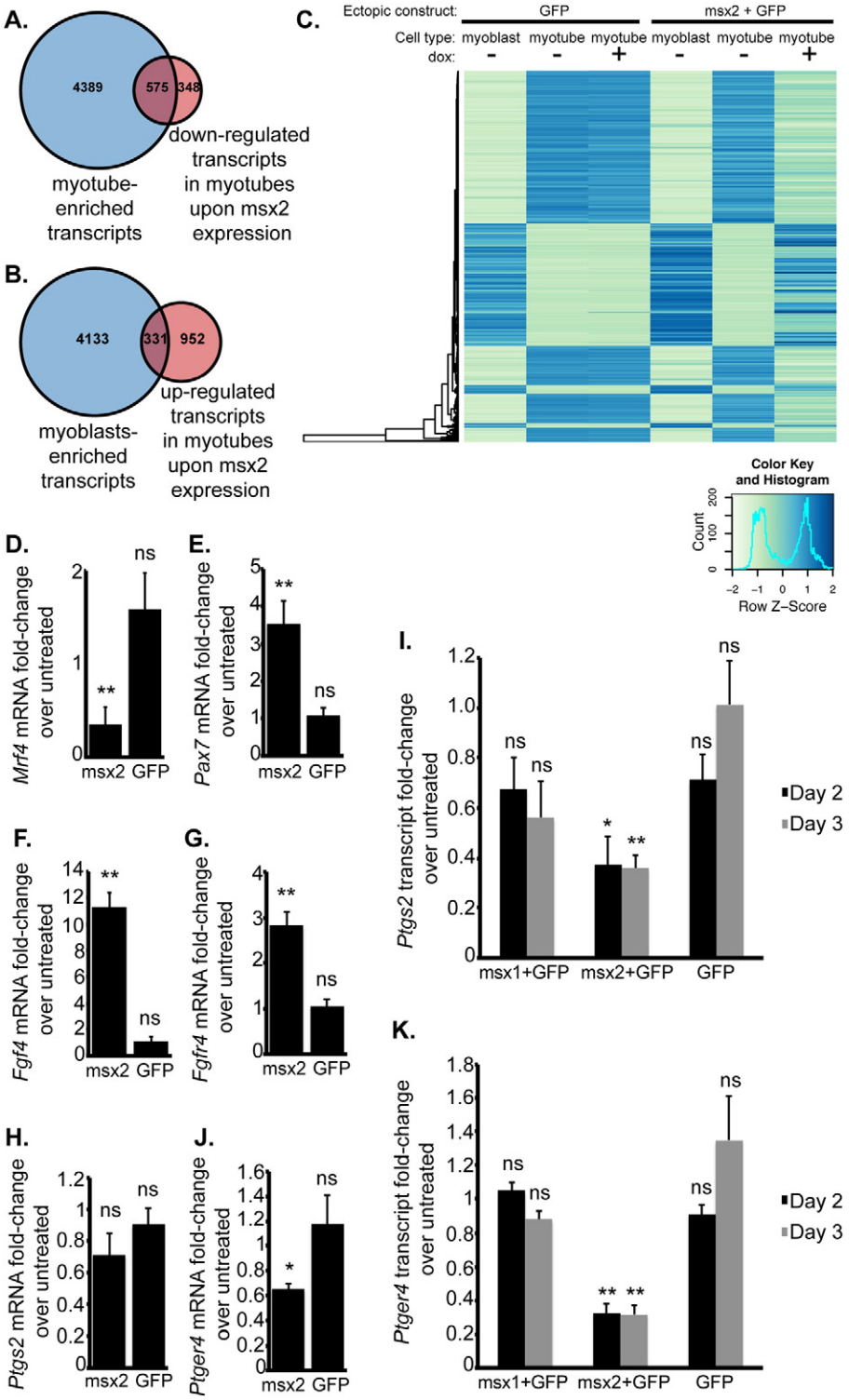

Fig. 2. Ectopic *Msx2* expression reprograms myotube nuclei. A. Myotube-enriched transcripts are downregulated upon *Msx2* expression. The number of upregulated transcripts in myotubes vs. myoblasts is shown in a Venn diagram as myotube-enriched transcripts (blue disk) and was compared with downregulated transcripts by MSX2 (red disk). Data are representative of three independent experiments. A false discovery rate (FDR)-corrected p-value of less than 0.01 and an at least 1.5-fold expression change were used as selection criteria to call genes differentially expressed between samples. B. Myoblast-enriched transcripts are upregulated upon *Msx2* expression. The number of downregulated transcripts in myotubes vs. myoblasts is shown in a Venn diagram as myoblast-enriched transcripts (blue disk) and was compared with upregulated transcripts by MSX2 (red disk). C. *Msx2*-expressing myotubes have a similar expression pattern to undifferentiated myoblasts. The displayed heatmap was generated for a total of 906 transcripts in the Venn diagram overlaps in A. and B. (D–G) Validation of RNA-seq results. Transcript levels of *Mrf4* (D), *Pax7* (E), *Fgf4* (F), *Fgfr4* (G), *Ptgs2* (H) and *Ptger4* (J) were measured by qRT-PCR upon *Msx2* expression for one day. Data are representative of three independent experiments and are represented as means ± SEM (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$; ns, not significant versus untreated conditions). (I, K) Selective downregulation of prostaglandin pathway by MSX2. *Msx2* was expressed in isolated myotube cultures up to 3 days. Transcript levels of *Ptgs2* (I) and *Ptger4* (K) were measured by qRT-PCR. Data are representative of three independent experiments and are represented as means ± SEM (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$; ns, not significant versus untreated conditions).

downregulated for cellularization, which is the reverse process of cell fusion. Therefore, we also measured transcript levels of *Ptgs2* and *Ptger4* for later time points. As shown in Fig. 2I, after two and three days of *Msx2* expression, *Ptgs2* transcript levels went down by 60–80%. Similarly, *Ptger4* transcript levels were downregulated by ~80% upon *Msx2* expression (Fig. 2K). GFP expression alone did not change transcript levels of both genes (Fig. 2 I–K). Surprisingly, *Msx1* expression in myotubes also did not lead to any decrease in *Ptgs2* and *Ptger4*, suggesting that these two transcripts were selectively downregulated by MSX2. Taken together, these results confirm the RNA-seq results and demonstrate that MSX2 reprograms the myotube transcriptome to become more similar to the myoblast transcriptome.

3.3. MSX2, but not MSX1, drives major cellularization events in mammalian myotubes through a cAMP pathway

A number of top-ranked downregulated transcripts in our RNA-seq results encode structural proteins for muscle cells (Table S3). Selective downregulation of structural proteins and the prostaglandin pathway prompted us to assess whether these gene expression differences would be reflected in a change of morphology of myotubes. Since cellularization is one of the hallmarks of muscle cell dedifferentiation during newt regeneration, we aimed to analyze potential cleavage events in isolated myotube cultures by live cell imaging (Brockes and Kumar, 2002). Fig. 3A shows that myotubes induced to ectopically

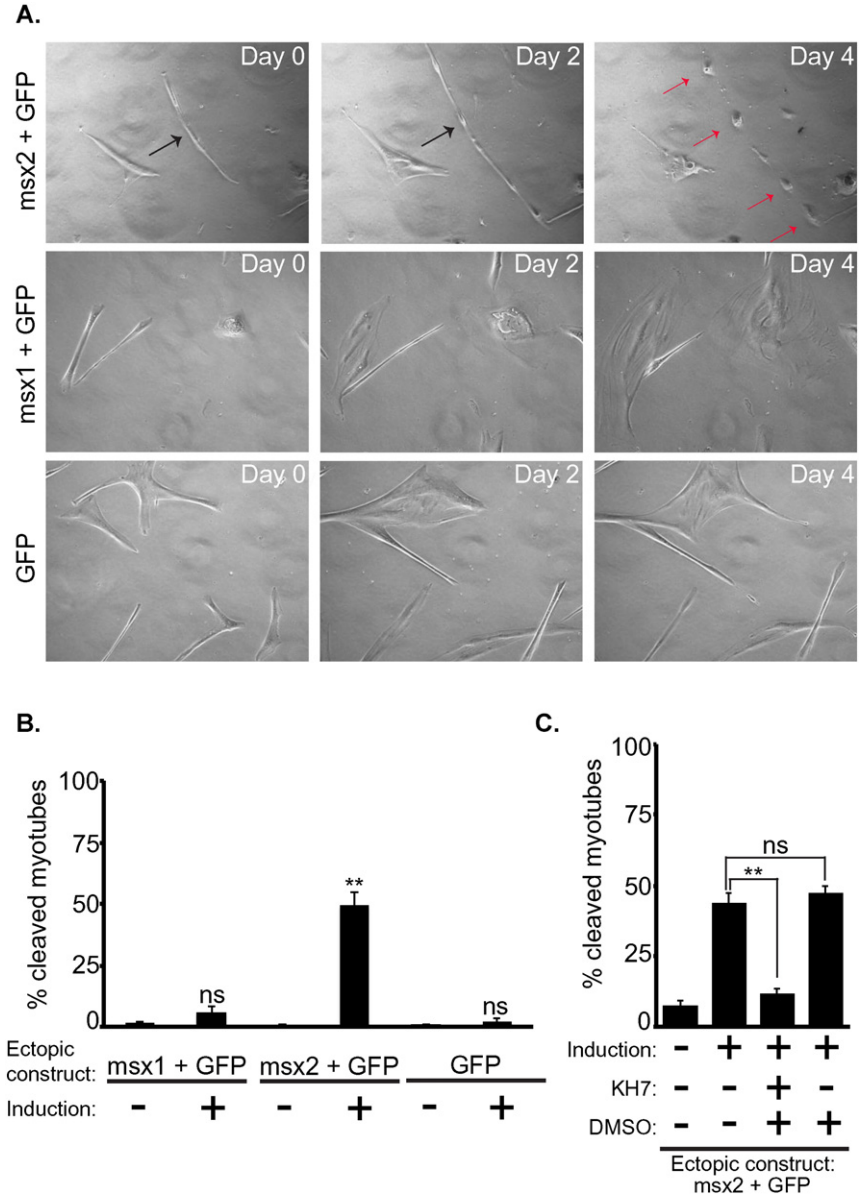

Fig. 3. Ectopic *Msx2* expression leads to significant morphological changes and cellularization in mammalian myotubes through cAMP pathway. A. *Msx2* expression leads to cleavage of myotubes. Isolated transgenic myotubes were induced with doxycycline and imaged for 4 days. Black arrow shows a myotube that undergoes cleavage. Red arrows mark the cleaved cells. Note that *Msx1* and *GFP*-alone expressing myotubes do not undergo cellularization. B. *Msx2*-induced cleavage is seen in nearly half of the myotubes. Cleaved myotubes upon *Msx1*, *Msx2* or *GFP* alone expression were quantified. Total number of myotubes analyzed for each condition is as follows: *Msx1*-uninduced (386); *Msx1*-induced (355); *Msx2*-uninduced (373); *Msx2*-induced (334); *GFP*-uninduced (350); *GFP*-induced (351). Data are representative of three independent experiments and are represented as means ± SEM (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$; ns, not significant versus untreated conditions). C. Inhibition of soluble adenylyl cyclase blocks MSX2-induced cellularization. Isolated transgenic myotubes were induced with doxycycline in the presence or absence of a selective inhibitor for soluble Adenylyl cyclase (KH7, 100 μM) and imaged for 4 days. Cleaved myotubes upon *Msx2* expression were quantified. Total number of myotubes analyzed for each condition is as follows: *Msx2*-uninduced (196); *Msx2*-induced (212); *Msx2*-induced + 100 μM KH7 (224); *Msx2*-induced + DMSO (212). Data are representative of three independent experiments and are represented as means ± SEM (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$; ns, not significant versus untreated conditions).

express *Msx2* form constrictions and cleave around days 3–4 (Movie S1). Quantification of the number of cleaved myotubes revealed that nearly 50% of *Msx2*-expressing myotubes cleave into smaller fragments (Fig. 3B). To our surprise, *Msx1*-expressing myotubes did not show significant numbers of cleavage events (Fig. 3 A–B). As expected, *GFP*-expressing control myotubes did not result in significant numbers of cleavage events. Hence, *Msx2* is a major driver of cellularization in mammalian myotubes.

Among the highest upregulated transcripts in our RNA-seq analysis we noticed several G-protein coupled receptors (GPCRs), such as the corticotropin-releasing hormone receptor 2 (*Crhr2*). GPCRs can activate cAMP signaling among others. This downstream secondary messenger signaling has various crucial roles in muscle physiology, including those during myoblast differentiation and fusion (Berdeaux and Stewart, 2012). Therefore, we reasoned that cAMP pathway could mediate MSX2-induced changes in myotubes, including cellularization and phenotypic dedifferentiation, and inhibition of this pathway would block the morphological and gene expression changes upon MSX2. To test this, we used a selective inhibitor for soluble adenylyl cyclase, KH7. When the myotubes were treated with KH7 during ectopic *Msx2* expression, myotube cellularization was blocked and returned to baseline levels (Fig. 3C). Furthermore, KH7 treatment during ectopic *Msx2* expression also reversed the increase in the transcript levels of the myoblast marker *Pax7* (Fig. S6B) and the cell cycle regulator *CyclinD1* (Fig. S6C). The decrease in the levels of *Ptger4* upon MSX2 was also reversed by KH7 (Fig. S6D). Thus, we conclude that cAMP signaling is necessary for the MSX2-induced cellularization and the phenotypic dedifferentiation of mammalian myotubes.

3.4. MSX2 leads the myotube nuclei into partial cell cycle re-entry

Besides morphological changes and downregulation of muscle-specific markers, myotube dedifferentiation also entails cell cycle re-entry of postmitotic myotubes (Duckmanton et al., 2005). Therefore, we wanted to assess the potential of *Msx2* expression in inducing cell cycle re-entry and compare it with that of *Msx1* expression. One of the main roadblocks for cell cycle re-entry in mammalian myotubes has been shown to be *CyclinD1* expression, which drives the myotubes from G0 to G1 (Latella et al., 2001). Our results in Fig. S4C and S6C showed that *Msx2* expression upregulates *CyclinD1* transcript levels. We then analyzed CyclinD1 protein and transcript levels in isolated myotube cultures after *Msx2*, *Msx1* and *GFP*-alone expression. As shown in Fig. 4A, *Msx2* expression upregulated *CyclinD1* transcript levels around 5.5-fold, as opposed to no change upon *GFP* expression alone. *Msx1* expression also had a more modest effect and increased the *CyclinD1* transcript levels by about 3-fold (Fig. 4A). We then immunostained myotubes for CyclinD1. While CyclinD1 was not detected in untreated *Msx2*-, *Msx1*- or *GFP* myotubes, *Msx2*-expressing myotubes were positive for nuclear CyclinD1 (Fig. 4B, arrows). As indicated by the transcript levels, *Msx1* expression led to somewhat less upregulation of CyclinD1 compared with levels induced by MSX2. As expected, control GFP expression did not change CyclinD1 levels.

Next, we tested whether *Msx2*- and *Msx1*-expressing myotubes proceed to S-phase and initiate DNA replication. Cyclin A is one of the well-characterized markers for S-phase entry and is not expressed in myotubes (Yoshida et al., 1998). Therefore, we first checked *Cyclin A* transcript levels upon *Msx2*, *Msx1* and *GFP*-alone expression for two and three days. Fig. S7A demonstrates that *Cyclin A* levels remained unchanged for three days upon MSX2 or MSX1 induction. We then tested DNA replication after four days of *Msx2* and *Msx1* expression in myotubes by EdU-incorporation assay. None of the myotube-associated nuclei was positive for EdU staining, indicating that myotube nuclei did not replicate their DNA (Fig. S7B, arrows). Together, we conclude that *Msx2* expression leads to a partial cell cycle re-entry, judged by the increase in CyclinD1 levels and failure to enter S-phase.

3.5. MSX2-induced myotube dedifferentiation can be recapitulated pharmacologically

Dedifferentiation protocols that will be ultimately used for therapeutic purposes would preferably be induced pharmacologically. Even though most signaling pathways are shared between salamanders and mammals, mammalian myotubes are known to be refractory to soluble factors that induce dedifferentiation in newt myotubes or within blastema (Yun et al., 2014). We reasoned that this refractory nature could be partially caused by chromatin features in mammalian cells. If the chromatin would be made more accessible, the same soluble factors could trigger similar responses in mammalian cells. To test this hypothesis, we took a candidate approach and chose molecules that would upregulate *Msx2* expression and are implicated to have a role in blastema. Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, and bone morphogenic protein 4 (BMP4) have been shown to upregulate MSX proteins (Mehra-Chaudhary et al., 2001; Wang et al., 2010; Binato et al., 2006). Fibroblast growth factor 1 (FGF1), on the other hand, has anti-apoptotic effects and induces partial dedifferentiation in adult mammalian cardiomyocytes (Engel et al., 2005). Non-transgenic heterogeneous myotube cultures of pmi28 line were treated with TSA, BMP4, FGF1 alone and their combinations for 24 h. As shown in Fig. S8A-B, *Msx1* and *Msx2* transcript levels increased by ~3- and ~17-fold, respectively, upon combinatorial treatment with TSA, BMP4 and FGF1 (TBF). Next, we checked the expression of two myotube markers upon different treatments. Transcript levels of *MHC* and *Mrf4* decreased by >90% and >80% upon TBF treatment, respectively (Fig. S8C-D). Interestingly, TBF treatment resulted in the maximum downregulation among the conditions tested. We also compared the *CyclinD1* transcript levels across different combinatorial treatments. *CyclinD1* was most upregulated by FGF1 alone (~12-fold) and BMP4-FGF1 combination (~13-fold). TBF treatment led to an ~5-fold increase in *CyclinD1* transcript levels (Fig. S8E). Western blot analysis demonstrated that TBF treatment downregulated protein levels of myotube markers, MHC and myogenin, in heterogeneous myotube cultures (Fig. S8F).

We next aimed to test the potential of TBF treatment to induce cellularization in myotubes. To test this, we isolated myotube cultures of pmi28 line and performed live imaging upon TBF treatment. Fig. 5A shows a TBF-treated multinucleated myotube that undergoes two consecutive cleavage events within the course of 4 days (black arrow), as opposed to no such morphological change in the control myotube. We also sought to confirm the TBF-induced expression changes seen in heterogeneous myotube cultures in isolated myotube cultures. As shown in Fig. 5B, TBF treatment increased the transcript levels of *Msx1* and *Msx2* in isolated myotube cultures by ~6- and ~15-fold, respectively. In accord with the response seen in heterogeneous myotube cultures, the myotube markers *MHC* and *Mrf4* were decreased by 50% in isolated myotube cultures treated with TBF for 24 h (Fig. 5C). Finally, we tested whether the CyclinD1 increase observed in heterogeneous cultures was a myotube response. Isolated and TBF-treated myotube cultures were immunostained for CyclinD1. TBF treatment increased CyclinD1 levels in myotube-associated nuclei (Fig. 5D, arrow). About 90% of the TBF-treated myotube-associated nuclei were positive for CyclinD1. Taken together, the pharmacological treatment increases the expression of *Msx* genes and recapitulates several aspects of myotube dedifferentiation induced by ectopic expression of *Msx2*.

4. Discussion

In this study we have shown that ectopic expression of a mammalian ortholog of the early amphibian blastema marker, MSX2, leads to dedifferentiation of mammalian myotubes with partial cell cycle re-entry. We propose a model in which MSX2 1) reprograms myotube nuclei by downregulating myotube-specific transcripts and upregulating progenitor myoblast-specific transcripts, 2) leads to cellularization of myotubes

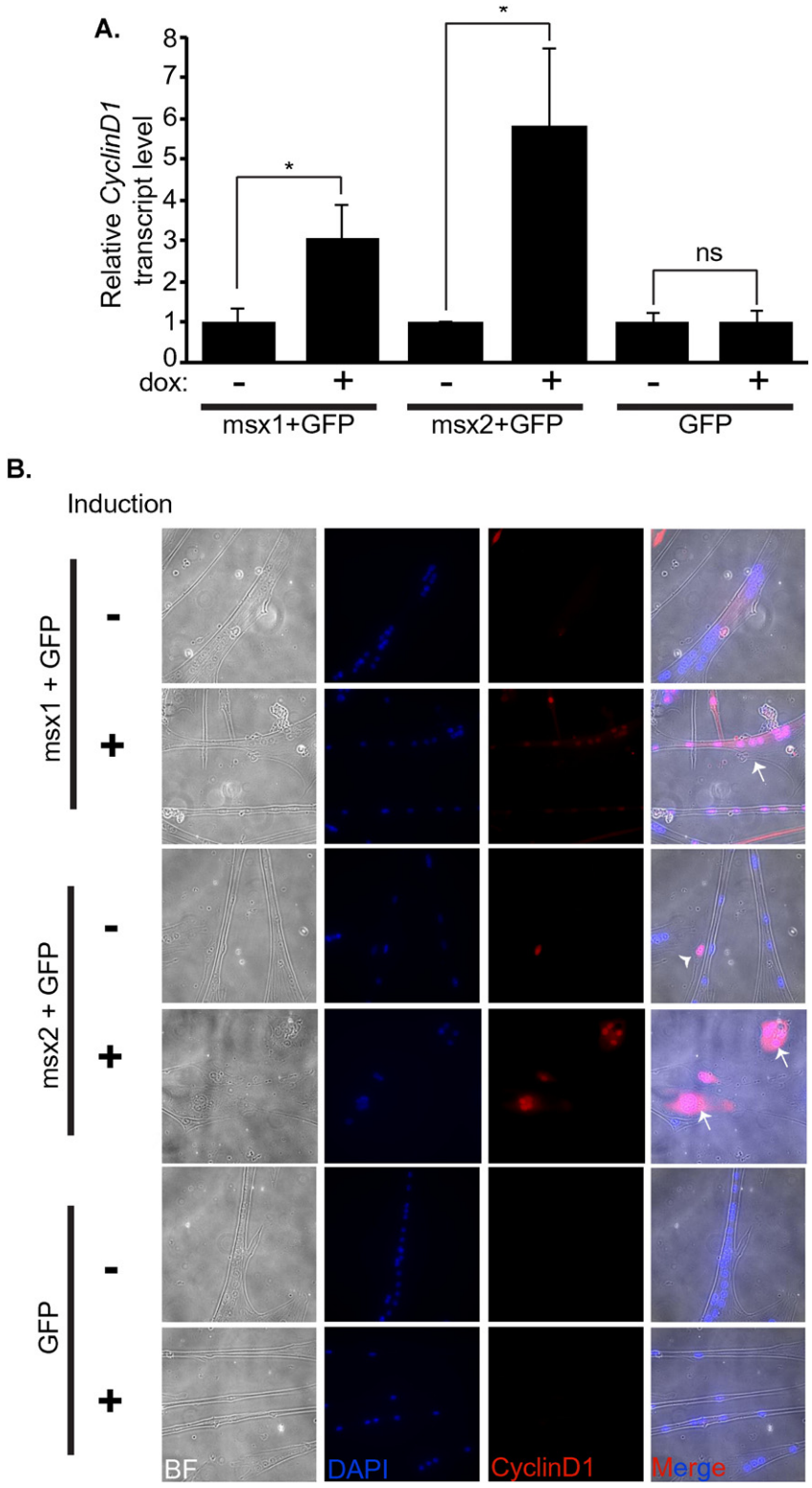

Fig. 4. Partial cell cycle re-entry by MSX2. A. *CyclinD1* transcript levels are upregulated upon *Msx2* and *Msx1* expression. Transcript levels of *CyclinD1* were measured by qRT-PCR upon *Msx1*, *Msx2* and *GFP* alone expression for one day. Data are representative of three independent experiments and are represented as means ± SEM (***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$; ns, not significant versus untreated conditions) B. *Msx2* and *Msx1* expression increases the protein levels of CyclinD1. Isolated myotubes were induced with doxycycline for three days and immunostained for CyclinD1. White arrows point to myotubes with CyclinD1-positive nuclei. Arrowhead points to a contaminating mononucleated myoblast. Data are representative of three independent experiments.

through cAMP pathway and 3) leads to partial cell-cycle re-entry by up-regulating CyclinD1 (Fig. 6).

Our results demonstrate that ectopic *Msx2* expression in mammalian myotubes leads to substantial morphological changes and eventually to the cleavage of nearly half of the myotubes into smaller cells. However, we did not observe proliferation of a mononucleated cleaved cell and colony formation. This indicates that cellularization is independent of cell cycle re-entry and that additional pathways need to be activated

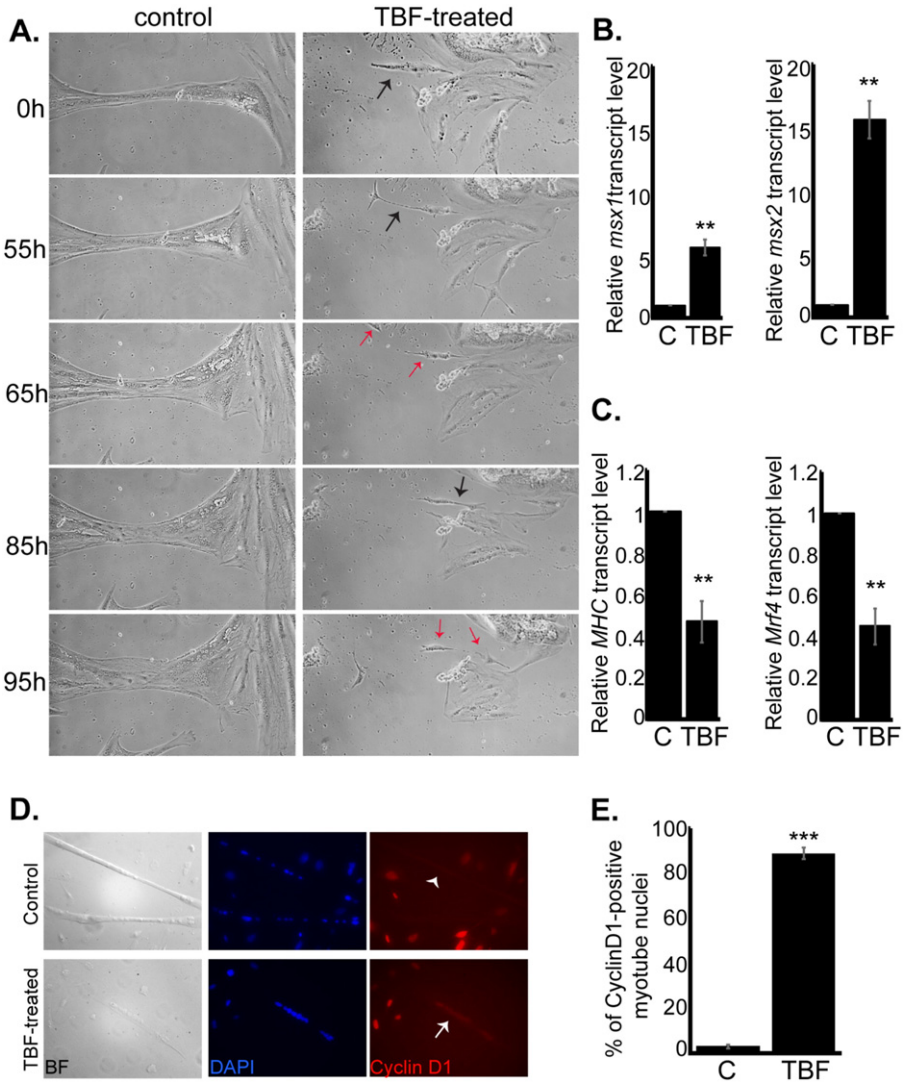

Fig. 5. A pharmacological treatment that recapitulates MSX2-induced dedifferentiation. A. TSA–BMP4–FGF1 (TBF) treatment leads to cleavage of myotubes. Isolated myotubes were treated with TBF (60 ng/ml TSA, 25 ng/ml BMP4 and 50 ng/ml FGF1 (in combination with 10 ng/ml heparin)) and imaged for 4 days. Black arrows show a myotube that undergoes two rounds of cleavage. Red arrows mark the cleaved cells. B. *Msx1* and *Msx2* transcript levels are increased upon TBF treatment. Transcript levels of *Msx1* and *Msx2* were measured by qRT-PCR in isolated myotubes treated with TBF for 24 h. Data are representative of six independent experiments and are represented as means ± SEM (***p < 0.0001; **p < 0.01; *p < 0.05; ns, not significant versus untreated conditions) C. *MHC* and *Mrf4* transcript levels are decreased upon TBF treatment. Transcript levels of *MHC* and *Mrf4* were measured by qRT-PCR in isolated myotubes treated with TBF for 24 h. Data are representative of six (*MHC*) and four (*Mrf4*) independent experiments and are represented as means ± SEM (***p < 0.0001; **p < 0.01; *p < 0.05; ns, not significant versus untreated conditions) D. TBF treatment increases CyclinD1 protein levels. Isolated myotubes were treated with TBF for 24 h and immunostained for CyclinD1. White arrow points to a myotube with CyclinD1-positive nuclei. Arrowhead points to a control myotube with CyclinD1-negative nuclei. Data are representative of three independent experiments. E. Quantification of CyclinD1 increase in myotubes. Data are from three independent experiments with a total of 414 control and 860 TBF-treated myotube nuclei analyzed.

to obtain proliferative cleavage products. In sharp contrast to MSX2, MSX1 did not lead to a significant number of cleavage events. Two previous studies that ectopically expressed *Msx1* in mammalian myotubes reported cleavage percentages of 9% and 1–5% in C2C12 myotubes and primary mouse myotubes, respectively (Odelberg et al., 2000; Yang et al., 2014). These studies also reported proliferation and colony formation of mononucleated cleaved cells. However, in 355 *Msx1*-expressing myotubes that we analyzed up until 7 days of imaging, we did not see any cleaved mononucleated cell that initiated proliferation and formed a colony. It is unclear whether the discrepancy between our study and the previous ones is due to different culture models or experimental artifacts such as contaminating mononucleated cells adjacent to myotubes, as any remaining mononucleated cell in the culture has the potential to form a colony. However, these differences underpin the necessity to further assess both qualitative and quantitative contributions of MSX1 and MSX2 in induced mammalian myotube dedifferentiation.

The finding that *MHC* and *myogenin* expression does not change upon ectopic *Msx1* expression but decreases drastically upon *Msx2* expression is another piece of evidence for the selective role of MSX2 in dedifferentiation. The previous two studies on MSX1 induction in myotubes did not characterize MHC levels upon *Msx1* expression. Our expression data show that *Mrf4* expression is significantly decreased by MSX1, suggesting that the extent of dedifferentiation-related events induced by MSX1 could be more limited than MSX2-induced changes in myotubes.

Myogenin expression was shown to be an early event during myoblast differentiation before cell cycle withdrawal and fusion (Andrés and Walsh, 1996). The early-established expression of myogenin was downregulated after other myotube markers such as MHC and MRF4 and right before cellularization, indicating that this myogenic factor serves as a gatekeeper for differentiation (Andrés and Walsh, 1996). Our results provide insights into the temporal order of events during myotube dedifferentiation and suggest that the loss of muscle markers

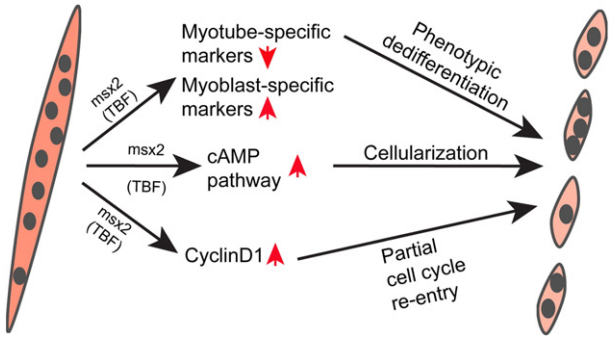

Fig. 6. Model for MSX2-induced or pharmacologically induced mammalian muscle cell dedifferentiation. Ectopic expression of *Msx2* or TBF treatment in mammalian myotubes lead to 1) downregulation of myotube-specific transcripts and an upregulation of myoblast-specific transcripts (phenotypic dedifferentiation); 2) cleavage of the myotubes into smaller cellular fragments through cAMP pathway (cellularization) and finally 3) upregulation of CyclinD1 (partial cell cycle re-entry).

is not simultaneous. MHC and MRF4 are downregulated as early as one day after MSX2 induction and their expression decreases gradually in the following days. On the other hand, we have not seen downregulation of myogenin until after three days of *Msx2* expression, suggesting that this effect is rather secondary of MSX2 transcription factor activity. The finding that myogenin downregulation precedes cellularization also suggests that dedifferentiation is not the exact reversal of differentiation, but rather has its own specific sequence of events.

Our transcriptome-wide characterization of MSX2-induced changes in myotubes demonstrates that the expression of key myotube markers is decreased by MSX2. We identified hundreds of myotube-enriched transcripts that were downregulated by MSX2. It is worthwhile to note that RNA-seq analysis was done on myotubes that expressed *Msx2* for one day. Our results showing gradual decreases in MHC and MRF4 would suggest that the transcriptome-wide changes are likely to happen gradually and more myotube markers are expected to be downregulated in the later time points. A previous study showed that BMP-induced MSX1 or MSX2 inhibits smooth muscle gene transcription (Hayashi et al., 2006). Taken together with our data in myotubes of skeletal muscle origin, this suggests that MSX2 acts on very distinct muscle types in a similar fashion and leads to loss of differentiated muscle markers. Similar activities across distinct muscle types also opens up the possibility that MSX2 can lead to dedifferentiation of cardiac muscle to a progenitor state.

Our transcriptome analysis further identified activated and inhibited signaling pathways during myotube dedifferentiation. These pathways not only include those that were implicated to have roles in salamander regeneration, such as FGF pathway (Ferretti et al., 2001), but also those that were not studied within the context of dedifferentiation, such as prostaglandin pathway. Fgfr4 transcripts were shown to be upregulated following tail amputation in newts (Ferretti et al., 2001). Similarly, FGF4 was shown to induce a regenerative response in wing bud regeneration in chick embryos (Kostakopoulou et al., 1996). Upregulation of both of these FGF pathway members upon *Msx2* expression suggests that they might also play a role during myotube dedifferentiation. On the other hand, two crucial members of prostaglandin pathway, namely the enzyme COX2 and the prostaglandin receptor PTGER4 are downregulated. Interestingly, *Msx1* expression in myotubes does not downregulate transcript levels of these prostaglandin pathway members. Myoblast fusion that leads to formation of multinucleated myotubes is regulated by prostaglandin E (PGE) (Zalin, 1977; Entwistle et al., 1986; David and Higginbotham, 1981). COX2 enzyme is responsible for PGE production and PTGER4 is a receptor for PGE. Interestingly, we found that downregulation of the PGE-synthesizing enzyme and its receptor precedes cellularization suggesting that downregulation of the prostaglandin pathway might be necessary for MSX2-driven cellularization.

The importance of the finding that the prostaglandin pathway is selectively downregulated by MSX2, but not MSX1, is 2-fold; i) this functional disparity between MSX1 and MSX2 provides a mechanistic insight into a cellularization event during muscle dedifferentiation and ii) it also demonstrates that these two homeodomain-containing homolog proteins are not completely redundant and have different functionalities. Previous studies and sequence comparisons have shown differential binding affinities of MSX1 and MSX2 homeodomains to DNA and differences in primary protein sequence at the N-terminus (Catron et al., 1996). Our results indicate that these differences lead to functional and qualitative distinction of MSX1 and MSX2. This idea is also strengthened by our observation that *MHC* transcript levels are decreased only by *Msx2* expression in myotubes, but not MSX1.

Our finding about the role of the cAMP pathway in myotube cellularization and phenotypic dedifferentiation connects MSX2-induced cytosolic signaling with the nuclear changes and opens up several questions about the downstream and parallel signaling events. Which GPCRs are activated upon MSX2-induction and are responsible for signaling to the nucleus for gene expression changes? What downstream effectors, secondary transcription factors and epigenetic modifiers mediate this signaling? How does the role of cAMP signaling during dedifferentiation compare with its role during differentiation? Previous studies have shown that intracellular cAMP levels decrease upon myoblast fusion and sustained cAMP signaling inhibits myoblast fusion and differentiation (Hu and Olson, 1988; Li et al., 1992; Tsai et al., 1997). Our results indicate that cAMP signaling is a major regulator of the downregulation of myotube-specific gene expression profile, as well as the consecutive morphological changes induced by ectopic *Msx2* expression.

Previous studies on newt myotubes undergoing dedifferentiation suggested that cell cycle re-entry was uncoupled from phenotypic dedifferentiation and cellularization. (Velloso et al., 2000). Furthermore, several reports aimed at inducing cell cycle re-entry in myotubes identified 1) CyclinD1 and CDK4 activation; 2) Rb and ARF downregulation, or 3) sustained ERK activity as necessary events (Pajcini et al., 2010; Latella et al., 2001; Yun et al., 2014). Our findings showing extensive phenotypic dedifferentiation and cellularization along with partial cell cycle re-entry suggest a similar uncoupling between the first two and the latter events for mammalian myotubes. On the other hand, the pathways leading to the events related to cell cycle re-entry are not entirely activated during *Msx2* expression, with the exception of CyclinD1 upregulation.

A recent study showed that a combination of BMPs and FGFs was sufficient to form the blastema in a newt limb regeneration model (Makanae et al., 2014). Although this study analyzed a complex heterogeneous tissue such as blastema, the results from our pharmacological treatments that recapitulate phenotypic dedifferentiation and cellularization events in isolated myotubes suggest that BMP and FGF signaling are likely to contribute to muscle dedifferentiation within blastema, as well.

Both the genetic and the pharmacological inducers that we used to engineer the amphibian dedifferentiation response into mammalian myotubes have been previously implicated in the inhibition of differentiation. Expression of *Msx* genes have been associated with multipotent progenitor cells and inhibition of differentiation of various mesenchymal progenitor cells including the muscle progenitor cells (Hu et al., 2001; Kodaka K et al., 2015; Takahashi et al., 2001). Similarly, BMP signaling stimulates muscle progenitor cell expansion and inhibits myogenic differentiation (Ono et al., 2011; Shi et al., 2011). Interestingly, *Msx2* is a downstream target gene for BMP signaling (Brugger et al., 2004). FGF signaling is also required to maintain the proliferative state of the muscle progenitor cells and blocks differentiation (Olwin and Rapraeger, 1992; Rando and Blau, 1994). These inhibitory functions of MSX2, BMPs and FGFs on differentiation and their association with the maintenance of the proliferative progenitor cells further strengthens their potential to reverse the differentiated state of myotubes when ectopically expressed or activated in those cells. Our single and

combinatorial treatments also suggest that when combined with molecules that allow for a more accessible chromatin, as in the case of TSA, mammalian myotubes exhibit an enhanced response to these signaling molecules for cell fate reversal.

5. Conclusions

The data presented here demonstrate that MSX2 induces dedifferentiation with partial cell cycle re-entry in postmitotic multinucleated mammalian muscle cells. Our pharmacological treatment recapitulates MSX2-induced dedifferentiation and exhibits potential for future therapeutic protocols aiming at the generation of muscle stem cells after limb injuries. One advantage of the skeletal muscle tissue for such clinical applications is its accessibility by intramuscular injections, which circumvent any potential toxic effects of systemic injections. In conjunction with the current protocol, future studies should target the question of cell cycle re-entry in myotubes in order to achieve proliferating cleaved cells. In summary, our study provides mechanistic insights into muscle dedifferentiation, which will also help salamander regeneration research where transcriptomic data are just starting to be produced (Looso et al., 2013).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.09.012.

Acknowledgments and author contributions

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