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Over-expression of the transcription factor, ZBP-89, leads to enhancement of the C2C12 myogenic program

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Abstract

Myogenesis involves the complex interplay between the down-regulation of non-muscle genes and the up-regulation of muscle-specific genes. This interplay is controlled by the myogenic regulatory factors Myf5, MRF4, MyoD and myogenin. To trigger the up-regulation of these muscle-specific factors, certain environmental cues, such as the removal of serum, signal C2C12 myoblast cells to withdraw from cell cycle, fuse and activate muscle-specific genes. Here, the level of ZBP-89 (zfp148), a Krüppel-like transcription factor, has been shown to increase during myogenesis. Over-expression of ZBP-89, via adenoviral infection, led to the enhancement of the myogenic program without requiring the removal of serum. Quantitative real-time PCR and ChIP assays documented that ZBP-89 promoted the down-regulation of Pax7 coupled with the up-regulation of MRF4 and MyoD to regulate C2C12 differentiation *in vitro*. In addition, ZBP-89 over-expression up-regulated p21 and Rb while promoting the down-regulation of cyclinA and cyclinD1. In converse, the diminution of ZBP-89 by siRNA promoted the retention of myogenic and cell cycle regulators at myoblast levels resulting in a concomitant delay of the myogenic program. From these studies we conclude that the transcription factor ZBP-89 plays an important role in the timing of the myogenic program.

Keywords

ZBP-89; myogenesis; C2C12; p21; MyoD; Mrf4; myogenin

1. Introduction

Myogenesis can be viewed as a two-step process consisting of determination, where precursor cells commit to a muscle lineage, followed by differentiation of committed myoblasts to myotubes [1]. Complex mechanisms have evolved for controlling the proper timing of gene expression during this process via two families of myogenic factors. The basic Helix-Loop-Helix proteins (bHLH), Myf5, MRF4, MyoD and myogenin, act by binding as heterodimers with E-box proteins to E-box sequences (CANNTG) within muscle gene enhancers [1,2]. The

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temporal, spatial, and regional expression of bHLH proteins contributes to terminal differentiation of the various skeletal muscles. The second set of myogenic factors, the MEF2 family, bind to AT-rich sequences, are common to all muscle types, and function to potentiate the effect of bHLH proteins [3]. Thus, both transcription factor families are critical for the myogenic program. The differentiation of C2C12 myoblasts *in vitro* mimics many of the gene expression patterns seen in the embryo and thus represents an excellent model system to study myogenic gene regulation.

The bHLH family members control the processes of determination and differentiation within a given somitic cell, the embryological origin of skeletal muscle cells. These cells become committed to the muscle cell lineage via expression of both Myf5 and MyoD. This expression is believed to be the committed step in muscle development, since disruption of both factors results in the absence of skeletal muscle [4–8]. However, MyoD and Myf5 may serve partially redundant roles in determination as deletion of either single gene does not eliminate muscle development, although continued expression of Myf5 does prevent terminal differentiation of C2C12 cells *in vitro* [8,9,10]. After somitic cells are determined and migrate to skeletal muscle locations, the cells proliferate and differentiate into striated myotubes where continued expression of MyoD together with MRF4 expression is important. MRF4 and MyoD are believed to play redundant roles in differentiation, although recent evidence suggests that MRF4 may also play a role in determination [11]. Finally, myogenin is known to act downstream of both MyoD and Myf5, is crucial to differentiation, and its absence results in a deficiency of muscle fibers despite muscle cell migration and commitment [12–13]. The temporal expression of these factors during embryogenesis suggests similar yet unique roles. Temporally, Myf5 is expressed first in the developing myotome. MRF4 displays a biphasic expression pattern during embryogenesis by up-regulating at embryonic day 9, tapering off by day 11.5 and then re-instating at day 16. MyoD expression begins at embryonic day 11.5. Further understanding the individual role of each factor during myogenesis is complicated by their ability to cross-regulate each other's expression compounded by intricate promoter/enhancer elements controlling spatial and temporal expression [14].

ZBP-89 (BFCOLI, BERF-1, ZNF-148, zfp148) is a Krüppel-like, zinc-finger transcription factor that binds to a GC-rich region and can activate or repress transcription. ZBP-89 activates such genes as human stromelysin [15], the T cell α- and β-receptor [16], $p21^{waf1/cip1}$ [17] and the lymphocyte-specific protein tyrosine kinase [18]. ZBP-89 functions as a transcriptional repressor for the human gastrin [19], human ornithine decarboxylase [20], rat β–enolase [21], bovine adrenodoxin [22] epithelial neutrophil-activating peptide-78 (ENA-78) [23], and vimentin [24] genes. ZBP-89 functions as an activator by recruiting the histone acetyltransferase p300 to the p21 promoter to up-regulate gene expression [25]. When ZBP-89 functions as a repressor, it either competes with Sp1 for binding to the same or overlapping sequences [19] or bind separately to its own DNA element as found for the vimentin gene [24,26].

Recent evidence suggests ZBP-89 may play a role in skeletal muscle development. During mouse embryogenesis, ZBP-89 (Zfp148) mRNA increases from day 12 to 14 in skeletal muscle and then declines after birth [21]. While in adult mice, there are low levels of ZBP-89 mRNA in the adult heart and testis, intermediate levels in skeletal muscle and spleen, and higher levels in brain, kidney, lung and liver [21]. Interestingly, in the adult rat equal levels of ZBP-89 mRNA exist in heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis [19]. A comparison of the ZBP-89 promoter region between the mouse and human genes revealed at least 8 specific binding sites for Sp1, TCF-1, Oct1, NF-κB, MyoD, Ets-1, Lyf-1 and ZBP-89 itself [27]. The ZBP-89 promoter contains MyoD and several MEF-2 binding sites, supporting previous mRNA studies documenting an increase in ZBP-89 levels during myogenesis. To further support a role for ZBP-89 in myogenesis, a ZBP-89 promoter-reporter construct containing

the MyoD site displayed a 3-fold increase in expression in C2C12 myotubes versus myoblasts [27]. ZBP-89 is also important for blood development in mice and zebrafish, serving cooperative functions with GATA-1 and FOG-1 to control erythroid development [28,29]. Here, we showed that ZBP-89 over-expression led to enhancement of the myogenic program via regulation of bHLH myogenic transcription factors and cell cycle regulators important to C2C12 differentiation *in vitro*. Moreover, these results were corroborated by siRNA elimination of ZBP-89 expression.

2. Materials and methods

2.1. Cell cultures

C2C12 skeletal myoblast cells (ATCC CRL-1772) were maintained in high-glucose DMEM (Invitrogen) with glutamine, supplemented with 10% FBS (GM), penicillin (100 units/ml) and streptomycin (50 μg/ml). Sub-confluent cells were switched to differentiation medium (DM) containing 1% FBS to initiate differentiation into myotubes.

2.2. RNA extraction and quantitative real-time PCR (qPCR)

For establishing a time course of RNA expression, C2C12 myoblast cells were plated and harvested 24 h later as myoblast cells or switched to DM and harvested every 24 h to follow differentiation *in vitro*. For adenoviral over-expression, cells were plated for 24 h and then left in GM or transferred to DM and then infected with Ad-empty, Ad-β-gal or Ad-ZBP-89. Total RNA was extracted using the TRIzol reagent (Invitrogen) 48 h after infection and prepared as mentioned previously [30]. For qPCR amplification, data acquisition and analyses was carried out with a LightCycler instrument using a sequence-detection system (model 7000; Applied Biosystems, Inc., Foster City, CA). The qPCR mixture consisted of 10 μl of SYBR green master mix, 3 μl of cDNA and primers plus water to a final vol of 20 μl. Primer sequences used were as follows: for Rb 5′-AAGCTAGCATGCCGCCCAAGCCCCG-3′ and 5′-TCTTCAACTCAAGCCTGGC-3′; for p21 5′-ATGTCCAATCCTGGTGATGTCC-3′ and 5′-TCAGGGTTTTCTCTTGCAGAAG-3′, for myogenin 5′-ACTCTTCGCCCCCGT-3′ and 5′-CCGCCCTGCCACTCAT-3′, for cyclinD1 5′-TCTACACTGACAACTCTATCCG-3′ and 5′-TAGCAGGAGAGGAAGTTGTTGG-3′, for ZBP-89 5′-CGCATTTAGAAGATGCGTCA-3′ and 5′-CCCCTCCTGCAAATTATCAA-3′, for Myf5 5′-TGTATCCCCTCACCAGAGGAT-3′ and 5′-GGCTGTAATAGTTCTCCACCTGTT-3′, for MRF4 5′-CTACATTGAGCGTCTACA GGACC-3′ and 5′-CTGAAGACTGCTGGAGGCTG-3′, for MyoD 5′-GACCTGCGCTTTTTTGAGGACC-3′ and 5′-CAGGCCCACAGCAAGCAGCGAC-3′, for cyclinA1 5′-TTCCCCAA TGCTGGTTGA-3′ and 5′-AACCAAAATCCGTTGCTTCCT-3′, for Pax7 5′-TGGAAGTG TCCACCCCTCTTGGC-3′ and 5′-ATCCAGACGGTTCCCTTTGTCGCC-3′. Gene amplification was carried out as follows: 95°C for 10 min, followed by 50 cycles in 3 steps: 95°C for 15 s, 55°C for 30 s, and 68°C for 45 s. At the end of the amplification cycles, dissociation curves were determined to rule out signal from primer dimers and other nonspecific dsDNA species. Data was normalized to U6 mRNA levels. Experiments were carried out in triplicate on three separately isolated RNA samples and the standard error was shown. The size of the PCR products was confirmed by analysis on a 2% agarose gel stained with ethidium bromide.

2.3. Plasmids and reagents

The Ad-ZBP-89 and a 2100 bp p21 promoter (in pGL3) were kindly provided by Dr. Juanita Merchant (Univ. of Michigan, Ann Arbor, MI). The cyclinD1 promoter construct was kindly provided by Dr. R.G. Pestell (Northwestern, Chicago, Il). Protease inhibitor cocktail (P-8340), monoclonal anti-vimentin antibody (V6630) and monoclonal anti-Flag antibody (F-3165) were

purchased from Sigma (St. Louis, MO). Anti-tubulin (sc-9104) and anti-MHC (sc-20641) antibodies were purchased from Santa Cruz Biotechnology.

2.4. Adenoviral infection and siRNA transfection

For adenoviral infection, C2C12 cells (5×10^4) were plated in each well of a six-well plate, incubated overnight at 37°C, media was changed to either 1% FBS for myotubes or 10% FBS for myoblasts and then infected with either adenoviral flag-ZBP-89 (Ad-ZBP-89), Ad-empty vector (Ad-empty) or Ad-β-galactosidase (Ad-β-gal) at an MOI of 5. Cells were visualized at successive time points using a light microscope and digital camera. All pictures were selected from three independent experiments. For siRNA transfection, C2C12 cells (8×10^4) were plated in each well of a 6-well plate, incubated overnight at 37°C and then the media was changed to either DM or GM. Cells were then transfected with siControl (20 nm) or siZBP-89 (20 nm) (Dharmacon) using Dharmacon transfection reagent 2. Cells were visualized at successive time points and pictures taken from three separate experiments with a representative experiment shown.

2.5. DNA transfection and luciferase assays

C2C12 cells (2×10^5) were plated in each well of a six-well plate, incubated overnight at 37° C, media was changed to either GM or DM, and then transiently transfected with plasmid DNA, either cyclinD1 or p21 promoter constructs (2 μg), using the MIRUS DNA transfection method (Fisher) and then co-infected with Ad-β-gal or Ad-ZBP-89. The Renilla plasmid was co-transfected to serve as an internal control for transfection efficiency. Myoblast cells were harvested 48 h after transfection. Cell lysates were prepared via the freeze-thaw method. Dual-luciferase assays were performed according to the established protocol of Promega. All transfections were performed in triplicate with at least two different DNA preparations for each plasmid.

2.6. Whole cell extract preparation and Western blotting

Whole cell extracts (WCE) were prepared from C2C12 cells infected with Ad-ZBP-89, Ad-empty or Ad-β-gal. Cells were washed in cold 1X PBS and then lysed in 4% SDS in 1X PBS with protease inhibitor cocktail at 10 μl/1 ml (Sigma). After brief sonication, an equal volume of 1X PBS was added to the lysate to reduce the SDS concentration to 2%. Lysates were then centrifuged for 10 min at 13,000 rpm, supernatants collected, and protein concentration measured using the BioRad D_c Protein Assay kit (BioRad, Hercules, CA). Equivalent amounts of protein (50 μg) were boiled in 1X SDS sample buffer for 5 min and analyzed on 4–20% gradient polyacrylamide gel (NuSep Inc., Austell, GA). Western blotting was carried out as previously described [31]. Antibodies (1:1000) used for Western blots are as follows: anti-flag, anti-β-tubulin, and anti-MHC.

2.7. BrdUrd staining and cell proliferation assays

C2C12 cells (1×10^3) were plated on coverslips and incubated overnight at 37°C. Cells were then infected with Ad-ZBP-89, Ad-empty vector, or Ad-β-gal and incubated overnight at 37° C. For siRNA transfection, C2C12 cells were also plated on coverslips (1×10^3), incubated overnight, and then transfected with Dharmafect reagent 2, siControl or siZBP-89 and incubated overnight at 37°C. 48 h after treatment, cells were washed in 1X PBS briefly and fixed in 1:1 methanol-acetone at −20°C for 10 min. The slides were washed briefly with 1X PBS, followed by permeabilization with 0.5% TritionX100 in 1XPBS for 30 min. Cells were washed twice in 1XPBS and blocked overnight in 1XPBS with BSA and fish gelatin. Coverslips were incubated for an hour with an antibody to MHC. Cells were washed three times in 1XPBS and then incubated with secondary antibody. Finally, cells were washed three times in 1XPBS with 1% Hoechst reagent. Cells were visualized using a fluorescent microscope. Cell nuclei

versus nuclei stained were quantitated by counting 250 cells. All quantitation was the result of three independent experiments and subjected to a student's t-test analysis.

Cell proliferation was monitored using the WST-1 reagent (Roche). WST-1 is a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to a formazan product by mitochondrial succinate-tetrazolium reductase in viable cells thereby offering a method to measure cell proliferation. C2C12 cells were plated in 96 well plates (5×10^2) and incubated overnight at 37°C. C2C12 cells were then washed in 1X PBS and media switched to either GM or DM. Cells were infected with Ad-ZBP-89, Ad-empty vector or Ad-β-gal and allowed to incubate overnight at 37°C. For successive days as indicated, media was removed, cells were washed in 1X PBS and then 10 μl of WST-1 reagent was added to 100 μl of serum-free media and allowed to incubate for 4 h. The optical density was read at 490 nm and results were averaged from three separate experiments. For siRNA transfection, C2C12 cells were also plated in 96 well plates (5×10^2), incubated overnight and media changed as described previously. Cells were then transfected with Dharmafect reagent 2, siControl or siZBP-89 and allowed to incubate overnight at 37°C. Cells were incubated with the WST-1 reagent as mentioned above and the results were quantified graphically.

2.8. ChIP assay

ChIP assays were performed as mentioned previously [26,30,32]. For qPCR, 2 μl out of the 20 μl of extracted DNA was used in 50 cycles of amplification in 3 steps: 95°C for 15 s, 55° C for 30 s, and 68°C for 45 sec. At the end of the amplification cycles, dissociation curves were determined to rule out signal from primer dimers and other nonspecific dsDNA species. Data was normalized to IgG immunoprecipitated DNA levels. Experiments were carried out in triplicate and one representative experiment was shown. The size of the PCR products was confirmed on a 2% agarose gel stained with ethidium bromide. The primer sequences were as follows: for p21 (−282 to +1) 5′-AATGTGTCCAGCGCACC-3′ and 5′-CCGGGCCTTTCTTTATGTT-3′, for MyoD (proximal promoter region) 5′-CGCCCCCAGCCTCCCTTTCCAG-3′ and 5′-TGTCAGAGGTGTGGTGAAGAAA-3′, for MyoD enhancer (18–22 kb upstream of the transcriptional start site) [33] 5′-CGGGGTCGGGATCCGGAC-3′ and 5′-GGTGTCGTA AACCCCCGTA-3′, for myogenin upstream region 5′-CAAGACCCCTTCCCGTCCGTC-3′ and 5′-CCTGCAGGCCTGCCCCTGG-3′, for myogenin proximal promoter region (−147 to +1) 5′-GAATCACATGTAATCCACTGGA-3′ and 5′-CGCCAACTGCTGGGTGCCA-3′, for MRF4 enhancer (20 kb upstream of the transcriptional start site) [34] 5′-TTGTGTTAATCCCCAGTTGT-3′ and 5′-CTCCTCCTTGGCCTCTGACT-3′, for cyclinD1 proximal promoter region 5′-GGTTTAAGAACAGGGTGTCC-3′ and 5′-GTTAAGCAGAGATCAAAGCC-3′, for Pax7 (−632 to −332) 5′-GAATTCCTCCCTTCAAGTCACC-3′ and 5′-TGAATGGGGCGGATCCGCCG-3′, Pax7 (−332 to +1) 5′-CCGGGATCGTCCCTCTCT-3′ and 5′-CAGGGCTGGACGG AGGAGAC-3′ [35].

3. Results

3.1. Over-expression of ZBP-89 leads to the enhancement of the myogenic program

To analyze the effect of ZBP-89 expression on the myogenic program, C2C12 myoblast cells were infected with adenovirus encoding ZBP-89 attached to the Flag-epitope (Ad-ZBP-89) or as negative controls adenovirus alone (Ad-empty) or containing β-galactosidase (Ad-β-gal) under the control of the CMV promoter to promote exogenous, sustained expression. Infected cells were either maintained as myoblasts (MB) in GM or promoted to differentiate to myotubes (MT) by a switch to DM (Fig. 1A). C2C12 myoblast cells differentiated into myotubes by forming long structures of fused cells detectable in culture whereas myoblast cells in GM

remained as single cells Myoblast cells alone or infected with either Ad-β-gal or Ad-empty displayed the same visual pattern of single cells over this time period (Fig. 1: MB+Ad-β-gal versus MB). By 48 h over-expression of ZBP-89 induced long structures of fused cells even in the presence of GM akin to myotubes or infected myotubes (Fig 1: compare MB+Ad-ZBP-89 to MB). ZBP-89 over-expression was confirmed via Western blot analysis using a Flag-tag antibody where β–tubulin served as a loading control (Fig. 1B). Endogenous ZBP-89 protein levels during C2C12 differentiation could not be monitored as a suitable, commercial antibody, which recognizes a protein of the correct molecular weight at low endogenous levels is not available. qPCR analysis of endogenous ZBP-89 mRNA levels at successive time points after serum withdrawal demonstrated that ZBP-89 mRNA levels rapidly increased during the first 24 h in DM, declined at 48 h, but gradually returned to the 24 h level by 120 h (Fig. 1C). A similar biphasic expression pattern has been detected for MRF4 during myogenesis [1,36]. The number of nuclei per fiber increased in myoblasts infected with AD+ZBP-89 similar to myotubes or infected myotubes (Fig. 1D). Staining for myosin heavy chain (MHC) indicated that large numbers (85%) of myoblast cells infected with Ad+ZBP-89 stain with MHC while myoblasts or Ad-β-gal controls did not (Fig. 1E). Quantification verified that 90% of Hoechst stained myoblasts infected with Ad-ZBP-89 also expressed MHC comparable to myotubes or Ad+ZBP-89 infected myotubes (Fig. 1F)

3.2. Over-expression of ZBP-89 affected cell proliferation and suppressed DNA synthesis

To determine if ZBP-89 over-expression affected cell proliferation, BrdUrd incorporation into myoblasts or treated cells was compared to similarly treated myotubes [37]. Myoblasts maintained in GM for 24 h continued to proliferate as a large percentage of cells (>70%) co-stained with DAPI and BrdUrd. Myoblast cells infected with Ad-β-gal demonstrated similar proliferation rates. Myoblast cells switched to DM for 24 h demonstrated a 15% reduction in BrdUrd incorporation. Interestingly, after 24 h of Ad-ZBP-89 infection there were fewer myoblast nuclei incorporating BrdUrd than in other differently treated cells. Whether kept as myoblasts in high serum or allowed to differentiate to myotubes, only 50% of Ad-ZBP-89 infected cells remained proliferative at 24 h.

In addition, the growth of myoblasts, myotubes or cells infected with either Ad-β-gal or Ad-ZBP-89 was compared using the proliferation reagent, WST-1 (Fig. 2B). Both myoblasts and control cells continued to proliferate for 4 days tapering off by day 5. Myoblast cells infected with Ad-ZBP-89 mimicked the growth rate of myotubes or myotubes plus Ad-ZBP-89 in DM.

3.3. ZBP-89 modulated the bHLH myogenic regulatory factors

The effect of ZBP-89 on the expression of bHLH myogenic regulatory factors was measured during C2C12 differentiation (Fig. 3). C2C12 cells were plated for 24 h and infected with either Ad-ZBP-89, Ad-empty vector or Ad-β-gal. Cells were harvested 48 h later and RNA isolated to determine the subsequent effect of ZBP-89 over-expression on the timed expression of several bHLH myogenic factors. Pax7 is a determination factor that down-regulates during differentiation and myotube fusion [10]. The mRNA levels of Pax7 declined in myoblasts with the addition of Ad-ZBP-89 akin to myotubes while infection with either Ad-empty or Ad-β-gal had no effect (Fig. 3A). On the other hand Ad-ZBP-89 expression in myoblasts induced MRF4 expression by 9-fold (Fig. 3A). Infection with Ad-ZBP-89 also increased MyoD expression in myoblasts akin to myotube levels [38] (Fig. 3A). Myogenin is another bHLH regulatory factor known to substantially increase during differentiation. The addition of Ad-ZBP-89 greatly up-regulated myogenin expression 1000-fold in myoblasts mimicking levels seen in myotubes with an additional 1.7-fold increase in myotubes plus Ad-ZBP-89 compared to myotubes alone (Fig. 3B). Thus ZBP-89 appeared to induce the myogenic program via modulating the expression of crucial bHLH myogenic regulatory factors.

ChIP assays were conducted on chromatin isolated from myoblasts, myotubes, or myoblasts plus Ad-β-gal or Ad-ZBP-89 to determine if ZBP-89 was directly binding to specific DNA elements within the promoters of these target genes. Experiments with myoblasts infected with Ad-empty vector or myotubes plus Ad-ZBP-89 were not reported, since the same result was detected as with myoblasts plus Ad-β-gal or Ad-ZBP-89, respectively. Interestingly, ZBP-89 was found to bind to an upstream region of the Pax7 promoter (Fig. 4A) previously suggested to contain a putative GC-box and other GC-rich regions [35]. Thus, ChIP assays supported our previous results (Fig. 3A) and suggested that ZBP-89 must be acting as a transcriptional repressor to promote the down-regulation of Pax7 during C2C12 myogenesis. ZBP-89 was also found to bind the MRF4 enhancer region (Fig. 3C) [34], substantiating our previous results (Fig. 3A). Interestingly, ZBP-89 did not bind the MyoD minimal promoter region (data not shown), but bound an upstream enhancer region known to contain an H4TF-1 element. Previously, a similar H4TF-1 element within the vimentin gene was shown to bind ZBP-89 (Fig. 3C) [32,33]. However, we did not detect ZBP-89 binding to either the myogenin minimal promoter (Fig. 4E) or an upstream myogenin enhancer (data not shown), both of which contain known GC-rich regions. Thus, ChIP assays corroborate that ZBP-89 was specifically binding to DNA elements within the promoters of some of these target genes, but not all, further supporting the selectivity of these ChIP results.

3.4. ZBP-89 affected the expression of downstream targets of the myogenic regulatory factors

In addition to the timed activation of key myogenic regulatory factors, skeletal muscle cells permanently withdraw from cell cycle during differentiation. Thus, the effect of ZBP-89 on crucial cell cycle regulators such as p21, Rb, cyclinD1 or cyclinA was investigated (Fig. 4). MyoD is known to indirectly activate p21 expression during myogenesis [39,40]. p21 mRNA levels, a known target for ZBP-89 regulation in other cell lines [25], increased 5.7-fold after infection with Ad-ZBP-89 while the negative controls (Ad-empty and Ad-β-gal) had no effect on p21 expression (Fig. 4A). Rb protein levels also increased during myogenesis to promote cell cycle withdrawal [42]. Myoblasts or infected controls demonstrated low levels of Rb mRNA; however, Rb levels were increased in myotubes (6-fold), and even more in myoblasts or myotubes infected with Ad-ZBP-89. Conversely, cyclinA mRNA levels declined during myogenesis and with infection of Ad-ZBP-89 whereas both negative controls (infection with Ad-empty vector or Ad-β-gal) demonstrated little effect on cyclinA mRNA levels (Fig. 5B). Finally, cyclinD1 down-regulation is also one of the key cell cycle regulators required to decline during myogenesis [4,37,43]. CyclinD1 mRNA was extremely low in myoblasts and myotubes infected with Ad-ZBP-89 (Fig 4A). Thus, ZBP-89 also apparently either directly or indirectly regulated key cell cycle regulatory proteins important for the myogenic program.

Previously, ZBP-89 has been shown to bind to the p21 promoter and activate its expression [25]. p21 expression is known to be up-regulated via Western blots and transient transfection analysis in the C2C12 cell line during differentiation [37,39]. Here, the effect of ZBP-89 expression was monitored on the activity of a full-length p21 promoter luciferase construct (p21Luc2300 bp) in myoblasts, myotubes or duplicate cultures infected with Ad-β-gal or Ad-ZBP-89 (Fig. 4B). As discussed earlier, transfection of myoblasts plus Ad-empty vector or myotubes plus Ad-ZBP-89 were not included, since identical results were obtained with myoblasts plus Ad-β-gal or myotubes plus Ad-ZBP-89, respectively (Fig. 3). Transient transfection of p21Luc into myotube cells resulted in a 1.6–fold increase in relative luciferase activity over that level detected in myoblast cells. Addition of Ad-ZBP-89 further increased p21 promoter activity 2-fold in myoblast cells while there was no effect with infection of Ad-β-gal alone. ChIP assays verified ZBP-89 binding on the p21 promoter (Fig. 4C). A cyclinD1 full promoter luciferase construct was also assayed via transient transfection analysis in C2C12 cells (Fig. 4D). Myoblast and myoblast+Ad-β-gal cells demonstrated high reporter gene

activity while myotubes and myoblast plus Ad-ZBP-89 demonstrated a 7.5-fold decrease in relative luciferase activity. ChIP assays were performed to determine if ZBP-89 binds directly to the cyclinD1 promoter during myogenesis (data not shown). No evidence for ZBP-89 binding to the cyclinD1 promoter was detected; thus, ZBP-89 must act through an indirect mechanism to repress cyclinD1 expression during C2C12 myogenesis. MHC is a known downstream target of MyoD and myogenin. MHC levels were assayed via Western blot analysis in extracts prepared from C2C12 cells as indicated. As expected MHC levels increased substantially in myotubes and myoblasts+Ad-ZBP-89 while no expression was detected in myoblasts or myoblast+Ad-β-gal (Fig. 5). Tubulin levels remained constant serving as a loading control. In summary, ZBP-89 modulated the expression of cell cycle regulators and an important downstream protein target (MHC) of the myogenic program.

3.5. Elimination of ZBP-89 via siRNA approaches

By a number of criteria, we have shown that ZBP-89 enhanced the myogenic program. We next determined the effect of ZBP-89 elimination via siRNA approaches. C2C12 myoblast cells were transfected with Dharmacon siControl or siZBP-89 and observed at 24 and 48 h (Fig. 6A). Myoblasts with siControl mimicked the same visual pattern seen earlier for myoblast cells as there was continued proliferation without the formation of long fused structures. Elimination of ZBP-89 appeared to also result in proliferation similar to that seen for the siControl. qPCR analysis of RNA isolated from myoblasts or myotubes plus siZBP-89 verified that ZBP-89 mRNA levels were barely detectable after siZBP-89 treatment, while ZBP-89 mRNA levels in the siControl showed normal levels of myoblast or myotube ZBP-89 mRNA expression, respectively (Fig. 6B). Further investigation into the effects of ZBP-89 elimination on the myogenic program was examined via Hoechst and MHC staining (Fig. 6C). Elimination of ZBP-89 in myotubes delayed myogenesis as there was a substantial decline in the number of MHC stained cells despite the removal of serum (Figs. 6C and D).

3.6. Effect of siZBP-89 on C2C12 proliferation

The proliferation capacity of myoblast, myotube, and similar cells transfected with siRNAs was assayed via BrdUrd incorporation and the proliferation reagent WST-1 (Fig. 7). Myoblast cells in GM continued to proliferate when stained with BrdUrd as a large percentage of cells (72%) co-stained with DAPI and BrdUrd (Fig. 7A) as seen previously (Fig. 2A). Myoblast cells treated with siControl or siZBP-89 demonstrated similar proliferation rates. The addition of siZBP-89 to myoblasts contrasted to earlier results (Fig. 2A) where over-expression of Ad-ZBP-89 reduced myoblast proliferation from 72% to 50% even in the presence of growth media. Here, BrdUrd incorporation in myoblasts remained constant with the addition of siZBP-89 similar to that of the myoblast controls. However, proliferation decreased to 60% for myotubes switched to DM for 24 h analogous to those results displayed by myotubes treated with siControl or siZBP-89. Interestingly, the addition of siZBP-89 to myotubes did not overcome this decrease in proliferation probably due to the reduced level of serum in DM. In addition, cell growth was measured using the WST-1 proliferation reagent. Again, myoblast cells treated with either the transfection reagent alone, with the siControl or with siZBP-89 all demonstrated similar proliferation rates that mimicked the rates seen for myoblast cells alone (Fig. 7B). Concurrently, myotubes transfected with the three aforementioned reagents mimicked the growth rates seen for myotube cells (Fig. 7C).

3.7. Elimination of ZBP-89 affected myogenic and cell cycle regulatory factors

C2C12 myoblasts were transiently transfected with siZBP-89 and RNA was isolated 48 h after transfection to monitor the levels of myogenic factors analyzed earlier. Pax7 mRNA levels declined in myotubes compared to myoblasts. An analgous decrease was noted in myoblasts or myotubes transfected with siZBP-89 but not with the siControl (Fig. 8A). Perhaps low levels

of ZBP-89 are required for some factor to up-regulate Pax7 and the lack of this factor is rate limiting for Pax7 expression. In contrast, MRF4 levels were low in myoblasts and increased at least 5-fold in myotubes. Similar results were seen upon transfection of the siControl. Transfection with siZBP-89 resulted in MRF4 levels equivalent to those detected in myoblast cells; thus, elimination of ZBP-89 prevented MRF4 up-regulation. MyoD mRNA levels were also low in myoblasts while there was a substantial increase in myotubes. Similar results were obtained with the siControl. Both myoblast or myotube cells treated with siZBP-89 showed statistically significant decreases in MyoD mRNA levels. Apparently elimination of ZBP-89 overcomes the ability of serum withdrawal to promote the up-regulation of MyoD. Similar results were obtained for myogenin (Fig. 8B) and p21 (Fig. 8D).. Thus, treatment with siZBP-89 blocked the normal increased expression of MRF4, MyoD, p21 and myogenin during C2C12 differentiation.

mRNA levels for additional cell cycle regulators were analyzed upon treatment with siZBP-89 (Fig. 8) and compared to previous results (Fig. 4). Myoblasts demonstrated low levels of Rb mRNA which increased in myotubes, analogous to results obtained previously (Fig. 8D compared to 4A). siZBP-89 drastically reduced Rb expression to levels even lower than those detected in myoblasts or controls thereof. A substantial decrease in cyclinA mRNA levels was noted in myotubes or myotubes plus the siControl compared to myoblasts and its similar control (Fig. 8C). Elimination of ZBP-89 further increased cyclinA levels in both myoblasts or myotubes. Previously, cyclinD1 mRNA levels were shown to decline after infection with ZBP-89; therefore, we determined what effect elimination of ZBP-89 would have on cyclinD1 (Fig. 8D). CyclinD1 mRNA levels were high in myoblasts and demonstrated a substantial decrease in myotubes. Interestingly, the level of cyclinD1 mRNA in myoblasts or myotubes treated with siZBP-89 was ½ that level of mRNA expressed in myoblast cells. Thus, the blockage of myogenesis by the elimination of ZBP-89 led to low levels of Rb mRNA and corresponding higher levels of cyclinA1 or cyclin D1 mRNA in both myoblasts and myotubes treated with siZBP-89.

4. Discussion

This current study presents compelling evidence that the transcription factor ZBP-89 is differentially expressed during C2C12 differentiation and contributes substantially to the C2C12 myogenic program. Infection with Ad-ZBP-89 induced the fusion of C2C12 myoblast cells in growth media and inhibited cell proliferation (Figs. 1 and 2). Over-expression of ZBP-89 induced the bHLH regulatory factors MRF4, myogenin, and MyoD, and the cell cycle regulatory proteins p21 and Rb (Figs. 3, 4 and 5), factors known to contribute to the differentiation of myoblasts into myotubes [37,44,45,46]. ZBP-89 down-regulated Pax7 (Fig. 3), as well as cyclinD1 and cyclinA (Fig. 5), all factors known to decline during myogenesis [43]. However, elimination of ZBP-89 via siRNA down-regulated the bHLH proteins and cell cycle regulators that were increased with ZBP-89 over-expression. Thus, ZBP-89 enhanced the myogenic program in C2C12 cells by affecting the expression of a variety of muscle-specific transcription factors and cell cycle regulatory proteins.

These results suggest the following model for the role of ZBP-89 in C2C12 myogenesis (Fig. 8). From qPCR and ChIP analyses, ZBP-89 can bind to the Pax7 promoter to repress Pax7 transcription (Figs. 3 and 4). Pax7 has been shown by others to up-regulate Myf5 expression and in turn together these factors inhibit C2C12 differentiation [10]. Thus, ZBP-89 repression of Pax7 mRNA expression would enhance myogenic progression. To further promote differentiation, ZBP-89 binds and activates the MRF4 enhancer and as well as a H4TF-1 element within the MyoD enhancer (Figs. 3 and 4) [33,34]. MyoD acts downstream to indirectly induce p21 expression by a previously unknown mechanism. [40]. Here, we show that ZBP-89 can bind to the p21 promoter via a binding site at −274 adjacent to a known E-box sequence.

Perhaps ZBP-89 enhances the recruitment of MyoD to the p21 promoter. In nonmuscle cell lines, ZBP-89 recruits p300 to the p21 promoter; therefore, MyoD, ZBP-89 and p300 may be part of a multi-protein complex to activate p21 expression during differentiation [25]. p21 and p57 then up-regulate myogenin expression [41]. Via a different mechanism, MyoD also activates myogenin expression during differentiation [40]. In summary, ZBP-89 indirectly up-regulates myogenin expression by up-regulating the bHLH factors that act upstream and can assist myoblast differentiation by down-regulating Pax7 and up-regulating MRF4, MyoD and p21.

By increasing the bHLH myogenic regulatory factors, ZBP-89 also indirectly regulated Rb and cyclinD1. The phosphorylation status of Rb correlates with cell proliferation where differentiation and hypophosphorylation is associated with the lack of cell growth [44]. Rb and MyoD have been shown to directly interact to contribute to Rb hypophosphorylation, suppression of myoblast cell growth, exit from cell cycle, and cellular differentiation [47]. Rb also cooperates with MyoD to activate the transcriptional activation domain (TAD) of MEF2 [4]. Thus, activation of Rb expression is part of the mechanism whereby ZBP-89 can contribute to myoblast differentiation. In contrast, cyclinD1 declines during myogenesis where it is the predominant cyclin controlling progression through the G1 phase by interacting with cdk4 [4,37,43]. Forced expression of cyclinD1 inhibits myogenesis [43,46,48]. ZBP-89 contributed indirectly to cyclinD1 down-regulation during myogenesis, since ChIP analysis demonstrated no detectable levels of ZBP-89 bound to relevant regions of the cyclinD1 promoter (from −635 to transcriptional start). ZBP-89 might regulate the expression of a yet unknown protein that regulates cyclinD1 expression. The indirect down-regulation of cyclinD1 is another mechanism whereby ZBP-89 contributes to muscle differentiation.

Several possibilities may explain how ZBP-89 functions as a transcriptional activator and repressor during differentiation. Previous studies suggest ZBP-89 is modified by post-translational modifications [49]. ZBP-89 contains multiple phosphorylation sites for a number of different kinases such as CamKII and PKC-ζ, which are important to the myogenic program [50]. We and others have detected multiple ZBP-89 bands in Western blots of extracts prepared from non-muscle cells grown on ^{32}P-orthophosphate (Wu unpublished observation) [19,30]. Thus, ZBP-89 could be selectively phosphorylated during myogenesis by a kinase such as CaMKII. CaMKII has been shown to phosphorylate and regulate HDAC4 localization to promote differentiation [50]. In non-muscle cells ZBP-89 repression involves HDAC1; however, similar studies have yet to be conducted in muscle cells [26]. The ability of ZBP-89 to function as an activator or repressor could depend on direct modification of ZBP-89 or its required co-activators or co-repressors. In each case, post-translational modifications might initiate this process at the onset of serum withdrawal. ZBP-89 and these factors could act in an auto-regulatory loop, since the mouse ZBP-89 (Zfp148) promoter contains a number of putative myogenic factor binding sites for MEF-2 and MyoD and promoter-Luc constructs demonstrated increased activity in myotubes versus myoblast cells [27].. These myogenic factors and ZBP-89 could act in a regulatory loop to further promote each other's expression during differentiation. Furthermore, ZBP-89 has been shown to be selectively sumoylated at two separate locations controlling its function as a repressor versus a weak activator of transcription [49]. However, in this case regulation by sumoylation was shown for DNA binding sites where ZBP-89 and Sp1 compete for binding to the same DNA element and not for examples where Sp1 and ZBP-89 bind to separate elements. Further studies are required to determine how post-translational modifications control the ability of ZBP-89 to function as an activator or repressor during C2C12 myogenesis.

While this study has been conducted within the C2C12 myogenic cell line, its correlation to *in vivo* embryogenesis warrants further investigation. ZBP-89 over-expression or elimination via siRNA regulates the activity of many proteins that are key regulators of the myogenic

program and cell cycle withdrawal, i.e. MRF4, MyoD, cyclinD1, Rb and p21. The manipulation of ZBP-89 during embryogenesis could have a widespread effect on skeletal muscle differentiation and embryogenesis, since knockout of ZBP-89 results in embryonic lethality by day 10.5. Previous Northern blots suggested that ZBP-89 mRNA is up-regulated at day 12–14 in mouse embryos and then declines in newborns and adults once differentiation is complete [21]. In our study notable differences in ZBP-89 mRNA levels were found during differentiation. These results were supported by previous studies from a group that found a 3-fold increase in the activity of a transiently transfected ZBP-89 promoter construct in C2C12 myotubes compared to myoblasts [27]. However, this same group found there were no changes in ZBP-89 mRNA levels via Northern blot analysis [21]. Our results may differ due to the sensitivity of qPCR compared to Northern blots where it is difficult to accurately quantitate the total level of the three ZBP-89 mRNA isoforms. Our ZBP-89 primers were designed to a conserved 5′-region, thus all ZBP-89 mRNA molecules are included in our measurements. Since ZBP-89 can be either an activator or a repressor of gene expression, ZBP-89 protein levels are not required to decline during differentiation to decrease expression of a target gene like β-enolase [21]. Changes in ZBP-89 activity may occur by protein modification and/or differential interaction with other known ZBP-89 partners such as Sp1, HDAC1 or p300 on different promoters [19,25,26,49]. Furthermore, preliminary data suggested ZBP-89 also affects cardiac development (Salmon unpublished observation) [21] and hematopoiesis [28, 29]. Finally, ZBP-89 may also be important in gamete production as suggested by mouse knockout studies, although this result may be dependent on the knockout mechanism [28,51]. In conclusion ZBP-89 appears to play key roles in several developmental programs and conditional knockouts are required to further investigate these mechanisms *in vivo*. However, ZBP-89 plays a major role in differentiation of the C2C12 myogenic cell line, which likely extends to myogenesis *in vivo*.

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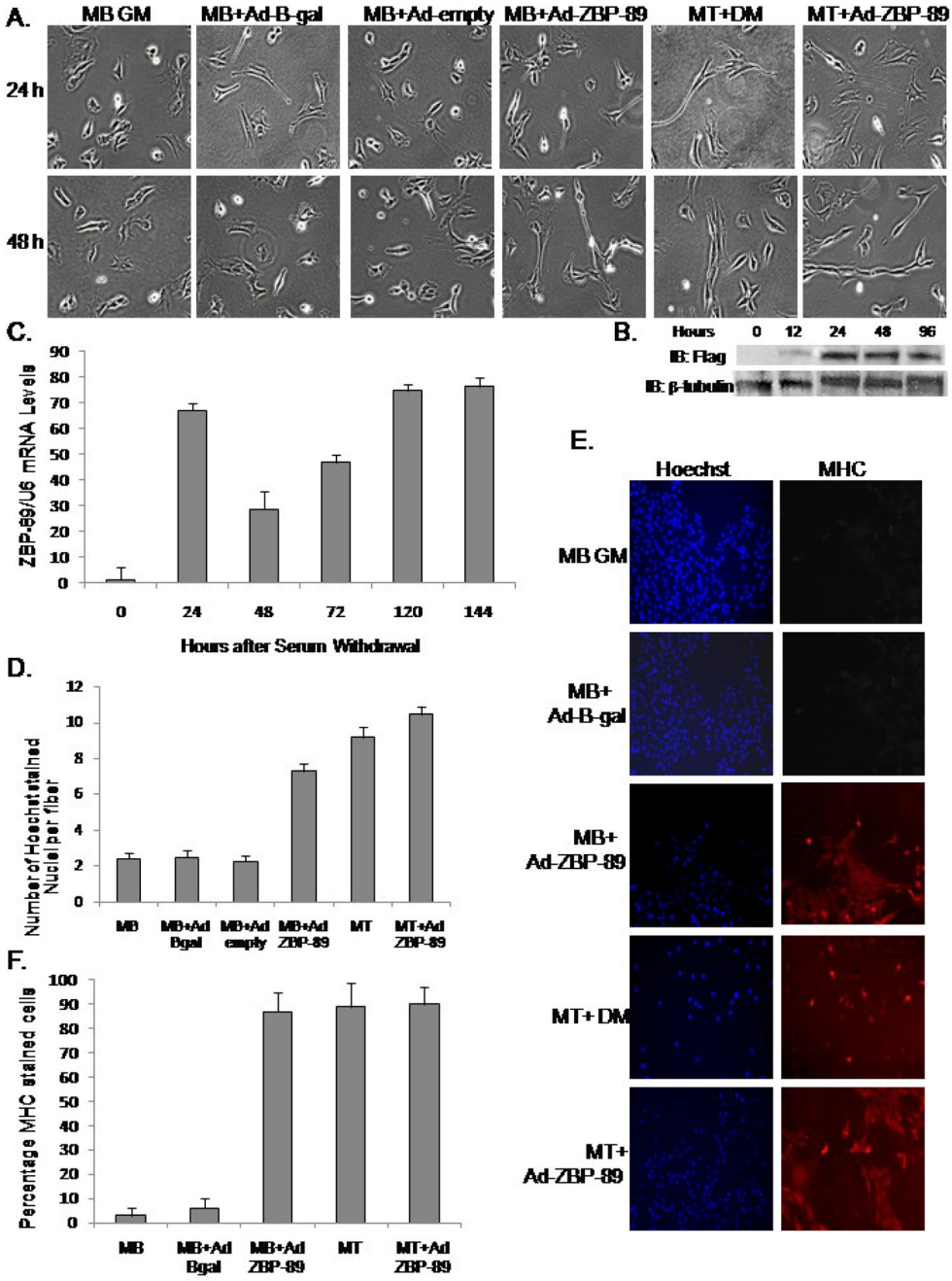

Fig. 1.

Over-expression of ZBP-89 enhances the myogenic program in C2C12 skeletal muscle cells. (A) C2C12 myoblast cells (MB) were plated at 1×10^4/well, incubated in GM (DMEM plus 10% FBS) or differentiated to myotubes (MT) in DM (DMEM plus 1% FBS) plus either Ad-β-gal, Ad-empty vector or Ad-ZBP-89 for 24 and 48 h, and visualized using a light microscope at 20X magnification. Representative fields from 3 independent experiments are shown. (B) Western blot analyses of WCEs (50 μg) isolated from C2C12 cells as grown in Fig. 1A for 0, 12, 24, 48 and 96 h as described in Materials and methods. Antibodies used for immunoblotting (IB) are indicated at dilutions of 1:1000 and β-tubulin is included as a loading control. (C) qPCR analysis of endogenous ZBP-89 mRNA levels in MBs plated for 24 h and harvested or

MTs switched to DM and harvested at the indicated times. Details of qPCR conditions are discussed in Materials and methods. The y-axis represents the relative ZBP-89 mRNA levels normalized to U6 expression. Results are graphed with MB set as 1x and are the average of three separate experiments performed in triplicate with bars representing the standard error of the means (S.E.). (D) Graphical analysis of cells infected with Ad-β-gal, Ad-empty or Ad-ZBP-89 and stained with Hoechst to determine the number of nuclei per muscle fiber. Results are from three separate, independent experiments. (E.) MBs, MTs and Ad-infected cells were co-stained with Hoechst and MHC and then visualized via fluorescence microscopy. Results are representative of three independent experiments. (F.) Quantitation of microscopy results from part E. Results are graphed as the percentage of Hoechst stained cells that also express MHC.

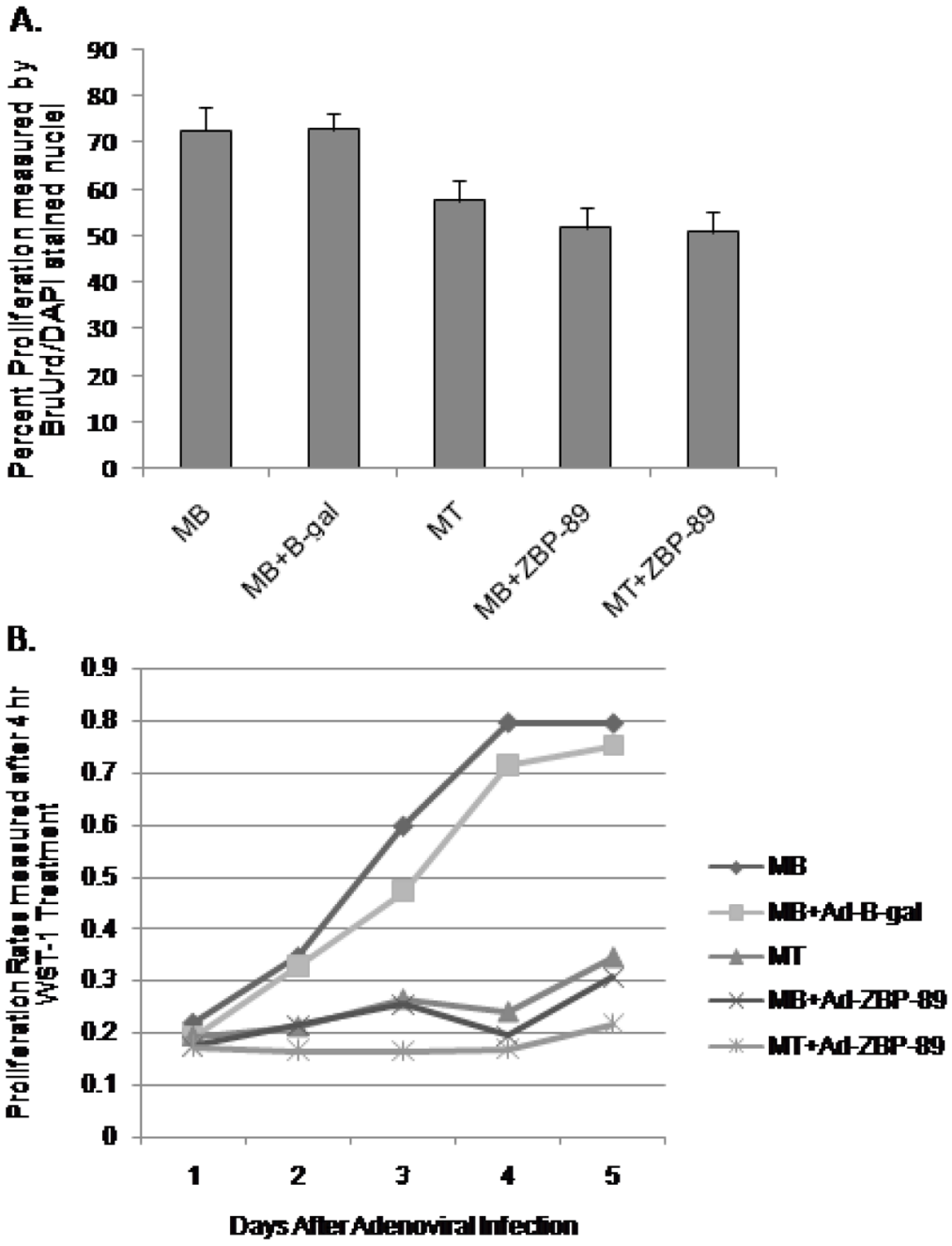

Fig. 2.

Over-expression of ZBP-89 in C2C12 myoblasts suppresses DNA synthesis and proliferation. (A) C2C12 MBs were plated at 1×10^4 and incubated in GM or DM and infected with either Ad-ZBP-89 or Ad-β-gal for 24 h. DNA synthesis was monitored following incubation of cells with BrdUrd for 5 h. Cells were grown on coverslips, followed by fixation with methanol-acetone and anti-BrdUrd staining. Cell nuclei were visualized by DAPI. BrdUrd-labeled cells were counted relative to the number of nuclei. The results presented were derived from the counting of at least 250 cells from three independent experiments and bars represent the S.E. (B) C2C12 MBs were plated at 5×10^2 and incubated in GM or DM infected with either Ad-ZBP-89 or Ad-β-gal. Cell growth was measured at 1, 2, 3, 4, and 5 days using the WST-1

proliferation reagent incubated for 4 h and recorded as the optical density (OD) at 490nm (y axis). The mean from three independent experiments was graphed.

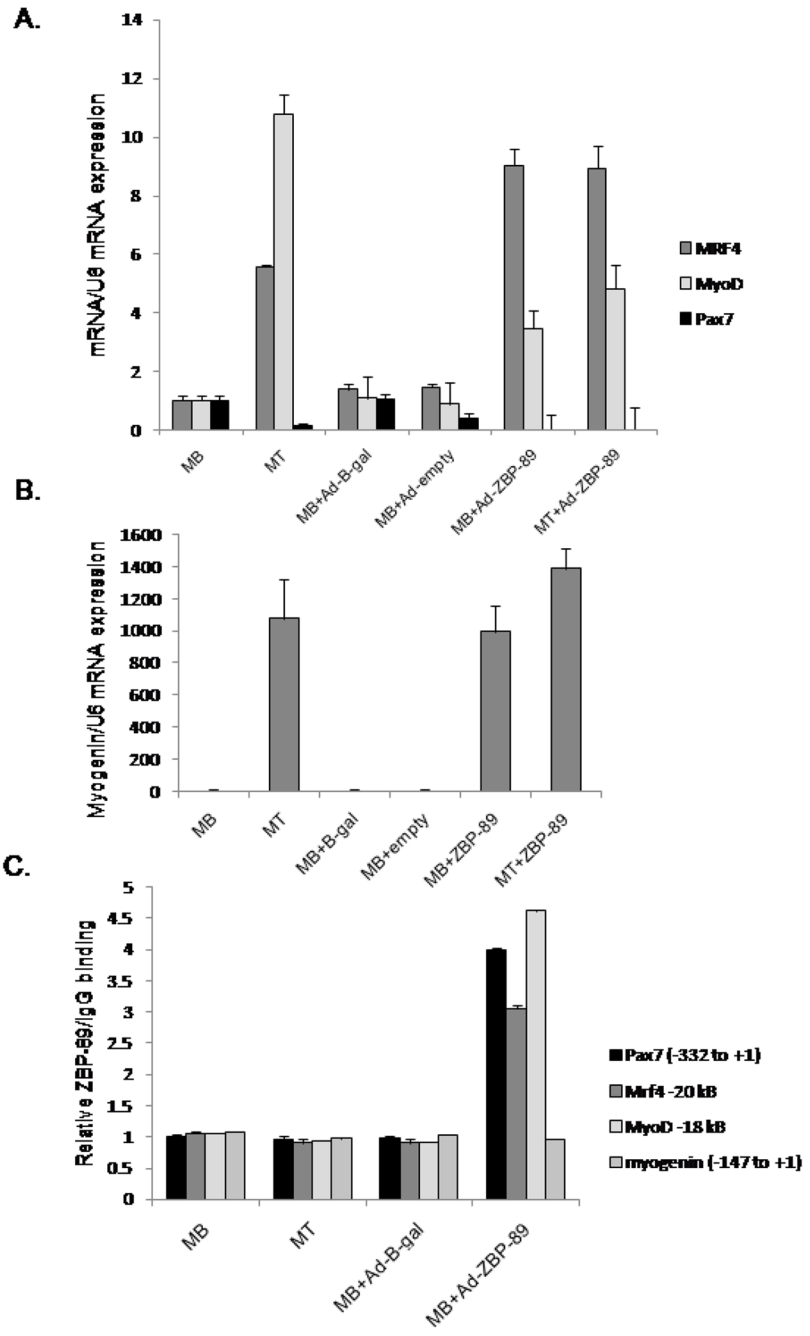

Fig. 3.

ZBP-89-dependent modulation of myogenic regulatory factor expression. (A) qPCR analysis of endogenous MRF4, Pax7, and MyoD mRNA levels in C2C12 MBs, MTs, MBs plus Ad-β-gal, Ad-empty vector, or Ad-ZBP-89 plus Ad-ZBP-89 isolated 48 h after infection as described in Materials and methods. The y-axis represents the relative mRNA levels normalized to U6 expression and set relative to MBs as 1x. Results are the average of three separate experiments performed in triplicate with bars representing the S.E. (B) qPCR analysis of endogenous myogenin mRNA levels as described in part A. (C) ChIP analysis of ZBP-89 binding over the Pax7 promoter region (−632 to −333), the MRF4 enhancer region [34], the MyoD enhancer region [33], and the myogenin proximal promoter region (−332 to +1) in MBs, MTs, and MBs

plus Ad-ZBP-89 or Ad-β-gal 48 h after infection as described in Materials and methods. Results obtained with IgG versus Flag-tagged antibody immunoprecipitation are shown.

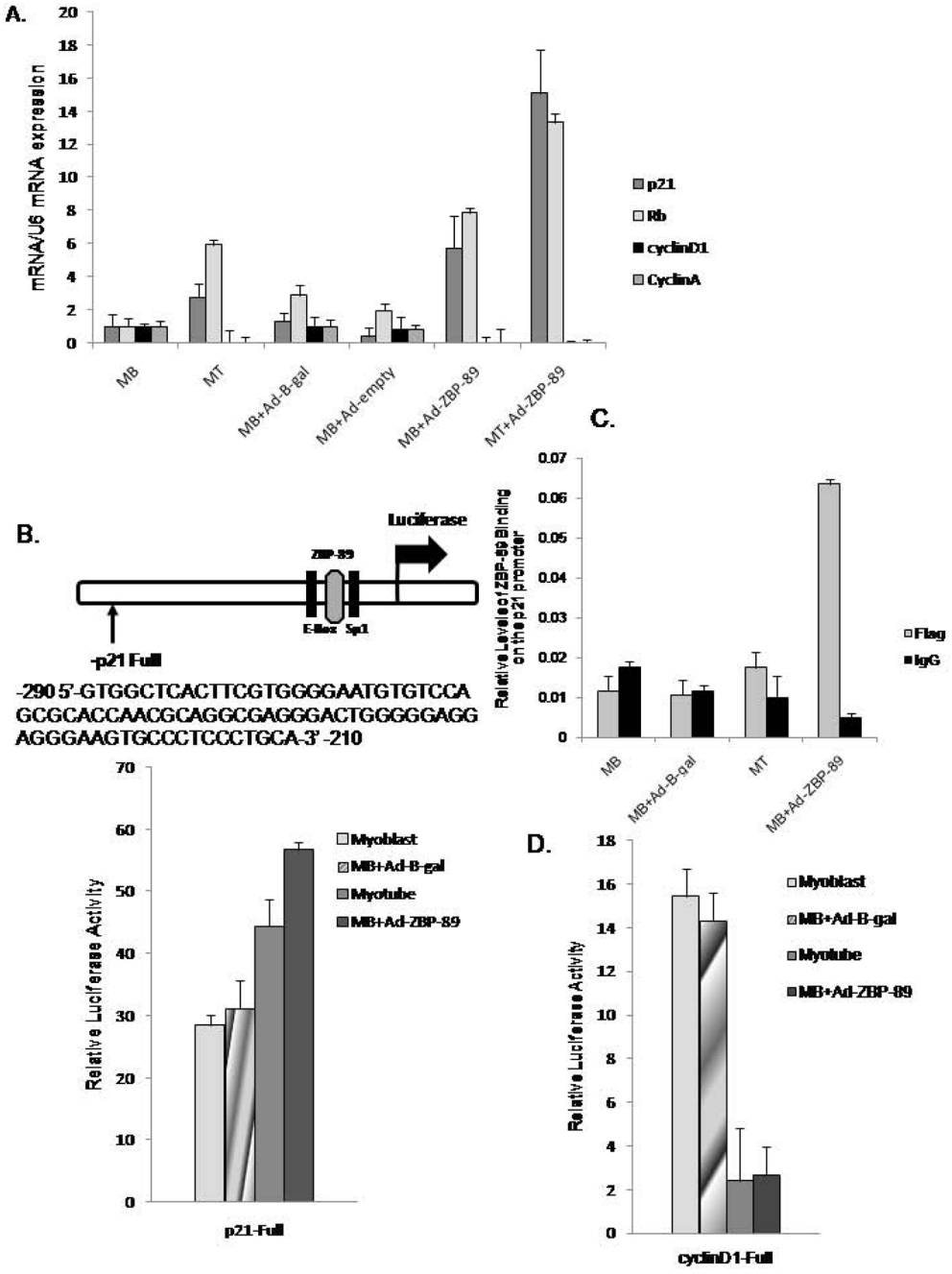

Fig. 4.

ZBP-89 regulates cell cycle protein expression and binds to DNA control regions of the p21 promoter. (A) qPCR analysis of endogenous p21, Rb, cyclinD1 and cyclinA as prepared in Fig 3A. mRNA was normalized to U6 mRNA expression. Results are graphed with MBs set as 1x and are the average of three separate experiment performed in triplicate with bars representing the standard errors of the mean. (B) A p21 promoter (full promoter containing a E-box, ZBP-89 and Sp1 binding sites as shown) luciferase (p21Luc) construct was transiently transfected into C2C12 cells and reporter gene activity measured in MBs, MBs+Ad-β-gal or Ad-ZBP-89 and MTs as described in Material and methods. Results are the average of three separate experiments performed in triplicate on two separate plasmid preps and bars represent the S.E.

(C) ChIP analysis of ZBP-89 binding over the endogenous p21 promoter in MBs, MTs, and MBs plus Ad-β-gal or Ad-ZBP-89 as described in part A. (D) A cyclinD1 (full promoter) luciferase construct was transiently transfected into C2C12 cells and reporter gene activity measured as described in Material and methods. Results are the average of three separate experiments performed in triplicate on two separate plasmid preps and bars represent the S.E.

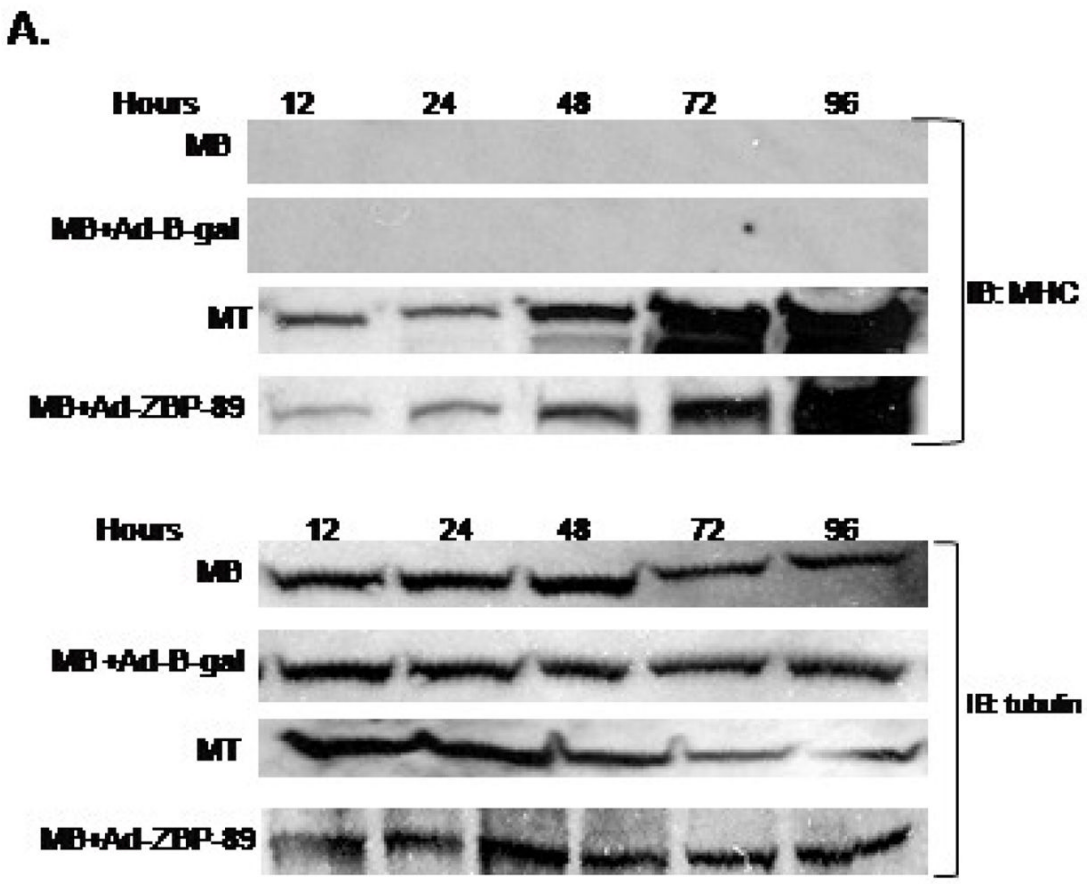

Fig. 5.

ZBP-89 modulates MHC expression during myogenesis. (A) Western blot analysis of WCEs (50 ug) isolated from C2C12 cells as grown in panel 1A for 12, 24, 48, 72 and 96 h as described in Materials and methods. Antibodies used for immunoblotting (IB) are indicated at dilutions of 1:1000. β-tubulin served as a loading control.

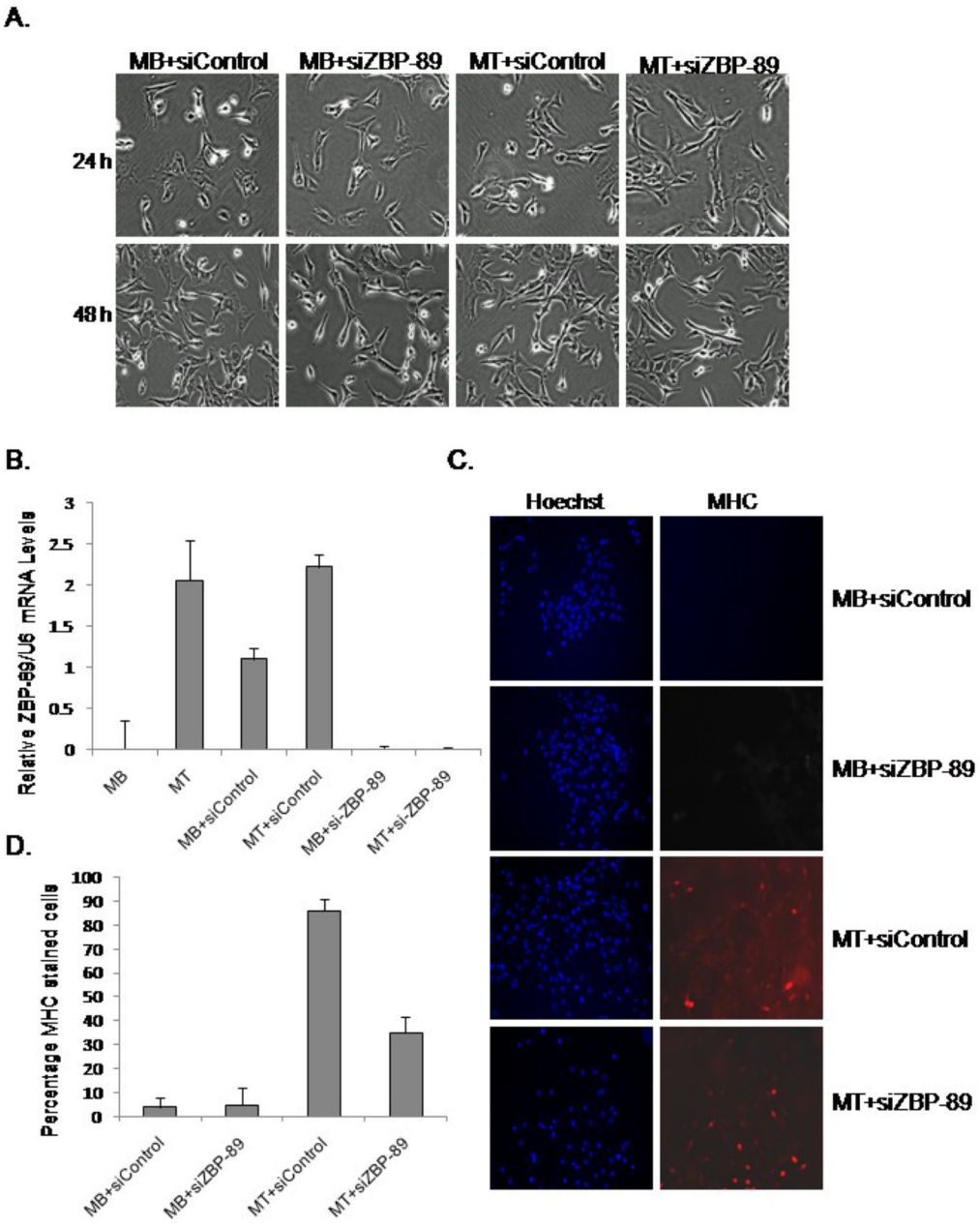

Fig. 6.

Elimination of ZBP-89 via siRNA in C2C12 myoblast cells. (A) C2C12 MBs were plated at 8×10^4, incubated in GM or DM, transfected with siControl or siZBP-89 for 24 and 48 h, and visualized using a light microscope. Representative fields are shown from three independent experiments. (B) qPCR analysis of endogenous ZBP-89 mRNA levels prepared as described in Fig. 1C. The relative ZBP-89 mRNA levels were normalized to U6 expression and graphed relative to MBs set as 1x. Results are the average of three separate experiments performed in triplicate with bars representing the S.E. (C) MB, MT and infected cells were co-stained with Hoechst and MHC and then visualized via fluorescence microscopy. A representative image from three independent experiments is shown.. (D) Quantitation of microscopy results from part C graphed as the percentage of Hoechst stained cells that also express MHC.

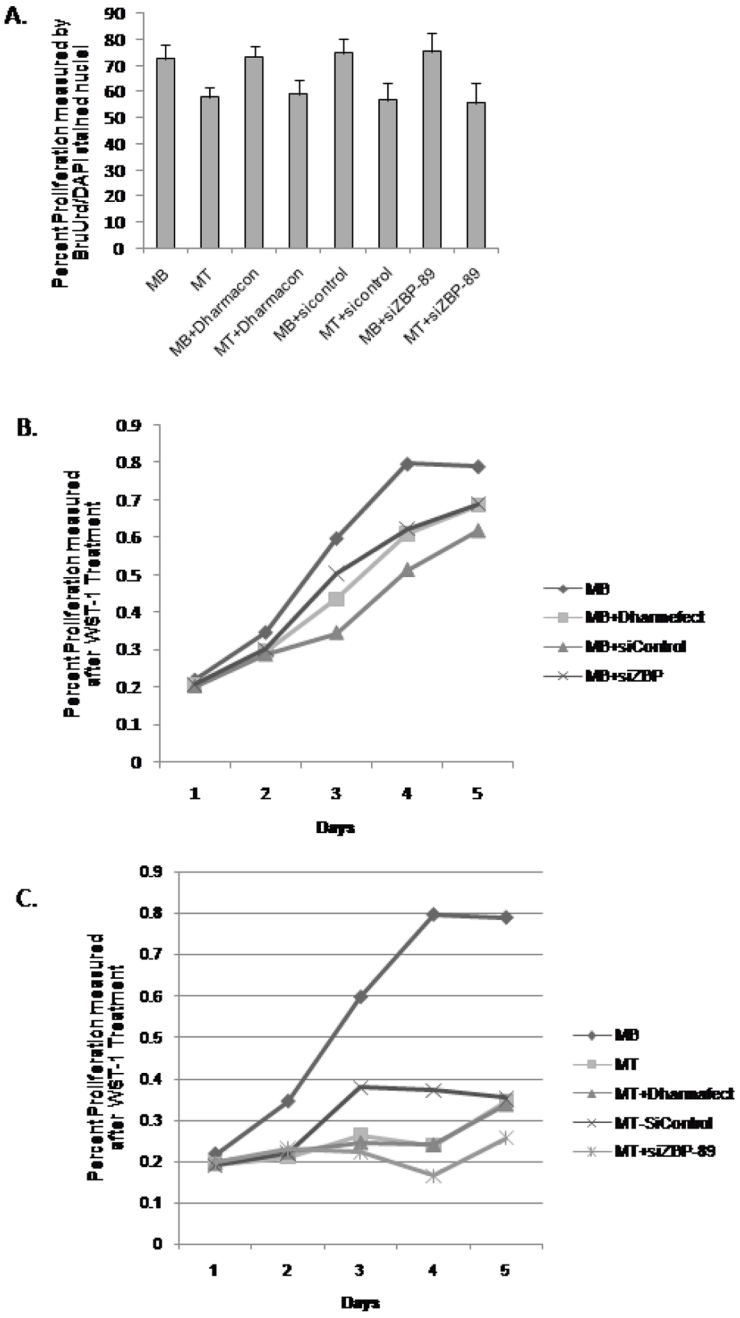

Fig. 7.

Effect of siZBP-89 on C2C12 proliferation. (A) C2C12 MBs were plated at 1×10^4 and incubated in GM alone or transfected with either transfection reagent alone (Dharmafect), siControl or siZBP-89 for 24 h and DNA synthesis was determined following incubation of cells with BrdUrd for 5 h as described previously in Fig. 2A. (B) C2C12 MBss were plated at 5×10^2 and incubated in GM for MBs and transfected with transfection reagent alone, siControl or siZBP-89. Cell growth was determined at 1, 2, 3, 4, and 5 days after infection by the addition of the WST-1 proliferation reagent for 4 h. The mean from three independent experiments was graphed. (C) C2C12 MBs were plated at 5×10^2 and incubated in GM for MBs or switched to

DM for MTs and transfected with either transfection reagent alone, siControl or siZBP-89 as described in B.

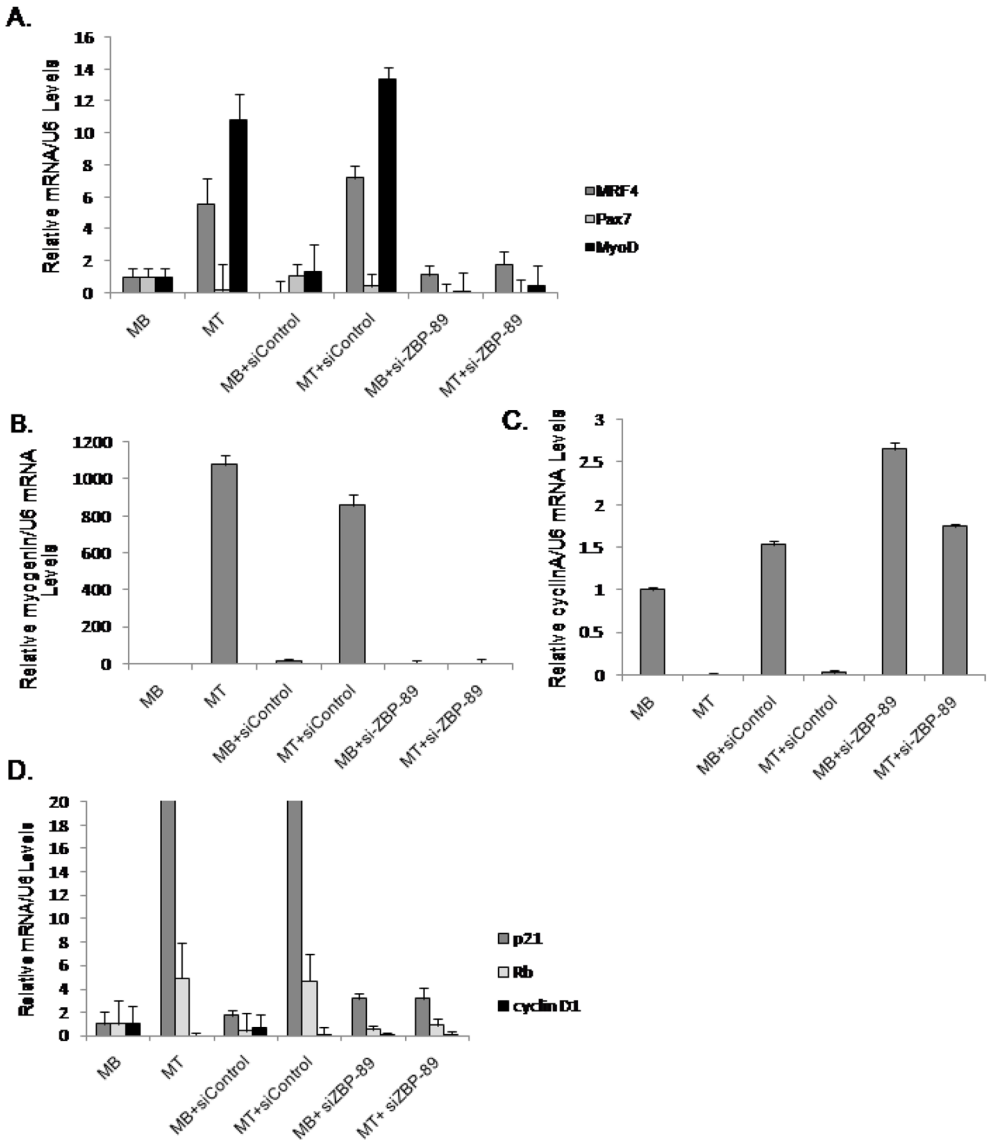

Fig. 8.

Elimination of ZBP-89 affects expression of bHLH regulatory factors and cell cycle proteins (A) qPCR analysis of endogenous MRF4, Pax7 and MyoD mRNA levels prepared as described in Fig. 3A. The relative mRNA levels were normalized to U6 expression and graphed relative to MBs set as 1x. Results are the average of three separate experiments performed in triplicate with bars representing the S.E. (B) qPCR analysis of endogenous myogenin mRNA levels prepared as mentioned in part A. (C) qPCR analysis of endogenous cyclinA mRNA levels prepared as mentioned in part A. (D) qPCR analysis of endogenous p21, Rb and cyclinD1 mRNA levels prepared as mentioned in part A.

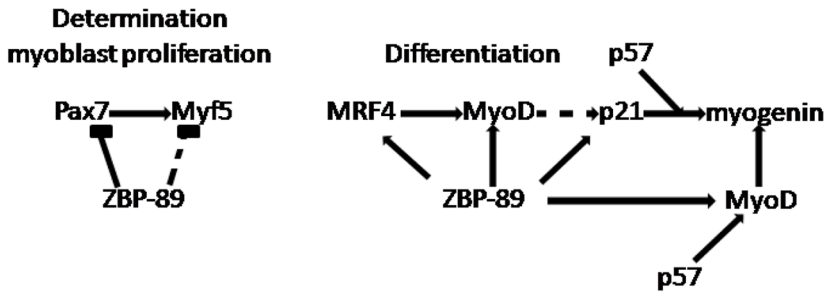

Fig. 9.
Model of ZBP-89 regulation during myogenesis. (A) Proposed model of ZBP-89 regulation during *in vitro* myogenesis of C2C12 cells.