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The brain expressed x-linked gene 1 (Bex1) regulates myoblast fusion

Chunhui Jiang[#1], **Jing-Hua Wang**[#1], **Feng Yue**[1], and **Shihuan Kuang**[1,2,*]

[1] Department of Animal Sciences, Purdue University, West Lafayette, IN 47907

[2] Center for Cancer Research, Purdue University, West Lafayette, IN 47907

[#] These authors contributed equally to this work.

Abstract

Skeletal muscle development (myogenesis) is a complex but precisely orchestrated process involving spatiotemporal regulation of the proliferation, differentiation and fusion of myogenic progenitor cells (myoblasts). Here we identify brain expressed x-linked gene 1 (Bex1) as a transient, developmentally regulated gene involved in myoblast fusion. Bex1 expression is undetectable in adult muscles or in quiescent muscle stem cells (satellite cells). During embryonic myogenesis, however, Bex1 is robustly expressed by myogenin^{+} differentiating myoblasts, but not by Pax7^{+} proliferating myoblasts. Interestingly, Bex1 is initially localized in the cytoplasm and then translocates into the nucleus. During adult muscle regeneration, Bex1 is highly expressed in newly regenerated myofibers and the expression is rapidly downregulated during maturation. Consistently, in cultured myoblasts, Bex1 is not expressed at the proliferation stage but transiently expressed upon induction of myogenic differentiation, following a similar cytoplasm to nucleus translocation pattern as seen in vivo. Using gain- and loss-of-function studies, we found that overexpression of Bex1 promotes the fusion of primary myoblasts without affecting myogenic differentiation and myogenin expression. Conversely, Bex1 knockout myoblasts exhibit obvious fusion defects, even though they express normal levels of myogenin and differentiate normally. These results elucidate a novel role of Bex1 in myogenesis through regulating myoblast fusion.

Keywords

skeletal muscle; myogenesis; myoblasts; regeneration

Introduction

Under normal situation, mammalian adult skeletal muscle is relatively stable with minimal nuclei turnover, no more than 1-2 percent per week (Schmalbruch and Lewis, 2000).

[*]Correspondence: Shihuan Kuang, Ph. D., 174B Smith Hall, 901 West State Street, West Lafayette, IN 47907, USA, Phone: 765-494-8283, Fax: 765-494-6816, skuang@purdue.edu.

However, skeletal muscle is susceptible to a variety of injuries. Upon injury, skeletal muscle has the outstanding capacity to initiate a rapid and extensive repair process, namely muscle regeneration, to prevent further muscle loss and maintain muscle mass. Of note, muscle stem cells, or satellite cells, play an indispensable role in muscle regeneration (Sambasivan et al., 2011; von Maltzahn et al., 2013). In the early stage of muscle regeneration, satellite cells are activated from quiescence and proliferate as myoblasts to generate a sufficient number of cells. Subsequently, the proliferating myoblasts withdraw from the cell cycle and fuse to the injury sites to repair muscle damage. As muscle regeneration is a complex and highly orchestrated process, unraveling the regulatory network governing muscle regeneration has drawn intense research attention in regenerative biology.

Myoblast fusion is a crucial cellular process contributing to muscle regeneration as well as muscle growth and development. Myoblast fusion is characterized by cell attraction, migration, adhesion, and alignment followed by the membrane rearrangement and finally resolution (Doberstein et al., 1997). The fusion process occurs through two phases. The first stage leads to the formation of nascent myotubes with few nuclei from myoblast-myoblast fusion. The second stage results in the formation of large syncytia with increased nuclear number and augmented myotube size from myoblast fusion with nascent myotubes (Horsley and Pavlath, 2004). Much progress has been made in unraveling signaling pathways underlying myoblast fusion in Drosophila, that occurs between two genetically different cell subpopulations of founder and fusion-competent myoblasts (Baylies et al., 1998). Of note, the ELMO - Myoblast city - Rac pathway has been shown to play an essential role in myoblast fusion (Duan et al., 2012; Geisbrecht et al., 2008; Rushton et al., 1995). Intriguingly, this signaling pathway is well conserved between Drosophila and vertebrates. It has been reported that ELMO - DOCK1 (ortholog of Myoblast city) - Rac also coordinately control the myoblast fusion in mice (Laurin et al., 2008). Furthermore, the ELMO-DOCK1-Rac pathway is under the control of brain-specific angiogenesis inhibitor (BAI) family members, including BAI1 and BAI3, both of which have been corroborated to promote myoblast fusion (Hamoud et al., 2014; Hochreiter-Hufford et al., 2013). Recently, a muscle-specific plasma membrane protein, myomaker, has been identified to directly participate in the myoblast fusion process (Millay et al., 2013). Although these factors have significantly filled the gap in understanding the fundamental process of myoblast fusion, the regulatory network controlling myoblast fusion in vertebrates remains largely elusive.

Bex1 belongs to a small growing family including six members with high homology in gene sequences and structures but distinct in their expression patterns and subcellular localization (Alvarez et al., 2005). Until now, the functions of Bex1 have been largely unknown. Bex1 has been recently proposed to play key roles in the formation of multiple signaling network hubs (Fernandez et al., 2015). In particular, Bex1 has been identified as a regulator of neuron regeneration, as Bex1 knockout mice are deficient in axon regeneration after sciatic-nerve injury (Khazaei et al., 2010). In addition, Bex1 levels are cell-cycle dependent in PC12 neuronal cells, with the lowest expression level in G1 phase and the highest level in S phase. Moreover, down-regulation of Bex1 is necessary for the cell cycle exit of neural progenitor cells, as overexpression of Bex1 results in sustained proliferation even under growth-arresting conditions. Further studies have confirmed that Bex1 regulates cell cycle by interacting with p75 neurotrophin receptor (p75NTR) to regulate the downstream

signaling pathway (Vilar et al., 2006). Besides its roles in the nervous system, Bex1 has been identified as a candidate tumor suppressor gene because its inactivation is associated with the development of various types of tumors (Foltz et al., 2006; Karakoula et al., 2014; Lee et al., 2013).

The implication of Bex1 in muscle regeneration was discovered by microarray analysis aimed to identify genes whose expression is altered by cardiotoxin (CTX)-induced muscle injury, a well-established model to study muscle regeneration (Goetsch et al., 2003; Yan et al., 2003). A potential role of Bex1 in muscle regeneration is further suggested by the observation Bex1 expression is dysregulated in the mdx mice (Turk et al., 2005), which undergo progressive muscle degeneration and regeneration and are widely used as a model to study Duchenne Muscular Dystrophy. Furthermore, Bex1 knockout mice displayed defective muscle regeneration, manifested by prolonged proliferation and delayed differentiation of myogenic cells (Koo et al., 2007). However, how Bex1 regulates myoblast behavior and function is still unclear.

In this study, we first examined the expression pattern of Bex1 during embryonic and adult myogenesis. We found that Bex1 is transiently expressed by newly differentiated myoblasts and its expression pattern undergoes dynamic cytoplasmic-nuclear trafficking. We further used gain- and loss-of-function studies to determine how alternations of Bex1 levels affect myogenesis. We found that Bex1 promotes myoblast-myotube fusion without affecting myogenic differentiation per se. These data suggest that Bex1 is temporally regulated during myogenesis to promote myoblast fusion.

Materials and methods

Animals

All procedures involving animal maintenance and experimental use were performed based on the instructions established by Purdue University's Animal Care and Use Committee. Bex1 KO mice were provided by Prof. Frank L. Margolis (Koo et al., 2007). Bex1 heterozygous mice were bred to generate Bex1 null and wild type littermates used as controls in the experiments. The PCR genotyping was done as previously described (Koo et al., 2007).

Muscle injury and regeneration

Muscle regeneration was induced by intramuscular injection of Cardiotoxin (CTX; Sigma-Aldrich, St. Louis, MO). Mice were anesthetized with a ketamine-xylazine cocktail, then 50μl of 10 mM CTX was injected into the Tibialis Anterior (TA) muscle. Muscles were harvested at day 5 and 14 post injection for histological studies. Age- and gender-matched control mice were used for each experiment.

Culture of skeletal muscle derived primary cells

Primary cells were isolated from limb skeletal muscles of 2-month old mice. Muscles were minced and digested with a cocktail of type I collagenase and dispase B mixture (Roche Applied Science) and subsequently cultured in growth media (F-10 Ham's medium

supplemented with 20% fetal bovine serum, 4 ng/mL basic fibroblast growth factor, and 1% penicillin-streptomycin) on collagen-coated dishes. Upon confluence, cells were differentiated in myogenic differentiation medium (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin).

Cryosection

Fresh muscles were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek) and immediately frozen in dry ice-cooled isopentane. Muscle blocks were cut at 10μm with a Leica CM 1850 cryostat instrument. The sections were placed on Superfrost Plus glass slides (Electron Microscopy Sciences).

Immunostaining and image capture

Muscle tissue sections and cell cultures were first fixed in 4% PFA solution and blocked in the blocking buffer containing PBS, 5% goat serum, 2% bovine serum albumin, 0.2% Triton X-100 and 0.1% sodium azide for 1 hour. Next, the sections or cell cultures were incubated with primary antibodies diluted in blocking buffer overnight at 4°C, then incubated with secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI) diluted in PBS for 30 minutes at room temperature, and finally mounted with Dako fluorescent mounting media (Glostrup, Denmark). Fluorescent pictures were taken with a Coolsnap HQ CCD camera (Photometrics, USA) driven by IP Lab software (Scanalytics, USA) in a Leica DMI 6000B fluorescent microscope (Mannheim, Germany). As the analysis of the immunofluorescence was qualitative, identical image handling and fluorescence scoring criteria were applied in all the experiments.

Recombinant adenovirus construction and infection

Recombinant adenoviruses expressing Bex1 were constructed using the Adeasy system, including the adenoviral plasmid (pAdEasy-1) and the shuttle vector (pAdTrack-CMV), both of which were kindly provided by Prof. Yongxu Wang. The pAdTrack-CMV vector contains two separate CMV promoters driving the expressions of GFP and Bex1 independently. The sequence of Bex1 was PCR amplified and cloned into the pAdTrack-CMV vector. The recombination with the Adeasy1 plasmid and transfection to HEK293 cells as well as amplification of the recombinant adenovirus were performed as previously described (He et al., 1998). For infection experiments, myoblasts were grown to 80% confluence and infected with virus for 48h at 37°C.

Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted and purified from muscles or cell cultures using Trizol, followed by the digest with Turbo DNase (Ambion). Random hexamer primers were used for the reverse transcription from RNA to cDNA. qPCR was performed with a Light Cycler 480 machine (Roche). 18s was used as housekeeping gene for normalization. For qPCR result analysis, $2^{-\Delta\Delta ct}$ method was applied to calculate the fold change.

Protein Extraction and Western Blots Analysis

Total protein was extracted from muscles or cells using RIPA buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). Protein concentrations were measured by Pierce BCA protein assay reagent (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes (Millipore Corp., Billerica, MA), and probed with specific antibodies (Bex1, 1:5000 dilution, provided by Prof. Frank L. Margolis; Pax7, 1:10 culture supernatant, DSHB, U Iowa; myogenin, 1:1000 dilution, Santa Cruz Biotechnology; GAPDH, 1:1000 dilution, Santa Cruz Biotechnology), then detected by chemiluminescence with FluorChem™ R System (ProteinSimple, Santa Clara, CA, USA).

Statistical analysis

Data are displayed with mean ± s.e.m. P-values were calculated by two-tailed Student's t-test. P-values <0.05 were considered to be statistically significant.

Results

Transient expression of Bex1 during muscle development and regeneration

As the first step to understand the function of Bex1 in muscle development and regeneration, we sought to determine the expression of Bex1 using a previously validated polyclonal antibody (Koo et al., 2005). As Pax7 and Myog have been widely used to mark undifferentiated and differentiated myogenic progenitor cells, respectively (Cheng et al., 1992; Relaix et al., 2005; Venuti et al., 1995), we co-stained these proteins with Bex1 by immunohistochemistry to determine which cell population expresses Bex1 at different stages of myogenesis in mice. At embryonic day E10.5 when primary myogenesis begins (Biressi et al., 2007), most progenitors in the somites (dermomyotome) were $Pax7^+$, but none of them expressed Bex1 (Fig. 1A). However, Bex1 immunofluorescence was co-localized to a subpopulation of $Myog^+$ cells (Fig. 1B). Inspection of intracellular localization indicates that Bex1 is predominantly located in the cytoplasm at this stage (Fig. 1B'). Interestingly, at stage E12.5 when the primary myogenesis peaks (Biressi et al., 2007), Bex1 immunofluorescence signal is mostly localized in the nucleus (Fig. 1C). This nucleocytoplasmic dynamics is consistent with the previous study showing both nucleus and cytoplasm locations in Bex1-transfected HEK 293 cells (Behrens et al., 2003).

In addition to the myotome, Bex1 immunofluorescence was also detectable in the neural tube and gut of E12.5 embryos (Supplemental Fig. S1A). Furthermore, a small portion of $Bex1^+$ cells in the myotome were $Myogenin^-$ (Fig. 1B), indicating a distinct identity. Interestingly, we detected the co-localization of Bex1 and PDGFRa in the myotome of E15.5 embryos (Supplemental Fig. S1B), suggesting that Bex1 is expressed in the fibroadipogenic lineage cells. This is further confirmed by Bex1 mRNA quantification showing 3 times more abundance in fibroblasts compared to myoblasts (Supplemental Fig. S1C). Bex1 signal was undetectable in the muscle after E15.5 stage throughout embryonic and perinatal development (Supplemental Fig. S2). These results indicate that Bex1 is only

transiently expressed in differentiating or newly differentiated muscle cells during embryonic myogenesis.

We also examined the expression pattern of Bex1 during postnatal muscle regeneration. In the absence of muscle injury, Bex1 signal was undetectable in resting muscles (Fig. 2A, Day 0), suggesting that Bex1 is not required for maintaining normal muscle function. Upon CTX-induced muscle regeneration, Bex1 was highly induced in the cytoplasm of central-nucleated regenerating myofibers at Day 5 post injury, but its expression was again undetectable at Day 14 (Fig. 2A). Importantly, Bex1 was also expressed in mononuclear myogenic cells at Day 5, indicated by the co-localization of Bex1 and Myogenin (Fig. 2B). In contrast, Bex1 was not detected in any Myogenin$^+$ mononuclear cells at Day 3 (Fig. 2C), suggesting that Bex1 is turned on after Myogenin induction. This is in line with the expression pattern of Bex1 during embryonic muscle development. Consistent with this observation, Bex1 mRNA levels also exhibited a similar temporal expression pattern that peaks at Day 5 post CTX injury (Fig. 2D), when myoblasts have been extensively expanded and myoblast fusion peaks (Robertson et al., 1990). After this time point, Bex1 expression dropped rapidly and return to an undetectable level at Day 14 (Fig. 2D), when the muscle regeneration is largely completed. These data demonstrate that Bex1 is transiently expressed in nascent myotubes or newly regenerated myofibers during skeletal muscle development and regeneration, and suggest a role of Bex1 in myoblast fusion.

Bex1 is exclusively expressed in differentiated myoblasts during myogenic differentiation

To further model the dynamics of Bex1 expression in myogenesis, we examined the expression pattern of Bex1 in satellite cells during their activation, proliferation and differentiation in vitro under culture conditions. Under growth conditions, Bex1 is barely detectable in proliferating myoblasts (Fig. 3A, left). Within 24 h upon serum withdrawal induced differentiation, Bex1 is highly upregulated in the cytoplasm of a subpopulation of myoblasts (Fig. 3A, middle). At 3 days after differentiation, Bex1 expression is mainly located in the nucleus of newly formed myotubes (Fig. 3A, right). Thereafter, Bex1 expression gradually declines (data not shown). Quantitative PCR results indicate that Bex1 mRNA levels increased by nearly 10-fold within 24 h and by over 40-fold within 3 days after induced differentiation (Fig. 3B). The relative levels of Bex1 protein exhibited a similar trend of increase to Bex1 mRNA during differentiation of primary myoblasts (Fig. 3C). These results confirm that Bex1 expression is induced at the early stages of myogenic differentiation.

In addition, we performed single myofiber culture experiments to mimic satellite cell activation, proliferation and differentiation in vivo. After 72 hours in culture, satellite cells attached on their host myofibers typically form clusters of cells containing both proliferating and differentiating cells that are distinguishable by markers (Fig. 3D-F). Co-staining these clusters with Bex1 and Pax7 reveals that Bex1 was never expressed in undifferentiated Pax7$^+$ myoblasts (Fig. 3D). Similarly, Bex1 signal was never found in proliferating myoblasts that were Ki67$^+$ (Fig. 3E). However, Bex1 signal was readily detectable in both the cytoplasm and nucleus of Myog$^+$ differentiating cells (Fig. 3F). These data again support

the notion that Bex1 expression is absent in proliferating myoblasts but rapidly induced in newly differentiated myoblasts.

Bex1 promotes myoblast-myotube fusion without affecting differentiation

To directly investigate the role of Bex1 in myogenesis, we first performed gain-of-function studies on primary myoblasts through adenovirus-mediated overexpression. This approach led to roughly a 30 times increase in the Bex1 mRNA level in proliferating myoblasts (Fig. 4A). Overexpression of Bex1 had no effect on the proliferation of myoblasts, as the proportions of cells at G1, S, G2/M phase were indistinguishable between control and overexpression myoblasts (Supplemental Fig. S3A). After being induced to differentiate for 24 h, both GFP control and Bex1 overexpressed myoblasts started to form myosin heavy chain (MHC)-expressing nascent myotubes (Fig. 4B). However, the myotube size and differentiation index were indistinguishable between the control and Bex1 overexpression groups (Fig. 4B-C), suggesting that Bex1 does not affect the differentiation of primary myoblasts. Interestingly, the fusion indexes were also identical between the control and Bex1 overexpression groups (Fig. 4D), indicating that Bex1 does not regulate myoblast-myoblast fusion and the initial formation of myotubes.

Accompanying the first stage of myoblast-myoblast fusion is the second stage involving fusion of myoblasts to nascent myotubes, leading to increases in myotube size (hypertrophy). We next asked if Bex1 regulates myoblast-myotube fusion by examining myotubes at 96 h after induced differentiation. Strikingly, after differentiated for 96hs, Bex1 overexpression caused a dramatic accumulation of nuclei in myotubes, leading to apparent myotube hypertrophy (Fig. 5A). Using a quantitative measurement, we found that the percentages of myotubes with 5 or more nuclei was significantly increased by Bex1 overexpression, with a concomitant reduction of small myotubes containing 2-4 nuclei/myotube (Fig. 5B). The myotube hypertrophy was correlated to a higher level of Bex1 protein, but the levels of Myog or MHC protein were comparable between the control and Bex1 overexpression groups (Fig. 5C). We further corroborated the results in C2C12 myoblasts using electroporation mediated gene transfer, followed by 6 days of differentiation. Again, Bex1 overexpression led to apparent hypertrophy of the myotubes (Fig. 5D). The average number of myonuclei per myotube was 4.7 in the control cells, but this number increased to 9.4 in the Bex1 overexpressing myotubes (Fig. 5E). Altogether, these data suggest that Bex1 promotes myoblast-myotube fusion without affecting myogenic differentiation per se.

Bex1 knockout myoblasts are defective in myoblast-myotube fusion in vitro

We also carried out loss-of-function studies to confirm the role of Bex1 in myoblast fusion. To do this, we compared the fusion of primary myoblasts isolated from littermate wildtype (WT) and Bex1 knockout (KO) mice, which have been shown to have regeneration defects (Khazaei et al., 2010; Koo, 2010; Koo et al., 2007). The primary myoblasts from the Bex1 KO mice were normal in terms of proliferative capacity, manifested by normal growth rate and comparable abundance of $Ki67^+$ cells between WT and KO groups (Supplemental Fig. S3B). After being differentiated for 24 h, the Bex1 KO myoblasts formed nascent myotubes normally, indistinguishable from WT control in terms of nuclei number per myotube and

myotube size (data not shown). However, after being differentiated for 96 h, the WT myoblasts formed large myofibers with multiple nuclei, whereas the Bex1 KO myoblasts failed to form large myotubes and had fewer myonuclei per myotube (Fig. 6A). On average, the percentage of large myotubes containing 5 or more nuclei was significantly lower in the Bex1 KO (24%) than that of the WT (43%), and the percentage of small myotubes containing 2-4 nuclei/myotube was significantly higher in the KO group (Fig. 6B). The reduced myotube size in the KO cultures was not associated with reductions in the levels of Myog or MHC proteins (Fig. 6C), suggesting that Bex1 KO does not affect myogenic differentiation per se. Collectively, these gain- and loss-of-function data provide compelling evidence that Bex1 promotes myoblast-myotube fusion and myotube hypertrophy.

Discussion

In this study, we show that Bex1 is expressed following myogenic differentiation during embryonic muscle development as well as being transiently induced during muscle regeneration. We also find that Bex1 is specifically expressed in differentiated myocytes in vitro. Of note, Bex1 undergoes translocation from the cytoplasm to the nucleus as differentiation proceeds. We used gain- and loss-of-function studies to demonstrate that Bex1 functions to promote myoblast - myotube fusion without affecting myogenic differentiation. These results collectively elucidate a transient role of Bex1 in embryonic and postnatal myogenesis.

Previous studies suggested that Bex1 plays roles in myoblast cell cycle withdrawal as knockout myoblasts had prolonged proliferation and delayed differentiation compared to WT mice during muscle regeneration (Koo et al., 2007). The conclusion was drawn based on analyzing the proliferation marker PCNA and cyclin-dependent kinase inhibitors (CDKIs) in whole muscle tissues, but not specifically in myoblasts. Evidently, regenerating muscles contain various types of cells including inflammatory cells, endothelial cells and fibroblasts (fibroadipogenic cells), whose proliferation could also be affected by Bex1. To test whether Bex1 regulates cell cycle withdrawal in myoblasts, we carried out cell cycle analysis using flow cytometry after adenovirus mediated Bex1 over-expression. However, Bex1 over-expression did not shift cell cycle distribution (Supplemental Fig. S3A), suggesting that Bex1 does not regulate cell cycle in myoblasts. We also conducted Ki67 immunostaining on Bex1 KO and WT myoblasts and calculated the percentage of Ki67 positive cells. We did not detect any differences in the abundance of Ki67 positive cells between WT and KO myoblasts (Supplemental Fig. S3B). Moreover, we showed that Bex1 did not regulate myogenic differentiation per se (Fig. 4).Collectively, these data demonstrated that Bex1 did not regulate the cell transition from proliferation and differentiation.

It has been well documented that Bex1 is abundantly expressed in neurons with a dynamic nucleocytoplasmic distribution pattern. This nucleocytoplasmic trafficking was associated with neuron growth factor (NGF) induced p75NTR signaling pathway (Lee et al., 2013). Here, we also observed the nucleocytoplasmic distribution pattern in myogenic cells. Bex1 was initially diffusely expressed in the cytoplasm but then translocated to the nucleus at later stages of differentiation. This nucleocytoplasmic trafficking may also be related to the

p75NTR signaling pathway as p75NTR has been reported to have high expression levels in developing rat myoblasts as well as in rat and chicken muscles (Ernfors et al., 1988; Lomen-Hoerth and Shooter, 1995; Raivich et al., 1985, 1987; Schecterson and Bothwell, 1992; Yamamoto et al., 1996). Another possible signaling pathway is the Ca^{2+} dependent signaling pathway, which plays indispensable roles in myogenic differentiation. It has been reported that Bex1 can directly interact with Calmodulin (CaM) (Koo et al., 2007), the ubiquitous Ca^{2+} -binding protein, which mediates the uptake of various nuclear proteins (Sweitzer and Hanover, 1996), such as NFAT isoforms. It would be interesting to investigate in future studies the underlying mechanisms and functional significance of the nucleocytoplasmic trafficking of Bex1 during myogenesis.

Mammalian skeletal muscle is generated through the proliferation, differentiation and fusion of myoblasts into multinucleated myofibers. The spatiotemporal expression pattern of Bex1 suggests that Bex1 plays a critical role during myogenic differentiation. Here, we verified that Bex1 could positively regulate myoblast fusion with myotubes. Interestingly, the primary fusion was not affected by either Bex1 overexpression or knockout. How Bex1 regulates myoblast-myotube fusion remains largely unknown. Recently, it was reported that apoptotic cells can induce the ELMO/DOCK1/Rac pathway through the cell surface protein BAI1 to enhance myoblast fusion (Hochreiter-Hufford et al., 2013). Besides, Bex3 has been identified to interact with p75NTR to mediate the apoptosis pathway (Mukai et al., 2000). Given that Bex1 and Bex3 both share similar sequences required for p75NTR binding, it is plausible to hypothesize that Bex1 could promote apoptosis through interaction with p75NTR, which consequently initiates the ELMO/DOCK1/Rac signaling pathway and mediates the fusion. Although Bex1 knockout myoblasts are defective in myoblast-myotube fusion in vitro, the mutant mice have normal muscle development and regeneration. This might be attributable to the compensatory effect of other Bex family members in the absence of Bex1. Alternatively, if Bex1 regulates fusion through apoptosis, massive muscle degeneration could overwhelm the effect of Bex1 on cell apoptosis, and mask any defects of myoblast fusion in the Bex1 KO mice. Generating Bex1 transgenic mice to overexpress Bex1 or Bex1/Bex3 compound KO mice may provide additional clues to the in vivo role of Bex1 in muscle development and regeneration. Nevertheless, our study contributes to our understanding the cellular and molecular mechanism of myogenesis, especially the molecular regulation of myoblast fusion. These data may have implications in the development of therapeutic strategies to treat muscle diseases primarily caused by fusion defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Bex1 is transiently expressed in Myog+ cells during embryonic myogenesis
- Bex1 expression is upregulated during muscle regeneration
- Bex1 overexpression promotes myoblasts fusion without affecting differentiation
- Bex1 knockout myoblasts have fusion defects
- Bex1 regulate myoblast fusion with nascent myotubes

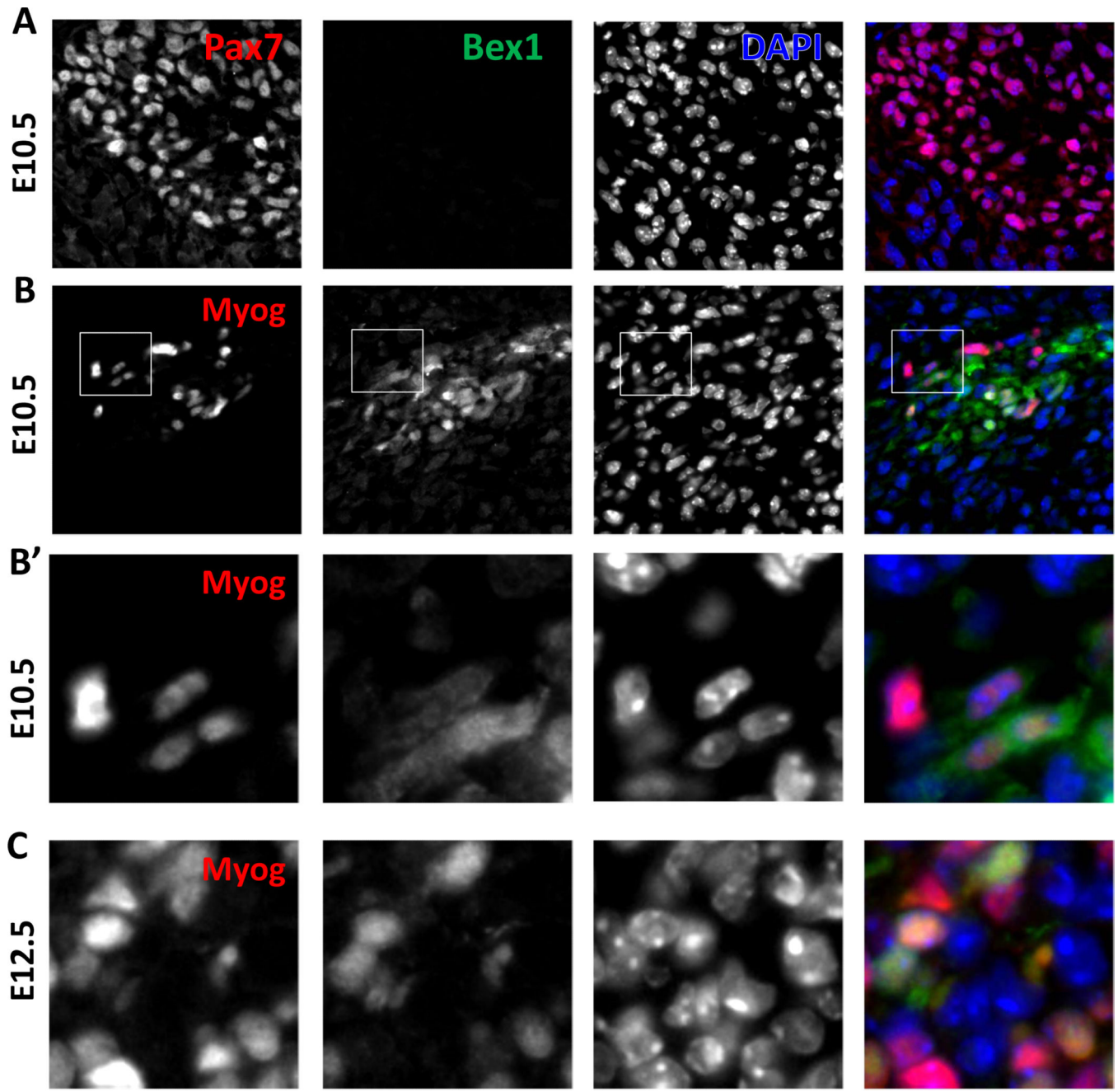

Figure 1.

Expression pattern of Bex1 in muscle progenitors during embryonic development. (A-B) Cross sections of E10.5 embryos at the level of the somites, using antibodies recognizing Bex1 together with Pax7 (A), or Bex1 and Myogenin (B) showing a concurrent expression of Bex1 and Myogenin. B' shows an enlarged view of the boxed area in B. Nuclei were counterstained with DAPI (blue). (C) Representative images of myogenic cells in somites at E12.5, using antibodies recognizing Myogenin (red) and Bex1 (green), showing that Bex1 undergoes cytonucleotrafficking from cytoplasm at E10.5 to nucleus at E12.5. Nuclei were counterstained with DAPI (blue).

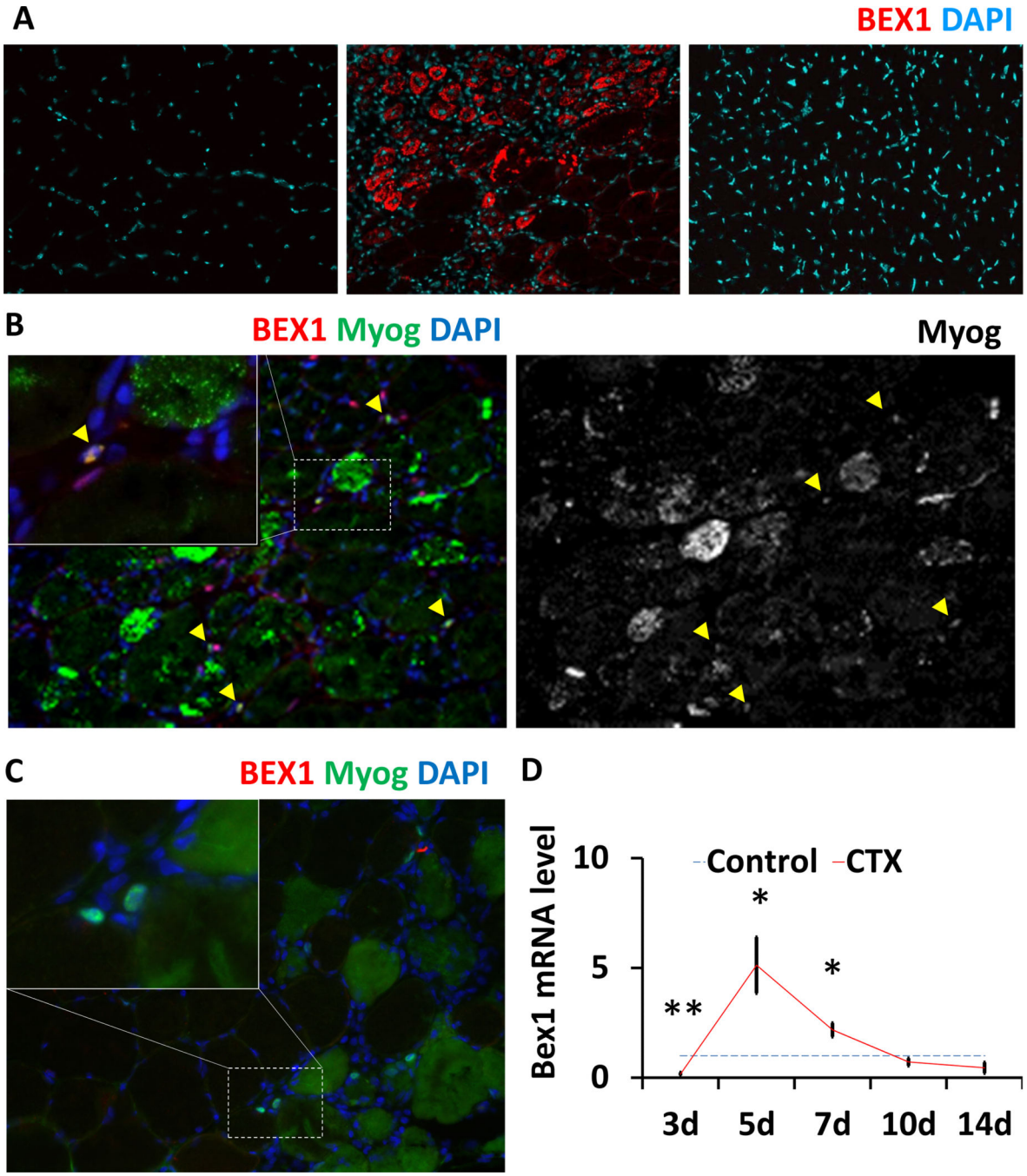

Figure 2.

Bex1 is spatiotemporally induced during muscle regeneration. (A) Cross sections of Tibialis anterior (TA) muscles at Day 0, Day 5 and Day 14 post CTX induced muscle injury, using antibody recognizing Bex1 (red). Nuclei were counterstained with DAPI (cyan) for easy visualization. (B) Higher magnification images of cross sections of TA muscles at Day 5 post CTX induced muscle injury using antibodies recognizing Bex1 (red) and Myogenin (green). Nuclei were counterstained with DAPI (blue). The yellow arrowheads indicated Myogenin^{+}Bex1^{+} mononuclear cells. Myogenin staining only image was shown in black

and white. (C) Higher magnification images of cross sections of TA muscles at Day 3 post CTX induced muscle injury showing the lack of Bex1 staining signal. Nuclei were counterstained with DAPI (blue). The asterisks indicated Myogenin^{+}Bex1^{-} cells. (D) Quantitative PCR analysis of the expression level of Bex1 transcript at different days during muscle regeneration. The dot line indicates the expression level of Bex1 in rested muscles. N=3. Error bars represent s.e.m. *P<0.05 compared with non-injured muscles.

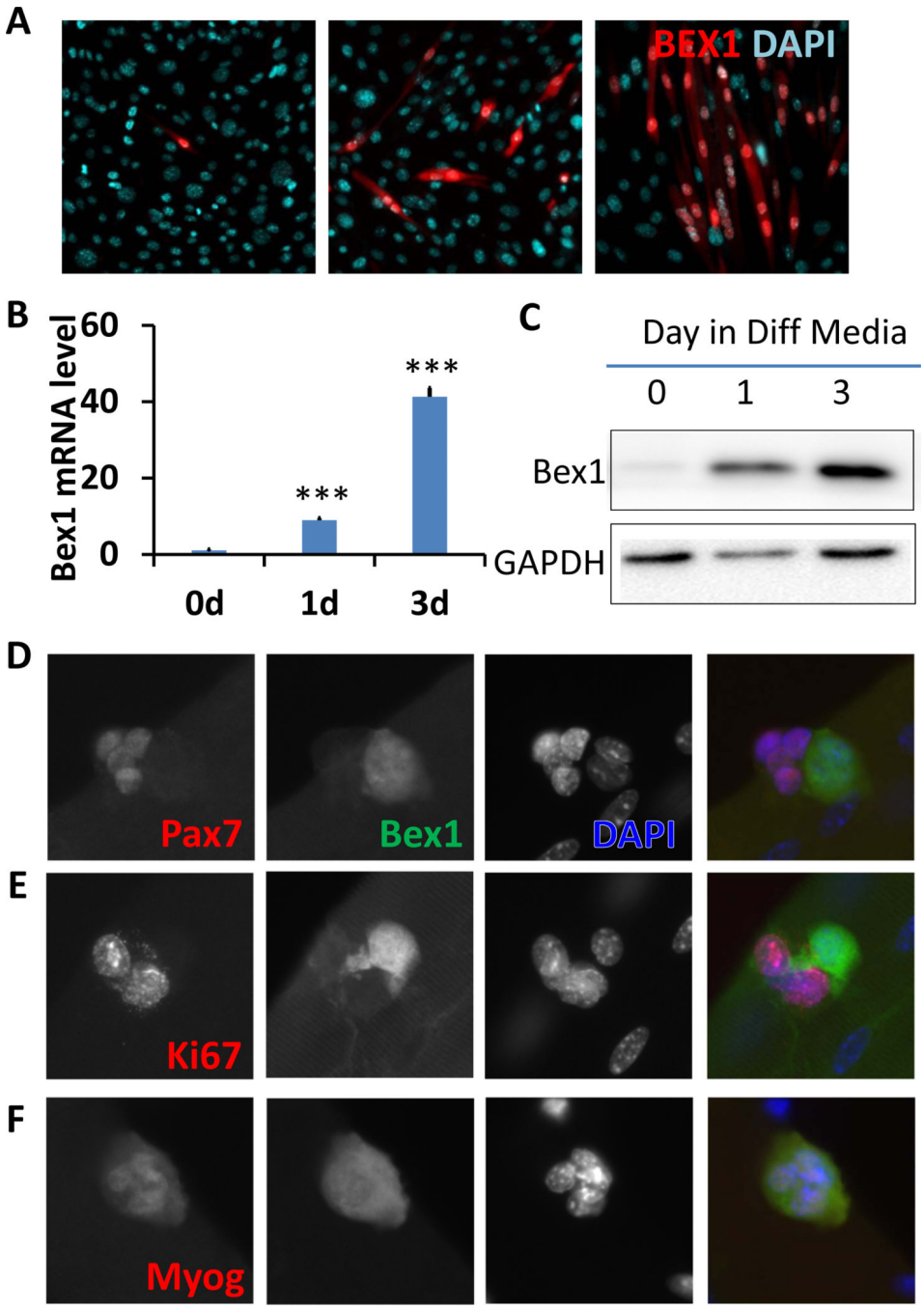

Figure 3.

Bex1 is only expressed in differentiating myoblasts but not in proliferating myoblasts. (A) Immunostaining of primary cell culture at different days post differentiation induction, using antibody recognizing Bex1 (red). Nuclei were counterstained with DAPI (cyan). (B) Quantitative PCR analysis of the expression level of Bex1 transcript at different days post differentiation induction. n=3. (C) Western blot results showing the expression level of Bex1 protein at different days post differentiation induction. n=3. (D-F) Representative images of satellite cells in single EDL muscle fibers after 72hs culture, labeled with Bex1 (green) with

Pax7 (red) (D), KI67 (red) (E) and Myogenin (red) (F). Nuclei were counterstained with DAPI (blue). Error bars represent s.e.m. *P<0.05 compared with undifferentiated myoblast.

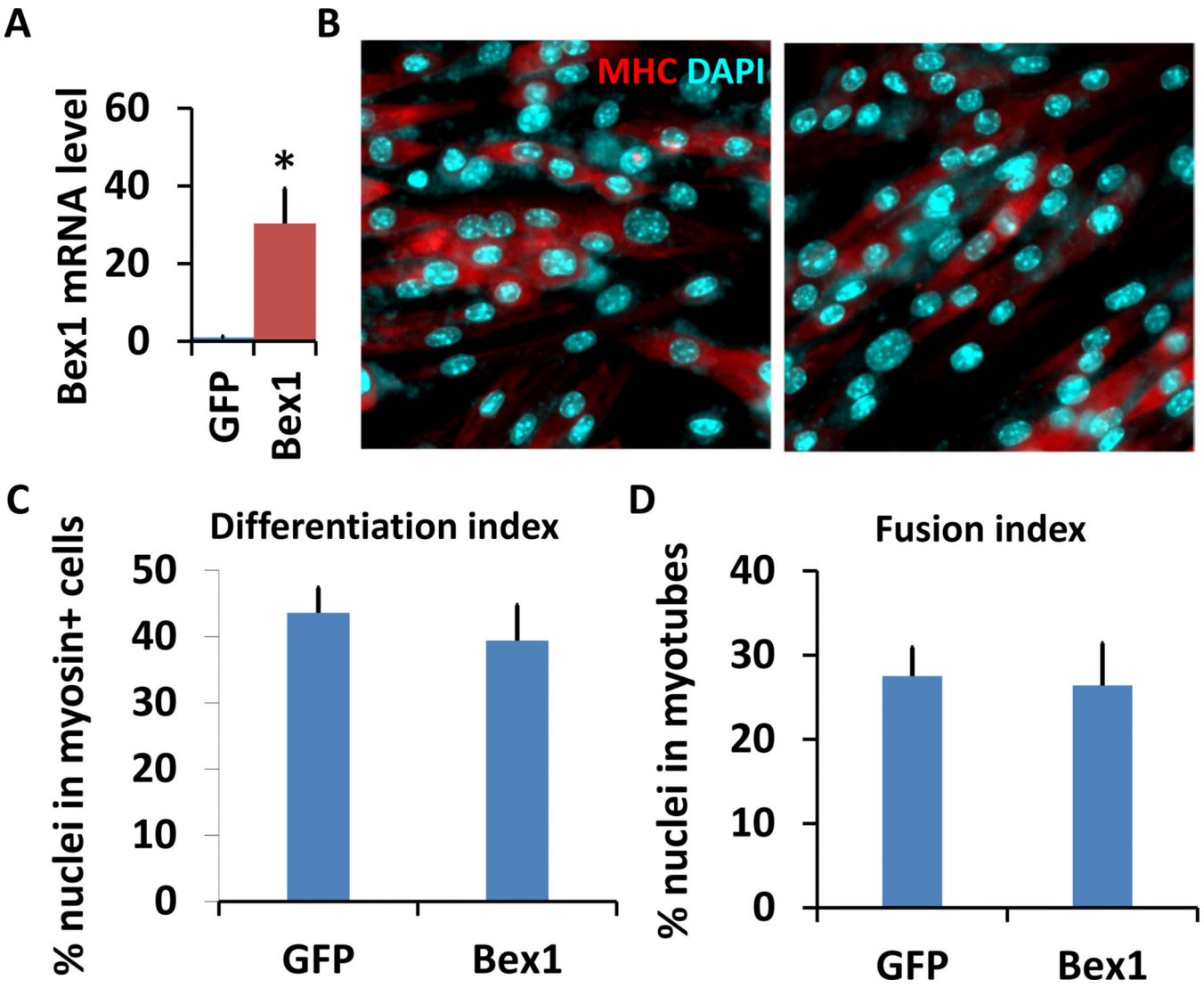

Figure 4.

Bex1 overexpression does not affect myoblast-myoblast fusion. (A) Quantitative PCR analysis of the expression level of Bex1 transcript in primary myoblasts after infection with adenovirus expressing GFP alone or GFP plus Bex1, labeled as GFP and Bex1, respectively. n=3. (B) Primary myoblasts were differentiated for 24hs with infection of adeno-virus expressing GFP and Bex1. Immunostaining with antibody recognizing MHC (red). Nuclei were counterstained with DAPI (cyan). (C) Differentiation index, calculated as the ratio of nuclei in Myosin+ cells. n=3. (D) Fusion index, calculated as the ratio of nuclei in Myosin+ myotubes. n=3. Error bars represent s.e.m. *P<0.05 compared with GFP-infected cells.

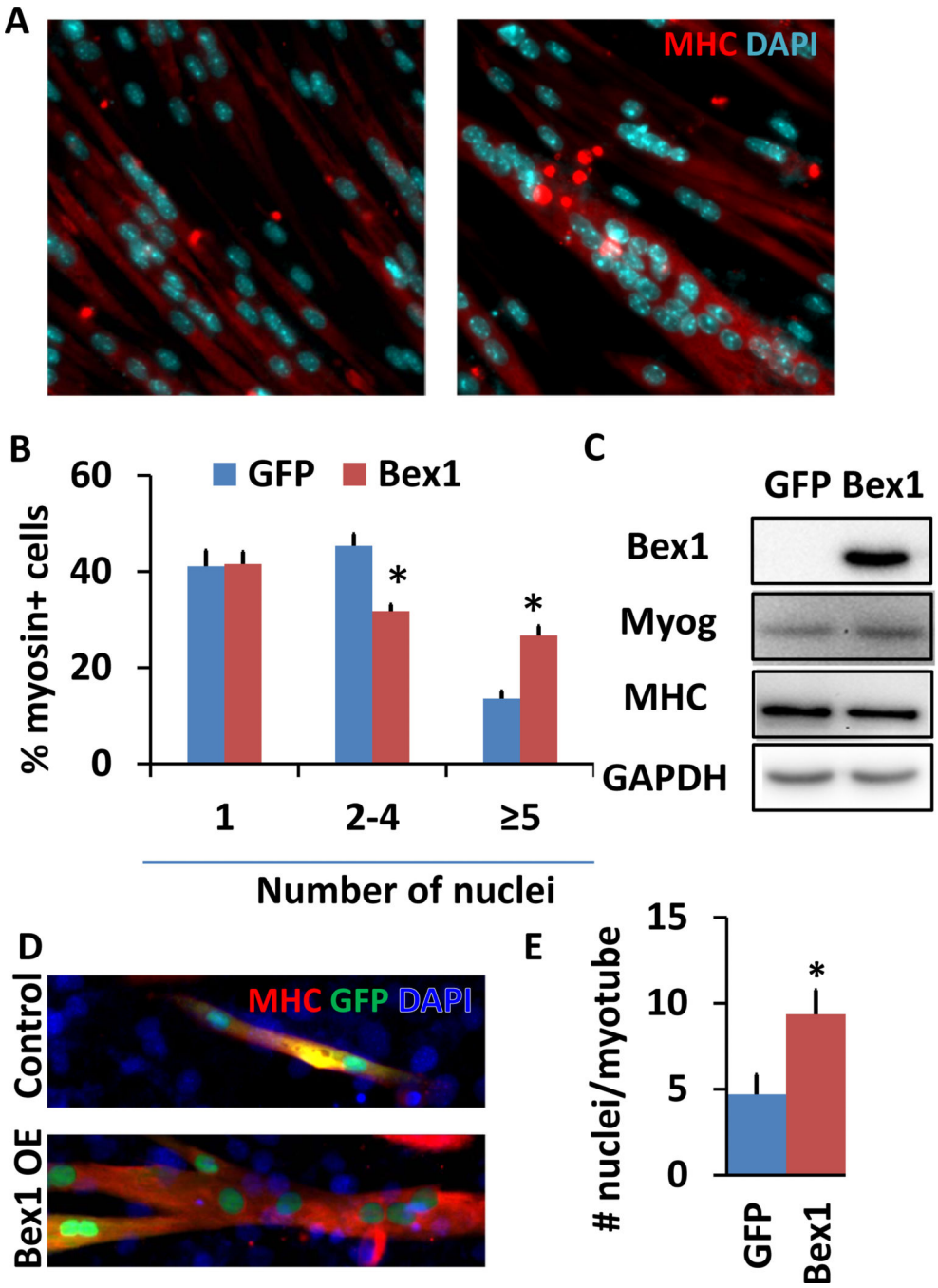

Figure 5.

Bex1 promotes myoblast-myotube fusion and myotube hypertrophy. (A) Primary myoblast was cultured and differentiated upon confluence. After 24hs differentiation, adeno-virus expressing GFP and Bex1 was added with 96 more hours differentiation. Immunostaining with antibody recognizing MHC (red). Nuclei were counterstained with DAPI (cyan). (B) Quantification of ratio of myosin+ cells with different nuclei number in each field. ≥ 200 cells were analyzed with 5 independent experiments. (C) Western blot results showing the expression levels of Myogenin and MHC in primary myoblast culture after differentiation

with GFP and Bex1 virus infection. GAPDH worked as control. n=3. (D) C2C12 myoblasts were electroporated with plasmids expressing GFP (top panel) and GFP-Bex1 (bottom panel) then induced for differentiation. Immunostaining of myotubes with antibody recognizing MHC (red). Nuclei were counterstained with DAPI (blue). (E) Quantification of the number of nuclei in GFP^+ and $GFP\text{-}Bex1^+$ myotubes. n=3. Error bars represent s.e.m. *P<0.05 compared with GFP-infected cells.

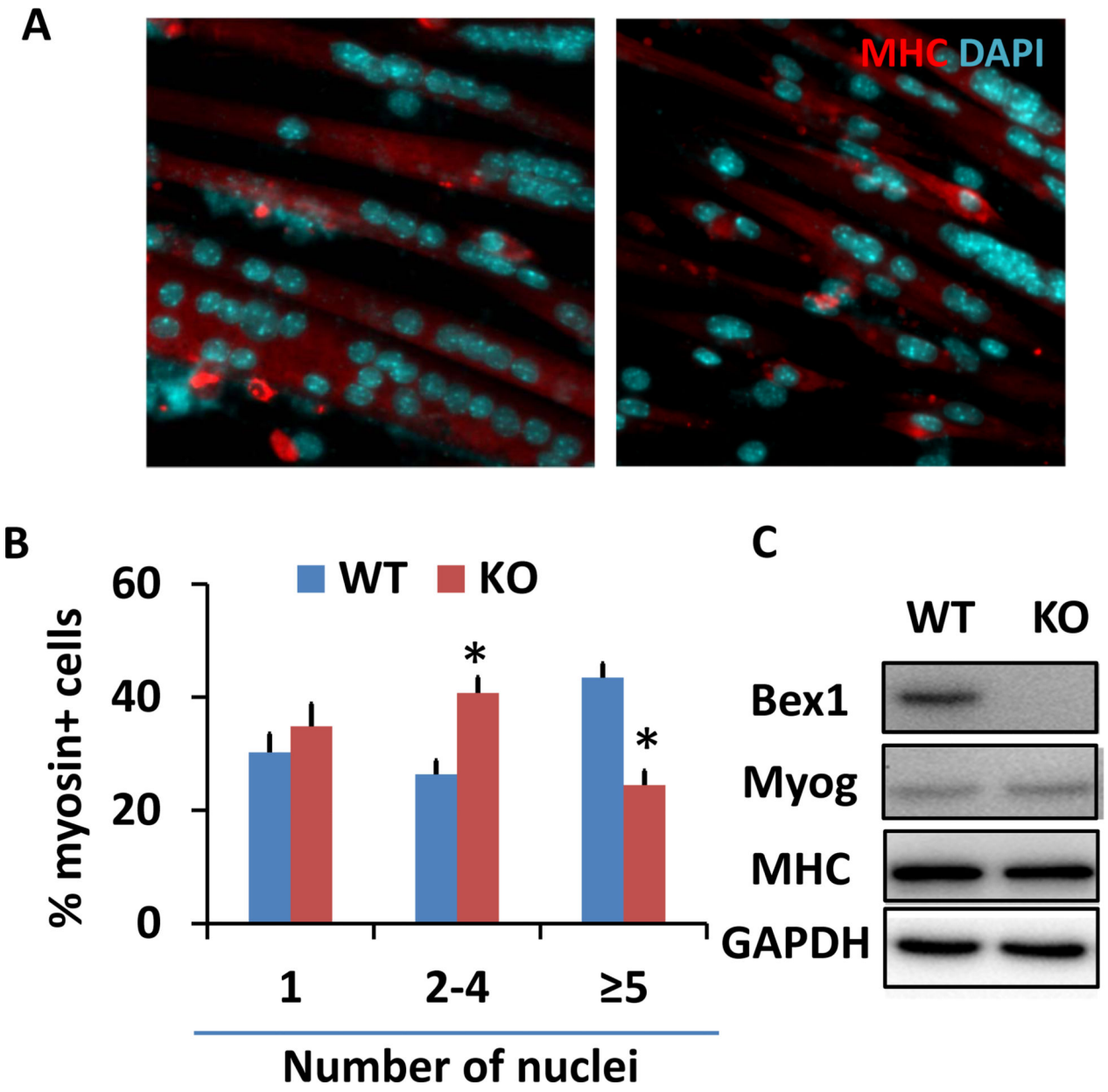

Figure 6.

Bex1 knockout myoblasts are deficient in fusion with myotubes. (A) Primary myoblasts were cultured from WT and Bex1 knockout mice and differentiated for 48hs upon confluence. Immunostaining with antibody recognizing MHC (red). Nuclei were counterstained with DAPI (cyan). (B) Quantification of ratio of Myosin+ cells with different nuclei number in each field. ≥ 200 cells were analyzed with 3 independent experiments. (C) Western blot results showing the expression levels of Myogenin and MHC in primary myoblast culture from WT and Bex1 knockout mice after differentiation. GAPDH worked as control. n=3. Error bars represent s.e.m. *P<0.05 compared with WT.