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## Prostaglandin F<sub>2</sub> $\alpha$ promotes muscle cell survival and growth through upregulation of the inhibitor of apoptosis protein BRUCE

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### Abstract

During skeletal muscle growth and regeneration, the majority of differentiating myoblasts undergoes cell-cell fusion to form multinucleated myofibers, while a proportion of myoblasts undergoes apoptosis. The treatment of myoblasts with prostaglandin F<sub>2</sub> $\alpha$  (PGF<sub>2</sub> $\alpha$ ) during myogenesis *in vitro* leads to the formation of large myotubes, but the mechanism by which PGF<sub>2</sub> $\alpha$  promotes myotube growth has not been investigated. Here, we demonstrate that PGF<sub>2</sub> $\alpha$  reduces cell death during myogenesis *in vitro* and *in vivo*. In addition, we show that PGF<sub>2</sub> $\alpha$  increases expression of the inhibitor of apoptosis protein (IAP) BRUCE through a pathway dependent upon the nuclear factor of activated T cell 2 (NFATC2) transcription factor. Importantly, PGF<sub>2</sub> $\alpha$ -mediated reduction in muscle cell death is dependent upon BRUCE, and overexpression of BRUCE is sufficient to promote muscle cell survival and growth. These results establish a previously unrecognized link between NFAT signaling and regulation of IAP expression and are the first to identify a signaling pathway that increases BRUCE expression. In addition, our results provide evidence that increasing the pool of muscle cells available for fusion by inhibiting cell death enhances myotube growth.

### Keywords

PGF<sub>2</sub> $\alpha$ ; BRUCE; myoblast survival; apoptosis; inhibitor of apoptosis; myogenesis; muscle growth; prostaglandin

### Introduction

During skeletal muscle myogenesis, muscle precursor cells, or myoblasts, undergo a phase of rapid proliferation, followed by cell cycle exit, myogenic differentiation, and finally cell fusion. These events lead to the formation of multinucleated myofibers *in vivo* and myotubes *in vitro*. Skeletal muscle growth and regeneration are dependent upon an adequate pool of myoblasts. Therefore, the identification and characterization of molecules that promote the expansion, survival, and fusion of myoblasts is important not only for gaining a better understanding of myogenesis, but also for developing treatments for the loss of muscle mass that occurs with disease and aging.

A number of studies have suggested that prostaglandins (PGs), signaling molecules derived from arachidonic acid by cyclooxygenase (COX) and PG synthase enzymes, play important roles during muscle growth (1). Several PGs have been implicated in multiple phases of

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myogenesis (1–4). For example, PGF2 $\alpha$  is secreted by skeletal muscle cells *in vitro* and *in vivo* (5,6) and promotes myotube growth during later stages of myogenesis by increasing the number of nuclei within myotubes (3). PGF2 $\alpha$  signals via the FP receptor to increase levels of intracellular calcium, activating the nuclear factor of activated T cells 2 (NFATC2) transcription factor in muscle cells. PGF2 $\alpha$ -induced myotube growth is dependent upon NFATC2, suggesting that this growth may be dependent upon a transcriptional target of NFATC2. A known target of NFATC2 transcription, the cytokine interleukin-4 (IL-4), also promotes muscle growth (7). However, PGF2 $\alpha$  does not require IL-4 to exert its growth-promoting affect on muscle cells, as IL-4 null myotubes undergo growth similar to that of wild-type cells in response to PGF2 $\alpha$  treatment (8).

During myogenesis, a majority of myoblasts exits the cell cycle and undergoes terminal differentiation. Meanwhile, a proportion of differentiating myoblasts undergoes cell death (9–13). Interestingly, signaling pathways required for the initiation and execution of programmed cell death, or apoptosis, are activated during myogenesis (10,14–16). For example, the cysteine protease caspase 3 is not only activated during myogenesis, but also its activity is required for the initiation of myogenic differentiation (14). The mechanism by which the majority of muscle cells undergoes caspase-dependent differentiation but escapes caspase-induced apoptosis is not clear.

Control of apoptosis is critical for development and homeostasis and the balance between life and death is tightly controlled by multiple pathways within the cell. The activity of apoptosis-promoting factors like caspases is in part regulated by a class of proteins known as inhibitor of apoptosis proteins (IAPs) (17). Members of the IAP family contain at least one baculovirus IAP repeat (BIR) domain and most also contain domains associated with the ubiquitin degradation pathway. BRUCE (for BIR ubiquitin-conjugating enzyme) is an approximately 530 kDa mouse IAP that is thought to inhibit apoptosis by facilitating the ubiquitin-dependent degradation of multiple caspases (18,19). BRUCE null mice are embryonic lethal, while overexpression of either BRUCE or its human ortholog, Apollon, is sufficient to inhibit apoptosis induced by a variety of stimuli (18,20–22). Despite the significant role for BRUCE in blocking apoptosis, very little is known about the regulation of BRUCE expression.

Here, we show that PGF2 $\alpha$  reduces cell death during myogenesis and increases expression of BRUCE in an NFATC2-dependent manner. BRUCE is required for PGF2 $\alpha$ -mediated muscle cell growth, as siRNA-mediated knockdown of BRUCE expression blocks the ability of PGF2 $\alpha$  to induce myotube growth. In addition, overexpression of BRUCE is sufficient to inhibit muscle cell death during myogenesis and thereby leads to the formation of larger myotubes *in vitro*. These results not only provide important information regarding the mechanism by which PGF2 $\alpha$  promotes muscle growth, but also introduce a novel regulator of BRUCE expression.

## Results

### PGF2 $\alpha$ reduces cell death during myogenesis

We previously reported that PGF2 $\alpha$  promotes myotube growth during myogenesis *in vitro* (3). To confirm this observation, primary mouse muscle cells were differentiated for 24 hours, treated with PGF2 $\alpha$ , and assessed for changes in myotube size 24 hours after treatment. As expected, cells treated with PGF2 $\alpha$  formed larger myotubes after 48 hours of differentiation (Figure 1a). In addition, the proportion of myotubes containing  $\geq 5$  nuclei increased with PGF2 $\alpha$  treatment (Figure 1b). Control myotubes did not grow as large even at 72 hrs suggesting that PGF2 $\alpha$  does not promote myotube growth by accelerating the rate of growth. Our previous studies indicated PGF2 $\alpha$  did not induce gross differences in total DNA content as measured using a spectrophotometer assay (3). However, in the current study we consistently noted small

changes in cell density following PGF2 $\alpha$  treatment (Figure 1a). Therefore, we hypothesized that the change in myotube size following PGF2 $\alpha$  treatment might be a consequence of increasing the number of cells available for fusion. Consistent with this hypothesis, PGF2 $\alpha$  treatment led to an 18% increase in the total number of nuclei following 48 hours of differentiation (Figure 1c). The change in cell number following PGF2 $\alpha$  treatment was not transient, as the total number of nuclei remained elevated, even after 72 hours of differentiation (Figure 1c). To determine whether the increase in cell number following PGF2 $\alpha$  treatment was a result of a change in the fraction of cells undergoing proliferation, cells were incubated with the thymidine analogue BrdU at the time of PGF2 $\alpha$  treatment. Twenty-four hours later, cells were assessed for BrdU incorporation. PGF2 $\alpha$  did not increase the percentage of BrdU<sup>+</sup> cells (Figure 1d), suggesting that PGF2 $\alpha$  does not increase myotube size by promoting cell proliferation.

Because PGF2 $\alpha$  increases cell number without altering proliferation, we speculated that PGF2 $\alpha$  treatment reduces cell death during myogenesis. To quantify the extent of cell death that occurs during differentiation *in vitro*, we collected floating cells from the media following 48 hours of differentiation and incubated the cells with propidium iodide (PI) to stain dead cells. Approximately 12% of control cells died during this time period (Figure 1e), but PGF2 $\alpha$  reduced the number of PI<sup>+</sup> cells following 48 hours of differentiation by 62% (Figure 1e). To determine whether a proportion of the cell death that occurs during myogenesis results from programmed cell death, or apoptosis, differentiating muscle cells were analyzed for sub-diploid DNA content by flow cytometry. Myoblasts were cultured in differentiation media (DM) at low density for 24 hours to induce differentiation but restrict cell fusion, treated with vehicle or PGF2 $\alpha$ , and fixed 16 hours later. As a positive control for apoptosis, muscle cells were treated with 1  $\mu$ M staurosporine for 4 hours. PI staining was analyzed by flow cytometry and revealed a 25% reduction in apoptosis following PGF2 $\alpha$  treatment (Figure 1f). Together, these results indicate that PGF2 $\alpha$  reduces apoptosis during myogenesis.

To analyze whether PGF2 $\alpha$  could also alter the extent of muscle cell death *in vivo*, we assessed myoblast survival following transplantation. We chose myoblast transplantation as a model for muscle cell death *in vivo* because a high percentage of donor myoblasts undergoes death within several days of transplantation into recipient muscle (23). PGF2 $\alpha$  pre-treated male myoblasts were transplanted into the tibialis anterior (TA) muscles of female mice (Figure 1g). One week after transplantation, host muscles were collected and the extent of donor cell survival was estimated by performing real-time PCR with a Y-chromosome specific gene. PGF2 $\alpha$  pre-treatment increased the quantity of male DNA present in host muscles one week after transplantation approximately 2.6-fold (Figure 1f), indicating that PGF2 $\alpha$  pre-treatment increased the number of male donor cells that survived one week after transplantation. Although we cannot conclude that PGF2 $\alpha$  acts in the same manner *in vitro* and *in vivo*, our data do support a model in which PGF2 $\alpha$  protects myoblasts from cell death.

### PGF2 $\alpha$ increases BRUCE expression during myogenesis

To determine the mechanism by which PGF2 $\alpha$  mediates myotube growth and muscle cell survival, we performed microarray analyses of RNA isolated from differentiating muscle cells 6 hours after treatment with PGF2 $\alpha$ . Twenty known genes and several uncharacterized transcripts were identified as differentially expressed in response to PGF2 $\alpha$  through this microarray (Figure 2a). We were especially interested in the upregulation of BRUCE mRNA by PGF2 $\alpha$ , given the reported role for BRUCE in inhibition of apoptosis (18, 21). Therefore, we confirmed by real-time RT-PCR that BRUCE mRNA expression increases approximately two-fold in response to PGF2 $\alpha$  treatment (Figure 2b). To determine whether PGF2 $\alpha$  treatment increases BRUCE protein expression, we utilized flow cytometry (Figure 2c) and determined

that following 6 hrs of PGF2 $\alpha$  treatment, the proportion of BRUCE<sup>+</sup> muscle cells increased  $9.8 \pm 0.9\%$  relative to vehicle (n=4, p<0.001).

Expression of BRUCE during myogenesis has not been previously reported. We therefore performed RT-PCR and western blot analyses to examine BRUCE expression during myogenesis *in vitro*. BRUCE mRNA was present in proliferating myoblasts (0 hr, Figure 3a) and following the onset of differentiation (24 and 48 hr). However, BRUCE protein was detected only in differentiating myotubes, and not proliferating myoblasts (Figure 3b), suggesting that post-transcriptional regulation of BRUCE expression may occur in myoblasts. Immunostaining of muscle cells after 48 hours of differentiation revealed that BRUCE protein was present in both mononucleated cells and myotubes (Figure 3c). To determine when BRUCE is expressed during myogenesis *in vivo*, muscle injury was induced by injection of BaCl<sub>2</sub> into the TA muscles of adult mice. RNA and protein were collected from uninjured muscles or after 3, 5, 7, or 14 days of regeneration. RT-PCR determined that BRUCE mRNA is expressed throughout myogenesis *in vivo* (Figure 3d), but BRUCE protein was detected only at later stages of regeneration (Figure 3e). Together, these results demonstrate that BRUCE is expressed during skeletal muscle myogenesis.

### NFATC2 is required for upregulation of BRUCE by PGF2 $\alpha$

We have previously shown that PGF2 $\alpha$  does not promote growth of NFATC2 null myotubes (3), suggesting that the transcription factor NFATC2 is required for the growth-promoting effects of PGF2 $\alpha$ . Although PGF2 $\alpha$  treatment of wild-type cells led to an 18% increase in cell number during myogenesis (Figure 1b), no such effect was observed for NFATC2 null cells (Figure 4a). Similarly, PGF2 $\alpha$  did not increase expression of BRUCE mRNA in NFATC2 null myotubes (Figure 4b). To determine if basal expression of BRUCE is regulated by NFATC2 in muscle cells, we assessed BRUCE protein levels in wild-type and NFATC2 myotubes after 24 or 48 hours of differentiation (Figure 4c). Western blot analyses revealed that expression of BRUCE protein was not significantly altered in the absence of NFATC2. Together, these results indicate that while NFATC2 is not essential for maintenance of basal levels of BRUCE expression during myogenesis, the PGF2 $\alpha$ -mediated increase in BRUCE expression and muscle cell number are dependent upon NFATC2.

### BRUCE is required for PGF2 $\alpha$ -mediated reduction in muscle cell death

Because PGF2 $\alpha$  increases expression of BRUCE, an inhibitor of apoptosis, we hypothesized that the reduction of muscle cell death induced by PGF2 $\alpha$  is dependent upon upregulation of BRUCE. To test this hypothesis, we used RNAi to reduce BRUCE expression in primary myoblasts. One of two siRNA oligonucleotides corresponding to unique regions of the BRUCE transcript (BRUCE-1 and BRUCE-2) or a scrambled control siRNA oligonucleotide were transfected into proliferating myoblasts. BRUCE mRNA (Figure 5a) and protein (Figure 5b) were significantly reduced in differentiating cells by both siRNA oligonucleotides. To determine if BRUCE is required for the growth promoting effects of PGF2 $\alpha$ , myoblasts transfected with control or BRUCE siRNA were differentiated for 24 hours, treated with vehicle or PGF2 $\alpha$ , and fixed 24 hours later. Although PGF2 $\alpha$  increased the size of control myotubes, cells transfected with either BRUCE siRNA and treated with PGF2 $\alpha$  remained similar in size to vehicle treated cells (Figure 5c). Consistent with this observation, the percentage of BRUCE siRNA-transfected myotubes containing  $\geq 5$  nuclei did not increase in response to PGF2 $\alpha$  (Figure 5d). In addition, while PGF2 $\alpha$  increased the total number of control cells, BRUCE siRNA blocked this increase in cell number (Figure 5e). PGF2 $\alpha$  was also unable to reduce the death of cells transfected with BRUCE siRNA (Figure 5f). Together, these results support a model in which PGF2 $\alpha$  increases myotube size and reduces cell death by upregulating expression of BRUCE.

## Overexpression of BRUCE in muscle cells is sufficient to promote myotube growth and reduce cell death during myogenesis

If increased expression of BRUCE is required for PGF2 $\alpha$  to promote myotube growth, we speculated that increasing expression of BRUCE in the absence of PGF2 $\alpha$  would be sufficient to reduce muscle cell death and increase myotube size. To test this hypothesis, primary myoblasts transfected with either a BRUCE expression vector (18) or a control vector were differentiated for 48 hours. Western blot analysis of protein lysates collected after 24 hours of differentiation indicated that BRUCE was highly expressed in cells that had been transfected with the BRUCE expression vector (Figure 6a). BRUCE overexpressing myotubes were larger (Figure 6b) and the proportion of myotubes containing  $\geq 5$  nuclei increased 28% over control (Figure 6c). In addition, overexpression of BRUCE increased the total number of nuclei by 22% (Figure 6d) and reduced cell death after 48 hours of differentiation by 36% (Figure 6e). To determine if increasing BRUCE expression reduces cell death *in vivo*, we transplanted male muscle cells overexpressing BRUCE into the TA muscles of female mice. One week after transplantation, the quantity of male DNA present in the host muscles that received BRUCE overexpressing myoblasts was approximately 8-fold higher than muscles that received control myoblasts (Figure 6f), suggesting that BRUCE expression increased muscle cell survival *in vivo*. Together, these results demonstrate that increasing expression of BRUCE is sufficient to promote myotube growth and reduce cell death during myogenesis.

## Discussion

During muscle development and regeneration from injury *in vivo*, a subpopulation of myoblasts undergoes apoptosis (11,12,24,25). During myogenesis *in vitro*, as many as 50% of myoblasts reportedly undergo apoptosis (9,10,15), although the extent of cell death likely depends on origin of the myoblasts and the culture conditions used for each study. Myoblasts may undergo apoptosis during myogenesis as a means of regulating muscle size. Accordingly, excess myoblasts may be eliminated by apoptosis when the muscle reaches a predetermined size. Alternatively, a portion of myoblasts may undergo apoptosis as a consequence of the cellular stress involved in transitioning from a highly proliferative to terminally differentiated cell type. Our data show that PGF2 $\alpha$  reduces the extent of apoptosis that occurs during myogenesis *in vitro* (Figure 1f). Furthermore, our transplantation results suggest that PGF2 $\alpha$  treatment promotes muscle cell survival *in vivo* (Figure 1h). PGF2 $\alpha$  also increases myotube size (Figure 1a; (3)), suggesting that reducing cell death during myogenesis increases the pool of myoblasts available for fusion, and thus enhances myotube growth. In support of this concept, overexpression of Necdin reduces the number of apoptotic cells during skeletal muscle regeneration and promotes the formation of larger myofibers (24). Therefore, a change in the proportion of myoblasts that undergoes apoptosis during myogenesis influences muscle size.

PGs have been implicated in myoblast proliferation, differentiation, migration, and fusion (1), but the current study is the first study to demonstrate a function for a PG in myoblast survival. Studies in other cell types, however, have linked PG signaling to a reduction in apoptosis. Treatment of several cell types with PGF2 $\alpha$  reduces cell death in response to apoptotic stimuli (26–28), but induces apoptosis in others (29). Thus, the relationship between PG signaling and apoptosis is cell type specific. Interestingly, the deregulation of apoptosis that occurs with tumorigenesis is often accompanied by increased expression of COX-2 and PGs (30). Our results provide an additional link between PG signaling and the inhibition of cell death and may have important implications for therapeutic strategies to control the reduction of apoptosis associated with tumorigenesis. COX-2 inhibitors have been the subjects of clinical trials for the prevention of cancer, but the increased rates of cardiovascular complications associated with the use of COX-2 inhibitors have raised serious concerns.

Selective inhibitors of specific PGs may reduce the broad range of effects caused by COX-2 inhibition and therefore may be better candidates for chemoprevention.

Control of apoptosis is critical for tissue development and maintenance. The two convergent signaling cascades, the extrinsic and intrinsic pathways, which initiate apoptosis have been studied extensively. Both pathways lead to the activation of the caspase enzymes via cleavage of pro-caspase zymogens. The protease activity of caspases ultimately results in the execution of cell death. Interestingly, the activation of several caspases occurs during myogenesis (14, 15,31). Caspase-3 is activated early in muscle differentiation and promotes the activation of upstream regulators of the MAP kinase p38, a key initiator of myogenic differentiation (14). Caspase-12 is also activated during muscle differentiation, and has been implicated in the initiation of apoptosis that occurs during myogenesis (15). Activated caspases are inhibited by the IAP family of proteins, thus the IAPs serve as a shield to the final execution of apoptosis (17). Expression levels of IAPs are dynamic, and can be regulated at the transcriptional, post-transcriptional, and post-translational levels (17). Interestingly, increased expression of IAPs has been reported in several types of cancer cells and tumors (17). Although regulation of IAP transcription has not been well characterized, expression of the IAP survivin is cell cycle dependent (32) and NF- $\kappa$ B can modulate expression of c-IAP2 and xIAP (33,34). Interestingly, PGE<sub>2</sub> increases expression of survivin in multiple cell types, likely via activation of the E prostanoid (EP)<sub>2</sub>/EP<sub>4</sub> receptor and downstream of phosphatidylinositol-3-kinase activity (35, 36). Here, we have shown that PGF2 $\alpha$  increases expression of another IAP, BRUCE (Figure 2).

BRUCE, also known as Apollon in humans, is a 530 kDa mouse protein that is expressed in a variety of tissues (19). BRUCE not only binds caspases, but also promotes degradation of caspases by the ubiquitin proteasome pathway (18,20). BRUCE/Apollon expression protects cells from a number of apoptotic stimuli (18,20). Interestingly, caspase activity during spermatogenesis is regulated by the *Drosophila* homologue of BRUCE, which protects the differentiating sperm from apoptosis (37). Multiple groups have generated BRUCE deficient mice, and all mice are embryonic lethal (20–22). However, whether embryonic lethality associated with loss of BRUCE function is caused by an increase in apoptosis is unclear. Ren et al. reported the abundance of apoptotic cells in the placenta and yolk sac in mice lacking the C-terminal half of BRUCE. Surprisingly however, Lotz et al. did not detect significant changes in the extent of apoptosis in the developing BRUCE null embryo. Embryonic lethality in the BRUCE null mice may arise from a potential role for BRUCE in the regulation of cell proliferation (21,38). In our hands, siRNA knock-down of BRUCE in myoblasts does not alter cell proliferation (data not shown). However, siRNA knock-down of BRUCE expression blocks the ability of PGF2 $\alpha$  to reduce cell death during myogenesis (Figure 5). Unexpectedly, knock-down of BRUCE does not significantly alter basal levels of cell death. Possibly, other IAPs may compensate for this decrease in BRUCE expression during embryogenesis and myogenesis, thereby protecting cells from death in the absence of BRUCE function. Importantly, overexpression of BRUCE in myoblasts is sufficient to protect cells from undergoing death during myogenesis and leads to the formation of larger myotubes (Figure 6). Our results are consistent with previous studies and suggest that while loss of BRUCE does not induce spontaneous apoptosis, increasing BRUCE expression above basal levels is sufficient to protect cells from a variety of apoptotic stimuli.

Despite the importance of BRUCE in the inhibition of apoptosis, signaling pathways involved in the regulation of BRUCE expression have not been identified. Our results suggest that NFATC2, a transcription factor that is activated in response to increases in intracellular Ca<sup>2+</sup> levels, plays a key role in the regulation of BRUCE expression in muscle cells. PGF2 $\alpha$  signals through the G-protein coupled FP receptor to activate intracellular Ca<sup>2+</sup> signaling (35). Previously, we showed that treating myotubes with PGF2 $\alpha$  stimulates NFATC2 translocation

to the nucleus (3). Here, we have demonstrated that  $\text{PGF2}\alpha$  increases cell number and BRUCE expression, but only in the presence of NFATC2 (Figure 4). NFATC2 is not required for basal BRUCE expression during myogenesis. Interestingly, this is not the first example of gene expression that is regulated in an induced, but not basal manner by NFATC2. For example, while NFATC2 is required for increased expression of CD95 ligand in response to T cell receptor activation in T cells, constitutive expression of CD95 ligand in Sertoli cells is not dependent upon NFATC2 (39). Two NFAT binding sites in the CD95 ligand promoter direct inducible, but not constitutive gene expression. Whether the transcriptional activity of NFATC2 directly regulates BRUCE expression in muscle, or if an intermediate pathway is involved, remains to be determined once the promoter region of BRUCE is defined. Importantly, this work identifies a hitherto unrecognized relationship between NFAT signaling and regulation of IAP expression.

The identification of factors that increase the pool of myoblasts available for fusion and therefore promote muscle growth may lead to novel therapies for the treatment of muscle disorders. We have shown that  $\text{PGF2}\alpha$  and BRUCE support myoblast survival *in vitro* and *in vivo*, which may be valuable for increasing the success of myoblast transplantation strategies for muscular disorders. In addition, this work has identified  $\text{PGF2}\alpha$  and NFATC2 as novel regulators of BRUCE expression. To date, few factors regulating IAP expression have been identified and the discovery of additional regulators could provide new targets for chemoprevention.

## Materials and Methods

### Primary muscle cell culture

Primary myoblasts were derived from the hindlimb muscles of 6–8 week old female wild-type or NFATC2<sup>-/-</sup> Balb/C mice, unless otherwise noted, and cultured to >99% purity as previously described (7). Cells were maintained in growth media (GM: Ham's F10, 20% FBS, 5 ng/ml bFGF, 100 U/ml penicillin G, 100  $\mu\text{g}/\text{ml}$  streptomycin) in a humidified 5%  $\text{CO}_2$  incubator at 37°C on collagen-coated dishes. Cells were differentiated in differentiation media (DM: DMEM, 1% Insulin-Transferrin-Selenium A (Invitrogen, Carlsbad, CA) 100 U/ml penicillin G, 100  $\mu\text{g}/\text{ml}$  streptomycin) on 6-well plates coated with Entactin-Collagen IV-Laminin (E-C-L, Millipore, Billerica, MA) at a density of  $2 \times 10^5$  cells per well. For prostaglandin experiments, myoblasts were differentiated for 24 hrs prior to treatment with vehicle (0.095% ethanol) or 1  $\mu\text{M}$   $\text{PGF2}\alpha$  (Sigma, St. Louis, MO).

### Analysis of cell proliferation, death, and fusion

To assess cell proliferation, differentiating myotubes were incubated in 25  $\mu\text{M}$  5-Bromo-2'-deoxyuridine (BrdU, Sigma) for 24 hrs. Cells were subsequently fixed in 2% paraformaldehyde and immunostained using a BrdU antibody (Accurate Chemical and Scientific Corp, Westbury, NY) at a dilution of 1:500. At least 500 myoblasts from three independent isolates were analyzed for each condition.

To analyze cell death, the medium was collected following 48 hrs of differentiation and spun at  $1400 \times g$  for 2 min to collect floating cells. These cells were then resuspended in PBS containing 0.5  $\mu\text{g}/\text{ml}$  propidium iodide (PI, Sigma) and incubated for 5 min. PI<sup>+</sup> cells were counted on a hemocytometer to quantify the number of dead cells in each condition. Apoptosis of differentiating muscle cells was analyzed as described previously (40,41). Myoblasts were differentiated at low density ( $10^6$  cells per 150 mm dish) for 40 hrs. The medium was collected and spun at  $1400 \times g$  for 2 min to collect floating cells and adherent cells were collected by trypsinization. Cells were fixed for 1 hr in 100% ethanol on ice and subsequently suspended in 50 mM  $\text{Na}_2\text{HPO}_4$  and 10 mM citric acid, pH 7.8, diluted in PBS for 5 min at room

temperature. Cells were then suspended in 0.5  $\mu\text{g/ml}$  PI and analyzed by flow cytometry. Data from all flow cytometry experiments were analyzed using FlowJo software (Version 8.7, TreeStar Inc., Ashland, OR).

To assess myoblast fusion, cells were differentiated for 48 hrs, fixed in 3.7% formaldehyde, and immunostained for embryonic myosin heavy chain (F1.652, Developmental Studies Hybridoma Bank, Iowa City, IA) as described previously (3). The number of nuclei in each myotube ( $\geq 2$  nuclei) was counted and the percentage of myotubes with  $\geq 5$  nuclei was calculated. Fusion assays were performed using myoblasts from three independent isolates and  $\geq 100$  myotubes were analyzed for each experiment.

### Myoblast transplantations

Primary wild-type myoblasts from 6–8 week old male C57Bl/6 mice were prepared for transplantation by differentiating at low density ( $10^6$  cells per 150 mm E-C-L-coated plate) for 24 hrs while being treated with vehicle or 1  $\mu\text{M}$  PGF2 $\alpha$  at 0 and 12 hrs of differentiation. Alternatively, myoblasts transfected with control or BRUCE vectors (as described below) were differentiated at low density for 24 hrs. Prior to transplantation, the cells were suspended at a concentration of  $6 \times 10^4$  cells/ $\mu\text{l}$  in PBS with 0.1% BSA. A 25  $\mu\text{l}$  Hamilton syringe was used to inject a 5  $\mu\text{l}$  cell suspension into the tibialis anterior (TA) muscles of 8 week old female C57Bl/10ScSn-*Dmd*<sup>*mdx*</sup>/J mice (Jackson Labs, Bar Harbor, ME). Seven days after transplantation, TA muscles were collected and DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA isolated from each muscle was suspended in 200  $\mu\text{l}$  elution buffer.

Real-time PCR was used to quantify male DNA content in each recipient female muscle using the iCycler iQ Real-Time Detection system (Bio-Rad, Hercules, CA). PCR reactions were performed using 4  $\mu\text{l}$  DNA with 6 min denaturation at 95°C followed by 45 cycles of 95°C for 15 sec, 58°C for 20 sec, and 60°C for 35 sec. SYBR Green fluorescence was measured after the end of the extension step of each cycle. Primers for the Y-chromosome-specific gene *Zfy1* were used to amplify the male DNA (accession no. NM\_009570; sense, 5' TGGAGAGCCACAAGCTAACCA, antisense, 5' TCCCAGCATGAGAAAGATTCTTC). The starting quantity of male DNA template in each sample was determined by comparing the threshold cycle of PCR amplification ( $C_T$ ) to a standard curve. To normalize samples for total DNA content, real-time PCR was performed with primers for an autosomal gene (*NFAT5*, accession no. NM\_018823; sense, 5' GGGCTATAGACATGCACCACCACACAG, antisense, 5' AAGGGCTTCTCCAGAATGG). All real-time PCR reactions were performed in triplicate.

### Microarray analysis

Primary myoblasts were differentiated for 24 hrs prior to treatment with vehicle (0.095% ethanol) or 1  $\mu\text{M}$  PGF2 $\alpha$  (Sigma). Six hrs after treatment, RNA was isolated in a two-step process. Total RNA was first isolated using TRIzol reagent (Invitrogen), followed by further purification using the RNeasy kit and DNase treatment (Qiagen) according to the manufacturer's instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA integrity number of all samples was above 9.0. The Chemiluminescent RT-IVT Labeling Kit (Applied Biosystems, Foster City, CA) was used to amplify 1  $\mu\text{g}$  of total RNA according to manufacturer's instructions. For each sample, 10  $\mu\text{g}$  of cRNA was hybridized to a Mouse Genome Survey Microarray (Applied Biosystems) and the 1700 Chemiluminescent Microarray Analyzer (Applied Biosystems) was used to scan the microarrays and generate raw data. Quantile normalization of all data was performed using Bioconductor ([www.bioconductor.org](http://www.bioconductor.org)) and probes with the signal to noise ratio higher than 3 and flags lower than 5000 were used for further analysis. Probes with  $p < 0.05$  and a fold change



of  $\geq 2$  were considered significant. RNA was isolated from three independent cell isolates. Microarray analyses were performed by the Winship Cancer Institute Microarray Core at Emory University.

### Analysis of BRUCE mRNA expression

Muscle regeneration was induced in the TA muscles of 8 week old female Balb/C mice by injection of 50  $\mu$ l of 1.2% BaCl<sub>2</sub>. Differentiation of primary muscle cells was performed as described above. Total RNA was isolated from tissue or cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and cDNA was generated by reverse transcription using 1.5  $\mu$ g of total RNA. For semi-quantitative RT-PCR, BRUCE cDNA was amplified by 5 min denaturation at 95°C, followed by 32 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec. Primers specific to BRUCE (accession no. NM\_007566; sense, 5' CTCAGTCAGTCCTGCCTCATC, antisense, 5' CTCAGCAGTCCTCTGGATGTC) were designed to span intron and exon boundaries to control for any contaminating DNA in the RNA samples. Amplicons were separated by electrophoresis in a 1% agarose gel and visualized with ethidium bromide. To analyze BRUCE mRNA expression by quantitative real-time RT-PCR, BRUCE cDNA standards were first generated by performing RT-PCR with primers described above and gel purified with the QIAquick gel extraction kit (Qiagen). Real-time PCR was performed as described above with 40 cycles of 95°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec. The primers used for real-time RT-PCR amplified a region of the BRUCE transcript within the amplicon that had been generated for the standard curve (sense, 5' ATCGTGCTCTGCTAGAGTTGC, antisense, 5' ATCTTAGGCGGTTGGTGTAGG). Amplification of 18S ribosomal RNA using QuantumRNA primers (Ambion, Foster City, CA) was used as an input control for all reactions. Total RNA was isolated from three independent cell isolates and all real-time PCR reactions were run in triplicate.

### Analysis of BRUCE protein expression

For flow cytometry analysis, myoblasts were differentiated at low density (10<sup>6</sup> cells per 150 mm dish) for 24 hrs, treated with vehicle or PGF2 $\alpha$ , and collected by trypsinization 6 hrs later. Cells were fixed in 100% methanol for 10 min on ice and subsequently washed with PBS containing 0.1% bovine serum albumin (BSA). Cells were then incubated for 30 min on ice in BRUCE antibody (BD, Franklin Lakes, NJ) or control mouse IgG (Jackson ImmunoResearch, West Grove, PA) diluted to a concentration of 2.5  $\mu$ g/ml. Following three washes in PBS, cells were incubated on ice for 30 min in R-Phycoerythrin-labeled goat-anti-mouse (Invitrogen) antibody diluted 1:250. Following three additional washes, cells were analyzed by flow cytometry.

For western blot analysis, total protein was isolated from primary muscle cells or muscle tissue using M-PER reagent (Pierce, Rockford, IL) supplemented with protease inhibitors (Mini complete, Roche, Basel, Switzerland). Cells were lysed at 4°C for 15 min and lysates were cleared of insoluble material by spinning at 21,000 $\times$ g for 15 min. Protein concentration was determined by Bradford analysis (Bio-Rad) and equal amounts of protein ( $\geq 15\mu$ g) were loaded on each lane of a 5% polyacrylamide gel. Samples were separated by SDS-PAGE and transferred at 400 mA for 2 hrs to a nitrocellulose membrane. Immunoblotting was performed using BRUCE (BD, Franklin Lakes, NJ) or  $\alpha$ -tubulin (Sigma) antibodies diluted 1:500.

For immunostaining, primary muscle cells were differentiated for 0, 24, or 48 hrs and fixed in 3.7% formaldehyde for 10 min. Cells were first incubated for 1 hr in block buffer (5% donkey serum in PBS with 0.25% Triton X-100), followed by BRUCE or control IgG antibody diluted 1:100 in block buffer for 1 hr at room temperature. Following three washes in PBS + 0.2% Tween-20 (PBS-T), biotin-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch) diluted 1:250 in block buffer was added to cells for 1 hr and subsequently

washed as before. Antibody binding was detected with Texas Red-streptavidin (Jackson ImmunoResearch) diluted 1:500 in block buffer. Nuclei were visualized with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma).

### Transfection of primary myoblasts

Stealth RNAi (Invitrogen) was used for siRNA 'knock-down' experiments. Myoblasts were plated in GM at a density of  $10^5$  cells/well on a collagen-coated 6 well plate and twenty-four hrs later, duplexed RNA was added at a dilution of 80nM. Cells were then transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hrs after the transfection, cells were re-plated and differentiated as described above. Total RNA and protein were isolated after 24 hrs in DM and knock-down was assessed by real-time PCR and western blot. Two siRNA oligonucleotides corresponding to base-pairs 9676–9700 (BRUCE-1, (5'-3') CCU GAC AAU GCA GAA GGA AUC CAU A) or 11313–11337 (BRUCE-2, (5'-3') GAA AGA UUG GCU UGG UGG UUC UGA A) of the BRUCE mRNA transcript or a control scrambled oligonucleotide (Stealth RNAi Low GC negative control) were used for each transfection. Knock-downs were performed with myoblasts from three independent cell isolates.

A mammalian expression vector encoding FLAG-tagged full-length BRUCE cDNA (18) was used to overexpress BRUCE in primary myoblasts. In order to select for transfected myoblasts, the cells were co-transfected with a plasmid containing a neomycin resistance gene (pEXPR-IBA3, IBA Biotagnology, Hanover, Germany) at a ratio of 9 parts BRUCE-FLAG to 1 part pEXPR. Lipofectamine LTX with Plus Reagent (Invitrogen) was used to transfect  $2 \times 10^5$  cells with 2.5  $\mu$ g total DNA according to the manufacturer's instructions. Cells were subsequently grown in GM containing 50  $\mu$ g/ml G418 (Invitrogen) for approximately 2 weeks to ensure adequate selection and to allow for recovery from transfection. Overexpression was confirmed by western blot using BRUCE or FLAG (Sigma) antibodies diluted 1:500. Transfections were performed with myoblasts from three independent cell isolates.

### Statistical analyses

Comparisons between two groups were made using *t* tests. Analyses of multiple groups were performed using a two-way ANOVA with Bonferroni's posttest. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad) for Macintosh or Sigma-Stat 2.03 (SPSS). For all statistical tests, a confidence level of  $p < 0.05$  was accepted for statistical significance.

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### Abbreviations

<b>PGF2<math>\alpha</math></b>	prostaglandin F2 alpha
<b>IAP</b>	inhibitor of apoptosis protein
<b>NFAT</b>	nuclear factor of activated T cells
<b>PG</b>	

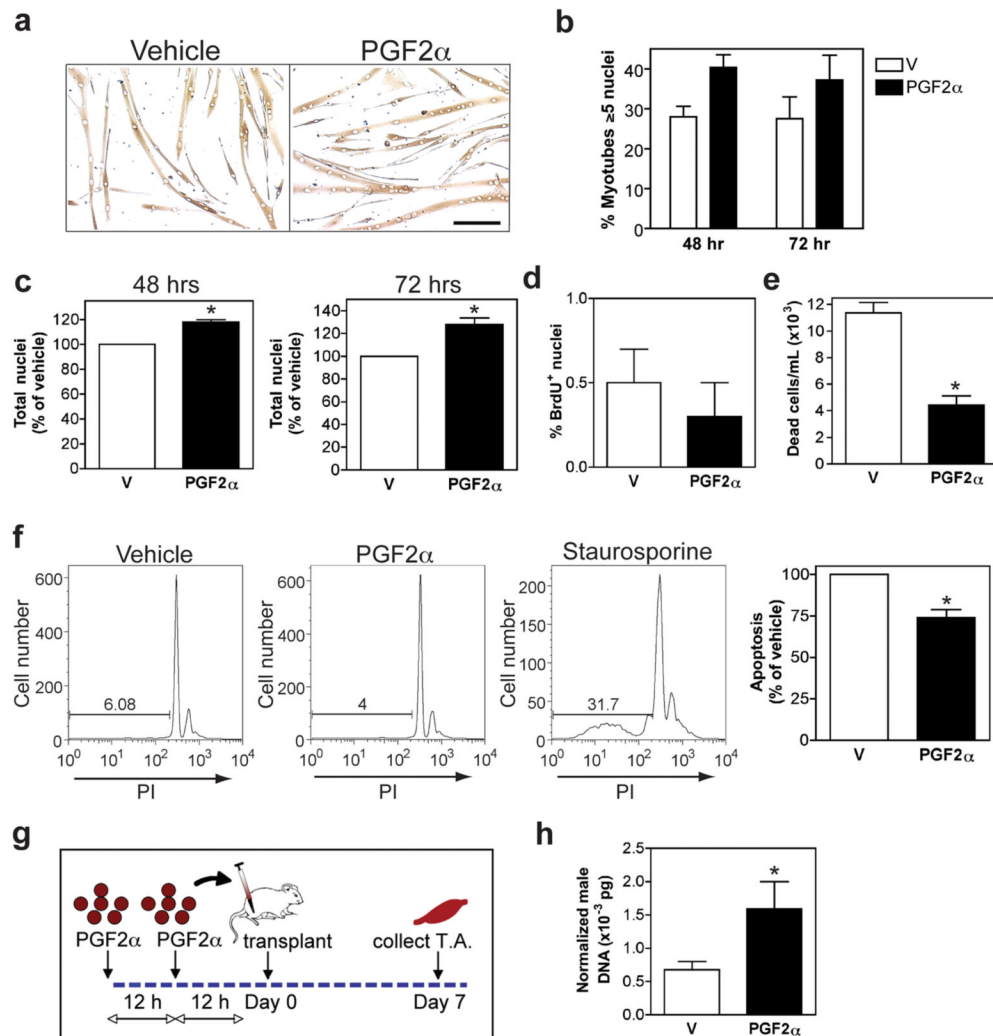
	prostaglandin
<b>COX</b>	cyclooxygenase
<b>IL-4</b>	interleukin-4
<b>BIR</b>	baculovirus IAP repeat
<b>BrdU</b>	5-bromo-2-deoxyuridine
<b>TA</b>	tibialis anterior
<b>PI</b>	propidium iodide

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**Figure 1. PGF2 $\alpha$  reduces cell death during myogenesis**

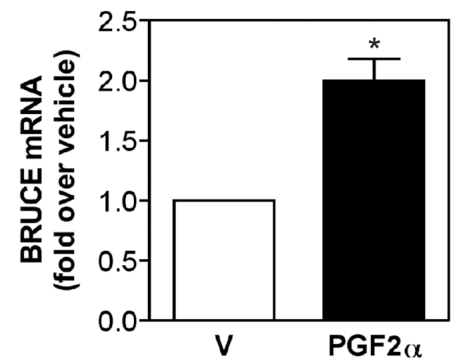
(a) Primary myoblasts were differentiated for 24 hrs, treated with vehicle (V) or 1  $\mu$ M PGF2 $\alpha$ , and 24 hrs later fixed and immunostained for eMyHC. Bar: 50  $\mu$ m. (b) Myoblasts were treated as in (a) and fixed after 48 and 72 hrs of differentiation. The number of nuclei per myotube was counted and the percentage of myotubes containing  $\geq 5$  nuclei was calculated. PGF2 $\alpha$  increased the percentage of myotubes with  $\geq 5$  nuclei. Control myotubes did not increase in size with time. (c) The average number of nuclei from 5 fields of each condition was calculated. After 48 hrs of differentiation, PGF2 $\alpha$  increased the number of nuclei per field by approximately 18% whereas after 72 hrs a 22% increase occurred ( $n=3$  at each timepoint). (d) Primary myoblasts were differentiated for 24 hrs, treated with V or PGF2 $\alpha$  and simultaneously incubated with BrdU. Cells were fixed 24 hrs later, immunostained for BrdU, and nuclei were visualized with DAPI. No difference was observed in the percentage of BrdU $^+$  nuclei ( $n=3$ ). (e) Primary myoblasts were differentiated for 24 hrs, treated with V or PGF2 $\alpha$ , and 24 hrs later dead cells in the media were stained with PI. PGF2 $\alpha$  reduced the number of PI $^+$  cells by 62% ( $n=3$ ). (f) Myoblasts were differentiated for 24 hrs at low density, treated with V or PGF2 $\alpha$ , and collected 16 hrs later. Analysis of DNA content by flow cytometry revealed a 25% reduction in sub-diploid DNA content following PGF2 $\alpha$  treatment. (g) Schematic representation of myoblast transplantation experiment. Briefly, male myoblasts were pre-treated with V or PGF2 $\alpha$  24 hrs prior to transplant into the TA of recipient female mice. One

week after the transplant, DNA from each TA was collected and real-time PCR was performed with primers for a Y-chromosome gene. (h) Pre-treatment with PGF2 $\alpha$  led to an approximately 2.6-fold increase in male DNA content within the recipient TA muscles 1 week after transplantation (n=11). All data are mean  $\pm$  SE. (\*significantly different,  $p < 0.05$ )

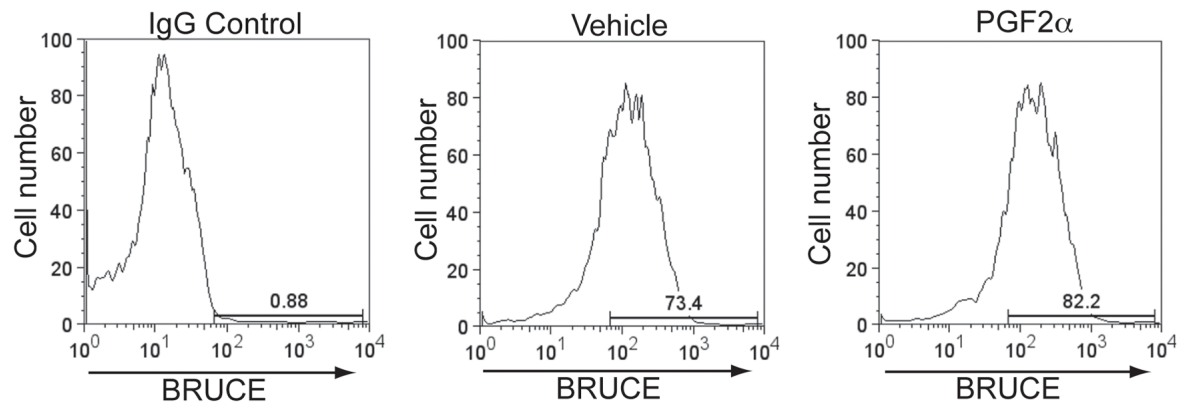
a

Gene Name	Fold Change (PGF2 $\alpha$ /Vehicle)
Baculoviral IAP-repeat containing 6 (BRUCE)	5.06
Olfactory Receptor 203	3.54
Secretory blood group 1 (Sec1)	3.41
Olfactory Receptor 643	3.27
Olfactory Receptor 895	3.25
Olfactory Receptor 700	3.16
Tetratricopeptide repeat domain 21B (Ttc21b)	3.02
Olfactory Receptor 132	3.00
Zinc finger, FYVE domain containing 26 (Zfyve26)	2.99
Flavin containing monooxygenase 4 (Fmo4)	2.88
Olfactory Receptor 434	2.88
5'nucleotidase, cytosolic II (Nt5c2)	2.80
Neurofilament 3 (Nef3)	2.64
Surfactant associated protein A1 (Sftpa1)	2.54
Proteasome 26S subunit 9 (Psm9)	2.51
Wingless-related MMTV integration site 3a (Wnt3a)	2.38
Periplakin (Pp1)	2.1
ATP-binding cassette family B, member 4 (MDR/TAP)	0.47
MAP kinase-activated protein kinase 3 (Mapkapk3)	0.42
Delta like kinase 1 (Dlk1)	0.31

b



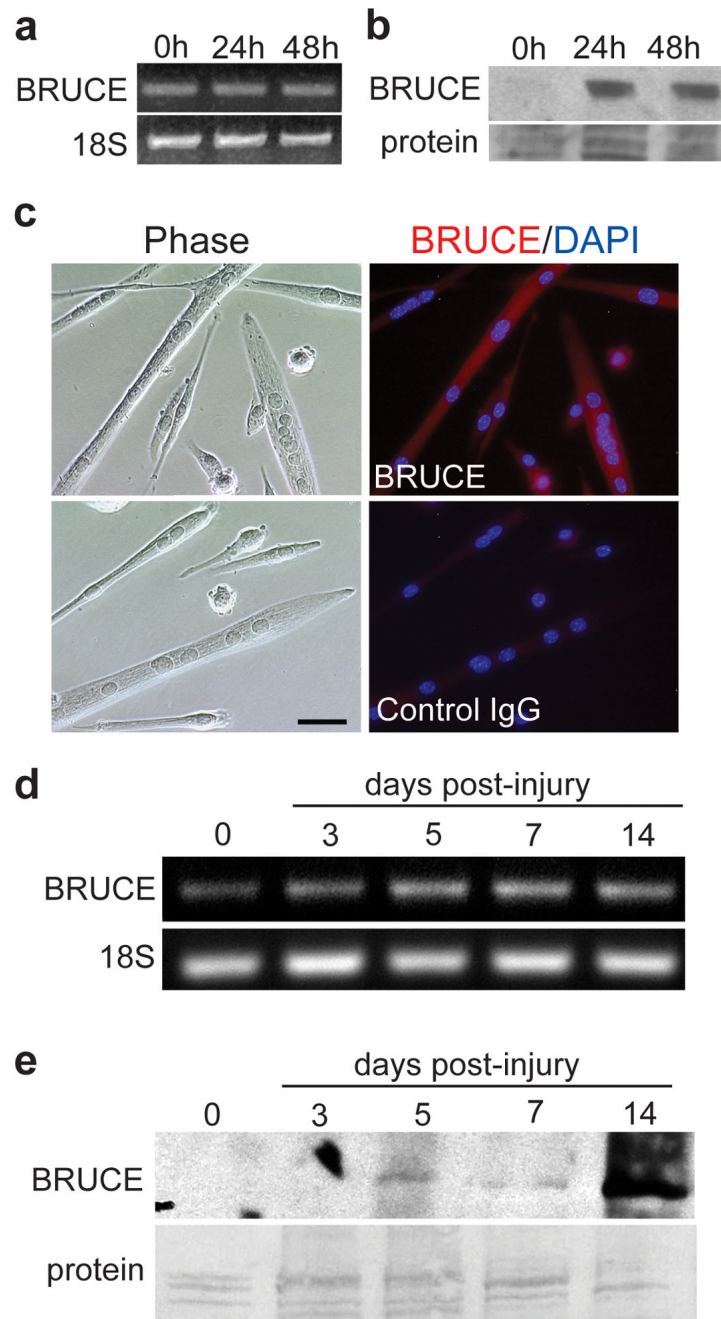
c



### Figure 2. PGF2 $\alpha$ increases expression of BRUCE

(a) Primary myoblasts were differentiated for 24 hrs, treated with vehicle (V) or 1 $\mu$ M PGF2 $\alpha$ , and 6 hrs later total RNA was collected. Microarray analyses were performed and genes with changes in expression of at least 2-fold with  $p < 0.05$  in response to PGF2 $\alpha$  treatment are listed. (b) Real-time RT-PCR confirmed a 2-fold increase in BRUCE mRNA following 6 hrs of PGF2 $\alpha$  treatment. Data are mean  $\pm$  SE,  $n = 3$ . (\*significantly different,  $p < 0.05$ ) (c) Representative histograms of BRUCE protein analysis by flow cytometry. The percentage of BRUCE<sup>+</sup> cells for each experiment was determined by setting the gate to include  $< 1\%$  of cells incubated with control mouse IgG. PGF2 $\alpha$  treatment increased the number of BRUCE<sup>+</sup> cells by  $9.8 \pm 0.9\%$  relative to vehicle ( $n = 4$ ,  $p < 0.001$ ).

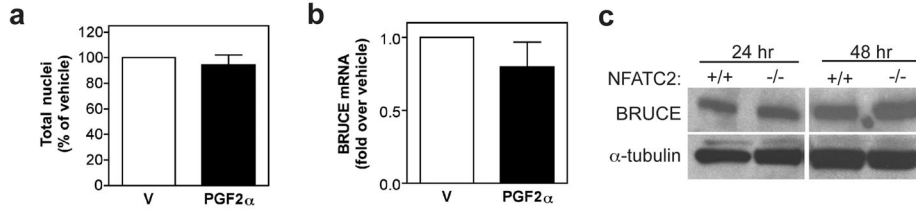




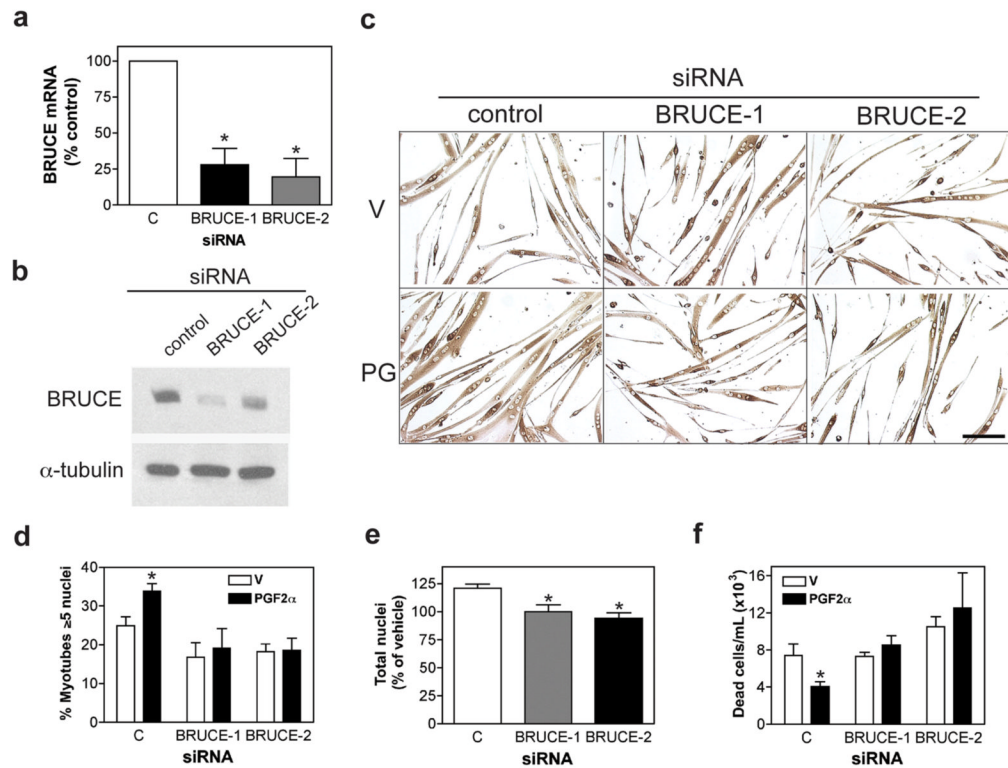
**Figure 3. BRUCE is expressed during myogenesis *in vitro* and *in vivo***

(a) Primary myoblasts were differentiated for 0, 24, or 48 hrs and BRUCE RNA was analyzed by RT-PCR. 18S ribosomal RNA was used as an internal control. BRUCE, 372 bp; 18S, 488bp. (b) Primary myoblasts were differentiated for 0, 24, or 48 hrs and BRUCE protein was analyzed by western blot. Ponceau S staining was used as a loading control. BRUCE, 530 kDa. (c) Representative fluorescent and phase-contrast images of muscle cells after 48 hrs of differentiation that have been immunostained for BRUCE and counterstained with DAPI. Bar: 25  $\mu$ m. (d) Muscle regeneration was induced by injection of BaCl<sub>2</sub> into the TA muscles of adult mice. Total RNA was isolated from uninjured muscles (0) or following 3, 5, 7, or 14 days of regeneration and BRUCE mRNA was analyzed by RT-PCR. cDNA was pooled from 2–3

mice for each timepoint. 18S ribosomal RNA was used as an internal control. BRUCE, 372 bp; 18S, 488bp. (e) Protein was isolated from muscle tissue following injury and BRUCE expression was analyzed by western blot. Ponceau S staining was used as a loading control. BRUCE, 530 kDa.

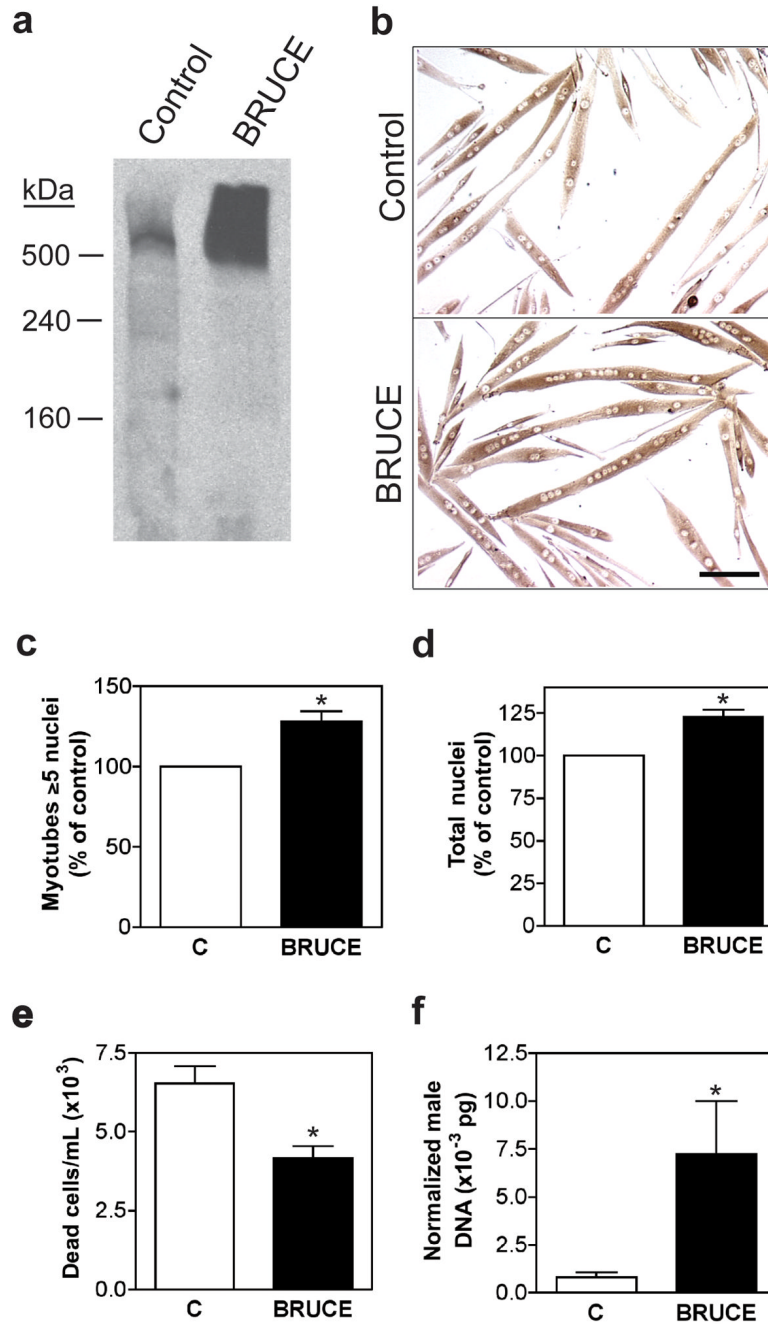


**Figure 4. NFATC2 is required for the regulation of BRUCE expression in muscle cells**  
 (a) NFATC2<sup>-/-</sup> myoblasts were differentiated for 24 hrs, treated with vehicle (V) or PGF2 $\alpha$ , and fixed 24 hrs later. The average number of nuclei from 5 fields of each condition was calculated. No difference in the number of nuclei per field following PGF2 $\alpha$  treatment was observed. (b) NFATC2<sup>-/-</sup> myoblasts were differentiated for 24 hrs, treated with V or PGF2 $\alpha$ , and 6 hrs later total RNA was collected. No difference in BRUCE mRNA was detected by real-time RT-PCR. (c) Wild-type or NFATC2<sup>-/-</sup> myoblasts were differentiated for 24 or 48 hrs and BRUCE protein was analyzed by western blot. No difference in BRUCE expression was observed between genotypes at either timepoint.  $\alpha$ -Tubulin was used as a loading control. BRUCE, 530 kDa;  $\alpha$ -tubulin, 55 kDa. All data are mean  $\pm$  SE, n=3.



### Figure 5. BRUCE is required for PGF2 $\alpha$ -mediated myotube growth

Primary myoblasts were transfected with one of two siRNA oligonucleotides for BRUCE (BRUCE-1 or BRUCE-2) or a scrambled control (C) and switched to DM 24 hrs later. (a) After 24 hrs in DM, BRUCE mRNA was analyzed by real-time RT-PCR. BRUCE mRNA expression was reduced greater than 70% by both siRNAs. (b) Western blots were performed with protein from transfected cells after 24 hrs in DM, which revealed that BRUCE protein expression was reduced by both siRNAs.  $\alpha$ -Tubulin was used as a loading control. BRUCE, 530 kDa;  $\alpha$ -tubulin, 55 kDa. (c) Myoblasts were differentiated for 24 hrs, treated with V or 1  $\mu$ M PGF2 $\alpha$ , and 24 hrs later fixed and immunostained for eMyHC. Bar: 50  $\mu$ m. (d) The number of nuclei per myotube was counted and the percentage of myotubes containing  $\geq 5$  nuclei was calculated. Although PGF2 $\alpha$  significantly increased the percentage of myotubes containing  $\geq 5$  nuclei, BRUCE siRNA blocked this increase in myotube size. (e) The average number of nuclei from 5 fields of each condition was calculated. Results are presented as the percentage difference between PGF2 $\alpha$  and V-treated cells. Although PGF2 $\alpha$  increased the number of control nuclei by approximately 20%, no such increase was observed for BRUCE siRNA-transfected cells. (f) Dead cells in the media were collected and stained with PI after 48 hrs in DM. Although the number of PI<sup>+</sup> control-transfected cells was reduced by 45% following PGF2 $\alpha$  treatment, PGF2 $\alpha$  did not alter the death of BRUCE siRNA-transfected cells. All data are mean  $\pm$  SE, n=3. (\*significantly different, p<0.05)



**Figure 6. BRUCE overexpression is sufficient to promote myotube growth and reduce muscle cell death during myogenesis**

(a) Primary myoblasts transfected with control (C) or BRUCE expression vectors were differentiated for 24 hrs and BRUCE protein was analyzed by western blot. BRUCE, 530 kDa. (b) Primary myoblasts transfected with C or BRUCE expression vectors were differentiated for 48 hrs, fixed, and immunostained for eMyHC. Bar: 50  $\mu$ m. (c) The number of nuclei per myotube was counted and the percentage of myotubes containing  $\geq 5$  nuclei was calculated. Overexpression of BRUCE increased the proportion of myotubes with  $\geq 5$  nuclei 28% over control. (d) The average number of nuclei from 5 fields of each condition was calculated. BRUCE overexpression increased the number of nuclei by 22%. (e) The number of PI<sup>+</sup> cells

in the media of BRUCE-overexpressing myotubes was reduced 36% compared with control. (f) Male myoblasts were transfected with a control or BRUCE expression vector and transplanted into the TA of recipient female mice. Real-time PCR of a Y-chromosome gene demonstrated that male DNA content within the host muscle one week after transplant was increased approximately 8-fold by BRUCE overexpression in donor muscle cells. All data are mean SE, n=3. (\*significantly different,  $p < 0.05$ )