Activation of non-myogenic mesenchymal stem cells during the disease progression in dystrophic dystrophin/utrophin knockout mice

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Abstract

Ectopic calcification as well as fatty and fibrotic tissue accumulation occurs in skeletal muscle during the disease progression of Duchenne muscular dystrophy (DMD), a degenerative muscle disorder caused by mutations in the dystrophin gene. The cellular origin and the environmental cues responsible for this ectopic calcification, fatty and fibrotic infiltration during the disease progression, however, remain unknown. Based on a previously published preplate technique, we isolated two distinct populations of muscle-derived cells from skeletal muscle: (i) a rapidly adhering cell population, which is non-myogenic, Pax7⁻ and express the mesenchymal stem cell (MSC) marker platelet-derived growth factor receptor alpha; hence, we termed this population of cells non-myogenic MSCs (nmMSCs); and (ii) a slowly adhering cell population which is Pax7⁺ and highly myogenic, termed muscle progenitor cells (MPCs). Previously, we demonstrated that the rapid progression of skeletal muscle histopathologies in dystrophin/utrophin knockout (dys⁻/⁻ utro⁻/⁻ dKO) mice is closely associated with a rapid depletion of the MPC population pool. In the current study, we showed that in contrast to the MPCs, the nmMSCs become activated during the disease progression in dKO mice, displaying increased proliferation and differentiation potentials (adipogenesis, osteogenesis and fibrogenesis). We also found that after co-culturing the dKO-nmMSCs with dKO-MPCs, the myogenic differentiation potential of the dKO-MPCs was reduced. This effect was found to be potentially mediated by the secretion of secreted frizzled-related protein 1 by the dKO-nmMSCs. We therefore posit that the rapid occurrence of fibrosis, ectopic calcification and fat accumulation, in dKO mice, is not only attributable to the rapid depletion of the MPC pool, but is also the consequence of nmMSC activation. Results from this study suggest that approaches to alleviate muscle weakness and wasting in DMD patients should not only target the myogenic MPCs but should also attempt to prevent the activation of the nmMSCs.

Introduction

Adult skeletal muscle possesses a remarkable regenerative ability dependent on muscle progenitor cells (MPCs) called satellite cells which reside beneath the basal lamina, closely juxtaposed to the muscle fibers (1–4). However, many studies have reported that in addition to satellite cells, a variety of other stem/progenitor cells can also be found in skeletal muscle and are a potential alternative cell source for muscle repair (5–10). Despite the presence of these muscle regenerative cell populations, skeletal muscle integrity can be debilitated by the deposition of adipose and fibrotic tissues in a variety of pathological conditions including Duchenne muscular dystrophy (DMD) (11,12).

DMD is one of the most common childhood muscular dystrophy, with an incidence of ~1 in every 3500 live male births (13). It is an x-linked, inherited disease caused by a lack of functional dystrophin, an essential transmembrane muscle protein...
within the dystrophin–glycoprotein complex in both skeletal and cardiac muscle cells (14,15). In dystrophic muscle, the damaged fibers degenerate and undergo necrosis and lose their ability to regenerate. Satellite cells are recruited to regenerate new myofibers, but this regeneration is often inefficient due to repeated cycles of degeneration and regeneration, which eventually leads to an exhaustion/depletion of the satellite cell population (16). Progressive muscle weakness and degeneration usually leads to the loss of independent ambulation by the middle of the patient’s second decade and a fatal outcome due to cardiac or respiratory failure by their third decade of life (17,18).

Recent evidence has emerged implicating adult stem cell dysfunction in the progression of DMD-associated histopathogenesis. These studies have reported that the rapid progression of muscle weakness in DMD might correlate with the decline in the number of functional MPCs (7,19,20). Of note, despite the lack of dystrophin from birth, the onset of the muscle weakness typically does not occur until patients reach 4–8 years of age, which happens to coincide with the exhaustion/depletion of the MPC pool due to the repeated cycles of degeneration and regeneration that the muscle fibers undergo (16,20).

One of the most striking pathological conditions in advanced cases of DMD is the accumulation of adipocytes, calcium deposits and fibrosis. Importantly, even with the occurrence of MPC depletion, we observed the formation of more adipose and fibrotic tissue in the skeletal muscle, heart and diaphragm of 6–8-week-old dKO mice (7,21). However, it remains unclear what cell population is responsible for the formation of these non-skeletal muscle tissues. Of note, although the mdx mouse is commonly used as an animal model of DMD, 6–8-week-old mdx mice exhibit only a mild dystrophic phenotype (muscle fiber degeneration and necrosis) and do not develop the severe histopathologies exhibited by age matched dKO mice, such as the accumulation of calcium deposits and fibrosis (7). Therefore, we focused this study on examining a population of cells in dKO mice we posited were responsible for the formation of the above dystrophic histopathologies.

Our research group has isolated two distinct populations of muscle-derived cells from the skeletal muscle of dKO mice utilizing a previously published preplate technique (22); (i) a rapidly adhering cell (RAC) fraction, and (ii) a slowly adhering cell (SAC) fraction. In previous publications, we characterized the SACs as a heterogeneous population of Pax7+ cells called muscle-derived stem cells (MDSCs) which are MPCs with high myogenic potentials, both in vitro and in vivo (8,22,23). We recently reported that MPCs isolated from 6- to 8-week-old dKO mice display a significant reduction in their proliferation capacity, resistance to oxidative stress and multilineage differentiation potentials, when compared with MPCs isolated from age-matched mdx and wild-type (WT) mice. These results suggest that in contrast to mdx and WT mice, rapid stem cell depletion occurs in the dKO mice (7). We therefore postulated that the decline in MPCs may be responsible for the progressive reduction in muscle regeneration and the rapid occurrence of the histopathology observed in the dKO mice (7). Since MPCs become impaired during the progression of the disease, it is unlikely that these cells are involved in the formation of ectopic non-muscle tissues. Until recently, we have considered RACs to be fibroblast-like cells that are highly positive for Sca-1 and CD34 (24), two cell surface markers that are also expressed by fibro/adipogenic progenitors (FAPs) (25); however, they have never been thoroughly characterized. Therefore, we hypothesized that the RACs might be responsible for the deposition of fibrotic tissue, ectopic bone and fat observed in dystrophic mice.

In this study, we show that the rapid accumulation of lipids, calcium deposits and fibrosis occurs in the skeletal muscle of dKO mice and worsens with age. RACs were isolated and characterized as non-myogenic mesenchymal stem cells (nmMSCs) with trilineage (adipogenic, osteogenic and chondrogenic) differentiation potentials. We determined that the proliferation and adipogenic/osteogenic potentials of nmMSCs isolated from 1-, 4- and 6–8-week-old dKO mice were progressively activated as the disease progresses, suggesting that the activation of nmMSCs is closely associated with the deposition of non-muscle tissues in the dKO mice. In addition, 6–8-week-old dKO-nmMSCs displayed significantly enhanced proliferation potentials, in vitro and in vivo, as was their adipogenic, osteogenic and fibrogenic differentiation capacities, compared with age-matched WT-nmMSCs. Our co-cultivation study further suggests that the activated dKO-nmMSCs exert a negative effect on dKO-MDSCs by inhibiting their ability to form myotubes, an effect we demonstrated to be, at least partially, mediated by the secreted frizzled-related protein 1 (sFRP1) released by the dKO-nmMSCs. Results shown here indicate that activation of nmMSCs is not only closely associated with the rapid occurrence of fibrosis, ectopic calcification and fat accumulation, but may also contribute to continuous muscle degeneration and weakness in the dystrophic dKO mice.

### Results

**Rapid accumulation of lipids, calcium deposits and fibrosis during the disease progression in the skeletal muscles of dKO mice**

Previously, we reported that the onset of muscular dystrophy in the dKO mice occurred as early as 5 days of age and dystrophic histopathology became progressively worse by 6–8 weeks of age. At this age, we also observed that the accumulation of connective tissue, ectopic calcium and fat deposits in the gastrocnemius muscles (GAS) of the dKO mice were substantially increased compared with that of age-matched WT-GAS (7,21). To determine the age of onset of these events, the GAS from 1-, 4- and 6–8-week-old dKO mice were examined. Oil Red O, Alizarin Red and Trichrome staining indicated that the accumulation of fat, calcium deposits and fibrosis started to occur in the GAS by 4 weeks of age and the severity of the histopathology increased substantially by 6–8 weeks of age in the dKO mice. There was a higher level of lipid accumulation observed in the 4-week-old dKO-GAS compared with that of the 1-week-old dKO-GAS, but lipid droplets were significantly increased in the GAS of 6-week-old dKO mice compared with that of the 1- and 4-week-old dKO mice (Fig. 1A). Alizarin Red staining showed more extensive calcium deposition in the GAS of 4- and 6–8-week-old dKO mice compared with that of the 1-week-old GAS (Fig. 1B). We observed larger areas of fibrosis between the muscle fibers of 6–8-week-old dKO-GAS compared with 1-week-old dKO muscle (Fig. 1C). Very small amounts of lipid, calcium and fibrosis accumulation was observed in the 1-week-old dKO-GAS (Fig. 1A–C). GAS of 1-, 4- and 6–8-week-old dKO mice were collected and analyzed for mRNA expression of adipogenic [peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα)], osteogenic (osteopontin (OPN), osteocalcin (OCN) and runt-related transcription factor 2 (RUNX2)] and fibrogenic [collagen type 1 alpha (Col 1α) and collagen type 3 alpha (Col 3α)] markers by real-time reverse transcriptase–polymerase chain reaction (RT–PCR) (Fig. 1D). The expression levels of the markers in the 4-week-old dKO-GAS were significantly higher than those in the 1-week-old dKO-GAS, except OCN. Adipogenic, osteogenic and fibrogenic markers in the 6–8-week-old dKO-GAS were significantly up-regulated compared
with those in the 1- and 4-week-old dKO-GAS. There were no significant differences in osteogenic (OPN and RUNX2) and fibrogenic (Col 1a) gene expressions between the 4- and 6-week-old dKO-GAS. Taken together, our results demonstrated that the dKO mice rapidly developed skeletal muscle abnormalities that worsened with age which was similar to DMD patients (11,12).

Non-myogenic RACs exhibit behaviors that are characteristic of MSCs

Next, we sought to determine what cell populations in the skeletal muscle were associated with the accumulation of lipid, calcium deposits and fibrosis in the dKO mice. Previously, we found that MPCs, including MDSCs isolated from 6- to 8-week-old dKO-GAS were defective in their proliferation capacities and adipogenic and osteogenic differentiation potentials (7); therefore, we searched for another population of skeletal muscle cells that were non-myogenic. Utilizing the preplate technique, RACs were obtained within 2 h of isolation, which is similar to the isolation method utilized to collect bone marrow-derived MSCs (BM-MSCs) (26). RACs were isolated from the skeletal muscle of 6-week-old WT and dKO mice and characterized by examining their cell surface markers and stem cell behaviors. The RAC population has a very similar morphology to that of BM-MSCs, which is substantially different from the MDSCs that are small, spherical and refractive under phase contrast microscopy (Fig. 2A). RT-PCR analysis and quantification of the relative gene expression levels in the WT-MDSCs and RACs showed that the MDSCs expressed significantly higher levels of Pax7 and Desmin, whereas the RACs expressed significantly higher levels of stem cell antigen-1 (Sca-1) and platelet-derived growth factor receptor alpha (PDGFRα), well-known markers of MSCs (27) (Fig. 2B and C). Immuno-fluorescent staining for Pax7 showed that nuclear-localized Pax7 expression was only observed in MDSCs and not in the RACs. Therefore, upon myogenic induction, only MDSCs were able to differentiate into myotubes (Fig. 2D). The biological property that most uniquely identifies MSCs is their capacity for trilineage mesenchymal differentiation (28). We confirmed that under appropriate induction conditions, WT-RACs exhibited osteogenic, adipogenic and chondrogenic differentiation potentials (Fig. 2E–G).

The phenotype of dKO-nmMSCs is similar to WT-nmMSCs

The immunophenotypes of the WT- and dKO-RACs were compared using fluorescence-activated cell sorting (FACS) analysis.

Figure 1. Severe skeletal muscle pathologies including lipid accumulation, ectopic calcification and development of fibrosis were observed in old dKO mice. (A) Oil Red O staining revealed the presence of lipid droplets in the gastrocnemius muscles of 1-, 4- and 6-8-week-old dKO mice. Severe lipid accumulation was observed in 6-8-week-old dKO muscle (n > 4). Scale bar = 100 µm. (B) Alizarin Red staining showed calcium deposits in the dKO muscle sections. More extensive calcium deposits were detected in old dKO muscle (n > 4). Scale bar = 100 µm. (C) Trichrome staining was performed to identify fibrotic regions of the gastrocnemius of dKO mice. Increased fibrotic areas were observed in old dKO muscle (n > 4). Scale bar = 100 µm. (D) RNA was extracted from freshly collected muscle tissues of 1-, 4- and 6-8-week-old dKO mice and real-time RT-PCR was performed (n > 4) for PPARγ, C/EBPα, OPN, OCN, RUNX2, COL1a and COL3a mRNA expression. Error bars indicate “mean ± SD”.*P < 0.05, **P < 0.001.
using multiple MSC markers. The FACS results indicated that both the WT- and dKO-RACs were positive for PDGFRα, CD90 and CD105 (markers reported to be expressed by mesenchymal cells) (28) and Sca-1. More than 90% of the RACs from both WT and dKO mice were positive for CD34 (an endothelial cell marker) and less than 1% of the RACs expressed CD45, a hematopoietic cell marker (Fig. 3A and B). Quantification of the FACS analysis indicated that there was no significant difference in the percentage of positive cells between the WT- and dKO-RACs for each of the markers tested (Fig. 3C). Based on the RAC’s marker profile and their trilineage differentiation capacities (Fig. 2), we hereafter referred to the RACs as nmMSCs. We further investigated if the nmMSCs were associated with the occurrence of ectopic non-muscle tissues in the skeletal muscle of the dKO mice.

**Proliferation and differentiation potentials of the nmMSCs are influenced by the disease progression in dKO mice**

To test whether the disease progression in dKO mice was associated with changes in nmMSCs function, nmMSCs isolated from 1-, 4- and 6–8-week-old dKO skeletal muscle were compared for their proliferation and differentiation potentials. Using Live Cell Imaging (LCI), we observed that nmMSCs isolated from 1-week-old dKO mice displayed significantly decreased proliferation rates compared with nmMSCs isolated from 4- and 6–8-week-old dKO mice (Fig. 4A). The population doubling time for 1-week-old dKO-nmMSCs was ∼44 and 36 and 30 h, respectively, for the 4- and 6–8-week-old dKO-nmMSCs. More importantly, our results revealed that the 4- and 6–8-week-old dKO-nmMSCs
displayed significantly increased adipogenic/osteogenic potentials when compared with the 1-week-old dKO-nmMSCs (Fig. 4B–D). In addition, immunohistochemistry for PDGFRα expression (red) showed that the number of PDGFRα expressing cells was significantly higher in the 4- and 6–8-week-old dKO-GAS when compared with the 1-week-old dKO-GAS (Fig. 4E and F). There were no significant differences in the number of endogenous PDGFRα+ cells, in vitro proliferation rates or differentiation potentials between the 4- and 6–8-week dKO-nmMSCs. Our results suggest that the nmMSCs are gradually activated with age in terms of their proliferation and adipogenic/osteogenic differentiation potentials as the disease progresses in the dKO mice. Earlier, we showed that the accumulation of lipids, calcium deposits and fibrotic tissue in the GAS of dKO mice started mildly at 1 week and became more severe as the mice grew older (Fig. 1A–C). Taking all these results into account, we propose that nmMSCs become activated as the histopathology of the dKO mice progresses, suggesting that they are involved in the deposition of the non-muscle tissues observed in the 6–8-week-old dKO mice.
dKO-nmMSCs show enhanced proliferation and adipogenic, osteogenic and fibrogenic differentiation potentials compared with age-matched WT-nmMSCs

Next, we compared the proliferation and differentiation potentials of nmMSCs isolated from 6- to 8-week-old WT and dKO mice (Fig. 5). Surprisingly, LCI analysis of the in vitro proliferation rates of 6-week-old WT- and dKO-nmMSCs showed that the dKO-nmMSCs displayed significantly greater proliferation rates than the WT-nmMSCs (Fig. 5A). We next examined the in vivo proliferation rates of the nmMSCs by performing immunohistochemistry for PDGFα and Ki67, a proliferation marker, in the GAS of 6-week-old WT and dKO mice. PDGFα⁺ nmMSCs were localized in the interstitial spaces between the muscle fibers (Fig. 5B). Our results indicated that the number of actively proliferating Ki67⁺PDGFα⁺ nmMSCs was ~15-fold higher in the dKO skeletal muscle when compared with the age-matched WT muscles (Fig. 5C). We next investigated if the actively proliferating dKO-nmMSCs demonstrated an increase in their multilineage differentiation capacities. Our results indicated significant increases in the adipogenic (Fig. 5D), osteogenic (Fig. 5E) and fibrogenic differentiation (Fig. 5F) capacities of the nmMSCs isolated from 6-week-old dKO mice compared with those isolated from age-matched WT mice. Upon quantification, dKO-nmMSCs exhibited a 4-fold higher adipogenic differentiation potential and 3.5-fold higher osteogenic potential when compared with the age-matched WT-nmMSCs. In addition, WT- and dKO-nmMSCs readily differentiated into Collagen I (Col I)- and Collagen III (Col III)-expressing cells under fibrogenic culture conditions. Adipogenic and fibrogenic differentiation were further
evaluated by immunostaining of Col I and Col III for fibrogenic cells, and PPARγ and C/EBPα for adipocytes (Fig. 5F). We found that there were more Col I- and Col III-positive cells in the dKO-nmMSC cultures compared with the WT-nmMSC cultures after fibrogenic induction. We also observed a significant increase in the number of PPARγ- and C/EBPα-positive cells in the dKO-nmMSC cultures compared with the WT-nmMSC cultures, after 6 days of adipogenic induction (Fig. 5F and G). Our results demonstrated that the function of the nmMSCs isolated from dKO mice were enhanced (increased proliferation and adipogenic, osteogenic and fibrogenic differentiation potentials) when compared with their WT counterparts.

Activated dKO-nmMSCs inhibit myotube formation of age-matched dKO-MDSCs

Since the numbers of endogenous proliferating nmMSCs in the interstitial spaces of the GAS of 6–8-week-old dKO mice were significantly increased compared with age-matched WT-GAS (Fig. 5B), we examined the possibility that these cells might play a role in the development of the dystrophic phenotype by influencing the MDSCs. Cross-talk between MDSCs and nmMSC was tested to see if the nmMSCs could influence the myogenic differentiation potential of the MDSCs through a paracrine mechanism. MDSCs isolated from 6–8-week-old dKO mice were co-cultured with age-matched WT- and dKO-nmMSCs, using a...
transwell system, and the fusion index of the dKO-MDSCs was monitored. WT-nmMSCs were used as the control. To quantify the reduction in myogenic differentiation potential after cocultivation, dKO-MDSCs were collected and immunostained for the terminal myogenic differentiation marker fast myosin heavy chain (fMyHC). When dKO-MDSCs were co-cultured with WT-nmMSCs, the myogenic potential of the dKO-MDSCs was significantly enhanced (Fig. 6A), as described previously (25,29–31). Surprisingly, the limited myogenic potential of the dKO-MDSCs was further exacerbated after co-culturing with the dKO-nmMSCs (Fig. 6A). fMyHC expressing cells were dramatically decreased when dKO-MDSCs were cultured in the presence of dKO-nmMSCs compared with the dKO-MDSCs alone (Fig. 6B). To rule out the possibility that the decrease in the dKO-MDSCs differentiation capacity was caused by the nmMSCs and not a secondary effect to a reduction in dKO-MDSC proliferation, we measured the effect of co-cultivation of the dKO-MDSCs with dKO-nmMSCs on cell proliferation of the dKO-MDSCs using an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphonylphenyl)-2H-tetrazolium (MTS) assay. No significant difference in proliferation was observed between the dKO-MDSCs alone and dKO-MDSCs co-cultured with dKO-nmMSCs during the first 2 days of co-culture (Fig. 6C). Next, we further analyzed changes in the expression of myogenic markers expressed by the dKO-MDSCs after 3–4 days of culturing in differentiation induction media with/without dKO-nmMSC co-cultivation, using real-time RT–PCR. We observed a significant reduction in the expression of the myogenic markers, MyoD, myogenin, eMyHC and fMyHC in the dKO-MDSCs in the presence of dKO-nmMSCs (Fig. 6D). These results suggest that nmMSCs isolated from 6–8-week-old dKO mice further inhibited the differentiation potential of age-matched dKO-MDSCs via an unknown secreted factor(s).

One interesting observation from our in vivo studies was that the number of endogenous PDGFRα+ nmMSCs was almost 3-fold higher in 6–8-week-old dKO skeletal muscle compared with that of the age-matched WT skeletal muscle (Fig. 5C). To mimic the in vivo dKO muscle micro-environment in our co-culture assay, we repeated the co-cultivation experiments above using 3:1, 1:1 and 1:6 ratios of dKO-nmMSCs to dKO-MDSCs. With a 3:1 ratio, we observed a larger effect of nmMSCs myogenic inhibition of the dKO-MDSCs compared with the 1:6 ratio. In fact, there was no significant difference in the formation of fMyHC expressing myotubes between the dKO-MDSCs only and dKO-MDSCs in the 1:6 ratio co-cultures (Fig. 6 E and F). Taken together, these results suggest that signals originating from nmMSCs contribute to the micro-environment’s ability to limit the myogenic differentiation capacity of the MDSCs in the dKO muscle.

Myotube formation of dKO-MDSCs is inhibited by the sFRP1 released by the dKO-nmMSCs

In search of a soluble factor responsible for functional interactions between nmMSCs and MDSCs from dKO mice, we considered sFRP1 as a potential candidate. In fact, it has been previously shown that sFRP1 inhibits myotube formation of C2C12 and satellite cells, with no significant effect on the cell cycle or apoptosis (32). When we compared sFRP1 mRNA expression isolated from the total GAS of WT and dKO mice, we observed a significant increase in sFRP1 gene expression in the dKO-GAS compared with that of the WT-GAS (Fig. 7A). Next, we examined if the dKO-nmMSCs expressed higher sFRP1 mRNA levels compared with that of WT-nmMSCs, which was confirmed by real-time RT–PCR. sFRP1 gene expression was shown to be increased ~4.5-fold in the dKO-nmMSCs when compared with age-matched WT-nmMSCs (Fig. 7B). sFRP1 is a secreted protein; therefore, we next investigated if higher amounts of sFRP1 was secreted by the dKO-nmMSCs compared with the WT-nmMSCs. We collected cell culture supernatants from WT- and dKO-nmMSC cultures and the levels of sFRP1 protein secreted from both cell types was assayed using a mouse-specific sFRP1 enzyme-linked immunosorbent assay (ELISA) kit. As expected, there was a significantly higher sFRP1 concentration in the cell culture supernatants isolated from the dKO-nmMSCs when compared with that of WT-nmMSCs (Fig. 7C). These results suggest that the secretion of sFRP1 from dKO-nmMSCs might be responsible for inhibiting the myogenic differentiation of dKO-MDSCs as described previously (Fig. 6).

Up-regulation of Wnt signaling in dKO muscle

The results above suggest that the up-regulation of sFRP1 in skeletal muscle may contribute to the dystrophic phenotype observed in dKO mice. In order to elucidate the mechanism by which the up-regulation of sFRP1 occurs, we investigated whether the Wnt signaling pathway was involved in the process, since sFRP1 is an antagonist of Wnt signaling (33,34). Western blot analysis revealed that the amount of dephosphorylated β-catenin (active form) was increased in 6–8-week-old dKO-GAS compared with age-matched WT-GAS (Fig. 8A). To further validate this finding, β-catenin expression was examined in dKO and age-matched WT muscle tissue sections. We observed higher β-catenin expression in dKO-GAS compared with WT-GAS, as determined by the quantification of β-catenin staining (Fig. 8B and C). Finally, by real-time RT–PCR, we observed an increase in Axin2 (Fig. 8D) and LGR5 (Fig. 8E) gene expressions, which are downstream targets of the Wnt signaling pathway (35,36), in 6–8-week-old dKO-GAS compared with age-matched WT-GAS. Our results suggest that Wnt signaling is also up-regulated in dystrophic dKO muscle.

Discussion

Despite the fact that the underlying pathogenesis of DMD has been well studied, treatment options remain limited. Traditionally available treatments for DMD patients include cell, gene and protein-mediated therapies to restore dystrophin; however, these technologies have major limitations (37–39). For instance, accumulation of intramuscular fat, calcium deposits and fibrotic tissue significantly limits the success of these regenerative approaches for DMD patients (27,40–43). In the current study, we showed that the accumulation of lipid, calcium deposits and fibrotic tissue coincide with the progressive reduction in muscle regeneration in the skeletal muscle of dKO mice. The histopathology and skeletal muscle abnormalities remain mild by the age of 1 week, but progressively worsens by 4 and 6–8 weeks of age and consequently leads to death by 8–10 weeks of age. The development of intramuscular non-muscle tissue reduces muscle homeostasis and limits the potential for muscle regeneration. Therefore, the use of dystrophin replacement alone to treat the primary defect in DMD patients may not be successful for rescuing muscle from progressive degeneration and wasting, especially when performed at later stages of the disease (38,39).

When searching for potential cell sources responsible for the accumulation of ectopic non-muscle tissues in dystrophic muscle, we considered resident skeletal muscle nmMSCs, which can be isolated from the tissue via the preplate technique. We have previously reported that MPCs isolated from aged dKO mice progressively become defective in their proliferation and differentiation capacities, including myogenic, osteogenic,
Figure 6. Limited myogenic potential of 6-week-old dKO-MDSCs was further exacerbated by co-culturing the cells with 6-week-old dKO-nmMSCs. (A) Myogenic differentiation capacities were tested by co-cultivating WT- or dKO-nmMSCs with dKO-MDSCs using collagen type I-coated transwell inserts. Immunohistochemistry for fMyHC was then performed for the detection of myotube formation. DAPI was used for counterstaining. Scale bar = 50 µm. (B) Quantification of the percentage of myotubes formed by the dKO-MDSCs after 3 days of myogenic differentiation with/without WT/dKO-nmMSCs co-cultivation (n > 4). Error bars indicate the “mean ± SD”. *P < 0.001. (C) MTS-based cell proliferation assay of dKO-MDSCs during first 48 h of co-cultivation with/without dKO-nmMSCs. (D) Real-time RT-PCR analysis was performed and myogenic gene expression was compared between the dKO-MDSCs co-cultivated with/without dKO-nmMSCs (n > 4). Error bars indicate the “mean ± SD”. *P < 0.05, **P < 0.001. (E) Co-culture myogenic differentiation experiments were done using 3:1, 1:1 and 1:6 ratios of dKO-nmMSCs to dKO-MDSCs. Scale bar = 50 µm. (F) Quantification of the percentage of myotubes formed by the dKO-MDSCs after 3 days of myogenic differentiation with 3:1, 1:1 and 1:6 ratios of dKO-nmMSCs to dKO-MDSCs co-cultivation (n = 3). Error bars indicate the “mean ± SD”. *P < 0.05, **P < 0.001.

Figure 7. Higher sFRP1 expression was detected in dKO skeletal muscle and secreted by dKO-nmMSCs compared with WT muscle and WT-nmMSCs. RNA was extracted from frozen muscle tissue and real-time RT-PCR was performed. Higher sFRP1 gene expression was observed from (A) dKO-GAS compared with age-matched WT-GAS (n > 4) and (B) 6-week-old dKO-nmMSCs compared with age-matched WT-nmMSCs (n > 4). Error bars indicate the “mean ± SD”. *P < 0.05. (C) Concentration of secreted sFRP1 from WT- or dKO-nmMSCs was measured with a mouse sFRP1 ELISA kit. The graph represents sFRP1 concentration per every 100 000 WT- or dKO-nmMSCs (n = 6). Error bars indicate the “mean ± SD”. **P < 0.0001.
adipogenic and chondrogenic capacities (7). In the current study, we demonstrated that the function of the nmMSCs in 6–8-week-old dKO mice appeared to be enhanced during disease progression. Six-week-old dKO mice displayed a significant increase in nmMSCs in their GAS compared with age matched WT mice. Moreover, nmMSCs isolated from 6-week-old dKO mice displayed increases in their in vitro proliferation and adipogenic, osteogenic and fibrogenic differentiation potentials compared with age-matched WT-nmMSCs. More importantly, the proliferation and differentiation potentials of dKO-nmMSCs isolated from 1-week-old dKO mice appeared to not be affected, but gradually became activated by 4 weeks and started to extensively proliferate and demonstrate an increase in adipogenic/osteogenic differentiation capacities by 6–8 weeks of age. These results taken

Figure 8. Enhanced Wnt signaling in dKO muscles. (A) Representative western blots of active β-catenin (92 kDa) and α-Tubulin (55 kDa) in GAS of 6–8-week-old dKO and WT mice. α-Tubulin was used as a loading control (n = 4). (B) β-catenin (β-Cat) staining of muscles from 6–8-week-old dKO and WT mice (n > 4). Scale bar = 50 μm. (C) The average pixel intensity corresponding to β-catenin staining was evaluated with Image J. Error bars indicate the "mean ± SD". "*P < 0.0001. RNA was extracted from frozen muscle tissue and real-time RT-PCR was performed. Higher (D) Axin2 and (E) LGR5 gene expression was observed in dKO-GAS compared with age-matched WT-GAS (n > 4). Error bars indicate the "mean ± SD". "*P < 0.0001. 
together suggest that the activation of the nmMSCs in the dKO mice might be involved with the occurrence of lipid and calcium deposition, and the formation of fibrotic tissue in the dKO muscle. Although it is not clear which event occurs initially, we posit that the activation of the nmMSCs is responsible for the accumulation of non-muscle tissues during the disease progression in dKO mice, and in turn, this influences the muscle micro-milieu, which further alters the myogenic potential of the MDSCs.

Furthermore, the difference in proliferation and differentiation potentials among the 1-, 4- and 6- to 8-week-old dKO-nmMSCs suggests that the activation of nmMSCs in the aged dKO mice may not be caused by intrinsic differences, but by the changes in their micro-environment. This result is supported by a recently published study which reported that the expression of transforming growth factor-β2 was induced in mdx muscle and this in turn further affected the behavior of muscle stem cells to become more profibrogenic (44), which suggests that the local changes in the micro-milieu are responsible for stem cell dysfunction in dystrophic muscle. Therefore, we believe that the muscle environmental cues in the dKO mice influence the nmMSCs and likely play a role in determining stem cell fate.

Recently, using an FACS-based cell isolation method, two research groups identified two populations of resident skeletal muscle MPCs; (i) FAPs which express Sca-1 and CD34 and (ii) PDGFRα+ MSCs/mesenchymal progenitor cells. Both the FAPs and PDGFRα+ cells displayed high adipogenic and fibrogenic potentials in a glycerol-injected fatty degeneration model and in mdx mice (25,27,46). However, since mdx mice have a relatively normal life span and mild clinical features of the disease until very late in their life (45), our group utilized the dKO mouse model, which exhibits numerous clinically relevant manifestations and life-threatening features similar to DMD patients. The current study results utilizing the dKO mouse model, which exhibits numerous clinically relevant manifestations and life-threatening features similar to DMD patients. The current study results utilizing the dKO mouse model support the contention that PDGFRα and Sca-1 expressing nmMSCs are highly proliferative and have highly enhanced adipogenic/osseogenetic potentials, which are likely associated with the deposition of lipid, calcification and fibrosis in the dystrophic muscle of the mice during the progression of the disease.

Woszczyk et al. (46) also reported that functionally and phenotypically similar muscle-resident mesenchymal progenitor cells expressing the angioptein receptor (Tie2), PDGFRα and Sca-1 surface markers exhibit robust BMP-dependent osseogenetic activity and mediate heterotopic ossification in mice that have experienced a traumatic injury. Knowing that Tie2+ cells (46), FAPs (25), PDGFRα+ MSCs (27) and nmMSCs share the same surface markers, anatomical location in the muscle and tri-lineage differentiation potentials, we postulate that they may represent the same population of cells.

Recently, a number of studies revealed that in addition to their role in muscle pathogenesis, nmMSCs play a crucial role in muscle regeneration by regulating satellite cell differentiation (25,27,30). Joe et al. (25) showed that although FAPs do not regenerate myofibers, they enhance the rate of terminal differentiation of primary myoblasts in co-culture experiments. In addition, FAPs can facilitate the function of histone deacetylase inhibitors to promote satellite cell-mediated regeneration during early stages of the disease in mdx mice (30). Conversely, Uezumi et al. (27) demonstrated that satellite cell-derived myofibers strongly inhibit the proliferation and adipogenic potentials of nmMSCs, suggesting that the interaction or balance between MPCs and nmMSCs is a key to maintaining muscle integrity and homeostasis.

Co-culture experiments showed that proliferating dKO-nmMSCs significantly limited the myogenic potential of the dKO-MDSCs, at least in part, by down-regulating genes that are important for myoblast maturation and terminal myogenic differentiation, including MyoD, myogenin, eMyHC and MyHC.

Interestingly, by decreasing the number of nmMSCs co-cultured with dKO-MDSCs, the MDSCs could be rescued from the inhibiting effects of the nmMSCs. Therefore, knowing that dKO mice suffer from constant muscle wasting and degeneration, we posit that the extensive proliferation of the nmMSCs in the dKO mice may be a source of trophic signals for hindering muscle regeneration during the chronic disease progression of DMD. FAPs’ involvement in inhibiting the MPC myogenic potential had also been described in a study by Mozzetta et al. (30) where they reported that FAPs isolated from old mdx mice repressed satellite cell-mediated myofiber regeneration. Of note, although young mdx mice exhibited successful muscle regeneration and very mild symptoms of DMD, old mdx mice (18 months and older) exhibit severe muscle fibrosis (47), which is similar to what is seen more rapidly in the dKO mice and is more similar to what is seen in DMD patients. Our results suggest that nmMSCs isolated from 6-week-old dKO mice are functionally equivalent to that of 18-month-old mdx mice, which supports our previous findings that propose the dKO mouse as a severe model of muscular dystrophy with accelerated disease progression compared with mdx mice (46).

Recent studies suggest that skeletal muscle fibroblasts maybe the main source of intramuscular adipocytes in pathological conditions, including obesity, sarcopenia and muscular dystrophies (49) and that their interactions with satellite cells are important for muscle regeneration (31). Murphy et al. (31) showed that during muscle regeneration after injury, cells expressing transcription factor 4 (Tcf4)—a marker for fibroblasts—extensively proliferate in close proximity to satellite cells and regenerating myofibers and that the ablation of Tcf4+ cells leads to premature satellite cell differentiation and depletion of the early satellite cell pool. In turn, ablation of Pax7+ cells results in a complete loss of muscle regeneration capacity and a significant increase in connective tissue (31), suggesting that interactions between satellite cells and fibroblasts are crucial for maintaining normal muscle homeostasis during regeneration. Agley et al. (49) showed that TE-7 human skeletal muscle fibroblasts, but not myogenic cells, readily undergo adipogenic differentiation. Interestingly, both Tcf4 and TE-7 expressing fibroblasts are RACs (adhering to the culture dish in 2–72 h) and are also positive for the PDGFRα antigen. When we compare the morphology, early adhering characteristics, marker profile, anatomical locations, differentiation potentials and the role that the TE-7+/Tcf4+ fibroblasts and nmMSCs/FAPs play in muscle regeneration, it appears that fibroblasts and MSCs share much more in common than previously identified. Taking this into account, these populations of cells may actually represent the same cell population; however, further studies need to be performed to compare the relationship between fibroblasts and nmMSCs.

In order to decipher the mechanisms behind the inhibitory effects the nmMSCs impart on the myogenic potential of MDSCs, we postulated that the sFRP1 might be involved in the process. sFRP1 is known to be an antagonist of Wnt signaling and the constitutive ectopic expression of sFRP1 in preadipocytes disrupts Wnt/β-catenin signaling, which results in the promotion of adipogenesis in vitro and adipose tissue expansion in vivo (32–34,50). Moreover, sFRP1 has also been reported to inhibit the myogenesis of satellite cells by down-regulating the expression of myogenin and Mrf4, both of which play roles in myoblast fusion and myofiber maturation (32). Immunostaining showed decreased myotube formation and real-time PCR showed a down-regulation of terminal myogenic differentiation genes (myogenin, eMyHC and MyHC) in the dKO-MDSCs after co-culturing the
cells with the dKO-nmMSCs. We also observed higher sFRP1 mRNA levels in the dKO-nmMSCs compared with age-matched WT-nmMSCs. ELISA results further confirmed that the dKO-nmMSCs were secreting more sFRP1 protein than the WT-nmMSCs, further validating our hypothesis that sFRP1 secreted by the dKO-nmMSCs contributes, at least in part, to the microenvironment that inhibits the myogenic differentiation of the MDScs in dKO muscle. Only weak expression of sFRP1 has been reported in skeletal muscle (34); yet, we observed an approximately 10-fold increase in sFRP1 mRNA expression from the GAS of 6-8-week-old dKO mice compared with age-matched WT mice. These results suggest that sFRP1 may be associated with increased proliferation and the adipogenic potential of the nmMSCs isolated from dKO mice. Furthermore, it also appears that sFRP1 may play a role in the elevation of adipogenic gene expression and the accumulation of lipids observed in the dKO skeletal muscle. Therefore, we propose that not only does the sFRP1 released by the nmMSCs hinder muscle regeneration, but it also contributes to establishing a pro-adipogenic muscle micro-environment, which promotes fatty degeneration in the skeletal muscle of the dKO mice.

It has been reported that the Wnt signaling is up-regulated in both aged and dystrophic mdx skeletal muscle, which consequently lead to an alteration of muscle stem cell differentiation from myogenosis to fibrogenesis (44,51). Indeed, the up-regulation of dephosphorylated β-catenin and increased mRNA expressions of Axin2 and LGR5, well-known canonical Wnt signaling target genes (25,36) suggests that Wnt is also elevated in 6-8-week-old dKO muscle compared with age-matched WT muscle. Increased Wnt signaling in dKO muscle may also be closely associated with enhanced fibrogenic or adipogenic programs in stem/progenitor cells, including nmMSCs. It is also possible that sFRP1 is activated in dKO muscle to antagonize the over-expression of Wnt signaling; however, the severe dystrophic muscle micro-milieu in dKO mice may also cause the dysregulation of sFRP1, resulting in a micro-environment that promotes adipogenesis. Further studies required to increase our understanding of how sFRP1 interacts with Wnt signaling that promotes the differentiation of nmMSCs toward an adipogenic lineage, which may contribute to the severe dystrophic phenotype observed in the dKO mice.

Overall, our data suggest that compared with age-matched WT-nmMSCs, nmMSCs isolated from 6-8-week-old dKO mice are highly proliferative and their propensity for adipogenic, osteogenic and fibrogenic differentiation is greatly increased. We therefore believe that the activation of nmMSCs coincides with the occurrence of fatty infiltration, ectopic calcification and fibrotic tissue accumulation in the skeletal muscle of dKO mice. More importantly, we demonstrated that the nmMSCs represent a major contributor to the formation of ectopic fat, calcification and fibrosis in the skeletal muscle of dKO mice and that the activation of the nmMSCs may exacerbate the regeneration of the dystrophic muscle by inhibiting the myogenic potential of MDScs. Results from this study could provide insights into new approaches to alleviate muscle weakness and wasting in DMD patients by targeting nmMSCs during the disease process.

Materials and Methods

Animals

C57BL/10 (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Dystrophin/utrophin knockout (dKO) mice, originally characterized by Deconinck et al. (45), were generated by crossing heterozygous dystrophin<sup>−/−</sup> utrophin<sup>−/−</sup> mice (48,52). Genotyping was performed by PCR analysis of tail samples. Mice ranged in age from 5 days to 8 weeks. Specific ages for each experiment are described below. All animal protocols used for these experiments were approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee.

Cell isolation and culture

Primary WT and dKO nmMSCs and MDSCs were isolated from 1- to 8-week-old WT and dKO mice using a modified preplate method described previously (7,22,53). Briefly, after enzymatic digestion of skeletal muscle tissue, muscle-derived cells were replated on collagen type I (C9791, Sigma-Aldrich) coated flasks over a period of days. Two hours after isolation, an RAC population was collected and characterized as nmMSCs. Seven days after preplating, an SAC population was obtained, which has been described to contain the MDSsc fraction of cells (8). nmMSCs and MDSCs were cultured in proliferation medium (PM) containing 10% fetal bovine serum (FBS), 10% horse serum, 0.5% chick embryo extract and 1% penicillin-streptomycin in Dulbecco’s modified Eagle’s medium (DMEM, 11995-073, Invitrogen).

Immunophenotyping

Flow cytometry was performed on WT- and dKO-nmMSCs at the end of their third expansion passage. One-hundred thousand WT and dKO-nmMSCs were collected, washed with phosphate buffered saline (PBS) containing 2% FBS, centrifuged and then placed on ice. The cells were then resuspended in a 1:10 dilution of mouse serum (M5905, Sigma-Aldrich) in PBS and incubated for 10 min. Phycoerythrin (PE)-conjugated rat anti-PDGFRα (12140181, eBioscience), PE-conjugated rat anti-Sca-1 (553108, BD), fluorescein isothiocyanate (FITC)-conjugated rat anti-CD34 (553733, BD), allophycocyanin-conjugated rat anti-CD90 (553007, BD), PE-conjugated rat anti-CD105 (562759, BD) and FITC-conjugated rat anti-CD45 (553080, BD) were added to each tube (1 µl/100 000 cells) and incubated for 30 min on ice. Only one antibody was used in each tube. The cells were then rinsed in 300 µl of cold washing buffer (2% FBS in PBS, 4 degrees). A single-color antibody was used to optimize fluorescence compensation settings for multicolor analyses.

Immunofluorescence and histology

Muscle cryosections were fixed with 5% formalin for 8 min and blocked with 10% donkey serum for 1 h. Slides were then incubated with rabbit anti-beta catenin (1:200, Abcam), goat anti-PDGFRα (1:100, R&D) and rabbit anti-mouse Ki67 (1:200, Abcam) or chicken anti-mouse laminin (1:500, Abcam) in 5% donkey serum. Next, sections were incubated with secondary antibodies including Alexa594-conjugated anti-rabbit iGg (1:300, Invitrogen), Alexa594-conjugated anti-chicken iGg (1:300, Invitrogen) or Alexa488-conjugated anti-goat iGg (1:300, Invitrogen) in PBS for 45 min.

mRNA analysis was performed via RT–PCR

Total RNA was obtained from nmMSCs, MDSCs and gastrocnemius muscle tissues isolated from 6- to 8-week-old WT animals using TRIzol reagent (Invitrogen) and a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Reverse transcription was performed using a Maxima first strand cDNA synthesis kit (Fermentas) according to the manufacturer’s protocol. PCRs were performed using an iCycler Thermal Cycler.
and primers used in the study included β-actin: CCACACCCGGCA CCAAGTTGG (sense) and TACAGCCCCGAGGAGCATCTG (antisense); desmin: AAACCTGATAGACGACCTGCAG (sense) and GCTTGGCA GTAGTCCACATCTCA (antisense); Pax7: GTGCTGCTAGTGATTTG CAT (sense) and CACACATCTGGACCCCTCATCC (antisense); Sca-1: CCTACTGTTGACAGAAAGGC (sense) and CAGGAAGTCTTGCG (antisense) and CCACATCTGAGCCCTCATCC (antisense); LGR5 TCGCCTTCCCCAGGTCCCTTC GCCGTGGTCCACACCCCGAT 79 Col3a ACGTAAGCACTGGTGGACAGA ACGTAAGCACTGGTGGACAGA 20 Col1a TCATCGTGGCTTCTCTGGTC GACCGTTGAGTCCGTCTTTG 142 OPN AGCAAGAAACTCTTCCAAGCAA GTGAGATTCGTCAGATTCATCCG 111 C/EBP eMyHC GGAGGCTGATGAACAAGCCA GCTAGAGGTGAAGTCACGGG 141 PPAR MyHC AGGAGCTGACGCAGACCGAAT CCCTCTGCAGTTCAGCCTTTACTTCC 157 Myogenin AGATTGTGGGCGTCTGTAGG CTACAGGCCTTGCTCAGCTC 199 MyoD TACCCAAGGTGGAGATCCTG CATCATGCCATCAGAGCAGT 200 Myf5 GTCAACCAAGCTTTCGAGACG CGGAGCTTTTATCTGCAGCAC 306 Pax7 GTGCCCTCAGTGAGTTCGAT CCACATCTGAGCCCTCATCC 168 Pax3 ACCCAAGCAGGTGACAACG CTAGATCCGCCTCCTCCTCT 203 βGene Forward primers Reverse primers Size

Table 1. Primers used for real-time RT–PCR

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<th>Reverse primers</th>
<th>Size</th>
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Differential assays

Adipogenic differentiation assay
A total of 35 000 nmMSCs were cultured in 24-well collagen type I-coated plates for 21 days in adipogenic differentiation medium (PT3004, Lonza). When the cells reached 100% confluence, three cycles of induction/maintenance medium were applied to the cells to induce optimal adipogenic differentiation. Each cycle consisted of incubating the nmMSCs with supplemented adipogenic induction medium for 3 days, followed by 2–3 days of culture in supplemented adipogenic maintenance medium. At the end of the third cycle, the cells were incubated in adipogenic maintenance medium for an additional 5–7 days. Adipogenesis was assessed using Oil Red O (00625, Sigma-Aldrich) to stain for intracellular lipid accumulation. For adipocyte differentiation quantification, cells were stained with AdipoRed (NC9049267, 30 µl/ml, Fisher) and 4,6-diamidino-2-phenylindole (DAPI), and fluorescence intensities were analyzed with a spectrophotometer. The AdipoRed fluorescence emission readings were normalized to the total number of cells in each well. After differentiation, adipogenic cells were stained with rabbit anti-PPARγ (1:200, Cell Signaling) or goat anti-C/EBPs (1:50, Santa Cruz Biotechnology) in 5% donkey serum in PBS.

Osteogenic differentiation assays
A total of 35 000 nmMSCs were cultured in osteogenic medium which contained DMEM, 10% FBS, supplemented with dexamethasone (D2915, 0.1 µM, Sigma-Aldrich), ascorbic-acid-2-phosphate (A8960, 50 µg/ml, Sigma-Aldrich), β-glycerophosphate (G6251, Sigma-Aldrich) and BMP2 (M11306AAB, 100 ng/ml, Medtronic). Osteogenesis was assessed using a Fast Blue Alkaline Phosphatase (ALP) kit (041M4338, Sigma) and by staining mineralized matrix with alizarin red. ALP activity was quantified with an ELISA-based assay according to the manufacturer’s protocol (54).

Chondrogenic differentiation assays

Fibrogenic differentiation
Ten thousand cells were plated on 24-well plates in DMEM supplemented with 2% FBS (fusion medium, FM) to stimulate myotube formation. Three days after differentiation, the cells were fixed with 10% formalin and stained with 1% alcian blue (pH 1.0) for 30 min. For quantification, 250 000 cells were cultured in pellets for 4 weeks in chondrogenic induction media. Sulfated glycosaminoglycans production was quantified using a Blysan assay kit (Bicocor, Carrickfergus, Northern Ireland) according to the manufacturer’s protocol.

Cell proliferation
To compare the proliferative potentials of the WT and dKO-nmMSCs, an LCI system (Kairos Instruments LLC) was used as...
Co-culture experiments

dKO-MDSCs were plated in the lower compartment of Transwell Permeable Supports (Costar) in PM media at a density of 30,000 cells per well. WT- and dKO-nmMSCs were seeded onto 6.5 mm transwell membrane inserts at the same density in PM media and placed above the dKO-MDSCs. As a control, each plate contained wells of dKO-MDSCs without transwell membrane inserts. To measure differentiation, the PM media was switched to FM. After 3 days, myogenic differentiation of the dKO-MDSCs was analyzed via immunostaining for MyHC, as described above.

Detection of sFRP1 secretion in nmMSCs

The amount of sFRP1 from WT- and dKO-nmMSC cultures was measured with a mouse sFRP1 ELISA kit (MB576370, MyBioSource) according to the manufacturer’s instructions. Briefly, nmMSCs from 6-week-old WT or dKO mice were plated on 6-well plates, in triplicate, and incubated in PM. Twenty-four hours after initial plating, the cells were washed three times with PBS and PM was switched with serum-free DMEM. Twenty-four hours after switching to the serum-free medium, the cell culture supernatants were collected and used to perform the ELISA for the sFRP1 protein. The results were immediately determined by comparing the optical density at 450 nm using a microplate reader (Infinite M200, TECAM, Inc.).

Western blot

Tissue lysates were prepared in Radioimmunoprecipitation assay buffer (Sigma) supplemented with protease and phosphatase inhibitors (1:100, Sigma) and quantified using the Bio-Rad Protein Assay (500-0001, Bio-Rad). Immunoblotting was performed as described previously (57). Membranes were incubated with mouse monoclonal antibody to active β-catenin (0.5 µg/ml, Millipore, Temecula, CA, USA) or rabbit polyclonal antibody to alpha tubulin (Abcam) at 4°C overnight in 5% milk in tween 20-Tris-buffered saline. Alpha tubulin was used to evaluate equal loading.

Statistical analysis

Data from ≥4 samples from each subject were pooled for statistical analysis. Results are given as the mean ± SD. Statistical significance of any difference was calculated using Student’s t-test. Values of P < 0.05 were considered statistically significant.

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Conflict of Interest statement. The authors do not have any conflicts of interest to disclose other than the corresponding author who receives consulting fees and royalties from Cook MyoSite Inc.

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