Dynamic Phosphorylation of the Myocyte Enhancer Factor 2Cχ1 Splice Variant Promotes Skeletal Muscle Regeneration and Hypertrophy

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ABSTRACT

The transcription factor MEF2C (Myocyte Enhancer Factor 2C) plays an established role in the early steps of myogenic differentiation. However, the involvement of MEF2C in adult myogenesis and in muscle regeneration has not yet been systematically investigated. Alternative splicing of mammalian MEF2C transcripts gives rise to two mutually exclusive protein variants: MEF2Cχ2 which exerts a positive control of myogenic differentiation, and MEF2Cχ1, in which the χ1 domain acts as trans-repressor of the MEF2C pro-differentiation activity itself. However, MEF2Cχ1 variants are persistently expressed in differentiating cultured myocytes, suggesting a role in adult myogenesis. We found that overexpression of both MEF2Cχ1/χ2 proteins in a mouse model of muscle injury promotes muscle regeneration and hypertrophy, with each isoform promoting different stages of myogenesis. Besides the ability of MEF2Cχ2 to increase differentiation, we found that overexpressed MEF2Cχ1 enhances both proliferation and differentiation of primary myoblasts, and activates the AKT/mTOR/S6K anabolic signaling pathway in newly formed myofibers. The multiple activities of MEF2Cχ1 are modulated by phosphorylation of Ser98 and Ser110, two amino acid residues located in the χ1 domain of MEF2Cχ1. These specific phosphorylations allow the interaction of MEF2Cχ1 with the peptidyl-prolyl isomerase PIN1, a regulator of MEF2C functions. Overall, in this study we established a novel regulatory mechanism in which the expression and the phosphorylation of MEF2Cχ1 are critically required to sustain the adult myogenesis. The described molecular mechanism will represent a new potential target for the development of therapeutical strategies to treat muscle-wasting diseases. Stem Cells 2016; 00:000–000

SIGNIFICANCE STATEMENT

A deep understanding of the mechanisms that control adult muscle mass is crucial for developing strategies to counteract muscle wasting-associated disorders. Our work demonstrate that MEF2C transcription factor promotes a balanced muscle growth by acting at two cellular levels: by stimulating muscle differentiation of stem cell-derived muscle precursors and by activating the protein synthesis pathway in differentiated myofibers. We show that the timely coordination of these activities is ensured by a molecular mechanism involving the cross-talk of alternative splicing and protein phosphorylation. Our findings supply evidence that modulating MEF2C function might be a valuable therapeutic strategy for muscle wasting therapies.

INTRODUCTION

Skeletal muscle regeneration and hypertrophy are key adaptive responses to both pathological and physiological stimuli. Both processes are sustained by a population of resident self-renewing muscle stem cells, referred to as Satellite Cells (SC), located under the basal lamina [1–4]. SC are quiescent in the adult steady state, when triggered by signals resulting from exercises or injuries, they become activated and give rise to a population of myogenic precursor cells (myoblasts) that proliferate, migrate and fuse with the host fibers or generate new myofibers leading to muscle growth/repair. SC self-renewal also ensures the maintenance of a stem cell pool [5, 6]. During the myogenic progression of SC, a controlled balance between proliferation, differentiation and self-renewal is required, to assure efficient...
stimulation of SC expansion and subsequent terminal differentiation and activation of the PI3K/AKT-dependent protein synthesis pathway in adult myofibres. The switch between these multiple mechanisms is associated with phosphorylation of the Ser98 and Ser110 residues that regulates its interaction with PIN1. We thus conclude that, the timely inclusion of α1 exon in Mef2c transcripts and phosphorylation of the corresponding MEF2C isoform determines its protein interactions: integration of these mechanisms leads to a coordinated modulation of MEF2C function during adult myogenesis.

Materials and Methods

Cell Lines, Primary Cultures, and Single Myofibers

C2C12, COS1 and NIH 3T3 cells were maintained in DMEM/10% FBS. To induce C2C12 cells differentiation, confluent cells were maintained in DMEM/2% Horse Serum (HS). Primary cultures of SC were prepared from adult muscles of Pax3Δ34/+ mice as described [25] and grown in SC plating medium (40% F12/40% DMEM/20% FBS, Ultroser). DMEM, F12 and FBS Gibco were provided by Life Technologies, Thermofisher, https://www.thermo.com, FBS by Gibco, HS Hyclone by GE Healthcare, http://www.gelifesciences.com, Ultroser Bioserpa by Pall Corporation, http://www.pall.com. Adult (4 weeks old) C57BL6 mice were killed by cervical dislocation, and the extensor digitorum longus (EDL) muscles isolated and digested in collagenase as described [26]. Myofibres and associated SC were isolated and cultured in suspension or in adhesion on matrigel-coated plates (BD) in SC plating medium. To promote differentiation of primary SC-derived myoblasts, after 4-5 days of culture they were kept in differentiation medium with low serum [27].

Muscle Regeneration

Mouse studies were performed in accordance with the current version of the Italian Law on the Protection of Animals and approved by the local ethics committee. Tibialis anterior (TA) muscles of C57BL6 mice (Charles River, http://www.crivel.com/) were chemically injured using cardiotoxin (CTX) and after 2, 5 or 15 days of recovery, the injured and the contralateral control muscles were collected and used for protein and RNA extraction or embedded in OCT compound, frozen in isopentane cooled with liquid nitrogen and then sectioned (cross-section of 8 μm thickness) for immunofluorescence staining. To induce protein overexpression during muscle regeneration, both TA muscles were injured and, after 48 h of recovery, lentiviruses (multiplicity of infection-MOI-25) encoding MEF2C isoforms were injected into one of the regenerating TA muscles, while the contralateral was injected with an empty lentivirus. Muscle samples were collected at day 5 and 15 postinjury. Changes in distribution of fiber size was assessed measuring the cross-sectional area (CSA) of myofibers. Counting was performed using NIH ImageJ and the distribution of muscle-fibers CSA was obtained for each size range.

Immunofluorescence and Bimolecular Fluorescence Complementation Assay

Isolated myofibers, muscle sections or plated cells were fixed with 4% paraformaldehyde, permeabilized (0.2% Triton X-100,
50 mM NH₄Cl in PBS) and then blocked for 1 hour with 2% HS, finally incubated overnight at 4°C with primary antibodies. Samples were stained with Hoechst (Sigma-Aldrich, Italy, Milan http://www.sigmaaldrich.com) and secondary antibody conjugated to a fluorochrome. After immunostaining cover-slips were mounted in Mowiol mounting medium and observed under a Zeiss Axioskop 40 fluorescence microscope equipped with an Axiocam HRC camera for image acquisition. For the bimolecular fluorescence complementation (BiFC) assay, isolated myofibers cultured in adhesion were transfected with the indicated plasmids using Lipofectamin LTX (Life Technologies), then treated as described [24].

Western Blot and Coimmunoprecipitation Assay

Western Blot assays were performed as described previously [21]. When shown, the results were quantified by densitometry using ImageJ software. CoIP assays were performed as described previously [24].

Transcription Reporter Assays

C2C12 and COS1 cells were cotransfected with pGL3(des-MEF2)3, pRSVβ-gal, and the MEF2C expression vectors, then analyzed as described [24].

Antibodies. Mouse monoclonal antibodies were directed against: PAX7 and MHC MF20 (Developmental Studies Hybridoma Bank, IA, Iowa City, Iowa, http://dshb.biology.uiowa.edu/), MYOD 5.8A (M3512 Dako, Milan, Italy, http://www.dako.com), MYOGENIN FSD (M3559, Dako), DESMIN (D33 Dako), VINCULIN (V4505 Sigma-Aldrich), DYSTROPHIN (ab 7164, Abcam, Milan, Italy, www.abcam.com), LAMININ (L9393 Sigma-Aldrich), CAVEOLIN-1 (a gift from A. Fanzani, Brescia), Ki67 (15580 Abcam), and RFP (1R10367 Life Technologies). Rabbit Monoclonal anti-MEF2C (D80C1, Cell Signaling). Anti-pSer98 MEF2C and anti-pSer110 MEF2C were developed and/or characterized in our laboratory (Supporting Information Fig. 1H) [30]. Immunoblot analysis confirmed the presence of MEF2C expression vectors during the myogenic progression of murine SC by semiquantitative PCR analysis. For this purpose, we used exon-specific or common primers, whose location is shown in Figure 1A and Supporting Information Fig. 1A. Both strategies show that the α2 exon is ubiquitously expressed in quiescent, proliferating and differentiated SC-derived myoblasts. Inclusion of the α1 exon, almost undetectable in quiescent SC, is enhanced in activated SC-derived myoblasts, both α1 and α2 exons are highly expressed in differentiating cells (Fig. 1A and Supporting Information Fig. 1B). To monitor the dynamics of α exon at the protein level, we cultured primary SC for 0, 48 and 72 hours on floating myoblasts from EDL muscles and we performed double immunolabeling for SC specific markers and MEF2C using a generic anti-MEF2C and two α1 isoform-specific antibodies developed and/or characterized in our laboratory (Supporting Information Fig. 1C-G). We show that, although all quiescent SC express MEF2C (n = 9 mice, >200 cells/mouse) (Fig. 1B day 0), only MYOD positive-dividing and MYOGENIN (MGN) positive-differentiating cells (Fig. 1C and 1D, days 2 and 3), express the α1 domain. We confirmed that 97% of SC on freshly isolated myoblasts were quiescent, CAVEOLIN-1 positive (Supporting Information Fig. 1H) [30]. Immunoblot analysis confirmed the presence of MEF2Cx1.1 protein in proliferating and differentiated C2C12 cells (Supporting Information Fig. 1I). Next, we tested the function of MEF2Cα1 in SC myogenic progression by overexpression. As Me2C transcripts have two alternative alternatively spliced regions, β exon and γ domain [31–33] (Fig. 1A, left panel), we investigated their expression in SC and found exclusive expression of the exon β.
skipped isoform, whereas both γ+ and γ- variants are equally expressed (Supporting Information Fig. 1L). Therefore, we decided to evaluate the activities of the α1 and α2 exons (24). Freshly isolated SC were transduced with a lentiviral vector carrying RFP and mouse MEF2C α1 cDNAs and their proliferation and differentiation potential were compared to those of control cells (schematized in Supporting Information Fig. 2A, >80% of RFP+ infected cells, Supporting Information Fig. 2B). We observed a 38% increase in the capacity of the cells to proliferate, upon overexpression of

Figure 1. MEF2Cα1 promotes SC proliferation and differentiation. (A): Left: structure of the coding exons of the mouse Mef2c transcript. Arrows show the primers annealing sites used to evaluate the expression of α1 and α2 exons. Right: expression levels of α exons in quiescent (Q), proliferating (P) or differentiating (D) SC. Negative controls: −RT, −RNA, PCR reaction mix. Expression vectors of the MEF2C isoforms were used as control templates. Rplp0 was used as a control of RNA quantities. Similar results were obtained from two sets of samples. (B-D): Single myofibers isolated from extensor digitorum longus muscles were cultured in floating conditions and immunostained for MEF2C, PAX7, MYOD or MYOGENIN. MEF2C was detected with a general antibody (total) (B), or two isoform-specific antibodies (MEF2Cα1* and MEF2Cα2) (C and D). Bars, 50 µm. (E, F): Primary SC were transduced with either control EV or MEF2Cα1 coding lentiviruses. 48 hours later, nuclei were stained with DAPI (E), 72 hours later cells were immunostained for MYOGENIN (F) and counted. The number of control transduced cells was taken as one. Three independent experiments with duplicate samples were carried out for each measurement. Error bars represent SEM. *, p < 0.05. Abbreviations: EV, empty vector; MEF2C, myocyte enhancer factor 2C; Rplp0, Ribosomal protein large P0; −RT, nonreverse-transcribed RNA; −RNA, reverse transcription mixture; SC, satellite cells.
Figure 2. Dynamics of Ser98/110 Phosphorylation. (A): MEF2C protein structure and positioning of Ser98/110 residues. Sequences of the mouse MEF2C α exons were aligned with ClustalW software, similar and identical residues are marked with dots and asterisks. Ser98/110 residues in α1 domain and the equivalent residues in α2 are marked in red. (B): Extracts were prepared from Mb or cells differentiated at different times (Mt 0-24 hours) and immunoblotted for MHC, MEF2C, MEF2C phosphorylated on Ser98 and Ser110 (pSer98, pSer110). VINCULIN was used as loading control. (C, D) Single myofibers were immediately fixed (Quiescence), cultured for 48 hours (Proliferation) (C) or plated on gelatin-coated dishes and immunolocalized for pSer98/pSer110, and PAX7 or MYOD or MYOGENIN proteins at different times after isolation. Bars, 50 μm (D). (E): C2C12 cells were transiently transfected with vectors encoding RFP and MEF2C wild type and mutated on Ser98/110 (2SA) or MEF2Cα2 vectors. GFP+ transfected cells were FACS-sorted, Plk2, JunB, and TnnC1 expression levels were analyzed by qPCR. Data are expressed as fold induction relative to control cells (EV). (F): Chromatin Immunoprecipitation analysis showing MEF2 recruitment to the MRE in the regulatory region of Plk2 gene in C2C12 myoblasts. Values are represented as % of Input DNA ± s.e. Statistical analysis (unpaired t test) was performed to compare the enrichment of MEF2 versus anti-FLAG control antibody. (G): Upper panel: transactivation assays on C2C12 cells transfected with MEF2Cα1 or the 2SA mutant on a MEF2-dependent reporter. Luciferase activities were normalized for transfection efficiency by using β-Galactosidase. Lower panel: Western blot analysis of the expression levels of ectopically expressed FLAG-tagged MEF2C proteins in transiently transfected C2C12 cells using an anti-FLAG antibody. Vinculin was used as loading control. *, p < 0.05; **, p < 0.01. Abbreviations: Mb, myoblasts; MEF2C, myocyte enhancer factor 2C; MHC, myosin heavy chain.
MEF2Cα1, as measured by cell number 48 hours postinfection (Fig. 1E). The ability of MEF2Cα1 to promote cell proliferation was also observed in nonmuscle cells such as NIH 3T3 mouse fibroblasts (Supporting Information Fig. 2C) and colon cancer cells [17]. We used a similar approach to study the role of MEF2Cα1 overexpression on terminal differentiation. For that purpose, 3 days post-infection cells were fixed and immunostained for MYOGENIN. SC-derived myoblasts were kept subconfluent and in growth medium, to dissect the effect of MEF2C overexpression from that of density-dependent cell cycle exit. In these conditions we observed a 25% increase of MYOGENIN positive cells compared to controls (Fig. 1F), suggesting that MEF2Cα1 up-regulation promotes differentiation in addition to the pro-proliferative effect described above.

**Phosphorylation of Ser98 and Ser110: A Regulatory Switch**

To gain insight into the molecular mechanisms responsible for switching-on alternatively the pro-proliferative and the pro-differentiative activities of MEF2Cα1, we focused our attention on two phosphoacceptor sites, Ser98 and Ser110, located in α1 domain, whose phosphorylation regulates MEF2C function in C2C12 myoblasts, allowing its association with PIN1, an inhibitor of muscle terminal differentiation (Fig. 2A) [17, 24]. As a first step, we characterized the level of phosphorylation of MEF2Cα1 during differentiation of C2C12 cells with phosphospecific antibodies, that specifically recognize MEF2C phosphorylated on Ser98 (anti-pSer98) or Ser110 (anti-pSer110) (Supporting Information Fig. 2D). We found that Ser98/110 are phosphorylated exclusively in myoblasts (Fig. 2B). Next, we performed the same analysis in SC retained in their niche on isolated myoblasts. Again we observed Ser98/110 phosphorylation in proliferating MYOD+ primary myoblasts (Fig. 2C, phosphorylation). As concern differentiation, we plated single myoblasts on Matrigel: Ser98 and Ser110 phosphorylation was detected in MYOD+ proliferating SC-derived myoblasts (Fig. 2D, phosphorylation), but not in differentiating MYOGENIN+ cells (Fig. 2D, differentiation). Overall our data indicate that MEF2Cα1 phosphorylation on Ser98/110 is restricted to proliferating myoblasts and absent in terminally differentiated cells. To test how the phosphorylation of PIN1 binding sites in MEF2Cα1 could affect proliferation and/or differentiation of SC, we investigated its transcriptional potential in promoting the expression of different target genes: two immediate early genes, polo like kinase 2 (Plk2) and JunB and troponin C1 (TnnC1), a myogenic target. We found that MEF2Cα1 induced the expression of both Plk2 and JunB endogenous genes and that this activation depends on the presence of intact Ser98/110 phosphoacceptor sites, given that equivalent levels (Supporting Information Fig. 2E) of the non-phosphoacceptable 2SA mutant had no activity (Fig. 2E). Conversely, the non-phosphoacceptable 2SA mutant stimulated the expression of TnnC1 more efficiently (32-fold) than the pro-myogenic MEF2Cα2 isoform (28-fold) (Fig. 2E). In order to investigate whether MEF2Cα1 directly regulates the transcription of Plk2 and JunB genes, we looked at putative MEF2 recognition elements (MRES) around their transcription start sites by bioinformatic approaches. We found putative MRES in the regulatory regions of both genes (Supporting Information Fig. 2F). Chromatin Immunoprecipitation (ChIP) experiments, with chromatin from proliferating myoblasts, showed a significant enrichment of MEF2 binding over the control antibody in the Plk2 amplicon containing the predicted MRE. The anti-NF-YB antibody, which is able to recognize the CCAAT-binding factor NF-Y, was used as negative control antibody (Fig. 2F). Oppositely, a significant percentage of input DNA was immunoprecipitated by the anti-NF-YB antibody, but not by the anti-MEF2, when qPCRs were performed with oligonucleotides specific for the JunB CCAAT-regulatory region (Supporting Information Fig. 2G). These results suggest a direct involvement of MEF2Cα1 in the transcription of Plk2 and an indirect role in JunB regulation, possibly through the regulation of other transcription factors. Overall our data indicate that phosphorylation of Ser98 and Ser110 inhibits the transcriptional potential of MEF2Cα1 toward muscle-specific genes, while boosting the activation, directly or indirectly, of cell-cycle related target genes. Coherently, the 2SA mutant is more transcriptionally active (two-fold) than the wild type MEF2Cα1 protein toward a myogenic luciferase reporter gene in muscle (Fig. 2G) and non-muscle cells (Supporting Information Fig. 2H). These findings indicate a critical role for phosphorylation of Ser98 and Ser110 in α1-domain-mediated transcriptional activation of proliferation—versus differentiation—target genes, suggesting that this covalent modification is the molecular switch of the bi-modal activity of MEF2C in SC.

PIN1/MEF2C Interaction in Primary Myoblasts

Next we determined the role of the peptidyl-prolyl cis-trans isomerase PIN1 in modulating MEF2C function in SC. First, we observed that Pin1 transcripts are upregulated in proliferating SC-derived myoblasts by RT-PCR (Fig. 3A) and that all MYOD-positive proliferating myoblasts express PIN1 by immunofluorescence on single myofibers (Fig. 3B). To demonstrate that the MEF2Cα1/PIN1 interaction takes place in adult myoblasts, we used the BiFC approach [34]. To this end, MEF2Cα1 and PIN1 were fused to the amino- or carboxyl-terminal fragment of YFP, respectively, and transfected into SC-derived myoblasts, the fusion proteins Jun-YN and Fos-YC were used as positive controls (Fig. 3C, panels ii, Fig. 3D, panels v). Coexpression of FLAG-tagged MEF2Cα1 and HA-tagged PIN1-YC in plated SC resulted in complementation of the YFP in the nucleus of MYOD positive proliferating SC (Fig. 3C, panels iii, Fig. 3D, panels vi). Given that adult myoblasts also express Mef2c transcripts including the α2 exon, we next investigated whether PIN1 distinguishes between the two isoforms by coimmunoprecipitation experiments in transfected COS cells. As shown in Figure 3E, we found that only MEF2Cα1 interacts with PIN1, unlike the muscle-specific MEF2Cα2 isoform, devoid of the Ser110 residue (Fig. 2A). Altogether our data indicate that the MEF2Cα1/PIN1 interaction might play a role in regulating the proliferative potential of adult myoblasts. To investigate this, equal numbers of freshly isolated adult SC were transduced with combinations of lentiviral vectors carrying PIN1 and/or MEF2Cα1 cDNAs, and their proliferation potential was assayed by evaluating the number of cells after 3 days in culture. We show that PIN1 and MEF2Cα1 synergize to promote proliferation, with an average 60% increase of cell number compared to control cells and about 15% increase versus MEF2Cα1 overexpressing cells (Fig. 3F). These data indicate that association with PIN1 increases the pro-proliferative activity of MEF2Cα1 in primary myoblasts. To reinforce these observations, we used lentiviral vectors encoding MEF2Cα1 mutated in the PIN1 binding sites located in the
α1 exon (Ser98/110) and in the C-terminus (Ser254 and Ser388) [24] (SA mutant) and we evaluated the proliferation and differentiation potential of transduced SC. We found that the SA mutant is not able to efficiently stimulate myoblasts expansion (Fig. 3G) but retains the ability to promote their differentiation, expressed as an increase of the percentage of MGN+ (50%) and TROPONIN T+ (TNT+) (60%) cells (Fig. 3G). Thus, phosphorylation of the PIN1 binding sites
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represents a switch to turn-on and turn-off alternatively the pro-proliferative or the pro-differentiative activities of MEF2C1 in cultured adult myoblasts.

Phosphorylation of the α1 Domain in Muscle Regeneration

We aimed to confirm the relevance of MEF2C1 phosphorylation in SC function in vivo during muscle regeneration. We first validated that this regulatory mechanism is active during skeletal muscle regeneration upon cardiotoxin (CTX)-induced injury. Western blot analysis showed that the expression level of the α1 domain increased in regenerating muscle at 2 days post-injury (p.i.) and, although it gradually decreased, it remained expressed throughout the course of regeneration (Fig. 4A, left and middle panels), together with a remarkable level of phosphorylation at Ser98/110 at 2 and 5 days p.i., a time frame concomitant with proliferation of activated SC (Fig. 4A, right panel). Next we investigated the cellular source of MEF2C protein by immunofluorescence analysis on cryosections of regenerating TA muscles at 5 days p.i.: MEF2C protein is expressed in 100% of centrally located nuclei of newly formed myofibers and in 30% of mononucleated interstitial cells (Fig. 4B). In summary our results indicate that the α1 domain is expressed throughout muscle regeneration and is subjected to a dynamic phosphorylation on Ser98/110, suggesting multiple roles played by MEF2C1 in vivo.

Effects of MEF2C Splice Variants Overexpression in Adult Muscle

To investigate the role of MEF2C1α1/α2 splice variants and of MEF2C1 phosphorylation in SC function in vivo, we performed a muscle regeneration assay. Due to the high functional redundancy of MEF2 proteins [7], we decided to adopt a gain of function strategy. TA muscles were subjected to a single CTX injury and 2 days later they were injected with lentiviruses encoding the MEF2Cα1 splice variants and the mutant MEF2C1 protein or the RFP cassette alone. Muscles were analyzed 5 or 15 days later (as schematized in Supporting Information Fig. 3A). Infection efficiency was >60%-70% for all vectors (Supporting Information Fig. 3B) and qRT-PCR analysis confirmed the ectopic expression of transcripts encoding RFP and MEF2C (Supporting Information Fig. 3C). Immunofluorescence analysis confirmed that MEF2C protein is expressed and phosphorylated on Ser98 in activated, DESMIN-positive SC (Supporting Information Fig. 3D). Examination of serial sections of muscles overexpressing MEF2C proteins revealed an increase in the overall size throughout the body of the muscle that is particularly pronounced with the 2SA mutant at 15 days p.i. (Fig. 5A). Evaluation of the cross-sectional areas (CSA) of regenerated muscles showed the presence of a number of hypertrophic myofibers, in muscles that overexpress all MEF2C proteins, in comparison to control injected muscles (Fig. 5B). However, the hypertrophic effect is particularly pronounced upon ectopic expression of the non-phosphorylatable 2SA mutant, already at an early stage of regeneration (5 days p.i.). The upper panels of Figures 5B show the frequency distribution of myofiber CSA in the transduced regenerating muscles. Table C shows the mode and median values inferred from the histogram and the calculated shifts of CSA versus the EV control injected muscles. The modal values at 15 days reflect the left-shift (880 μm²) with MEF2C1 wild type and the right shift (1440 μm²) with MEF2C2α and the non-phosphorylatable 2SA mutant, in comparison to control regenerating muscle, where the majority of fibers were 1280 μm² in size. Similarly, we found that the median cross-sectional area of myofibers in muscles overexpressing MEF2C1α and 2SA were respectively 1536 μm² and 1684,53 μm² versus 1348 μm² for the control muscles and 1314,81 μm² for muscles that overexpress the wild type protein. The hypertrophic effect of the 2SA mutant was already evident at the very early stages of muscle regeneration as indicated by the observation that the median CSA of myofibers was higher (660 μm²) than those of the other samples, with a shift of +202,88 μm², the shift is more evident at later stages of regeneration (+336,51 μm²). At the 5-day time point regenerating muscle that overexpress MEF2C1α2 is instead characterized by a high proportion of smaller newly regenerating myofibers, with a shift of ~80 μm² and ~97,64 μm² of the mode and median CSA, probably due to the ability of this splice variant to promote myotube formation [16].

Exclusively in the case of muscles overexpressing MEF2C1α1, at 15 days p.i., we observed the coexistence of hypertrophic fibers (>2400 μm², 9% of totally counted myofibers) with small, regenerating ones (<200 μm²) suggesting that, in addition to the hypertrophic effect, a regenerative process persists at a stage in which this process has normally ended. This observation suggest that MEF2C1α1 might stimulate SC proliferation also in vivo. To address this issue, we quantified the number of mononucleated interstitial cells expressing Ki67, a marker of proliferation, in transduced adult muscles 5 days p.i. In agreement with the in vitro data, we found that the percentage of Ki67+ nuclei was increased in MEF2C1α1 (6.5%) compared to MEF2Cα2 overexpressing (5.38%) and control muscles (4.8%), whereas ectopic expression of the 2SA mutant

Figure 3. PIN1/MEF2Cα1 interaction in primary myoblasts. (A): Expression level of Pin1 in quiescent (Q), proliferating (P) or differentiating (D) SC. Negative controls: ∼RT, ∼RNA, PCR reaction mix. Rplp0 was used as a control of RNA quantities. Similar results were obtained from two sets of samples. (B): Single myofibers were cultured for 48 hours and immunolocalized for Pin1 and MYOD. Bar, 50 μm. (C, D): Bimolecular Fluorescence Complementation (BiFC) assay. Myoblasts were transfected with FLAG-YN and HA-PIN1-YC negative controls (panels i and iv), FLAG-JUN-YN and HA-FOS-YC positive controls (panels ii and v) and FLAG-MEF2Cα1-LYN and HA-PIN1-YC (panels iii and vi). Cells were stained with anti-FLAG (red), anti-HA (blue) or anti-MYOD antibodies and DAPI. The fluorescence of the YFP (green stain) results from the bimolecular complementation assay. Scale bar, 50 μm. (E): COS-1 cells were cotransfected with HA-tagged PIN1 and FLAG-tagged MEF2C vectors. CoIP was performed with anti-FLAG antibody and immunoblotted for FLAG or HA tags. As shown in the representative immunoblot, HA-PIN1 is coimmunoprecipitated exclusively with the wild type MEF2Cα1 isoform. These findings were replicated over three independent experiments. (F): Myoblasts were transduced with the indicated combinations of lentiviruses. 48 hours later, DAPI-stained nuclei were counted. Three independent experiments were carried out for each measurement. Error bars represent SEM. Abbreviations: CoIP, coimmunoprecipitation; MEF2C, myocyte enhancer factor 2C; ∼RT, nonreverse-transcribed RNA; ∼RNA, reverse transcription mixture; SC, satellite cells TnT, troponin T.
correlates with an earlier reduction of proliferating interstitial cells (2.5%) (Fig. 5D). Overall our findings indicate that both MEF2Cα1 and MEF2Cα2 splice variants promote muscle regeneration and hypertrophy in adult muscles. Furthermore, we present evidence indicating a bimodal activity of MEF2Cα1 for which the level of phosphorylation of the PIN1 binding sites tips the balance toward its ability to promote cell proliferation, differentiation and muscle growth.

**Targets of Overexpressed MEF2C Splice Variants in Adult Muscle**

As MEF2C modulates alternatively muscle- and proliferation-related genes in a splice variant- and phospho-dependent manner in cultured myoblasts, we tested whether these regulatory mechanisms operate also in vivo. Indeed, qPCR analysis of RNA extracted from regenerated muscles (15 d.p.i.) showed that MEF2Cα1 overexpression promotes the expression of the immediate early gene PIk2 in adult muscle more efficiently than the 2SA mutant or the MEF2Cα2 splice variant, which in contrast are stronger activators of the muscle-specific gene TnnC1 (Fig. 6A). In addition, we observed that both the MEF2Cα2 isoform and the 2SA mutant protein and the controlateral TA muscles were used as control tissues. For all analysis, VINCULIN was used as control of the quantity of proteins. (B): Cryosections of regenerating TA muscle 5 d.p.i.; nuclei were stained with DAPI, MEF2C α1 is expressed during muscle regeneration and is dynamically phosphorylated on Ser98 and Ser110. (A): Left: Western blot analysis of protein lysates from murine injured (CTX) TA muscles analyzed at 2, 5, and 15 days postinjury with MEF2Cα1. The controlateral TA muscles were used as control (CTRL). Middle: densitometric analysis of the immunoblot experiment for MEF2Cα1 expression. Each time point, after normalization versus VINCULIN, was compared with the correspondent baseline level in the controlateral leg considered as 1. Right: representative Western Blot analysis of protein lysates from injured TA muscles at 2, 5, and 15 days postinjury with pSer98 and pSer110 antibodies. The controlateral TA muscles were used as control tissues. For all analysis, VINCULIN was used as control of the quantity of proteins. (B): Cryosections of regenerating TA muscle 5 d.p.i.; nuclei were stained with DAPI, MEF2C α1 is revealed by anti-MEF2C antibody and localizes in central nuclei (white arrowhead) and in mononucleated interstitial cells (red arrowhead). Scale bars are 50 μm. Abbreviations: CTX, cardiotoxin; CTRL, control tissues; MEF2C, myocyte enhancer factor 2C.

**DISCUSSION**

Our major finding is that MEF2Cα1, a MEF2C splice variant, is a strong stimulator of skeletal muscle hypertrophic growth, an effect that depends on two mechanisms, including the activation...
Figure 5. Overexpression of both MEF2Cα1 and MEF2Cα2 splice variants stimulates muscle regeneration and hypertrophy. (A): Representative anatomical sections of TA muscles transduced with lentiviral vectors encoding the indicated proteins removed from mice at 15 days after cardiotoxin injection. Bar: 50 \( \mu \)m. (B): Top: histograms showing the frequency distributions of myofiber cross-sectional areas in regenerating TA muscles transduced with EV or lentiviruses bearing the coding sequences of MEF2Cα1 and MEF2Cα2 or MEF2Cα1 mutated on Ser98 and Ser110 (2SA) 5 or 15 days p.i. MEF2Cα2- and 2SA-dependent hyperthrophy are indicated by the displacement of the mode from 1280 \( \mu \text{m}^2 \) in the control group to 1440 \( \mu \text{m}^2 \). Bottom: representative images of regenerating TA muscle sections immunostained for LAMININ (green). (C): Tables reporting Mode, Median and shifts obtained from the histograms in B and C. (D): Double immunostaining for KI67 (green) and DYSTROPHIN (red) and nuclear staining with DAPI (blue) in muscle sections from TA regenerating muscles transduced with EV or MEF2Cα1, MEF2Cα2 or the 2SA mutant at 5 days p.i. Scale bar, 50\( \mu \)m. On the right are reported the relative percentages of total KI67-positive nuclei in regenerating TA muscles. Data are mean ± SEM. Abbreviations: EV, empty lentivirus; MEF2C, myocyte enhancer factor 2C.
Figure 6. MEF2C-dependent expression of proliferation-, differentiation- and growth- associated genes is regulated by alternative inclusion of α1/α2 exons in Mef2c transcripts and by Ser98/110 phosphorylation. The mRNA and protein lysates were obtained from 15 d.p.i. regenerating TA muscles transduced with the indicated lentiviruses and analyzed as described below. (A, B): Plk2, TnnC1, Igf1, Igf2 mRNA expression levels were analyzed by qPCR. Data are expressed as fold induction relative to control muscle transduced with the EV. At least three independent experiments with duplicate samples were carried out for each measurement. Error bars represent SEM.* p < 0.05, ** p < 0.01. (C): Representative Western blot analysis of protein lysates from transduced regenerating muscles with MyHC, P38, MYOD, and P21 antibodies. (D): Representative Western blot analysis of protein lysates from transduced regenerating muscles with, AKT1 and 70S6K protein, pAKT on Thr308 and Ser473, mTOR, S6K, and pS6K antibodies. Red Ponceau staining was used as a loading control in C and D. (E): The number of DAPI-stained nuclei within dystrophin-positive sarcolemma were counted in transduced regenerating muscles, they are expressed per 100 myofibers. Data are mean ± SEM. (F): A model depicting the potential roles played by MEF2C proteins in adult myogenesis. Phosphorylated MEF2Cα1 promotes satellite cells-derived myoblasts (red cells) proliferation via upregulating, directly or not, the immediate early genes Plk2 and JunB. Subsequent increase of MEF2Cα2 level drives cell cycle exit, terminal differentiation and myoblast fusion to give newly formed myofibers, by inducing the expression of the CDK inhibitor P21 as well as P38 and MyoD. Dephosphorylated MEF2Cα1 contributes to the activation of muscle genes in nascent myofibers and it promotes myofiber growth by activating the anabolic AKT/mTOR/S6K pathway. Abbreviations: EV, empty lentivirus; MEF2C, myocyte enhancer factor 2C; MyHC, myosin heavy chain; pAKT, phospho-AKT; S6K, ribosomal S6 kinase; pS6K, S6K phosphorylated on Thr389.
of the anabolic AKT/mTOR pathway and its downstream target pS6K and the addition of new myonuclei via the proliferation and further fusion of SC-derived myoblasts to the adult myofiber. These different activities are alternatively switched-on by a dynamic phosphorylation of Ser98 and Ser110, two phosphoacceptor sites encoded by the alternative α1 exon, their phosphorylation is paramount in the control of the pro-proliferative function, given that substitution of these two phosphoacceptor sites with non-phosphorylatable Alanine residues represses the pro-proliferative activity and at the same time enhances the pro- myogenic and hypertrophic abilities of MEF2C. Previous studies have implicated MEF2 proteins in the control of adult skeletal muscle regeneration, where they activate the differentiation gene expression program [7, 8]. In this study we present evidence to suggest that MEF2C, in addition to the pro-differentiating function, mainly due to the MEF2Cα2 isoform, also promotes expansion of primary myoblasts, an activity exhibited by the MEF2Cα1 protein in a phosphorylation-dependent manner. On the basis of our results, we hypothesize that the pro-proliferative activity of MEF2Cα1 on SC is related to its ability to direct the expression of Immediate Early Genes, including, JunB, Plk2, which are to be added to other cycle-related genes that are regulated by MEF2 proteins in muscle [17, 41] and nonmuscle cells [42, 43].

The observed MEF2Cα1-dependent upregulation of JunB is in line with the results of ChIP analyses performed by the Encyclopedia of DNA Elements (ENCODE) consortium in human cells, demonstrating that MEF2 binds to regions upstream of the JunB gene, suggesting a direct role of MEF2C in regulating this gene. We identified a putative MRE in the promoter region of the JunB gene, near one conserved CCAAT elements, however, in our experimental conditions, we have not detected a recruitment of MEF2 to the identified MRE in front of an efficient binding of the NFY transcription factor to its binding site localized in the same region. This data may then indicate an indirect role of MEF2Cα1 in regulating the expression of JunB, potentially through other transcription factors. Plk2 transcripts present a broad tissue distribution and have been also detected in mouse SC [44]. Our findings also show that MEF2 proteins directly bind to the putative MRE located in the upstream region from the transcription sites of Plk2 suggesting that MEF2Cα1 might actively regulate Plk2 transcription by binding to an active enhancer.

One process contributing to the pro-proliferative function of MEF2C in primary myoblasts is the phospho-dependent interaction with PIN1. In this study we show that PIN1 and MEF2C physically interact in primary myoblasts and we found not only that PIN1 inhibits myogenic differentiation [24] but also that this interaction synergistically stimulates their expansion. The use of phospho-specific antibodies allowed us to follow the in vivo kinetics of phosphorylation of the PIN1 binding sites in the α1 domain: we observed a strong phosphorylation at Ser98/110 within five days after injury, when the regeneration response is at its highest. Later on, during remodeling of regenerated muscle we found maintenance of MEF2Cα1 expression but we did not detect phosphorylation at the PIN1 binding sites, suggesting an important role for the dephosphorylated protein in late phases of adult myogenesis. Our in vivo studies revealed that ectopic expression of the non-phosphorylatable MEF2Cα1 mutant protein induces the expression of myogenic markers and of P38, whose activity is important for early phases of myoblast differentiation [45–49] and myocyte fusion [50]. However, the pro-differentiative activity of the 2SA mutant is less pronounced than that exerted by MEF2Cα2, which instead potently stimulates the expression of the CDK inhibitor p21 gene, a key event for cell cycle exit and myogenic terminal differentiation. Conversely we observed that the 2SA mutant robustly promotes a notable increase of the cross-sectional area of myofibers that correlates with the activation of the AKT/mTOR anabolic pathway and the phosphorylation of the downstream target S6K [40, 51, 52] and the concomitant increase in the number of myonuclei. Altogether our data suggest a model whereby MEF2C protein variants promote different stages of myogenesis, ranging from SC proliferation, terminal differentiation and myofiber growth. MEF2Cα1 stimulates muscle growth through two mechanisms, by controlling first SC proliferation when phosphorylated on the PIN1 binding sites, subsequently, upon dephosphorylation it stimulates myonuclear accretion and protein synthesis through activation of the mTOR/S6K pathway (Fig. 6F). In support for this model, fully regenerated muscles overexpressing MEF2Cα1 are characterized by the coexistence of hypertrophic as well as small newly formed myofibers and an increase of Ki67-positive cells and Plk2 expression, reflecting the pleiotropic function played by this protein. Given our data, dynamic phosphorylation of Ser98/110 plays a key role in adult myogenesis. Both Ser residues are predicted to be dynamically phosphorylated by proline-directed protein kinases, including CDKs and mitogen-activated protein kinases (MAPKs), a prediction supported by our finding that CDK6/Cyclin D1 phosphorylates MEF2C at Ser110 in vitro [17] and treatment of cultured cells with CDK6 inhibitors reduces MEF2C phosphorylation. Experiments directed toward the identification of these kinases are the matter of ongoing work. Although MEF2C has been already implicated in activation of genes that promote cardiac hypertrophy in response to IGF1 signaling, this is the first report of its implication in skeletal muscle hypertrophy [53–55]. We hypothesize that MEF2C indirectly activates the AKT/mTOR pathway by inducing an up-regulation of Igf1 and Igf2 gene expression. ChIP analyses performed by the ENCODE consortium demonstrate that MEF2 is bound to MEF2 sites located in the promoter as well in an upstream region of the Igf1 gene, suggesting a direct role of MEF2C in promoting the expression of this gene. Interestingly previous studies indicate that forced expression of IGF1 can induce Mef2c gene expression in muscle cells [56]. Our observations of a MEF2C-dependent induction of Igf1 gene expression suggest a positive-feedback loop underlying muscle growth [56].

**CONCLUSION**

In general, our data demonstrate that MEF2C promotes muscle regeneration and growth at two cellular levels: by means of activating SC proliferation and differentiation to provide myonuclei to growing myofibers and through promotion of the IGF1/AKT anabolic signaling pathway in differentiated myofibers to increase muscle mass. The timely coordination of these activities in adult myogenesis is ensured by a molecular mechanism in which alternative splicing and phosphorylation finely regulate the activity of MEF2C. Our data supply preliminary evidence that modulating MEF2Cα1 function might be a valuable therapeutic strategy for muscle wasting therapies.

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AUTHOR CONTRIBUTIONS

F.B.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript; D.M.: Conception and design, collection and/or assembly of data, data analysis and interpretation; C.N.: Collection and assembly of data; L.D.F.: collection and/or assembly of data; S.B., M.G., and R.B.: Data analysis and interpretation; V.B.: Collection and/or assembly of data, data analysis and interpretation; C.I.: Conception and design, data analysis and interpretation; A.M.: Conception and design, data analysis and interpretation; S.M.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of the manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES


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