The role of Notch signaling in muscle progenitor cell depletion and the rapid onset of histopathology in muscular dystrophy

Xiaodong Mu, Ying Tang, Aiping Lu, Koji Takayama, Arvydas Usas, Bing Wang, Kurt Weiss and Johnny Huard*

Stem Cell Research Center, Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA 15219, USA

*To whom correspondence should be addressed at: Stem Cell Research Center, Department of Orthopaedic Surgery, University of Pittsburgh, Bridgeside Point 2, Suite 206, 450 Technology Drive, Pittsburgh, PA 15219, USA. Tel: +1 4126482798; Fax: +1 4126484066; Email: jhuard@pitt.edu

Abstract

Although it has been speculated that stem cell depletion plays a role in the rapid progression of the muscle histopathology associated with Duchenne Muscular Dystrophy (DMD), the molecular and cellular mechanisms responsible for stem cell depletion remain poorly understood. The rapid depletion of muscle stem cells has not been observed in the dystrophin-deficient model of DMD (mdx mouse), which may explain the relatively mild dystrophic phenotype observed in this animal model. In contrast, we have observed a rapid occurrence of stem cell depletion in the dystrophin/utrophin double knockout (dKO) mouse model, which exhibits histopathological features that more closely recapitulate the phenotype observed in DMD patients compared with the mdx mouse. Notch signaling has been found to be a key regulator of stem cell self-renewal and myogenesis in normal skeletal muscle; however, little is known about the role that Notch plays in the development of the dystrophic histopathology associated with DMD. Our results revealed an over-activation of Notch in the skeletal muscles of dKO mice, which correlated with sustained inflammation, impaired muscle regeneration and the rapid depletion and senescence of the muscle progenitor cells (MPCs, i.e. Pax7+ cells). Consequently, the repression of Notch in the skeletal muscle of dKO mice delayed/reduced the depletion and senescence of MPCs, and restored the myogenesis capacity while reducing inflammation and fibrosis. We suggest that the down-regulation of Notch could represent a viable approach to reduce the dystrophic histopathologies associated with DMD.

Introduction

The rapid onset of muscle histopathology observed in Duchenne muscular dystrophy (DMD) patients has been related, at least in part, to the depletion of functional muscle stem cells, which is the result of the continuous degenerative/regenerative cycling that occurs in their skeletal muscles due to a deficiency of dystrophin (1–3). The widely utilized mdx mouse model of DMD is deficient for dystrophin, but in contrast to DMD, the muscle regeneration capacity of the mdx mouse is un-altered and muscle histopathology is very mild, which is potentially attributable to a lack of muscle stem-cell depletion (2,4,5). In support of this contention, mdx/mTR mice, that are dystrophin-deficient and have a telomere dysfunction/shortening specifically in their muscle progenitor cells (MPCs), develop a more severe dystrophic phenotype than mdx mice. Their phenotype also rapidly worsens with age, due to the rapid depletion of their MPCs (2). Hence, treatments directed exclusively at restoring dystrophin within the mdx muscle fibers may not be sufficient for treating DMD patients, especially older patients (2,6,7). Therefore, therapeutic modulation of muscle stem cell activities could represent a viable...
approach for alleviating muscle weakness in DMD (7). To achieve that goal, many questions remain unanswered about the molecular pathway involved in the regulation of muscle stem-cell activity in dystrophic muscle.

Mdx and dystrophin/utrophin double knockout (dKO) mice are both important mouse models of DMD (5,8–10); however, in contrast to the mild phenotype observed in mdx mice, dKO mice exhibit a similar phenotype to that observed in human DMD patients including a shorter life span (~8 weeks compared with 2 years), increased necrosis and fibrosis in their skeletal muscles, severe scoliosis/kyphosis of the spine and severe cardiac involvement (cardiomyopathy) (8,9). Although dKO mice are deficient in both utrophin and dystrophin, in contrast to DMD patients, the dKO mouse model represents an animal model that more closely recapitulates the DMD phenotype (4,8,11,12). It is important to note that utrophin/- mice do not develop major histopathological signs of disease (13). Our group has recently verified that the depletion of MPCs occurs in dKO mice, which correlates with their impaired muscle regeneration capacity (14).

The reports on the role that Notch plays in normal muscle regeneration and muscle stem-cell activation remains controversial. Notch has been shown to be involved in the maintenance of stem-cell quiescence and the stem-cell pool in skeletal muscle (15–17). Notch signaling declines during the aging process and correlates with the impaired muscle regeneration capacity of aged individuals (18–20); however, Notch signaling has also been shown to be a repressor of myogenesis and hence has an adverse effect on muscle regeneration (21–25). Moreover, constitutively activated Notch1 Intracellular Domain (NICD) has been shown to result in an impairment in skeletal muscle regeneration and an increase in the number of undifferentiated Pax7 expressing cells present in the muscle (26). Elevated Notch signaling has also been found in Strata13/– mice which have a defect in their muscle regenerative capacity that results in the development of fibrosis (27). Conversely, delta-like 1 (Dll1), a non-canonical Notch ligand that inhibits Notch signaling, was found to be required for proper skeletal muscle development and regeneration (23). It was suggested that the continuous activation of Notch signaling impairs muscle regeneration and that a temporal decline in Notch signaling in muscle stem cells is required for proper muscle regeneration and repair (28).

Several lines of evidence have suggested that activated Notch signaling may also play an important role in the development of the histopathologies observed in DMD, including increases in: (i) muscle atrophy; (ii) premature cellular senescence; (iii) inflammation and (iv) fibrosis formation (29–41). (i) Muscle atrophy: increased Notch1 activation has been observed in denervation-induced skeletal muscle atrophy (29,30), while both DMD patients (42) and dKO mice (9,10) experience massive muscle atrophy. (ii) Premature cellular senescence: dKO mice show multiple phenotypic similarities to progeroid animals (animals that age prematurely), including premature death, stem-cell defects and skeletal muscle histopathologies; notably, Notch activation has been observed to mediate the premature senescence of various cell types in progeroid animals (35–37,43–45). (iii) Inflammation: the skeletal muscle of dKO mice exhibits much greater inflammation than mdx mice (9,10) and Notch is often co-activated with pro-inflammatory signaling involving TNF-α and NF-κB (31–33). In fact, recent studies showed that the canonical NF-κB DNA-binding motif 5-GGRRNNYYCC-3 exists in the promoters of Notch genes in human cells (46) and the treatment of porcine satellite cells with recombinant NF-κB enhances Notch1 expression (47). Additionally, Notch signaling has also been shown to mediate inflammatory responses in cardiovascular disorders (48). Finally, both Notch signaling and TNF-α/NF-κB signaling are known to repress the myogenic potential of muscle stem cells by down-regulating MyoD (21,49–51). (iv) Fibrosis: extensive fibrosis has been observed in the skeletal muscle of both DMD patients and dKO mice (9,10); while the co-activation of TGF-β1 and Notch signaling pathways has been reported in various tissues and cell types (including C2C12 myoblasts) during fibrogenesis, supporting the fact that Notch signaling represents a potential target to block fibrosis (38–41).

Taken together, these observations suggest that Notch signaling may play an important role in the depletion and senescence of MPCs and the histopathology of dystrophic muscle of dKO mice. Therefore, the current study was conducted to determine whether the MPC depletion and senescence that occurs in severely affected dystrophic muscle in dKO mice correlates with the over-activation of Notch signaling, and the results were compared with mdx mice that have a mild phenotype. In addition, Notch inhibition experiments were conducted in MPCs isolated from dKO mice (in vitro) and in dKO skeletal muscle (in vivo) to determine whether the inhibition and senescence could be alleviated by inhibiting Notch signaling to improve the impaired muscle regeneration in the dKO mice. Our results not only reveal that over-activation of Notch signaling occurs in MPCs and skeletal muscle of dKO mice, but more importantly that the inhibition of Notch signaling delays MPC depletion and senescence, and restores myogenesis while reducing inflammation and fibrosis in the severely affected dystrophic muscle of dKO mice.

Results

The depletion and senescence of MPCs rapidly occurs in the skeletal muscle of dKO mice and is related to a reduction in cell proliferation capacity with age

Immunostaining of muscle tissues with antibody against Pax7 revealed that the number of Pax7 positive (Pax7+) cells (satellite cells/MPCs) generally remained unchanged in the gastrocnemius muscles (GM) of 1- to 8-week-old mdx mice (Fig.1A and B). However, although the number of Pax7+ cells was enriched in the GM of dKO mice from 1- to 4-weeks of age, a significant decrease in the number of Pax7+ cells was observed in 8-week-old dKO mice (Fig.1A and B). Notably, Pax7+ cells in the ‘younger’ dKO mice (1 or 4 weeks old) were generally more enriched than that of the age-matched mdx mice (Fig.1A and B). In support of this observation, when examining single myofibers isolated from the 4- or 8-week-old dKO mice, fewer Pax7+ cells were observed to reside in the myofibers of the 8-week-old dKO mice, compared with the 4-week-old dKO mice (Fig.1C).

We next investigated whether the rapid depletion of MPCs in dKO skeletal muscles correlated with a reduction in the proliferation of Pax7+ cells. Dual immunostaining of Ki67 (a proliferation marker) and Pax7 in the skeletal muscle of 4- and 8-week-old dKO mice revealed a reduction in cells co-expressing Ki67 and Pax7 (Ki67+/Pax7+) in the 8-week-old dKO mice compared with the 4-week-old dKO mice (Fig.1D and E), indicating a progressive reduction in the proliferation of Pax7+ cells in dKO skeletal muscle as the mice age.

To identify the cell types of Ki67+ cells (proliferating cells) in the skeletal muscle of 8-week-old dKO mice, we co-immunostained Ki67 and PDGFR-α (PDGFR-α, a marker for non-myogenic mesenchymal progenitor cells) (52) and observed that some of the Ki67+ cells were also PDGFR-α+ (Supplemental Material, Fig. S1A). There were more Ki67+/PDGFR-α+ cells than...
Ki67+/Pax7+ cells in the skeletal muscle of 8-week-old dKO mice (Supplemental Material, Fig. 1A and B).

During skeletal muscle regeneration, MyoD activation occurs for proper myogenesis, and Pax7+ cells remain in an undifferentiated state without MyoD activation (53). Although Pax7+ cells were more enriched in the skeletal muscle of 4-week-old dKO mice than 4-week-old mdx mice, we observed that the number of MyoD+ myogenic cells was greatly reduced in the 4-week-old dKO mice, when compared with age-matched mdx mice (Fig. 1F and G), suggesting the reduced myogenesis potential of the muscle cells in dKO mice.

Furthermore, the cell senescence assay revealed that there were also more β-Galactosidase + (β-gal+) senescent cells in the skeletal muscle of 8-week-old dKO mice, compared with 8-week-old mdx mice (Fig. 1H), suggesting that the depletion of the MPCs in dKO mice could be contributed by accelerated cell senescence.

Increased non-muscle tissues (heterotopic ossification, adipose tissue and fibrosis) and up-regulation of Notch and pro-inflammatory signaling molecules in the skeletal muscle of dKO mice

Heterotopic ossification (HO) has been described in the limb muscles of mdx mice previously (54,55), and our recent study demonstrated much more extensive HO in the limb muscles of dKO mice, compared with mdx and WT mice (56,57). Our current observations further revealed that, in comparison to mdx mice, 8-week-old dKO mice featured not only extensive intramuscular HO, but also much more peri-muscular adipose tissue (Fig. 2A). Meanwhile, trichrome staining showed extensive formation of fibrosis in the skeletal muscle of dKO mice (Fig. 2A) (8,9). The presence of extensive HO, adipose tissue, and fibrosis in the skeletal muscle of dKO mice suggests that the depletion of MPCs (Pax7+ cells) and myogenic cells (MyoD+ cells) could have been...
The expression of a group of genes directly regulating muscle stem-cell activity was compared among skeletal muscle tissues of 4-week-old mdx, dKO and wild-type (WT) mice. A semi-quantitative PCR study showed that, in comparison to mdx mice, the expression of the Notch genes (Notch1-3, Hes1, Hey1, Jagged2), TGF-β genes (myostatin/MSTN, BMP4 and TGF-β1), inflammation genes (TNF-α, IL-1β and IL-6) and Pax3 (a marker of undifferentiated muscle stem cells), as well as down-regulation of Klotho (an anti-inflammatory factor) and MyoD (myogenic factor) in dKO skeletal muscle, compared with both mdx and WT skeletal muscle. Bar charts show the ratio of mdx mice compared with WT mice (mdx/WT), and the ratio of dKO mice compared with WT mice (dKO/WT) for each gene. (C) Immunostaining revealed more Notch3+ cells and Notch3+/Pax7+ cells (arrows) in dKO skeletal muscle, compared with mdx skeletal muscle; Pax7+ cells in mdx skeletal muscle are generally Notch3- (arrowhead). Notch1+ cells were also more abundant in dKO skeletal muscle. Red: Pax7; green: Notch3 or Notch1; blue: DAPI. (D) Graph showing the number of Notch3+ and Pax7+/Notch3+ cells in the skeletal muscles of 4-week-old mdx and dKO mice. * indicates the P-value is < 0.05. (E) Western blot analysis revealed higher levels of Hes1 and NICD in dKO skeletal muscle, compared with mdx and WT mice. (F) Graph showing the levels of Hes1 and NICD proteins in the skeletal muscles of 4-week-old mdx, dKO and WT mice. * indicates the P-value is < 0.05, when comparing dKO to either mdx or WT.
The effects of Notch activation in MPCs and skeletal muscle of mdx mice

In addition to the role of Notch in repressing myogenesis, previous observations have shown a positive effect of Notch activation on the proliferation of muscle stem cells (34,60). We suggest that the lower Notch activity observed in the mdx MPCs may influence their proliferation and myogenic potentials. To verify this possibility, the effect of Notch activation on the proliferation and myogenic potentials of the mdx MPCs was studied by treating the in vitro cultured mdx MPCs from 4-week-old mdx mice with the peptide of Jagged1 (61–63), a Notch ligand known to activate Notch signaling in skeletal muscle (19). During 4 days of treatment, it showed that the proliferation of mdx MPCs was promoted, while the myogenic differentiation was reduced with Jagged1 treatment (Fig. 3A). Meanwhile, the percentage of Pax7+ cells in the mdx MPCs was increased after 2 days of Jagged1 treatment (Fig. 3B and C). In addition, the expression of the pro-inflammatory factors TNF-α and IL-6 in the mdx MPCs was up-regulated with Jagged1 treatment, suggesting an increased activation of inflammatory signaling (Fig. 3D); also, the expression of MyoD was down-regulated in the mdx MPCs with Jagged1 treatment (Fig. 3D). This result is consistent with previous observations that TNF-α/NF-κB signaling represses the myogenic potential of muscle stem cells via the down-regulation of MyoD (49–51).

To further investigate the potential effect of Notch activation in the skeletal muscle of mdx mice in vivo, we conducted intramuscular injections of the Jagged1 peptide into the GM muscles of mdx mice 4 days after cardiotoxin-induced injury. Three days after peptide injection (7 days after muscle injury), we observed a higher number of Pax7+ cells and a lower number of MyoD+ cells in the injected muscle, compared with the control muscles injected with the scrambled peptide (Fig. 3E and F). Also, the size of the regenerating myofibers was smaller 3 days after the injection in the Jagged1 treated muscles compared with the control muscles (Fig. 3G), which is indicative of delayed muscle regeneration. The effect of Notch activation 2 weeks after initiating the repeated injection of the Jagged1 peptide demonstrated an increase in HO and fibrosis in the skeletal muscle of the mdx mice receiving the Jagged1 injections (Supplemental Material, Fig. S4).

Previous studies have shown that pro-inflammatory TNF-α/NF-κB signaling reduces the myogenic potential of muscle stem cells, via the down-regulation of MyoD (49–51). As expected, we observed that the myogenic potential of the mdx MPCs was reduced when the cells were exposed to TNF-α (20 ng/ml). This effect could be inhibited when the cells were co-treated with the Notch inhibitor DAPT (γ-secretase inhibitor) (10 µM) (Fig. 3H). This observation indicates that Notch signaling interacts with pro-inflammatory signaling which repressed the myogenic potentials of the mdx MPCs was studied by treating the in vitro cultured mdx MPCs from 4-week-old mdx mice with the peptide of Jagged1 (61–63), a Notch ligand known to activate Notch signaling in skeletal muscle (19). During 4 days of treatment, it showed that the proliferation of mdx MPCs was promoted, while the myogenic differentiation was reduced with Jagged1 treatment (Fig. 3A). Meanwhile, the percentage of Pax7+ cells in the mdx MPCs was increased after 2 days of Jagged1 treatment (Fig. 3B and C). In addition, the expression of the pro-inflammatory factors TNF-α and IL-6 in the mdx MPCs was up-regulated with Jagged1 treatment, suggesting an increased activation of inflammatory signaling (Fig. 3D); also, the expression of MyoD was down-regulated in the mdx MPCs with Jagged1 treatment (Fig. 3D). This result is consistent with previous observations that TNF-α/NF-κB signaling represses the myogenic potential of muscle stem cells via the down-regulation of MyoD (49–51).

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capacity of the muscle stem cells, and Notch inhibition could rescue the pro-inflammatory factor-induced reduction in myogenesis.

The beneficial effects of Notch inhibition in dKO MPCs

Since Notch over-activation was observed in dKO MPCs, we suggest that repression of Notch signaling may influence their proliferation and the myogenic potential of the dKO MPCs. MPCs isolated from 4-week-old dKO mice were cultured in cell-growth medium containing DAPT (10 µM) to inhibit Notch activity (64). During 4 days of treatment, a reduction in proliferation and an increase in myogenic differentiation were observed in the dKO MPCs with DAPT treatment (Fig. 4A). Meanwhile, the expression of TNF-α and IL-6 was down-regulated, and the expression of MyoD was up-regulated in the DAPT-treated dKO MPCs (Fig. 4B).

In addition, an increase in the percentage of MyoD+ cells after 2 days of DAPT treatment was also observed in the dKO MPCs (Fig. 4C).

Notch activation is also known to promote the osteogenic potential of human bone-marrow stromal cells and vascular smooth muscle cells (65,66). Because MPCs are known to have an osteogenic potential (57,67), and due to the extensive HO observed in the dKO mice (57), we suggest that the over-activation of Notch signaling in the dKO MPCs may be related to the potent osteogenic potential of the dKO MPCs. To verify this hypothesis, an osteogenic assay was performed with the dKO MPCs isolated from 4-week-old dKO mice, with and without DAPT treatment (10 µM). The results indicated that there was a reduction in the osteogenic potential of the dKO MPCs with DAPT treatment (Fig. 4C). Finally, the increased myofibroblastic differentiation (α-SMA positive cells) of the dKO MPCs with TGF-β stimulation

Figure 4. Effects of in vitro inhibition of Notch on cell proliferation, myogenic differentiation, osteogenic differentiation and myofibroblastic differentiation of dKO MPCs. (A) With DAPT treatment, the proliferation of dKO MPCs was reduced while the spontaneous fusion/myotube formation was increased compared with dKO MPCs without treatment. Arrows: myotubes. (B) Gene expression assay showed that TNF-α and IL-6 were down-regulated, and MyoD was up-regulated by DAPT treatment of dKO MPCs. (C) The percentage of MyoD+ cells in dKO MPCs was increased after 2 days of DAPT treatment; the osteogenic potential of dKO MPCs in osteogenic differentiation medium, which is shown as Alizarin Red staining of calcium deposition, was increased by DAPT treatment; the myofibroblastic differentiation potential of dKO MPCs, which was determined with the TGF-β1 induced production of α-Smooth Muscle Actin (SMA) by the cells, was reduced by DAPT treatment. Statistics include the comparison of MyoD+ cells in the myogenesis assay, Alizarin Red-positive signal in the osteogenesis assay, and α-SMA+ cells in the TGF-β1 induced myofibroblastic differentiation assay. * indicates P < 0.05.
was reduced with DAPT treatment (Fig. 4C). Compared with the dKO MPCs treated with TGF-β1 stimulation only, Collagen type I and TGF-β1 was also down-regulated when the cells were co-treated with DAPT (Supplemental Material, Fig. S2G). Therefore, the osteogenic or myofibroblastic differentiation potential of the dKO MPCs is reduced with Notch inhibition.

The effect of Notch activation on the dKO MPCs was also studied by treating the dKO MPCs with Jagged1 peptide. Results showed that both the proliferation and myogenic potentials of dKO MPCs remain unchanged (Supplemental Material, Fig. S5). These observations indicated that Notch activation did not improve the function of dKO MPCs.

The beneficial effects of Notch inhibition in dKO skeletal muscle on stem-cell depletion, senescence and histopathology

In vivo inhibition of Notch signaling in dKO mice was conducted with the intraperitoneal (IP) injection of DAPT dissolved in 95% corn oil/5% ethanol (68–70). The dissolving vehicle without DAPT served as a control. The DAPT injections were performed in the dKO mice when the mice were 3 to 8 weeks of age, 3 times per week. Results showed that DAPT injections significantly altered the expression profile of the stem cell regulatory genes in the dKO skeletal muscle making their gene profile appear more like the profile observed in mdx skeletal muscle. The expression of Notch, TGF-βs, pro-inflammatory factors and Pax3 was down-regulated, and the expression of Pax7 and MyoD was up-regulated with DAPT treatment (Fig. 5A). Western blot analysis confirmed a decrease in the expression of the Hes1 protein in the skeletal muscle of dKO mice receiving DAPT injection (Fig. 5B).

Pax7 immuno-staining in the skeletal muscle of 8-week-old dKO mice injected with DAPT revealed a significant increase in the number of Pax7+ cells (Fig. 5C). Co-immunostaining of Notch3 and Pax7 revealed that the number of Notch3+ cells was decreased and the number of Pax7+/Notch3- cells was increased in the dKO mice injected with DAPT (Fig. 5D and E). There was also an increase in the number of Pax7- cells residing in the isolated single myofibers of DAPT-treated dKO mice (Fig. 5F and G). Meanwhile, the number of MyoD+ cells in the skeletal muscle of dKO mice was also increased with DAPT treatment (Fig. 5H), indicating an improvement in the myogenic potential of the muscle stem cells. Moreover, the number of β-gal+ senescent cells in the skeletal muscle of dKO mice was decreased with DAPT treatment (Fig. 5I).

**Figure 5.** Effect of in vivo inhibition of Notch on the expression of muscle regeneration-related genes, and stem-cell depletion and senescence in the skeletal muscle of dKO mice. (A) Gene expression assay showed that compared with muscle tissue without DAPT injection, muscle tissues with DAPT injection featured a down-regulation of Notch genes (Notch 1, 3, Hes1 and Hey1), TGF-β genes (myostatin/MSTN, BMP4 and TGF-β1), inflammation genes (TNF-α, IL-1β and IL-6) and Pax3, and an up-regulation of Klotho, Pax7 and MyoD. Bar charts show the ratio of mRNA in DAPT-injected mice to control mice for each gene. (B) Western blot analysis of Hes1 validated that DAPT treatment decreased the activation of Notch signaling in dKO mice. (C) Representative images of immuno-staining showing that DAPT treatment increased the number of Pax7+ cells in the skeletal muscle of 8-week-old dKO mice. Red: Pax7; blue: DAPI. Asterisks: necrotic myofibers. (D) Immunostaining showed that DAPT treatment decreased the number of Notch3+ cells, and increased the number of Pax7+/Notch3- cells in the dKO skeletal muscle. Red: Pax7; green: Notch3; blue: DAPI. (E) Statistics of the number of Pax7+ cells in dKO skeletal muscle with or without DAPT treatment. * indicates P < 0.05. (F) In vitro cultured single myofibers isolated from 8-week-old dKO mice with or without DAPT injection contain a different numbers of Pax7+ cells. Red: Pax7; blue: DAPI. (G) Statistics of the number of Pax7+ at single myofibers. (H) Immunostaining showed that DAPT treatment also increased the number of MyoD+ cells. Red: MyoD; green: Collagen IV; blue: DAPI. (I) Cell senescence assay showed that DAPT treatment decreased the number of β-gal+ cells. H.E. staining was performed at the same slides and improved myofiber formation can be observed.
Notch inhibition by DAPT also improved the histopathologies of the dystrophic skeletal muscle including an increase in the number of regenerating myofibers expressing embryonic-myosin heavy chain (E-MHC+), and a reduction in the number of inflammatory cells (CD68+) and necrotic myofibers (mouse IgG+) (Fig. 6A); also, the formation of HO and fibrosis was reduced in DAPT-treated dKO mice (Fig. 6B). In addition, the intramyocellular lipid accumulation (IMCL) that we reported previously to be increased in the myofibers of dKO mice (71), was also reduced with DAPT treatment (Fig. 6B).

A schematic diagram of the potential role Notch plays in regulating muscle stem cells, which are involved in creating the histopathology seen in the skeletal muscle of dKO mice (Fig. 6C).

Discussion

Our results demonstrated that chronic over-activation of Notch signaling occurs in the severely dystrophic muscles of dKO mice, which correlates with accelerated stem-cell senescence, depletion and impaired muscle regeneration. Repression of Notch signaling was found to be beneficial for rescuing stem-cell senescence and depletion, and it reduced the histopathological features of the dKO mice by controlling stem-cell proliferation, repressing pro-inflammatory signaling and reducing fibrosis and HO in their skeletal muscle. Decreased Notch activity appears to be necessary for improving muscle regeneration in severely dystrophic muscle, and we suggest that the therapeutic modulation of stem-cell activity by regulating Notch could represent a viable therapeutic target for treating DMD patients.

The adverse role of Notch in muscle regeneration has previously been demonstrated, and constant activation of Notch has been found to impair muscle regeneration (21–27). A temporal decline of Notch in muscle stem cells has been found necessary for proper muscle regeneration and repair (28). Dystrophic muscle undergoes repeated cycles of degeneration and regeneration (72), and our results revealed that this 'decline of Notch expression' during muscle regeneration is more properly regulated in the skeletal muscle of mdx mice but exacerbated in the dKO mice. It is well known that stem-cell depletion does not occur in the skeletal muscle of mdx mice during most of their lives (2); however, mdx mice lacking the RNA component of telomerase (mdx/mTR) develop stem-cell depletion and severe muscular dystrophy (2), very similar to the phenotype of dKO mice.

It has been reported that Notch promotes stem-cell proliferation in normal muscle and maintains muscle stem cells in an
undifferentiated state (20,26). Injection of the Jagged1 peptide (a Notch activation ligand) into mdx mice in this study revealed that Notch activation promoted the proliferation of Pax7+ cells and repressed myogenic differentiation (Fig. 3). Notch activation is also known to promote osteogenesis and calcification (65,66,73). The results of this study support these reports since Notch inhibition in vitro and in vivo repressed the osteogenic differentiation of dKO MPCs and HO formation in the skeletal muscle of the dKO mice (Figs 4 and 6). Therefore, we suggest that the rapid depletion of Pax7+ muscle stem cells in dKO mice (6–8 weeks of age) could be explained in two ways: (i) Notch-mediated increases in stem-cell proliferation at an early age in dKO mice (before 4 weeks of age) could exhaust the stem-cell compartment and accelerate their senescence; and/or (ii) Notch- and inflammation-mediated excessive formation of HO, fibrotic tissue and adipose tissue in the skeletal muscle of dKO mice could promote the differentiation of muscle stem cells toward non-myogenic lineages (i.e. osteogenic cells, myofibroblasts or adipogenic cells) (67,74,75).

Based on our observations, we also suggest that ‘stem cell depletion’ in the dKO mice is not restricted to early myogenic progenitor cells (i.e. Pax7+ cells) since a depletion of MyoD+ myogenic cells was also observed when the mice were 4 weeks of age (Fig. 2A and B), which occurs earlier than the depletion of Pax7+ cells. MyoD+ cells are more differentiated than Pax7+ cells and are responsible for muscle regeneration. Therefore, the depletion of functional myogenic cells (MyoD+ cells) could be responsible for the impaired muscle regeneration observed in the dKO mice. One recent study demonstrated that Notch activation promoted the proliferation of Pax7+ cells while it inhibited the proliferation of primary myoblasts (MyoD+ cells) (26). Based on this report and our observations, we suggest that increased Notch activity in the dKO mice promotes the proliferation of Pax7+ cells but not MyoD+ cells, and prevents Pax7+ cells from differentiating into MyoD+ cells. Thus in order to improve muscle regeneration, both properly controlled stem-cell proliferation and myogenic differentiation are required. The micro-environment in the dKO muscles, which is hypoxic, necrotic, fibrotic and inflammatory, is not conducive for myogenic differentiation; although Notch over-activation stimulates the proliferation of Pax7+ stem cells, the impaired myogenic differentiation leads to a reduction in muscle regeneration.

Another important question is whether the depletion of the MPCs in the dKO mice is caused by the compromised proliferation capacity of the cells. The cell senescence assay revealed the presence of β-gal+ senescent cells in the skeletal muscle of 8-week-old dKO mice (Fig. 1H), which indicates that at least some of the muscle cells were in a non-proliferative state. Also, as Figure 1D shows, although the total number of proliferating cells (Ki67+) was not reduced in the 8-week-old dKO muscle compared with that of the 4-week-old dKO muscle, the number of proliferating myogenic stem cells (Ki67+/Pax7+) was significantly reduced. Furthermore, co-staining of Ki67 and PDGFR-α demonstrated that there were more Ki67+/PDGFR-α+ cells than Ki67+/Pax7+ cells in the skeletal muscle of 8-week-old dKO mice which indicates that there were a greater number of proliferating non-myogenic mesenchymal progenitors than myogenic progenitors present in the muscles of the 8-week-old dKO mice. This supports the fact that the proliferation potential of the myogenic stem cells was lower than that of the non-myogenic muscle cells in the dKO mice (Supplemental Material, Fig. S1). Moreover, our recently published study demonstrated that both the telomerase activity and proliferation potential of the dKO MPCs were lower than that of the mdx and WT MPCs (14). Therefore, we suggest that a rapid depletion of the myogenic stem cell pool occurs in the skeletal muscle of dKO mice; however, the overall population of proliferating cells in the muscles does not decrease which is probably related to the proliferation of the non-myogenic cells.

Notch has been shown to act as a pro-inflammatory signal in mouse cells (48,76), and more recently the canonical NF-kB DNA-binding motif was found in the Notch promoter of human cells (46). Our results showed that disregulated Notch signaling in the skeletal muscle of dKO mice potentially correlates with the excessive pro-inflammatory cytokines found in the skeletal muscles of the mice; however, it is important to note that Notch is not simply an upstream signaling factor for TNF-α/NF-κB. In fact, the crosstalk between Notch signaling and TNF-α/NF-κB signaling is interactive. TNF-α/NF-κB ‘can activate Notch by inducing Jagged1 expression’, moreover, the over-activation of Notch signaling could be the result of the excessive expression of pro-inflammatory cytokines which in turn could sustain the prolonged activation of excessive pro-inflammatory signaling (33,48,76,77). Notch and TNF-α/NF-κB signaling have both been reported to repress muscle stem-cell differentiation by down-regulating MyoD (21,49–51), suggesting that these two signaling pathways likely interact in regulating myogenesis and are therefore probably involved in mediating the formation of the dystrophic pathologies seen in DMD; however, such a correlation between Notch and TNF-α/NF-κB signaling in either normal muscle or diseased muscle has never been described. Co-activation of Notch signaling and pro-inflammatory signaling was observed in the skeletal muscle and muscle stem cells of dKO mice, which is consistent with previous observations of the interactive correlation of Notch and TNF-α/NF-κB signaling (31–33). It is interesting to note that, in addition to TNF-α/NF-κB signaling, Notch effectors, such as Hey1 and Hes1, could also be directly involved in repressing myogenesis in muscle stem cells (22,78). In fact, TNF-α signaling was found to be able to induce Hes1 and Hey1 expression by activating their promoters (76), suggesting a potential interaction between Notch effectors and TNF-α in mediating Notch activation signaling in the skeletal muscle of dKO mice.

TGF-β signaling is the key regulator of fibrosis formation in dystrophic muscles (79,80). Our results revealed that the over-activation of Notch signaling in the dKO skeletal muscle appears to interact with TGF-β signaling, and contributes to muscle atrophy and the formation of fibrosis and HO in the dystrophic muscles of these mice. Interaction between Notch signaling and TGF-β family members (TGF-β1, myostatin and BMP2/4) has been previously demonstrated in a variety of tissue-related events (39,81) including TGF-β1-mediated fibrosis formation (38,82–84), myostatin-mediated muscle atrophy (85) and BMP-mediated osteogenesis (66,86). Our in vitro and in vivo results further validate a correlation between the Notch and TGF-β signaling pathways in mediating the histopathology observed in the muscle of dKO mice including fibrosis, muscle atrophy and HO.

Notch inhibition with γ-secretase inhibitors (e.g. DAPT) has previously been studied for their ability to prevent a variety of different types of organ fibrogenesis and cancers (41,87,88). Several phase I and II clinical trials are currently underway to investigate the therapeutic efficacy of various γ-secretases, which has revealed some promising results (89,90). Our study demonstrated that the γ-secretase inhibitor DAPT was effective for improving the myogenic potential of dKO M3SDCs in vitro, and for improving the muscle regenerative potential of dKO mice in vivo. Importantly, the life span of the DAPT-treated dKO mice was also slightly increased (data not shown). Our results suggest that Notch inhibition with a γ-secretase inhibitor could potentially be beneficial for treating DMD patients. Gene expression profiles early on in
the disease process of DMD have shown a progressive down-regulation of DLK1 (an inhibitory ligand of Notch) from 5 to 24 months after birth (91), indicating a potential increase in Notch activation during disease progression; however, the activation status and the role of Notch signaling in the skeletal muscles of DMD patients requires further investigation.

Our results showed that Notch signaling was activated in Pax7+ muscle stem cells in dKO mice; however, the cell source of activating Notch ligands in muscle remains un-identified. According to previous publications, it seems that such Notch activating ligands (i.e. DLLs and Jagged1/2) could potentially reside on the cell membranes of pro-inflammatory cells (i.e. macrophages), myofibroblasts or endothelial cells (48,92,93). Although Notch signaling is usually activated by intercellular action, it has been demonstrated that cell–cell contact is not the only way for transferring Notch activation from cell to cell, and Notch signaling may be activated via exosome-delivered Notch ligands (94). Further studies need to be performed to clarify the cell source responsible for activating Notch ligands.

In summary, our findings revealed that the over-activation of Notch signaling, in concert with pro-inflammatory and pro-fibrogenic cytokines, are involved in the formation of fibrosis and heterotopic ossification, and the depletion of Pax7+ and MyoD+ muscle progenitor cells in the skeletal muscle of dKO mice. Notch inhibition in severely dystrophic muscle helps repress cell senescence and delays/reduces stem-cell depletion. In addition, Notch inhibition can reduce inflammation, necrosis, Ho and fibrosis, while improving muscle regeneration. On the other hand, Notch activation exacerbates the dystrophic muscle pathology of the mdx mice. We suggest that the Notch signaling pathway represents a target for the development of therapeutic approaches to alleviate the muscle weakness observed in DMD patients.

Methods and Materials

Animals

Wild-type (C57BL/10J) mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The mdx (dys−/−) and dKO (dys−/−; utrn−/−) mice were derived from our in-house colony. At least eight mice were used in each experimental sample group. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

Stem cell isolation from skeletal muscle

The muscle progenitor cells (MPCs) were isolated from the skeletal muscle of dKO and mdx mice (4 or 6 weeks old) using the modified preplate technique (95). Mice were sacrificed in a carbon dioxide chamber according to the IACUC protocol. The cells were cultured in the growth medium [GM, DMEM supplemented with 20% fetal bovine serum (FBS), 1% penicillin–streptomycin antibiotics and 0.5% chicken essential extract (CEE)] at 37°C in 5% CO2.

In vitro Notch activation or inhibition

For Notch activation, MPCs from 4-week-old mdx mice (mdx MPCs) or dKO mice (dKO MPCs) were treated with the Jagged1 peptide (CDDYYGFNCNKFPR) or a control scrambled peptide (RCGPDCFDNYGRYKCYCF) (61–63) (GenScript USA, Inc.) (20 µg/ml) for 2 or 4 days in proliferation or myogenic differentiation assay. For Notch inhibition, dKO MPCs from 4-week-old dKO mice were treated with DAPT (N-[N-(3,5-Difluorophenacetyl)-l-alanyl]-S-phenylglycine t-Butyl Ester; Calbiochem) (γ-secretase inhibitor) (10 µM, solved in DMSO) for 2, 4 or 7 days in different assays; control cells were treated with carrier (DMSO) only.

In vivo Notch activation or inhibition

In order to observe the effect of Notch activation on muscle regeneration in mdx mice, cardiotoxin (Calbiochem) (10 µM in 40 µl of PBS) was intramuscularly injected into the GM muscles of 8-week-old mdx mice to generate muscle injury, followed by intramuscular injection of the Jagged1 peptide or the control scrambled peptide (5 mg/ml in 15 µl of PBS) on Days 3 and 5 post-muscle injury. Peptides were prepared and injected as previously reported (61–63). Four days after the injection of the Jagged1 peptide (7 days after cardiotoxin injection), the muscle tissues were harvested for analysis. To observe the effect of Notch activation at later stages, the intramuscular injection of Jagged1 was performed three times a week for 2 weeks, and then muscle tissues were harvested to analyze HO and fibrosis formation. In order to observe the effect of Notch inhibition on muscle regeneration in the dKO mice, DAPT solution (10 mg/ml in 95% corn oil/5% ethanol, 30 mg/kg) (68–70) was injected into dKO mice via intraperitoneal (IP) injection three times per week from the age of 3 weeks, and continued until the mice reached 8 weeks of age. dKO mice receiving the vehicle (95% corn oil/5% ethanol) only served as the controls.

In vitro proliferation/myogenic differentiation, osteogenic differentiation and myofibroblastic differentiation assays

For the proliferation/myogenic differentiation assay, MPCs from 4-week-old dKO mice were cultured in cell-growth medium (20% FBS serum in DMEM) for 4 days, with or without DAPT (10 µM) supplementation. The proliferation and myogenic differentiation potential was then tracked by observing cell number and myotube formation (spontaneous cell fusion). The osteogenesis assay was conducted with osteogenic medium (DMEM supplemented with 110 mg/l sodium pyruvate, 584 mg/l L-glutamine, 10% FBS, 1% penicillin/streptomycin, 10−7 M dexamethasone, 50 µg/ml ascorbic-acid-2-phosphate and 10−2 M β-glycerophosphate), supplemented with BMP2 (50 ng/ml) for 7 days, with or without DAPT. Calcium deposition assessed with Alizarin Red staining was used to determine the osteogenic potential. The fibrogenesis assay was conducted by supplementing growth medium with TGF-β1 (10 ng/ml) for 7 days, with or without DAPT. Cells positive for α-smooth muscle actin (SMA) were monitored to determine the myofibroblastic differentiation potential.

Cell senescence assay

A cell senescence assay was performed on muscle cells and muscle tissues using a Senescence β-Galactosidase (β-gal) Staining Kit (Cell Signaling Technology) following the manufacturer’s protocol. The number of cells positive for β-gal activity at pH6, a
known characteristic of senescent cells not found in pre-sen- cent, quiescent or immortal cells, was determined.

mRNA analysis with semi-quantitative reverse transcriptase–PCR

Total RNA was obtained from MPCs or the skeletal muscles of mice using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer’s instructions. Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences are listed in Table 1. PCR reactions were performed using an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc.). The cycling parameters used for all primers were as follows: 95°C for 10 min; PCR, 40 cycles of 30 s at 95°C for denaturation, 1 min at 54°C for annealing and 30 s at 72°C for extension. Products were separated and visualized on a 1.5% agarose gel stained with ethidium bromide. All data were normalized to the expression of GAPDH (glyceraldehydes 3-phosphate dehydrogenase).

Western blot

Frozen muscle tissues were lysed with a lysis buffer [ratio of β-mercaptoethanol to sample buffer (62.5 mm Tris–HCl, pH 6.8, 2% SDS, 25% glycerol) = 1:20], as previously reported (98). The nuclear fraction of MPCs was obtained with a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). After being boiled, the whole tissue/cell lysates were centrifuged at 3000 g for 5 min, and the supernatant was collected and stored at 4°C. The samples were analyzed on a 12% SDS–polyacrylamide gel. Protein concentrations were determined by using the Bradford method (99). Four micrograms of protein from each sample were loaded onto the gel and electrophoresed at 80 V for 3 h. We then transferred the total protein to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) via electroblot transfer at 60 V for 50 min. Membranes were washed in PBS for 5 min and blocked with 1% non-fat dry milk and 2% horse serum in Triton-PBS (T-PBS; 0.01%) for 1 h at room temperature (shaken at 60 cycles/min). A primary antibody of Notch1 (Abcam) or Hes1 (Abcam) was applied 1: 1000 and incubated overnight at 4°C. The blots were then washed four times in T-PBS (15 min/wash). A secondary antibody of anti-Rabbit IgG conjugated with horseradish peroxidase (1:5000, Pierce, Rockford, IL, USA) was applied for 1 h, at which time the blots again were washed four times in T-PBS (15 min/wash). After the blots were washed, they were developed using enhanced chemiluminescence (Supersignal West Pico Chemilluminescent Substrate, Pierce), and the positive bands were identified using X-ray film. Protein levels of GAPDH were also analyzed to serve as a control.

Table 1. PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
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<tr>
<td>GAPDH</td>
<td>Forward: TCCATGCAACATTTGGACATTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCACCGCCACAGCTTTCTCA</td>
</tr>
<tr>
<td>Notch1</td>
<td>Forward: GCGCCAAAGAGGCTTGGAGAT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAAGCTCTGGTGGACATGGTG</td>
</tr>
<tr>
<td>Notch2</td>
<td>Forward: GAGAAAAACCTGGTCAAGAATGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGTGAGATTTGGGCGCATCCTC</td>
</tr>
<tr>
<td>Notch3</td>
<td>Forward: TGCCAGAGTTCAGTGTTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACAGCGAAATCGGCCATC</td>
</tr>
<tr>
<td>Hes1</td>
<td>Forward: CCAGCGAGTGCTCAACAGCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: AATGCGGCGGAGTCTTCTCTCTT</td>
</tr>
<tr>
<td>Hey1</td>
<td>Forward: CCACAGGAGCCAGCTAATACAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCACGTGATCCACAGCTCATGCT</td>
</tr>
<tr>
<td>Jagged2</td>
<td>Forward: ACAGTGTATTGGTGGTCTTCT</td>
</tr>
<tr>
<td>Myostatin</td>
<td>Forward: AGTGATCTTAAATAGGAGGCATG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTTCCAGCAGCGCAGGTCAC</td>
</tr>
<tr>
<td>BMP4</td>
<td>Forward: ATCCCTCTGAGAATGGTCTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCGGCTCTCAAGTGATCAAACACTAC</td>
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<tr>
<td>TGF-β1</td>
<td>Forward: GGAGAGCCCTGGATACCAAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAACCCAGGCTCCTCTTTCA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward: GATTATGGCTCAAGGGTACCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTTCCTCTCGACAGCTCAACAC</td>
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<tr>
<td>IL-1β</td>
<td>Forward: GAGAACAACCAAGCAAGGAAAAATATA</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGGAAACTCTGAGCACTGCAAAC</td>
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<tr>
<td>IL-6</td>
<td>Forward: GGAAATCTGGTGAATGAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTTAGGCTCATAAACGACT</td>
</tr>
<tr>
<td>Klotho</td>
<td>Forward: CCAACAGCATCTATGAAAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTACCAATTTCTCATGCTTCT</td>
</tr>
<tr>
<td>Pax3</td>
<td>Forward: CACCCAGCTGTTGGCATC</td>
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<tr>
<td></td>
<td>Reverse: AATTCCTGAGAATGGTCTG</td>
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<tr>
<td>Pax7</td>
<td>Forward: CCTCAAGATGGTGGCATC</td>
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<td></td>
<td>Reverse: GGGGCTCTCCTCTCTTTATACCTC</td>
</tr>
<tr>
<td>MyoD</td>
<td>Forward: ACAGTGGCAGTCTAGATGCTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCTGACAGCTGCTCTGCAAACG</td>
</tr>
<tr>
<td>Collagen I</td>
<td>Forward: AAGAGTTTCTACATGCGCTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGACAGTCTCTTGGAAAACCTT</td>
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Histology

HO in the muscles was assessed by Alizarin Red staining: tissue sections of skeletal muscle were fixed with 4% formalin (10 min), and rinsed with double-distilled (dd) H2O; slides were then incubated with Alizarin Red working solution for 10 min before they were washed with ddH2O. Fatty infiltration was detected by Oil Red O staining: tissue sections of skeletal muscle were fixed with 4% formalin (10 min), and rinsed with ddH2O and 60% isopropanol; slides were then incubated with Oil Red O working solution for 15 min, before being rinsed with 60% isopropanol and ddH2O. The IMCL was detected by AdipoRed assay reagent (Lonza): tissue sections of skeletal muscle were fixed with 4% formalin (10 min), and rinsed with PBS; slides were then incubated with AdipoRed assay reagent for 15 min, before being washed with PBS. Slides were then rinsed and dehydrated prior to coverslip mounting using permanent mounting medium. For Masson trichrome staining, sections were incubated in Weigert’s iron hematoxylin working solution for 10 min, and rinsed under running water for 10 min. Slides were transferred to Biebrich scarlet-acid fuchsion solution for 15 min before incubation in aniline blue solution for another 5 min. Slides were then rinsed, dehydrated, and mounted as earlier. Muscle tissue was sectioned and slides were incubated for 5 min in Alizarin Red solution prior to counterstaining with eosin. Slides were rinsed, dehydrated, and all incubations were performed at room temperature. Immunofluorescent staining of tissue sections: the frozen tissue sections were fixed with 4% formalin. The primary antibodies Pax7 (DHSB), MyoD (Santa Cruz), Collagen IV (Abcam), Ki67 (Santa Cruz), PDGF-α (Abcam), Notch1 (Abcam), NICD (Notch1 intracellular domain, Abcam) and α-SMA (Abcam) were applied at 1:100 – 1:200. It is notable that Pax7 antibody from DHSB was developed in mouse, and in addition to specifically binding to Pax7+ cells, it non-specifically binds to necrotic myofibers; such necrotic tissue revealed by the Pax7 antibody was verified by staining with mouse IgG, which has been traditionally used to identify necrotic tissue. All slides were analyzed under fluorescence microscopy (Leica Microsystems Inc., IL) and photographed at ×40 magnification.
Measurements of results and statistical analysis

The quantitation of the results from images was performed using Northern Eclipse (version 6.0; Empix Imaging, Inc., Mississauga, ON, Canada) and Image J software (version 1.32; National Institutes of Health, Bethesda, MD, USA). Data from at least four samples from each subject were pooled for statistical analyses. Results are given as the mean ± standard deviation (SD). Statistical significance of any difference was calculated using Student’s t-test. When P-values were <0.05, the difference was considered statistically significant.

Supplementary Material

Supplementary Material is available at HMG online.

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