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Bex1 Participates in Muscle Regeneration by Regulating Myogenic Satellite Cell Differentiation

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Bex1 protein is upregulated in regenerating muscle and interacts with calmodulin, a Ca^{2+}-binding protein involved in cell cycle regulation. Following cardiotoxin-induced injury the regenerating muscle of Bex1 knock-out mice exhibits prolonged cell proliferation and delayed cell differentiation compared to wild-type mice. To gain insight into this process, we compared the regenerating myogenic morphologies of Bex1 knock-out and wild-type mice at several time points. Bex1-positive cells were identified by double immunofluorescence staining. These studies demonstrated that a population of cells that are Bex1-positive after injury are c-Met/basal lamina-positive and Mac-1-negative indicating that they are derived from at least a subset of myogenic progenitor/satellite cells but not invading immune cells. In addition, in regenerating muscle, Bex1 co-localizes with calmodulin in the cytoplasm of the late myoblast or early myotube stage of myogenesis. These results suggest that Bex1 participates in muscle regeneration through the regulation of satellite cell proliferation and differentiation by its interaction with calmodulin. Current studies of Bex1 may provide a new molecular tool for the identification of activated satellite cell and open the way to new or improved therapeutic regimens against progressive muscular atrophy.

Key words: Bex1, calmodulin, satellite cell, muscle regeneration

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Our previous studies demonstrated a dramatic induction of Bex1 mRNA and protein following muscle injury (Koo *et al.*, 2007). This is consistent with the identification of *Bex1* by functional genomic analyses as a gene with elevated expression following muscle injury (Goetsch *et al.*, 2003; Yan *et al.*, 2003). To obtain insight into the physiological significance and mechanism of action of Bex1 in skeletal muscle regeneration, the gene trapping method was used to generate Bex1-deficient mice and genomic-DNA PCR and western blotting was used to confirm the Bex1-gene deletion (Koo *et al.*, 2007). Bex2 protein, which has 87% amino acid sequence identity to Bex1 (Alvarez *et al.*, 2005; Koo *et al.*, 2005), is still expressed in Bex1 knock-out (Bex1-KO) mice demonstrating the specificity of the Bex1-KO (Koo *et al.*, 2007). Mice lacking Bex1 are viable and fertile, but display a functional deficit in exercise performance compared to wild type (WT) mice (Koo *et al.*, 2007). After intramuscular injection of cardiotoxin (CTX), which causes extensive and reproducible myotrauma followed by recovery, the regenerating muscle of Bex1-KO mice exhibits elevated and prolonged cell proliferation as well as delayed cell differentiation compared to WT mice (Koo *et al.*, 2007). In addition, Bex1 interacts with calmodulin (CaM) in a Ca^{2+} dependent manner as confirmed by an endogenous Bex1 pull-down assay with CaM-agarose (Koo *et al.*, 2007). The interaction of Bex1 with CaM suggests that it could contribute to the regulation of muscle regeneration. However, the mechanism by which Bex1 expression controls myogenic proliferation and differentiation is not known.

Genome-wide discovery tools have been used to identify highly coordinated molecular changes involved in skeletal muscle regeneration and to identify their relationships and biological responses (Goetsch *et al.*, 2003; Yan *et al.*, 2003). An enhanced understanding of the molecular mechanisms that are associated with responses to muscle injury, and associated with the regenerative process, would facilitate therapies directed toward the treatment of debilitating myopathies. The expression of molecules is altered in response

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to skeletal muscle degeneration and regeneration. It is hypothesized that many of these molecules act as modulators to control the proliferation and differentiation of satellite cells that are the skeletal myogenic progenitor cells (MPCs) responsible for postnatal growth and repair (Hawke and Garry, 2001; Charge and Rudnicki, 2004; Dhawan and Rando, 2005). In most myopathies, the poor clinical outcome is ultimately due to a failure of the myogenic satellite cells to maintain muscle regeneration after continuous degeneration-regeneration cycles (Burghes *et al.*, 1987; Cossu and Mavilio, 2000; Heslop *et al.*, 2000). Satellite cells are also characterized by the expression of surface markers such as c-Met, syndecan 3 and 4, and basal lamina (Cornelison *et al.*, 2001). Once the proliferation potential of satellite cells is exhausted, or some of the molecules that are involved in activation, proliferation, and differentiation of satellite cells to myofibers are depleted, there is no, impaired, or altered regeneration of the skeletal muscle. The molecular mechanisms controlling muscle regeneration are not fully understood, particularly regulation of the signaling cascade that leads to proliferation and differentiation of the satellite cells. Also, the paucity of satellite cell marker proteins constrains our ability to study the functions of satellite cells in muscle regeneration. We hypothesized that Bex1 expression in activated satellite cells, through its interaction with CaM, participates in muscle regeneration by modulating satellite cell proliferation and differentiation.

Materials and Methods

Tissue preparation

All animal procedures were approved by the University of Maryland, Baltimore (UMB) Institutional Animal Care and Use Committee. Animals were kept in the UMB animal facility and were given access to food and water *ad libitum*. Mice were anaesthetized (ketamine, 165 mg per kg and xylazine, 10 mg per kg) and transcardially perfused with 20 mL of ice cold PBS, followed by 30 mL of ice cold 4% paraformaldehyde (PFA) in PBS. *Tibialis anterior* (TA) muscle was dissected out, postfixed in 4% PFA for 2 hr at 4°C, and cryoprotected in 30% sucrose overnight at 4°C. Tissues were embedded in OCT (Tissue Tek, Sakura, Torrance, CA, USA) and snap frozen in a dry ice/isopentane bath. Muscle tissues were sectioned coronally at 10 μm and attached to Superfrost-plus microscope slides (Fisher, Pittsburgh, PA, USA), dried at 37°C for 15 min, and stored at −80°C until needed.

Cardiotoxin injection

Bex1-KO and littermate WT male mice (6 to 8 weeks old) were anesthetized (ketamine, 165 mg per kg, and xylazine, 10 mg per kg) and 100 μL CTX (CTX 10 μM in saline, SIGMA, USA) was injected into the right TA muscle with a 30-gauge needle. PBS-injected left TA muscles were used as a control.

Hematoxylin and eosin (H&E) staining

CTX-induced injury was performed as described previously (Koo *et al.*, 2007). At various times (0, 3, 5, 7, or 17 days) after CTX injection mice were euthanized by cervical dislocation and muscle tissue harvested. The harvested TA muscles were fixed in 4% PFA, frozen, sectioned, and stained according to the manufacturer's instructions (SIGMA-Aldrich, USA).

Immunofluorescence staining

Frozen sections were washed with cold PBS and fixed with 4% PFA in PBS for 10 min on ice. Excess fixative was removed by washing with PBS and sections blocked (5% normal horse serum in PBS containing 0.1% of Triton X-100) for 60 min and then incubated overnight at 4°C with appropriate antibodies diluted in blocking buffer. For single- and double-labeled immunofluorescence staining, rabbit anti-Bex1 antibody (Koo *et al.*, 2005) at 1:20,000, or rabbit anti-Bex1 antibody at1:10,000 and either anti-mouse Mac-1 (AbD Serotec, USA) at 1:1,000 or anti-c-Met (Santa Cruz, sc-162, USA) at 1:1,000 or anti-Laminin (Sigma, L-9393, USA) at 1:1,000, or anti-CaM (Santa Cruz, c-1989, USA) at 1:1,000 were used on TA muscle sections on slides after several washes, the tissues were incubated with Cy2-, and Cy3-conjugated 2nd antibodies and nuclei visualized with 4'-6-Diamidino-2-phenylindole (DAPI) at room temperature (RT) for 40 min. After another series of washes, the slides were mounted using Gel/Mount (Biomeda, Foster, CA, USA) and examined by confocal microscopy (Olympus Fluoview, BX50WI, Olympus America, Melville, New York, USA) with appropriate excitation and emission barrier filters.

Results and Discussion

Yan *et al.*, (2003) reported alterations in Bex1 mRNA expression in regenerating skeletal muscle by microarrays and RT-PCR analysis and proposed that Bex1 is one of the molecules associated with muscle differentiation. We recently generated and characterized a Bex1-KO mouse (Koo *et al.*, 2007) as well as an antibody against Bex1 (Koo *et al.*, 2005) and demonstrated that following myogenic trauma the regenerating muscle of Bex1-deficient mice shows prolonged cell proliferation and delayed cell differentiation, indicating altered muscle

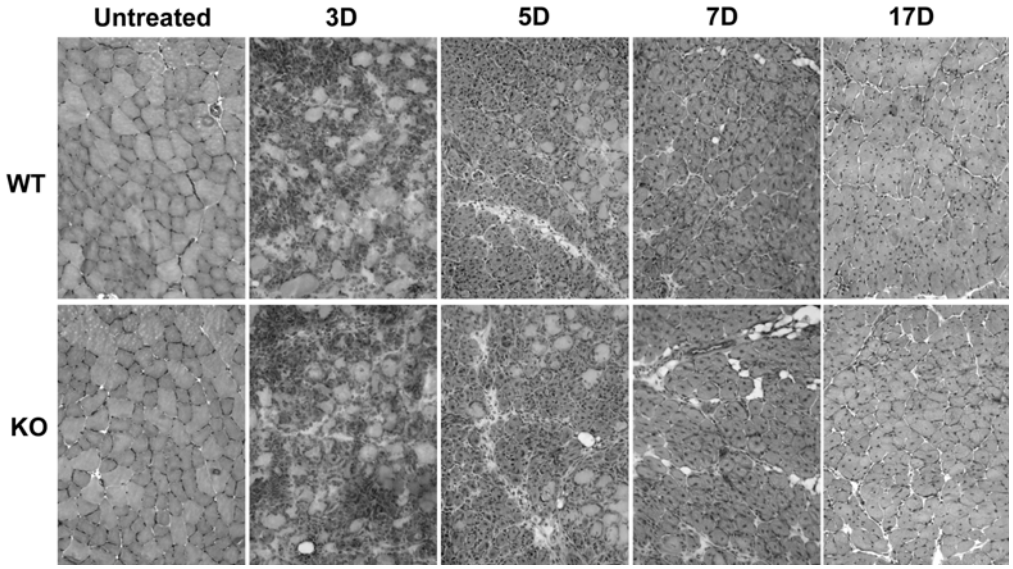

Figure 1. Cardiotoxin (CTX)-induced degeneration/regeneration shows similar histological appearances between Bex1-/- and wild type (WT) skeletal muscle by Hematoxylin and Eosin (H&E) staining. CTX was injected into the *Tibialis anterior* (TA) muscles of WT and Bex1-knock out (Bex1-KO) mice and harvested at 0 days, 3, 5, 7, 17 days (D) after injection. Although Bex1-KO exhibits altered muscle regeneration (Koo *et al.*, 2007) there is no obvious visible difference in muscle morphology by H&E staining between regenerating Bex1-KO and WT.

regeneration. The process of skeletal muscle repair is characterized by a degenerative phase followed by a regenerative phase. The impaired and altered skeletal muscle regeneration observed in many mice deficient in myogenic molecules are associated with a large number of mononuclear cells and the presence of degenerated myotubes (Floss *et al.*, 1997; Grady *et al.*, 1997; Garry *et al.*, 2000). To determine whether the regenerating myogenic morphologies of Bex1-KO mice are impaired or altered compared with those of WT mice after CTX-induced injury and to evaluate the importance of Bex1 for skeletal muscle regeneration, we performed H&E staining of regenerating muscle Bex1-KO and WT mice. CTX-injection in TA muscle induces extensive degeneration followed by complete regeneration, consisting of extensive proliferation within 2-3 days of injury, exiting from the cell cycle at approximately 5 days, forming differentiated myotubes, and largely restoring the damaged muscle within 2∼3 weeks (Garry *et al.*, 1997). The results of the present study demonstrate that CTX injected in Bex1-KO and WT mouse TA muscles induced an inflammatory response and mononuclear cell proliferation within 3 days of injection. Myogenic cell differentiation and new myotube formation were observed 5∼7 days post-injection and the damaged muscles were largely restored within 3 weeks. Most regenerated myofibers are smaller and display central myonuclei (Figure 1). We expected that Bex1-KO mice would show abnormal kinetics of myogenic morphologies during muscle regeneration but that subsequently their morphological and histochemical appearances would appear to be similar with those of WT mice. We predict that other mechanisms take over to compensate for the lack of Bex1 or else the sensitivity of the histology characterized by H&E staining is not adequate to detect the alteration of muscle regeneration of Bex1-KO mice, although these mice showed altered muscle regeneration after injury (Koo *et al.*, 2007).

To further investigate the role of Bex1 during the skeletal muscle repair process, we determined the critical period and the cellular localization of Bex1 expression in CTX-induced regenerating muscle using immunofluorescent staining with anti-Bex1 antibodies followed by H&E and DAPI staining. Skeletal muscle cells respond to regenerative cues, such as injury or excessive exercise, by proliferating to form myoblasts, which divide a limited number of times before terminally differentiating and fusing to form multinucleated myotubes (Morgan and Partridge, 2003). Our previous analysis by western blots demonstrated that Bex1 protein of regenerating skeletal muscle is maximally induced from 5 to 7 days post CTX-induced injury in WT mice (Koo *et al.*, 2007). With immunofluorescent staining, Bex1 expression was detected within 3 to 4 days and Bex1-positive cells were absent by 7 to 8 day after injury (Data not shown). Two or three different populations of Bex1-positive cells were identified (Figure 2). Bex1 is evenly expressed in cytoplasm and nucleus in some cells while other subsets of cells showed strong staining for Bex1 in the nucleus suggesting that Bex1 migrates in and out of the nucleus depending on its functional activity

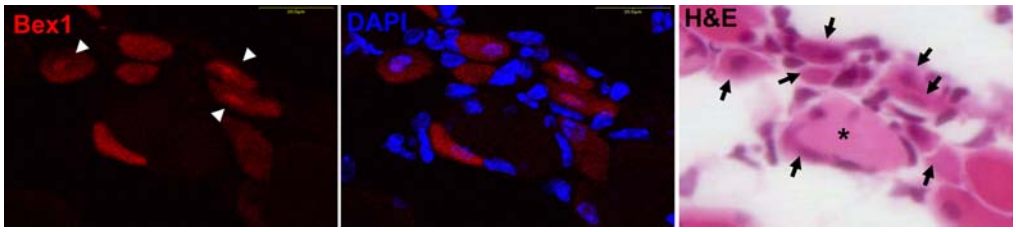

Figure 2. Bex1 is expressed in the late myoblast or early myotube stage of myogenesis. After cardiotoxin-injury, induced expression and localization of Bex1 in regenerating muscle of wild type mice was elucidated by immunofluorescent staining with the rabbit antibody against Bex1 followed by H&E staining. The arrows indicate Bex1-positive cells and the arrowheads illustrate that Bex1 is highly expressed in nuclei. Nuclei are counterstained with DAPI *: Large vacuolated cells.

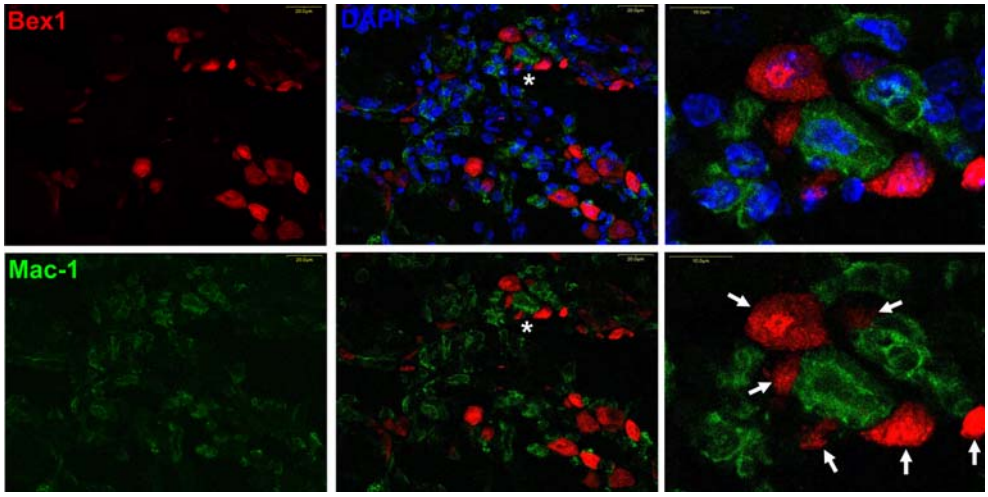

Figure 3. All Bex1 (+) cells are not macrophage (+) cells. The regenerating muscle cells of wild type mice after injury are identified by antibodies to Bex1 (red) and Mac-1 (green). Mac-1 staining represents inflammatory and immune cells including macrophages. Note the absence of double stained cells. Nuclei are counterstained with DAPI. *=the area of high magnification in panels to the right.

(arrowheads in Figure 2/Bex1). Similar observations were reported by Behrens *et al.,* (2003) in cultured cells overexpressing Bex protein. This phenomenon is also consistent with the report that NGF induces nuclear export of Bex1 in cells expressing the p75 neurotrophin receptor (NTR) (Vilar *et al.*, 2006). According to time period of Bex1 expression and a cell type of Bex1-positive, our results demonstrate that Bex1 protein is induced between late proliferation and early differentiation of regenerating muscle.

The process of skeletal muscle repair is a dramatic response to damage (Schmalbruch and Lewis, 2000). Regeneration is the result of many biological processes, such as inflammation, angiogenesis, arteriogenesis, and myogenesis, all of which lead to the reconstitution of functional skeletal muscle tissue (Charge and Rudnicki, 2004). It is well established that after the invasion of neutrophils into the muscle, an essential part of the innate immune system, macrophages become the dominant inflammatory cell type by 1 day with peak levels at day 3 post-injury (Orimo *et al.*, 1991; Tidball *et al.*, 1999). The early stage of Bex1 protein expression overlaps with the inflammatory and immune response to muscle damage at 3 days post CTX-induced injury. Therefore, we studied whether the Bex1-positive cells are inflammatory or immune cells. Mac-1 stains cells that are neutrophils, NK cells, and mononuclear phagocytes like macrophages. This analysis demonstrated that the population of Bex1-positive cells differed from that of Mac-1-positive cells and presumably represented non-inflammatory cells (Figure 3).

An important question that was not yet addressed in the current study is what cell types are expressing Bex1 in regenerating skeletal muscle. To clarify whether Bex1 is expressed in activated satellite cells of regenerating muscle, we performed immunostaining using adjacent cryostat sections of muscle tissue with antibodies against Bex1 and c-Met (a marker protein of satellite cells; Cornelison and Wold, 1997). Bex1 expression was detected in c-Met-positive satellite cells (Figure 4). These results demonstrated that CTX-injury induced Bex1 expression in activated satellite cells of WT mice. To support this result we used another marker protein of satellite cells, laminin. The satellite cells can unambiguously

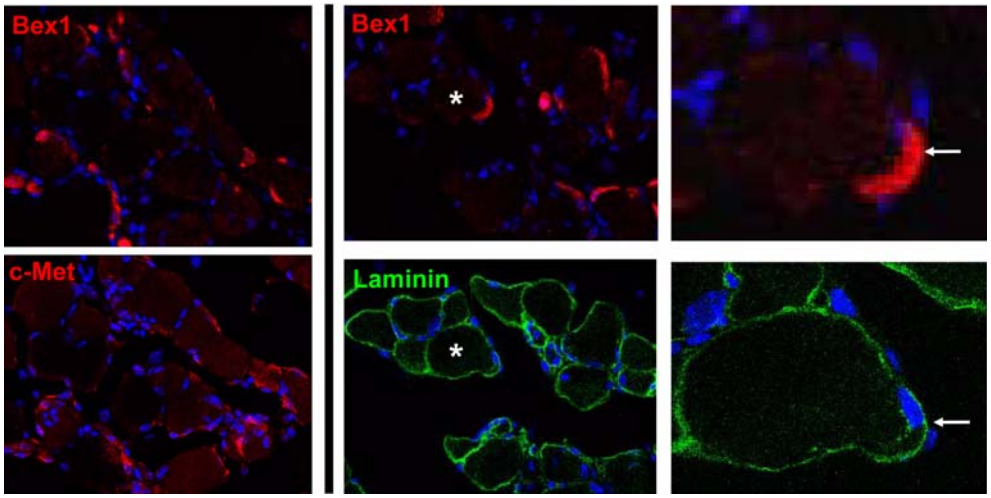

Figure 4. Bex1 is expressed in activated satellite cells identified with c-Met and are also surrounded by basal laminin at 4 days after CTX. Most of Bex1-positive cells are c-Met-positive cells and are surrounded by basal laminin (arrow). Nuclei are counterstained with DAPI. The arrows indicate Bex1-positive satellite cell (top arrow) surrounding by basal laminin (bottom arrow). *=the area of high magnification in the panel to the right.

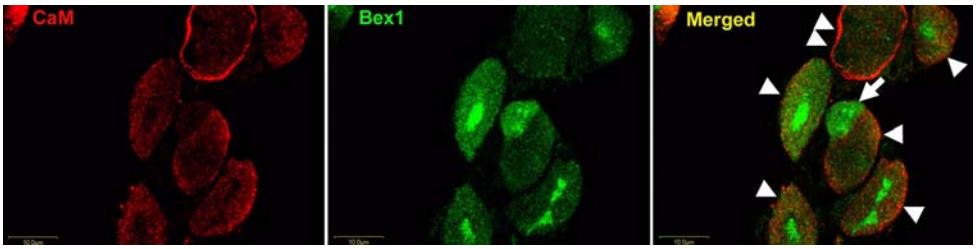

Figure 5. Bex1 co-localizes with calmodulin (CaM) in regenerating muscle cells of wild type mice. Co-localization of Bex1 (green) and CaM (red) illustrated at 7 days after cardiotoxin (CTX)-injury. Cryostat sections of muscle taken at 7 days after CTX were double-labeled with antisera to Bex1 and to CaM and were analyzed by confocal microscopy. The figure illustrates the presence of three different stages of the cellular responses of CaM and Bex1 expression after CTX treatment. First, Bex1 occupies the entire cell with no expression of CaM (arrow), Second, Bex1 is highly expressed in the nucleus and less intensely in the cytoplasm, whereas punctate staining for CaM occupies the entire cytoplasm and is absent from the nucleus (arrowhead), thirdly, in the last case CaM is localized to the plasma membrane and virtually no Bex1 staining is observed (double arrowhead). The cytoplasm of the second type of cells demonstrates the colocalization of Bex1 and CaM, which is the most abundant pattern seen 7 days after CTX treatment (arrowhead). The cell denoted by the arrow indicates a Bex1-positive cell that is CaM-negative which is the major type seen at 4 days after CTX treatment. The double arrowhead represents the predominant type seen in regenerating muscle at 10 days after CTX treatment.

be identified by electron microscopy due to their unique location within the basal lamina (Charge and Rudnicki, 2004). The satellite cells are separated from the myonuclei by a surrounding basal lamina and more abundant heterochromatin (Hawke and Garry, 2001). Following skeletal muscle injury, the satellite cells become activated and increase their cytoplasmic content. A detailed study of the expression of Bex1 and basal lamina in regenerating mouse muscle demonstrated that Bex1 was first detected in cytoplasm of activated satellite cells surrounding by basal lamina (Figure 4) but not in mitotically quiescent satellite cells in the absence of injury (Data not shown). Taken together, these data indicate the Bex1 is expressed in activated satellite cells and its absence results in altered properties of satellite cells.

Previous studies proposed that the interaction of Bex1 with CaM could have a physiological role during muscle regeneration (Koo *et al.*, 2007). In support of this hypothesis we performed *in vivo* co-localization of Bex1 and CaM by double labeling immunohistochemistry at several time points after intramuscular CTX injection. We have observed three developmental stages in the expression patterns of Bex1 and CaM in the regenerating muscle. To simplify the presentation we have selected a cell cluster that illustrates these three stages for Bex1 and CaM expression at 7 days after CTX-injury (Figure 5). First, Bex1 protein was highly induced in the cytoplasm but no CaM was apparent (arrow). Second, Bex1 is highly expressed in the nucleus and was also found in the cytoplasm with CaM, whereas no CaM was seen in the nucleus (arrowhead). Finally, the strong Bex1 signal in the nuclei disappeared and was replaced by a weak signal

for Bex1 in the cytoplasm. Surprisingly, CaM was highly localized to the plasma membrane (double arrowhead). These data indicate that Bex1 co-localizes with CaM in the cytoplasm of regenerating muscle. In the calmodulin-agarose pull down assay with CTX-injury TA muscle extract (Koo *et al.*, 2007), induced endogenous Bex1 protein is interacted and pulled down with CaM-agarose only in the presence of calcium. Taken together, the interaction of Bex1 with CaM is demonstrated by *in vitro* gel shift assay, direct pull down (Koo *et al.*, 2007), and co-localization analyses. Bex1 movement in and out of nucleus seems to be crucial for its effects on cell proliferation and cell-cycle arrest. In addition, intracellular CaM expression profiles were analyzed at the same time points for Bex1-KO and WT mice. In the western analysis against CaM (Koo *et al.*, 2007), even though CaM is abundantly present in skeletal muscle, the expression level of CaM was remarkably increased at 4 days after CTX injection, maintained the same level from 4 d through 10 d, and decreased to pre-injury level at 10 days. The expression level and cellular localization of CaM appear to be similar between KO and WT mice. These results indicate that an elevation in CaM seems to be crucially associated with Bex1 during muscle regeneration. However, it is not yet clear if Bex1 is involved in controlling the expression profile of CaM and the translocation of CaM from cytoplasm into plasma membrane. Perhaps Bex1 controls the efficacy of intracellular Ca/CaM by protein-protein interaction and thus plays a role in regulating the signal transduction cascade involved in muscle regeneration.

In summary, our data presented here indicates that Bex1 is induced in activated myogenic/satellite cells of regenerating skeletal muscle following CTX-induced injury. The interaction of Bex1 with CaM may control the balance between proliferation and differentiation of myogenic/satellite cells. Results from this work suggest a novel mechanism by which one of the genes transiently upregulated after CTX-injury modulates muscle regeneration. Understanding of the factors regulating myogenic satellite cells following muscle damage offers potentially useful strategies for the treatment of skeletal muscle myopathies such as Duchenne muscular dystrophy.

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